CONTENTS

PREFACE

The Scientific Committee would like to welcome you to this international workshop entitled "**Metabonomics: A new tool for exploring biocomplexity**" where a leading group of scientists in the field will explore most of the recent advancements and applications of metabonomics.

Metabonomics is a new systems biology approach that promises to add significant value to conventional disease diagnostics. It can also be applied to characterization of genetic modification in animal models of disease and it has a major role in improving the efficiency of the drug discovery and development process. The realization that obtaining the genome sequence of humans and other species is not sufficient to explain the fundamental nature of many disease processes has triggered a marked increase in interest in approaches that relate gene expression to phenotypic outcome. Several technologies have been, and continue to be, developed to achieve this end, namely genomics and transcriptomics (genetic complement and gene expression, respectively), proteomics (protein synthesis and cell signaling), metabolomics (metabolic regulation and fluxes in individual cells or cell types) and metabonomics (systemic metabolic profiling and regulation of function in whole organisms via analysis of biofluids and tissues).

In complex organisms, the levels of biomolecular organization and control are interdependent and moreover, can be affected by environmental events and stressors throughout life. All of the -omics technologies, which rely on analytical chemistry methods, result in complex multivariate data sets that require a variety of chemometric and bioinformatic tools for interpretation. The aim of such procedures, particularly for metabonomics, is to extract biochemical information that is of diagnostic or prognostic value and that reflects actual biological events rather than the potential for pathophysiology, which comes from the interpretation of gene expression and proteomic data. It is crucial to relate real-world or end-point observations to the measurements provided by the -omics technologies. This enables the understanding of the relationships between the inputs that change -omics responses and the outputs of those responses. As such, metabonomics provides a useful connection between the other -omics platforms and pathological end points, such as tissue histology or conventional clinical biochemistry measurements.

Metabonomics offers great potential in pharmaceutical drug discovery and development programmes. All such programmes must start from an understanding of what a "normal" physiological state looks like. From this perspective, it is then possible to understand other physiological states that deviate from "normal". This is possible because, by definition, any disease state will be abnormal. In this context, metabonomics can provide invaluable information in: a) the discovery and validation of potential targets for pharmaceutical intervention by determining the difference between normal and diseases tissues, b) the selection and optimisation of leads by

determining which lead candidate has a biochemical profile closest to a cell line in which the target is knocked out, c) the identification of lead candidates that have the fewest side-reactions in a model system, d) the development of drug candidates by ensuring that the end-point is achieved with the introduction of the fewest secondary effects, and e) the recovery of failed clinical candidates by a comparison of the biochemical profiles of both responsive and unresponsive patients to identify biomarkers of efficacy and safety.

Metabolic information can also be drawn from nutritional studies. Nutrigenomics aims to determine the influence of common dietary ingredients on the genome, and attempts to relate the resulting different phenotypes to differences in the cellular and/or genetic response of the biological system. More practically, nutrigenomics describes the use of functional genomic tools to probe a biological system following a nutritional stimulus. The application of metabonomics to such studies will permit an increased understanding of how nutritional molecules functionally affect metabolic pathways and homeostatic control.

Recent advances in Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectroscopy (MS) have made it possible to routinely make metabolic measurements. High resolution proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy is a very powerful, non-invasive technique that can provide information on a wide range of molecular processes. NMR has the ability to detect and characterize an abundance of metabolic components simultaneously, even when their identities are unknown. Most current techniques of analysis target only previously recognized and measurable substances, and any potential information that may be obtained from the unknown remainder is lost. All biological molecules contain protons, and are therefore represented somewhere within the proton-NMR spectra.

In conclusion, the workshop will offer an excellent opportunity to discuss the current applications of metabonomics in the diagnosis and prognosis of diseases, using complexity levels ranging from biofluids to cells and tissues. Special attention will be paid to the drug discovery applications regarding target identification, lead optimization, toxicity assessment of drugs, as well as other alternative applications of this methodology.

We sincerely hope that you enjoy our city and wish you a very fruitful workshop.

The Scientific Committee

SCIENTIFIC COMMITTEE

Dr Antonio Pineda-Lucena
Centro de Investigacion Pricipe Felipe, Valencia Spain

Dr Manfred Spraul
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Dr David MacIntyre
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SPONSORS

	21st October		22nd October		23rd October		24th October
		8:45	Opening Remarks				
			Session 1: Disease and Health I Ch: APL		***Session 5: Drug Discovery-Toxicity*** Ch: MS		***Session 9: Oral Presentations*** Ch: AM
		9:00	Claudio Luchinat	9:00	John C. Lindon	9:00	Claudio Santos
						9:20	Don Otter
		9:40	José María Mato	9:40	Donald Robertson		
							Session 10: Methods and Design
		10:20	*Refreshments/Break*	10:20	*Refreshments/Break*		Ch: JCL
			Session 2: Disease and Health II Ch: DR	10:50	Emmanuel Mikros	9:40	Teodoro Parella
		10:50	Bernardo Celda		***Session 6: Demonstrations***	10:20	*Refreshments/Break*
		11:30	Kathrin Renner	11:30	Carlos Cobas (MestreLab)	10:50	Manfred Spraul
		12:10	Hector Keun	12:10	Manfred Spraul (Bruker-Biospin)	11:30	**Open Discussion**
		12:50	*Lunch*	12:50	*Lunch*	12:30	**Closing Comments**
			Session 3: Oral Presentations Ch: WL		***Session 7: Oral Presentations*** Ch: HK		
		14:20	Gonçalo Graça	14:20	Ramón Campos-Olivas		
		14:40	Isabel García-Álvarez	14:40	Daniel Monleón		
		15:00	Rubén Ferrer-Luna	15:00	Kurt Boudonck		
		15:20	***Poster Session and afternoon tea***	15:20	***Poster Session and afternoon tea***		
			Session 4: Disease and Health III Ch: CL		***Session 8: Alternative Applications for Metabonomics*** Ch: KR		
17:00	***Registration***	17:00	Axel Meissner	17:00	Sunil Kochhar		
		17:40	Antonio Pineda	17:40	Wei Li		
19:00	***Welcome drinks***	19:00	***Touristic Bus***	21:00	***Banquet: Submarino Restaurant***		

INVITED SPEAKER ABSTRACTS

Session: Disease and Health I
Chairperson: Antonio Pineda-Lucena

IL1

Existence of different metabolic phenotypes and its implications

Claudio Luchinat

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We have recently shown that a thorough statistical analysis performed on NMR spectra of human urine samples reveals an invariant part characteristic of each person, which can be extracted from the analysis of multiple samples of each single subject[1].This finding (*i*) provides evidence that individual metabolic phenotypes may exist and (*ii*) opens new perspectives to metabonomic studies, based on the possibility of eliminating the daily ‘‘noise’’ by multiple sample collection.

This study has been extended to more subjects, confirming the original findings and showing that the phenotype space is far from being “saturated”. Furthermore, a new collection of samples from the same individuals after two-three years shows that the individual phenotype is relatively stable over this period at least in the examined cohort of young healthy adults.

In phenotyping diseases, blood-derived samples often yield better discrimination than urine samples, probably due to the lower day-to-day variability[2]. Our findings suggest that collection of multiple urine samples may alleviate this problem. Separation of the variable and the invariant parts of the urine NMR fingerprints may actually help add significance to disease-related features in the fingerprints themselves.

References

1. Assfalg, M., Bertini, I., Colangiuli, D., Luchinat, C., Schaefer, H., Schuetz, B., Spraul, M., Proc. Natl. Acad. Sci., 2008, 105, 1420-1424.
2. Bertini, I., Calabrò, A., De Carli, V., Luchinat, C., Nepi, S., Porfirio, B., Renzi, D., Saccenti, E., Tenori, L., submitted.

IL2

Metabolic Profiling as a Non-Invasive Tool for Non-Alcoholic Steatohepatitis Diagnosis

José M Mato[1], Cristina Alonso[2], Rubén Suero[2], Malu Martínez- Chantar[1], Jonathan Barr[2]

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Non-alchoholic steatohepatitis (NASH) is a critical stage in the process that spans from hepatic steatosis to cirrhosis and eventual liver failure. A potentially painful and hazardous biopsy is the only widely accepted test for distinguishing NASH from other forms of disease, and assessment is subjective and prone to sampling error. Here, a metabolomics approach using ultraperformance liquid chromatography coupled with electrospray time-of-flight mass spectrometry (UPLC-TOFMS) is evaluated as a possible non-invasive alternative for NASH diagnosis. Serum samples taken from different sets of patients were grouped according to the result of liver biopsy – grade of steatosis (1, 2 or 3) or NASH – and analysed together with a selection of healthy volunteers. Multivariate statistical analysis of the data show clear metabolic separation between NASH and other samples. Additionally, data mining techniques were used to highlight a number of key biomarkers differentially expressed with respect to progression of the disease.

IL3

In vivo and ex vivo MR metabonomics combined with ex vivo genomics for supporting brain tumour diagnosis, prognosis and treatment selection. FP6 eTUMOUR project

Bernardo Celda[1,2], Daniel Monleón[3], MCarmen Martínez-Bisbal[2], Rubén Ferrer[1], José Piquer[4], José Luís Llácer[4], Antonio Revert[5], Enrique Mollá[5], José Luís León[6], Vicente Benlloch[6], Salvador Campos[6], José González-Darder[7], Concepción López-Ginés[8], Miguel Cerdá-Nicolás[8], Adela Cañete[9], Federico V. Pallardó[10]

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Brain tumours are the leading cause of death in children under 15, and although in adults brain cancers are proportionately less common than other cancers, it is a devastating disease with high mortality. There is a great need to improve our understanding of brain tumour biology to improve diagnosis and to develop new treatments. ^{1}H MRS is currently the only non-invasive method that can be used to investigate molecular profile of brain tumours and also provide molecular images, more than six in one acquisition, of the distribution of chemicals in a tumour, which are also generally heterogeneous. A summary of the applications of ^{1}H MRS to the in vivo diagnosis and prognosis of brain tumours will be presented. In addition, examples of metabolite limits, infiltration and high cellularity location for neurosurgery applications by MRS molecular images will be shown. Likewise, recent ex vivo methods of studying the detailed biochemistry of tumour biopsies as metabolomic (HR-MAS) and transcriptomic (DNA microarrays) will be discussed as complementary to in vivo MRS (FP6 european project eTUMOUR). A preliminary comparison between molecular images from PET and 1H MRS will be also presented.

In conclusion, the combination among metabolic profiles from 1H MRS and HR-MAS and expression levels alterations from DNA microarrays, using multicenter protocols, has provided a set of biomarkers for a molecular classification of brain tumours, allowing the identification of tumour subtypes (as in GBM, meningiomas and oligoglial) and correlation with survival. This set of biomarkers can be used as well for improving the diagnosis and prognosis and for helping in a better selection and control of the therapy of brain tumours.

IL4

The application of flux analysis and metabolite pattern recognition to elucidate biological mechanisms

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Changes in the metabolism frequently reflect but also cause pathological alterations in a cell. The analysis of metabolite pattern but also of substrate flux provide useful information on the nature of the perturbation and offer new possibilities for interventions. This is shown by the mean of two completely different biological issues.

1. Flux analysis of monocytes- Two characteristics of tumors are an increased glycolytic activity, resulting in increased lactate concentration and lowered pH in the tumor microenvironment, and the ability to escape the immune answer. There is growing evidence, that the two phenomena are linked to each other. The increased lactate concentration in the tumor environment might influence the glycolysis dependent activation of immune cells and is – in part - responsible for the depressed immune answer. In a first step we measured the up-take of lactate by freshly isolated monocytes, using isotopically labelled lactate. Changes in glucose metabolism were determined by the use of 1,2 ^{13}C-glucose and its conversion to differently labelled lactate. The use of labelled glucose allows distinguishing between lactate produced by glycolysis or the pentose-phosphate pathway. Glycolytic activity was determined by measuring glucose up-take and lactate production. The tumor derived lactic acid is taken-up by monocytes, resulting in a drop in their glycolytic activity and cellular energy level and consequentially in a depressed immune function of the cells.

2. Detection of glucocorticoid induced changes on the mitochondrial level - Glucocorticoids are used in the therapy of childhood leukaemia for many years, but how they induce cell death is still unclear. The elucidation of the mechanism would offer new treatment possibilities with less severe side effects. We are currently investigating the underlying mechanism of the synergistic effect of glucocorticoids and 2-deoxy-glucose, as a potential new drug combination in the treatment of childhood leukaemia. Analysis of the energy metabolism revealed alterations in glycolysis and also mitochondrial oxidative phosphorylation, which can not be ascribed simply to substrate limitation. Therefore, changes in mitochondrial assembly under drug treatment are analyzed on different levels, e.g. the lipid composition by NMR spectroscopy. Mitochondria were isolated, tested for functional integrity and purity and metabolites were extracted by a methanol-chloroform protocol, samples were dried and resolved in chloroform D_4, one- and two-dimensional NMR experiments were performed.

Experimental set-up concerning flux analysis in cell culture and cell fractionation, metabolite extraction, data evaluation and interpretation are presented in the talk in detail.

IL5

Metabolic profiling of biofluids in oncology and toxicity studies

Hector Keun

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Metabolic profiling (metabonomics/metabolomics) is the untargeted analysis of metabolic composition in a biological sample, and is principally aimed at biomarker discovery. The frequent use of noninvasive biofluid analysis in metabonomics is suited to the clinic and facilitates dynamic monitoring. Analytical protocols for metabolic biomarkers are potentially robust because a metabolite is the same chemical entity irrespective of its origin, facilitating 'bench-to-bedside' translational research. Metabonomics can make an impact at several points in the drug-development process: target identification; lead discovery and optimization; preclinical efficacy and safety assessment; mode-of-action and mechanistic toxicology; patient stratification; and clinical pharmacological monitoring. In particular metabolic profiling presents new opportunities to personalise healthcare, illustrated by our current research in toxicology and oncology.

IL6

NMR-based metabonomics for diagnosis of parasitic infections and discovery of infection-related morbidity markers

Axel Meissner[a], Sibel Göraler, Crina Balog, Brigitte J. Vennervald, Oleg Mayboroda and André M. Deelder

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NMR-based metabonomics has over the last decade evolved to a well established methodology, adding to the arsenal of –omics techniques routinely applied in systems biology approaches for biomarker discovery, drug toxicity/safety assessment, metabolic phenotyping and disease diagnostics. While initially the emphasis of NMR-based metabonomics was placed on toxicology in preclinical drug development, its main focus has more recently shifted towards clinical research applications and nutritional science. As a consequence the average number of samples in metabonomic studies is rapidly growing. A typical study design in preclinical toxicology for testing of several compounds comprises not more than 200 to 300 samples, whereas clinical studies easily exceed 500 or more subjects giving rise to several thousands of samples for longitudinal study designs. This increased sample volume and sample collection over longer periods of time demands for high reproducibility and robustness of the methodology combined with a large degree of automation in order to ensure sufficient sample throughput for data acquisition as well as data processing. In addition, inhomogeneous cohorts in epidemiological studies present a challenge for multivariate data analysis, since relevant changes in the metabolic profile can easily be covered by larger non-relevant "background" variability within the data.

We conducted a NMR-based metabonomics study on 2000 urine samples from a well-characterized cohort from rural areas in Uganda near Lake Victoria where a high prevalence of a variety of parasitic infections occurs. Focus in this study was on schistosomiasis (also called bilharziosis) a parasitic infection that is endemic in 76 developing countries affecting more than 200 million people in rural areas and which ranks second behind malaria in terms of socio-economic and public health importance in the Tropics and Subtropics. Our particular interest was on *Schistosoma mansoni*, a parasite that is found in Africa, Madagascar and parts of South America. The goal of this study was to derive biomarkers of infection and infection related morbidity markers in order to monitor infection and morbidity status. Furthermore, the aim of this longitudinal study was to investigate the effect of Praziquantel treatment, treatment response and reoccurrence of infection which should provide valuable information to facilitate improved morbidity control.

Several technical aspects concerning NMR data acquisition and processing of large numbers of samples together with a general strategy for analysis of data from large inhomogeneous cohorts will be presented. Unbiased multivariate data analysis as well as targeted approaches on selected sub-groups will be used for identification of molecular markers or specific patterns that can serve as potential biomarkers of infection or morbidity related conditions.

IL7

Applications of ^{1}H-NMR Metabonomics in Disease Diagnosis and Regenerative Medicine: A Proof-of-principle Study

David A. MacIntyre[1], Beatriz Jiménez[1], Eloisa Jantus Lewintre[2], Sonia Prado López[3], Javier García Conde[2], Rubén Moreno Palanques[3], Miodrag Stojkovic[3] and Antonio Pineda-Lucena[1]

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Very recently, we have initiated several studies to evaluate the potential of metabonomics by NMR in the elucidation of the molecular basis of pathological processes, as well as in the identification of metabolic events relevant to the field of regenerative medicine. In particular, this presentation will focus on the application of ^{1}H-NMR metabonomics to the characterization of the serum profiles of B-cell Chronic Lymphocytic Leukemia (B-CLL) patients, and in the understanding of the role of hypoxia in stem cell differentiation.

B-CLL is an adult-onset leukemia characterized by the presence of elevated numbers of circulating clonal leukemic B cells. Clinical management of the disease is difficult as the individual prognosis of patients is extremely variable. This heterogeneity appears to be explained in part by differences in the mutational status of immunoglobulin heavy chain genes (IgV_H) in their clonal leukemic cells. Patients with unmutated (-mut) IgV_H genes exhibit an aggressive, usually fatal course, whereas patients with mutated (+mut) genes follow a comparatively indolent course. However, the determination of IgV_H mutations relies upon DNA sequencing methods that are time consuming and costly. We have evaluated the potential of metabonomics by NMR in B-CLL diagnosis by assessing the metabolic profiles of patients with B-CLL (±mut) and healthy controls. Initial results will be presented demonstrating that this technique represents a promising approach for the classification of B-CLL from serum samples.

Human embryonic stem cells (hESCs) are exposed to hypoxic environment *in vivo*. Evidence suggests that this environment promotes and maintains their ability to self-renew whilst inhibiting spontaneous differentiation. Despite this, the underlying role of hypoxia in stem cell differentiation and renewal is poorly characterised. The aim of this study was to use ^{1}H-NMR-based metabonomics to examine changes in metabolic profiles of hESCs associated with hypoxic conditions *in vitro*. Specifically our objectives were to identify and quantify metabolites that change in accordance with a hypoxic environment. Our results clearly show that ^{1}H-NMR based metabolomics is a useful tool for assessing changes in the metabolic pathways of hESCs *in vitro*. The metabolic changes identified in this study may reflect functional metabolic pathways underpinning the role of hypoxia in maintaining and promoting hESC renewal.

We acknowledge financial support from Bruker BioSpin (Spain) and technical support from Bruker BioSpin (Germany).

IL8

Some Recent Metabonomics Developments with Pharmaceutical Applications

John C. Lindon

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Now that the human and other genome sequences are known, there is a greater understanding of "druggable" targets and the characterisation of these has involved the measurement of gene expression (transcriptomics) and protein expression changes (proteomics). However, sometimes transcriptomics and proteomics do not provide evidence of end-point markers for disease diagnosis, or evaluation of beneficial or adverse drug effects in the way in which altered biochemistry provides the ultimate diagnostic information. Metabonomics holds out this possibility since many non-genetic factors can also affect the systems biology view of an organism with the recognition that environmental factors such as diet, age ethnicity, life-style and gut microfloral populations can have a large influence.

Metabonomics, as a key element in top-down systems biology, can provide real biological endpoints and is defined as "*the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification*". It involves the generation of metabolic databases, based on tissue or biofluid samples, for control animals and humans, diseased patients, animals used in drug safety testing, etc., allowing the simultaneous acquisition of multiple biochemical parameters on biological samples. Metabonomics is usually conducted on biofluids, many of which can usually be obtained non-invasively (e.g. urine) or relatively easily (e.g. blood), but other fluids such as cerebrospinal fluid, bile or seminal fluid can be used. It is also possible to use cell culture supernatants, tissue extracts and similar preparations

The two most information-rich techniques that give atom-specific molecular structural information are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, the latter including magic-angle-spinning NMR spectroscopy applied to tissue samples. For MS-based metabonomics, it is generally necessary to carry out a separation step, usually using liquid chromatography (LC or UPLC) or chemical derivatisation and gas chromatography (GC) before the MS stage. Whilst MS can be more sensitive than NMR spectroscopy and can give lower detection limits, there are problems of non-uniform detection caused by variable ionization efficiency. The metabolic response of an organism to a particular disease, toxin or pharmaceutical compound can then be extracted from the complex data sets, which are also subject to biological variation, by application of appropriate multivariate statistical analyses. Metabonomics also allows time-dependent patterns of change in response (metabolic trajectories) to stimuli to be measured.

Areas where metabonomics could impact pharmaceutical R&D include - validation of animal models of disease, including genetically-modified animals, preclinical evaluation of drug safety and ranking of compounds, assessment of safety in clinical trials and after product launch, improved understanding of idiosyncratic toxicity, improved differential diagnosis and prognosis of clinical diseases, better understanding of environmental population effects through epidemiological studies, patient stratification (pharmacometabonomics), ranking of the beneficial effects of pharmaceuticals both in development and clinically, the effects of interactions between drugs, and between drugs and diet, and identification of new drug targets

In this talk, some recently developed metabonomics data analysis methodologies based on NMR spectroscopy and mass spectrometry will be introduced and their use for sample classification and biomarker identification explored. The limits imposed by analytical, biological and environmental variation will be covered, including the evaluation of the effects of symbiotic gut microflora on metabolic profiles. Some pharmaceutical applications of the approach will be illustrated, including the COMET project for evaluating drug adverse effects, and the prediction of an individual's response to therapy before drug administration.

IL9

Metabonomics Technology in Drug Discovery and Safety Evaluation

Donald G. Robertson

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Metabonomics Technology (also known as metabolomics or metabolic profiling) is in a state of transition in the pharmaceutical industry. The technology has passed the "emerging technology" stage and now has made it past the value proposition phase of evaluation. The results of this evaluation have lead to the decline of internal efforts at metabonomics in many companies and lead to the expansion of the use of external service providers. Additionally, with the advent of the NIH Road Map initiative in the US, academic institutions are increasingly including metabonomics into there research efforts. Due to this changing landscape, platform application have also changed from primarily NMR based to largely MS (LC or CG) based. Within the industry, applications of metabonomic technology are also changing with decreased emphasis on screening applications and increased emphasis on mechanistic and biomarker applications and a growing interest in using the technology for model characterization. Importantly - combinations of metabonomics with other "omics", particularly transcriptomics, are being vigorously pursued. Several examples of "real world" pharmaceutical applications will be presented covering the landscape from biomarkers of efficacy and toxicity early in discovery, model characterization and mechanistic studies of a clinically relevant toxicity.

IL10

Application of NMR based Metabonomic Analysis in Cardiac and Hepatic Toxicological processes

Emmanuel Mikros

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NMR based metabonomics allows rapid analysis of the complex metabolic profiles of biological samples taking advantage of the degree of inherent biochemical similarities between samples. NMR in combination with multivariate chemometric analysis has been used successfully in the fields of drug toxicology and disease diagnosis. In this work we present the application of the metabonomic approach in different toxicity processes in liver and heart.

In order to delineate the mechanisms of liver disease, various experimental animal models of acute and chronic injury have been used. Among them, the carbon tetrachloride and thioacetamide (TAA) administration has been widely used to elucidate such mechanisms. The NMR metabolic profiles and the samples clustering after multivariate statistical analysis will be presented in cases like acute liver injury (CCl_4) and chronic (TAA) experimental fibrosis and cirrhosis. Differences between the toxic insult and regenerative state as well as between different stages of fibrosis have been also studied. The investigation was extended to liver regeneration rate after 70% partial hepatectomy (PH) in healthy as well as inotic and cirrhotic rats after the administration of thioacetamide (TAA).

Myocardial toxicity has been studied in the case of the administration of Adriamycin (doxorubicin-DXR). DXR is a commonly used antineoplastic agent, but its use is limited by the occurrence of dose dependent cardiotoxicity. ^{1}H NMR spectra of aqueous myocardium extracts showed alteration in heart energy metabolism and biomarkers like acetate and succinate relate to toxicity.

Finally in both liver (CCl_4 administration) and heart (DXR administration) toxicological processes free radicals is believed to be the main mechanisms of toxicity, thus metabolic alterations after co-administration of the antioxidant agent oleuropein has been also studied. Oleuropein is a phenolic constituent of olive tree present in olive fruits, olive leaves, and olive oil and several studies show that is an important factor of Mediteranean diet benefits. NMR metabolic profiling demonstrates the protective role of oleuropein contributing to the understanding of a more effective therapeutic approach.

IL11

Metabolic health assessment by nutritional metabonomics

Sunil Kochhar

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Metabonomics is also minimally invasive, and profiling of biological fluids by NMR or MS ensures a simultaneous analysis of a wide range of metabolites that are the endpoints of molecular regulatory processes, diet and gut microflora metabolism and environmental factors. By opening a direct biochemical window into the metabolome in a holistic fashion, metabonomics is uniquely suited in developing new generations of biomarkers that are capable of providing better understanding of complex metabolic phenomena as well as assessing intra- and inter-individual differences. This property makes metabonomics very efficient for the generation of biomarker patterns for the comprehensive characterization of metabolic health, the prognostics and the diagnostics of diseases, and the generation of new insights in the understanding of the interactions between diet and metabolism

Defining the metabolic phenotype or “metabotype” of human populations will offer a great opportunity to evaluate the metabolic response and the degree of this response to specific dietary modulations at the individual level allowing pre-dietary intervention metabolite profiling could be used to model and predict the responses of individual subjects to special foods. As an example the presentation will report results from a recent study where we determined the occurrence of a metabolic imprinting or memory in relation to regular consumption of chocolate. In a study of 22 human subjects based on their preference for chocolate and pattern of daily consumption, we generated metabolic profiles of plasma and urine samples and analyzed those employing multivariate statistics. A clear discrimination of subjects according to their chocolate liking was observed. The class separation using plasma metabolic profiles was present even from samples collected before the chocolate intake, suggesting the occurrence of a metabolic imprint or memory independent of the chocolate intake. Results indicate that subjects who do not like chocolate harbor statistically different lipoprotein profile in their postprandial phase suggesting differences in dynamics of the lipoprotein clearance in relation to regular consumption of chocolate. The class separation was also achieved on urine metabolic profiles due to metabolites most likely from gut microbiome metabolic activity. This could indicate differences in gut microflora functionality and metabolism in relation to regular intake of chocolate. In summary, nutritional metabonomics can then be foreseen as a powerful non invasive approach to address and optimize personalized nutrition.

IL12

Application of multi-dimensional HRMAS NMR in intact cells

Wei Li

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High-resolution magic-angle spinning NMR (HR-MAS NMR) is a hybrid technique from liquid state and solid state NMR and has been used in metabonomics studies using a variety of biological systems. However, due to the fast rotation and unique sample geometry, direct application of multidimensional NMR under MAS condition may not always work well. Using whole cell mycobacteria samples, we examined and optimized a number of 2D and 3D pulse sequences suitable to study mycobacteria cell wall structures. Parameters such as rotor synchronization, cell density, labeling strategies and temperature effects are investigated for optimal application of whole cell multidimensional HRMAS NMR. We applied these optimized condition to study metabolic changes during melanogenesis in melanoma cells, and demonstrated the different metabolic profiles associated with this process.

IL13

Simultaneous Acquisition of Multiple NMR Spectra

Teodor Parella

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New NMR experiments developed in our lab will be briefly presented and discussed. The concepts of time-sharing (TS) multiple-frequency evolution[1-4] and multiple-FID acquisition within the same scan (MA)[5-6] will be described as a means to improve NMR data collection. Examples of simultaneous acquisition of multiple and fully complementary NMR spectra from a one-shot NMR pulse sequence will be demonstrated in the structural characterization of small molecules in solution.

First, several time-sharing (TS) experiments based on the simultaneous evolution of different frequencies in the indirect dimension of a multidimensional NMR experiment will be demonstrated using inverse $^{13}C/^{15}N$ TS experiments, such as TS-HSQC, TS-HSQC-TOCSY, TS-HSQMBC and TS-HMBC pulse schemes, on different nitrogen-containing small and medium-sized molecules.

The combined principles of TS and multiple-FID acquisition into the same scan (MATS technique) will be proposed to obtain multiple and fully complementary NMR spectra in a single-shot acquisition. The principle is applied to collect simultaneously four different HMBC and HMBC-relayed experiments for ^{13}C and ^{15}N at natural abundance (see Figure 1). Important savings of 75% in measuring times can be achieved when compared to the individual acquisition. On the other hand, the concerted analysis of such data can be used for rapid structural characterization on a wide variety of compounds.

The main advantages in spectrometer time savings, sensitivity-per-time unit improvements and multiple information will be outlined and discussed. All these concepts can be of enormous interest in automation protocols for data acquisition, data processing and data analysis.

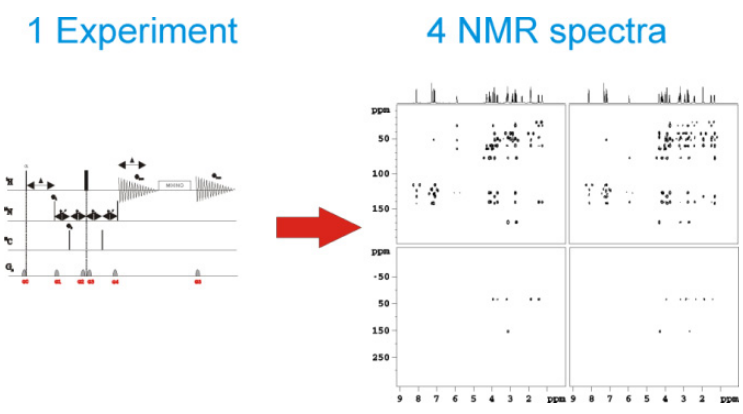

Figure 1: Separate C-HMBC, C-HMBC-COSY, N-HMBC and N-HMBC spectra can be separately obtained after proper data acquisition and data processing using the MATS technique.

References: [1]Nolis P.; Parella T., *Magn. Reson. Chem.* **2006**, *44*, 1031-1036. [2]Nolis P.; Parella T., *J. Biomol. NMR* **2007**, *37*, 65-77. [3]Pérez-Trujillo M.; Nolis P.; Parella T., *Org. Lett.* **2007**, *9*, 29-32. [4]Pérez-Trujillo M.; Nolis P.; Bermel, W.; Parella T., *Magn. Reson. Chem.* **2007**, *44*, 325-329. [5]Nolis P.; Pérez-Trujillo M.; Parella T., *Angew. Chem. Intl. Ed.* **2007**, *46*, 7495-7497. [6]Nolis P.; Gil, S.; Pérez-Trujillo M.; Parella T., *Magn. Reson. Chem.* **2008**, submitted.

IL14

Requirements for the successful use of NMR in Metabonomics

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NMR in Metabonomics has to operate under very stringent quality conditions due to the need to observe even smallest changes in the spectra, indicating for example ageing or disease progression. This means the internal variance of the analytical system has to be reduced as much as possible. This is as important for sample preparation, calibration, measurement and processing. Dealing reliably with large sample numbers needs a sample tracking system to avoid mix-up of samples and to allow rapid access to all relevant sample information. The system requirements are described, including also the addition of a mass spectrometry-chromatography coupling. To evaluate metabonomics spectra and results, the use of a reference compound database allows to identify and quantify many metabolites or assign outlier signals to biomarkers linking directly to statistics. The most important experiments used in Metabonomics are described as well as some of their important parameters.

Some remarks on experimental design and pitfalls are also mentioned.

ORAL PRESENTATIONS

Session I: 22/10/08
Chairperson: Wei Li

Presenting Author

14:20-14:40 **OP1: Rapid compositional profiling of Human Amniotic Fluid: Towards the diagnosis of pregnancy disorders.** Gonçalo Graça, Iola F. Duarte, Brian J. Goodfellow, António Barros, Isabel M. Carreira, Ana B. Couceiro, Manfred Spraul and Ana M. Gil

14:40-15:00 **OP2: ^{1}H MAS NMR Spectroscopy Reveals Metabolite Changes in C6 Glioma Cells after Treatment with Antimitotic Glycoside.** Isabel García-Álvarez, Leoncio Garrido, Ernesto Doncel-Pérez, Manuel Nieto-Sampedro, Alfonso Fernández-Mayoralas

15:00-15:20 **OP3: High-Resolution Magic Angle Spinning Proton Magnetic Resonance Spectroscopy of Oligodendroglial Tumours. Correlation with Histopathology and Genetic Subtypes.** Rubén Ferrer-Luna, Jose Manuel Morales, M.C. Martinez-Bisbal, Manuel Mata, Francisco Dasí, Horacio Martinetto, Jorge Calvar, Gustavo Sevlever, Daniel Monleón and Bernardo Celda

Session II: 23/10/08
Chairperson: Hector Keun

14:30-14:50 **OP4: Searching for metabolic markers of bladder cancer in urine by NMR.** Ramón Campos-Olivas, Julen Oyarzabal, Marta Sánchez-Carbayo, Pilar Gonzalez-Peramato, Manuel Urrutia, James K. Ellis, Hector Keun, Jeremy K. Nicholson, and Francisco J. Blanco

14:50-15:10 **OP5: Metabolite profiling of feces extracts from human colorectal cancers.** Daniel Monleon, José Manuel Morales, Antonio Barrasa, Bernardo Celda

15:10-15:30 **OP6: Mechanism-Based Biomarker Approach for Biomarker Discovery and Diagnostic Development.** Walt Gall, Jeffrey Shuster, Don Rose, Mike Milburn, Kurt Boudonck, John Ryals

Session III: 24/10/08
Chairperson: Alex Meissner

09:00-09:20 **OP7: NMR based Metabolic Profiling of *Saccharomyces cerevisiae* with High Levels of mRNA Mistranslation.** Claudio H Santos, João Paredes, Manuel AS Santos, Brian J Goodfellow

09:20-09:40 **OP8: Influence of Chromatographic Technique on Metabolite Identification.** Don Otter, D Rowan, H-M Lin, M Cao, K Fraser, and G Lane.

ORAL PRESENTATION ABSTRACTS

Session I 22/10/08
Chairperson: Wei Li

OP1

Rapid compositional profiling of Human Amniotic Fluid: Towards the diagnosis of pregnancy disorders

Gonçalo Graça[1], Iola F. Duarte[1], Brian J. Goodfellow[1], António Barros[2], Isabel M. Carreira[3], Ana B. Couceiro[4], Manfred Spraul[5] and Ana M. Gil[1]

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The metabolic profile of Human Amniotic Fluid (HAF) should be sensitive to the health state of mother and foetus and developing methods to monitor HAF composition may be the basis of new diagnostics and follow-up methods. Proton NMR spectroscopy is well established for metabolite profiling in biofluids[1, 2] and has been tentatively applied to HAF in healthy and disease pregnancies[3-5]. These studies have, however, lacked sensitivity, large enough number of cases and adequate statistical analysis. The aim of this work is to develop NMR/statistical models to evaluate HAF metabolic composition and use as diagnostic methods and biochemical probes.

HAF was collected for 75 women at amniocentesis (2nd trimester of pregnancy). After centrifugation, samples were analysed by NMR. 1D/2D NMR and LC-NMR/MS enabled the rapid (10-20 min) simultaneous identification of just over 70 metabolites in HAF, 7 of which detected in this biofluid for the first time, to our knowledge[6-8]. Spectral data was validated by multivariate methods: PCA and OPLS PLS-DA[8]. Multivariate analysis of the spectra showed that several foetus malformation cases are distinguished from controls in terms of HAF metabolic composition. The origin of metabolite changes is discussed.

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OP2

^{1}H MAS NMR Spectroscopy Reveals Metabolite Changes in C6 Glioma Cells after Treatment with Antimitotic Glycoside

Isabel García-Álvarez,[a] Leoncio Garrido,[b] Ernesto Doncel-Pérez,[a] Manuel Nieto-Sampedro,[a,c] Alfonso Fernández-Mayoralas*[,d]

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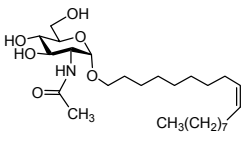

1
IC_{50} = 1,3 µM
(rat glioma line C6)

Recently we have obtained a family of *N*-acyl-glucosamine derivatives, some of which showed antimitotic activity against C6 glioma cells with low IC_{50} values, being oleyl glycoside **1** the most inhibitory compound.[1] Due to the high antiproliferative activity exhibited by **1**, it was of interest to obtain information about its mechanism of action. With this purpose, we have analyzed metabolite changes after treatment of C6 glioma cells with **1** using high-resolution magic angle spinning ^{1}H NMR. The assignment of relevant signals was based on the chemical shifts reported for metabolites of cultured glioma cell lines.[2]

The treatment of C6 cells with 10 µM of **1** gave a spectrum in which the signal for CoA metabolites drastically decreased and the concentration of choline-containing metabolites increased as compared to control (Figure 1). However, when the cells were incubated in the presence of higher concentrations of **1** (40 µM) the signal for CoA metabolites at 0.7 ppm was significantly high. In addition, the spectrum showed a profile for low cell density and viability, as evidenced by the small intensity of the peak from creatine (Figure 1C).

The data obtained from the ^{1}H NMR spectra of the different experiments suggest that oleyl glycoside **1** may be altering the metabolism of fatty acids in C6 glioma cells. At higher concentrations (above 40 µM) a significant ratio of cell death occurred though an apoptotic process. The typical characteristics of cell death was verified by the analysis of DNA-fragmentation induced by **1**.

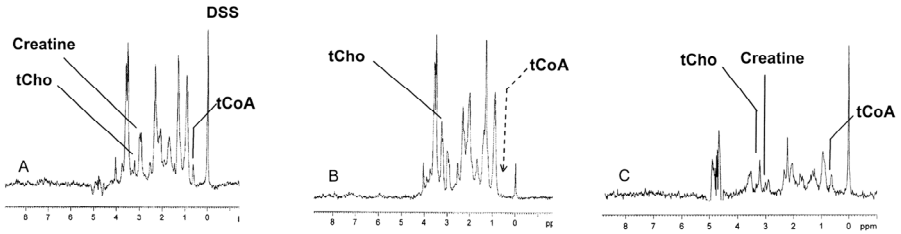

Figure 1. ^{1}H NMR spectra of control C6 (A), cultured C6 with 10 µM (B) and 40 µM (C) of **1**. Abbreviations: tCho, total choline metabolites; tCoA, total coenzyme A metabolites.

Acknowledgements: Financial support by the Servicio de Salud de Castilla la Mancha Community (SESCAM), CSIC (PIF, 200580F0062) and MEC (CTQ2007-67403/BQU) is greatly appreciated. **References** [1]García-Álvarez, I.; Corrales, G.; Doncel-Pérez, E.; Muñoz, A.; Nieto-Sampedro, M.; Fernández-Mayoralas, A. *J. Med. Chem.* **2007**, *50*, 364-373. [2]Govindaraju, V.; Young, K.; Maudsley, A. A. *NMR Biomed*. **2000**, *13*, 129-153.

OP3

High-Resolution Magic Angle Spinning Proton Magnetic Resonance Spectroscopy of Oligodendroglial Tumours. Correlation with Histopathology and Genetic Subtypes

Rubén Ferrer-Luna[1], Jose Manuel Morales, M.C. Martinez-Bisbal, Manuel Mata, Francisco Dasí, Horacio Martinetto, Jorge Calvar, Gustavo Sevlever, Daniel Monleón and Bernardo Celda.

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Oligodendroglial Tumours (OT) are constituted by pure Oligodendroglial tumors (OD), and mixed Oligoastrocytic tumors (OA) and their respective Anaplastic grade (AOD, AOA). According WHO classification, pure and mixed classes are considered differential tumour entities. Both classes share genetic-molecular alterations but at different frequency. Unfortunately, histopathological classification has a subjective component and it is limited in treatment management. It has been observed that Oligodendrogliomas are one of the most chemosensitive solid tumours and that loss of chromosome (LOH) 1p was tightly associated with chemotherapy response[1]. Nowadays, it is not clear if different Oligodendroglial Tumours molecular subtypes present differential metabolic profiles. In order to evaluate the potential benefit of metabolic profiles 19 Pure Oligodendrogliomas and 10 Mixed Oligoastrocytomas were analyzed.The main purpose was determine whether high resolution magic angle spinning (HR-MAS) proton (^{1}H) spectroscopy obtained through eTUMOR protocols[2] distinguish between histopathologic types, and genomic subtypes of oligodendroglial tumors. Spectra were acquired with a Bruker Avance DRX 600 and ARX 500 spectrometers. Tissue degradation was avoided keeping sample at 4ºC during experiment. CPMG sequence was used as T2 filter and monodimensional spectra were processed. Aliphatic (0.5 and 4.50 ppm) and Aromatic (4.5 and 9.5 ppm) regions were evaluated and normalized to total spectra area. Spectra were binned into 0.01 ppm buckets to reduce the impact of misalignment. Finally, 37 metabolites previously identified and assigned[3] were quantified and evaluated by Unsupervised (PCA and Hierachical Clustering) and supervised statistically analysis. Previous to final contrast we take into account effects caused by spectrometers and anatomical tumour location. OT with worst prognosis and lower survival presented increased levels of Phosphocholine, Choline, Fatty acids and Alanine. These metabolites provide biological insights related with behavior and tumour evolution, moreover mentioned metabolites could be used to monitor tumour progression "*in vivo*" by MRS as a non invasive diagnostic tool.
In the same way we are evaluating the correlations among Fatty acid, Phosphocholine, Choline, Glutamine, Glutamate and Alanine relative concentration and expression level of genes implied in their biosynthesis and degradation pathways.

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OP4

Searching for metabolic markers of bladder cancer in urine by NMR

Ramón Campos-Olivas[a], Julen Oyarzabal[b], Marta Sánchez-Carbayo[c], Pilar Gonzalez-Peramato[d], Manuel Urrutia[e], James K. Ellis[f], Hector Keun[f], Jeremy K. Nicholson[f], and Francisco J. Blanco[g].

[a]Spectroscopy and NMR Unit, [b]Computational Chemistry Section, and [c]Tumor Markers Group, Centro Nacional de Investigaciones Oncológicas (CNIO), Melchor Fernández Almagro 3, 28029 Madrid; [d]Pathology Department, Hospital General de Guadalajara; [e]Urology Department, Hospital Universitario de Salamanca, [f]Imperial College, London; and [g]Structural Biology Unit, CIC-bioGUNE, Bilbao. *rcampos@cnio.es*

Urine is an easily obtainable body fluid from human patients, and represents an ideal substrate for bladder cancer biomarker research. We are applying ^{1}H NMR spectroscopy to analyse the metabolic profile of urine in an initial set of 150 samples from healthy individuals, patients with benign urological diseases, and bladder cancer patients collected immediately before cystoscopy and urinary cytology at two independent clinical settings. Identifying metabolic signatures would represent a non-invasive method for bladder cancer detection which may complement current invasive and uncomfortable cystoscopic evaluation of malignancy. Multivariate data analysis methods are used to extract the relevant metabolic patterns from body fluids with a complex chemical composition like urine. These methods give a simplified and therefore interpretable view on these complicated metabolomics datasets. We have collected the ^{1}H NMR spectra of buffered urine samples at 600 MHz under a defined protocol using an internal standard for spectral alignment. The intensities of selected regions of the spectra were analysed using multivariate statistical methods: Principal Component Analysis (PCA) and Partial Least Squares-Discrimination Analysis (PLS-DA), in an attempt to uncover possible resonances/compounds that are associated with the presence of bladder cancer. The methods employed and the results obtained so far, together with future plans to extend and complement this study, will be presented.

OP5

Metabolite profiling of feces extracts from human colorectal cancers

Daniel Monleon[1], José Manuel Morales[2], Antonio Barrasa[3], Bernardo Celda[4].

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Purpose. Colorectal cancer is the second leading cause of cancer death in developed countries. Better preventive strategies are needed to improve the outcome of this disease. The increasing availability of high-throughput methodologies opens new possibilities for the screening of new markers. The application of NMR metabolic profiling to feces extracts has an interesting potential as diagnostic tool for detecting colorectal cancer patients on general population. We obtained NMR metabolic profiles of fecal extracts in colorectal patients and healthy individuals, to characterize possible differences between them and to identify potential diagnosis markers.

Subjects and methods. Samples: NMR spectra were collected for 25 water fecal extracts from 10 healthy colon controls and 15 colorectal cancer patients. Full length colonoscopy established the diagnosis in every patient. All stool samples were collected before surgery or endoscopic exam, and with no bowel preparation. Five grams of stools were taken and diluted into 10 ml. of bidestilated water. NMR spectroscopy: The whole study was performed at 10C to minimize sample degradation. The spectra were recorded in a Bruker-AVANCE600 spectrometer. A presat experiment was recorded for each sample. All spectra were preprocessed with 0.3Hz line broadening. Alanine doublet was used for spectral referencing. Data analysis: Statistical multivariate analysis was performed using in-house MATLAB scripts and the LIBRA library. Two orthogonal components were removed by OSC prior to any feature reduction. Robust Principal Component Analysis (RPCA) was applied to the set of spectral vectors. Principal components chosen explained at least 80% of the variance.

Results and discussion. All NMR spectra showed narrow line widths and adequate signal-to-noise ratios with well resolved spin-spin multiplicities. Measured NMR spectra show significant variability due to the lack of dietary control. Multivariate analysis performed over the NMR spectra allowed detecting some differences between the two groups. The loadings plot showed that the major metabolic differences correspond to some short chain fatty acids (SCFA) and some amino acids.

Conclusion. Our results demonstrate that metabolic profiling on fecal extracts is a cheap, reproducible and effective method for detecting colonic epithelium alterations and complement other stool screening methods. Lower SCFA levels, which have been previously associated with colorectal cancer development, seem to be the most effective marker between controls and patients fecal extracts. Some aminoacids also display some correlation with the disease. Differentiation between control and colorectal cancer patient fecal extracts by NMR spectroscopy opens new possibilities for the development of new screening protocols.

OP6

Mechanism-Based Biomarker Approach for Biomarker Discovery and Diagnostic Development

Kurt Boudonck, Walt Gall, Jeffrey Shuster, Don Rose, Mike Milburn, John Ryals.

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Mechanism-Based Biomarker Approach for Biomarker Discovery and Diagnostic Development: Historically, traditional diagnostic development has been a relatively inefficient, long-term process involving discovery of one biomarker at a time, resulting in product development timelines of several years. Genomics and proteomics approaches have experienced challenges with biomarker discovery for diagnostic development, partly due to the vast heterogeneity of populations studied and the requirement for detecting a strong, specific signal amidst wide biological variation. In contrast, a metabolomics approach* for identifying biomarker candidates includes measuring a more tractable number of analytes, as well as directly identifying metabolites which more closely reflect the molecular pathways involved in a physiological phenotype or disease mechanism under study. This presentation will offer insight into this application of metabolomics through the development of diagnostic tests including a diagnostic test that distinguishes indolent from aggressive prostate cancer tumors to help reduce the number of unnecessary biopsies and prostatectomies as well as an easy-to-administer, fasting blood test that provides a measure of a patient's insulin sensitivity.

Metabolomics Approach: This presentation will present the use of metabolomics in developing a diagnostic test. The process starts with the selection of appropriate clinical samples for identifying relevant biomarkers. These samples are analyzed using a metabolomics platform based on three independent analyses: LC-MS/MS (ESI +), LC-MS/MS (ESI -), and GC-MS. From the raw mass spectral data, biochemicals are identified and quantified using software to filter noise from relevant chemical data and match the spectra to a database generated from reference compounds. After data processing, statistical modeling approaches are implemented on the hundreds of small molecules identified in a single sample, to yield a select group of biomarker candidates that highly correlate to a gold standard reference method for the purpose of developing a diagnostic test. Different subsets of biomarkers are selected in a variety of combinations when developing a multivariate diagnostic algorithm that may reflect presence of a subclinical condition/disease or its progression. Further analyses of these subsets of small molecules are carried out using targeted, structural elucidation techniques, in preparation for subsequent analytical validation and clinical validation studies for diagnostic product development.

OP7

NMR based Metabolic Profiling of Saccharomyces cerevisiae with High Levels of mRNA Mistranslation

Claudio H Santos[a], João Paredes[b], Manuel AS Santos[b], Brian J Goodfellow[a].

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High mRNA translation fidelity is critical for the production of stable and functional proteomes and, therefore, mRNA mistranslation events that produce aberrant proteins are often linked to disease. In normal physiological conditions mRNA mistranslation errors are low (10^{-4} to 10^{-5}), however, the error frequency increases under stress, aging and certain diseases. In order to understand this phenotype at the molecular level, we have induced constitutive mRNA mistranslation in *Saccharomyces cerevisiae*, using tRNA engineering methodologies[1].

By using NMR spectroscopy at high field, the effect of mRNA mistranslation on the *Saccharomyces cerevisiae* metabolome was studied. Using an extraction method similar to that used in Lewis et al[2], low MWt (< 5kDa) metabolites were obtained and a series of 1D and, on selected samples, ^{1}H-^{13}C-HSQC spectra were acquired. Using a PCA approach, the separation of samples from control cells and samples from cells with different levels of mRNA mistranslation was achieved. The loadings from the PCA plots indicated that separation was due to differing levels of trehalose, glycerol, acetic acid and a number of amino acids. These results are consistent with previous studies that showed important differences in gene expression in mistranslating cells[1]. Comparison of these data from mistranslating cells with data from other types of stress, namely heat shock, will allow better characterization of the *Saccharomyces cerevisiae* stress response at the metabolome level.

In parallel, complete identification of the low MWt metabolites, visible by NMR, is being carried out using 1D/2D NMR in conjunction with LC-NMR methods. A previous study identified ca. 40 metabolites. This has now been expanded to ca. 60 using coupled NMR methods.

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OP8

Influence of Chromatographic Technique on Metabolite Identification

Don Otter[a], D. Rowan[b], H-M. Lin[b], M. Cao[a], K. Fraser[a] and G. Lane[a]

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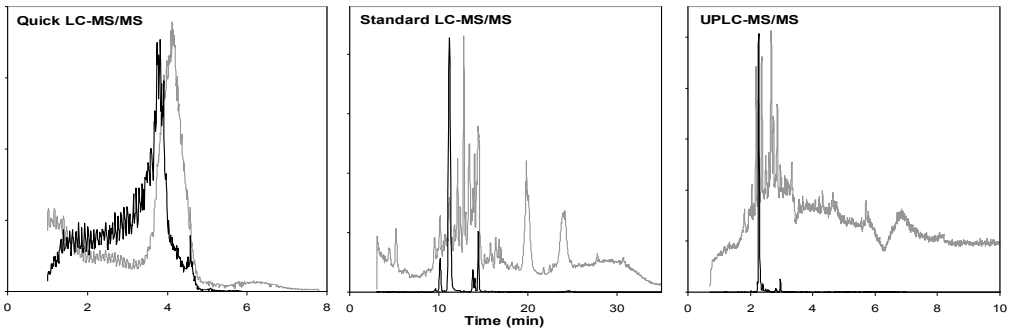
don.otter@agresearch.co.nz

Longitudinal metabolomic analysis is being undertaken with the goal of identifying robust, causally linked metabolite-based biomarkers functionally prognostic of gut inflammation and the health status of the IL10$^{-/-}$ gene-deficient mouse model of Inflammatory Bowel Disease. Xanthurenic acid (M_r 205 Da) has been identified as a possible biomarker in this model[1] (H-M Lin, unpublished results). Mouse urine was collected and three different chromatographic techniques were used to separate the metabolites prior to analysis by mass spectrometry. The performance of each chromatographic method was monitored by characterisation of the negative ion of xanthurenic acid (204 m/z).

LC-MS/MS with a short (20 mm) column was used as the initial longitudinal metabolic profiling method due to its high sample throughput, sensitivity and dynamic range whilst requiring small sample volumes (4□L) and minimal sample preparation[2]. The method, using a Thermo LTQ linear ion-trap MS, yielded semi-quantitative data on 500 to 1000 metabolites, together with qualitative fragmentation data on ca 200 ions, within 8 minutes. The 204 m/z ion trace was seen as a broad smear with a peak at 3.8 minutes. Possible candidate ions were then further characterised by standard LC with targeted MS-MS^n. There were at least three 204 m/z negative ions present (10.0, 11.2 and 14.4 min) but examination of the MS/MS spectra showed that only the 11.2 min peak corresponded to xanthurenic acid.

UHPLC-MS/MS was then investigated as a possible compromise method offering both high throughput and high ion resolution. There was good separation of individual ions by UHPLC with a chromatogram similar to that obtained by standard LC-MS/MS in a much shorter elution time. However the peak width for the 204 m/z ion was very narrow (approximately 3 sec) resulting in limitations in the MS and MS^n experiments which could be performed.



Chromatograms of mouse urine. Grey = TIC, black = 204 m/z.

In summary: quick LC-MS/MS gave excellent sample throughput and could be used to identify possible candidate ions but only provided MS/MS data for the more dominant ions due to its poor chromatographic separation. Standard LC-MS/MS was ideal for the more detailed, targeted analysis of limited numbers of samples and UHPLC-MS/MS, whilst giving good sample throughput and excellent ion separation, was often handicapped by the very sharp peaks being too narrow for the acquisition rate of the ion-trap MS.

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POSTER PRESENTATIONS

15:20-17:00hrs, 22/10/08

Presenting Author

P1 **Metabolic profile differences in GBM's by HR-MAS and findings in histology and *in vivo* MRS.** M.Carmen Martínez-Bisbal, Vicent Esteve, Beatriz Martínez-Granados, Miguel Cerdá, Francisco Sancho-Bielsa, Bernardo Celda

P2 **The potential of HRMAS NMR for assessing the metabolic phenotype of lung cancer.** Iola F. Duarte, Cláudia Rocha, Ana M. Gil, Brian J. Goodfellow, Lina Carvalho, Vitor Sousa, Isabel M. Carreira, E. Humpfer, M. Spraul

P3 **Metabolomics in pre-diabetes research.** Marianna Lucio, Rainer Lehmann, Hans-Ulrich Häring, Philippe Schmitt-Kopplin

P4 **A NMR-based metabonomics approach to the study of GSPE intake in rats fed a hyperlipidic diet.** Maria Vinaixa, Miguel A. Rodriguez, Juan Fernández-Larrea, Mª Cinta Bladé, Jesús Brezmes, Nicolau Cañellas, Xavier Correig, Lluis Arola, Manfred Spraul

P5 **NMR as a tool for investigating congenital disorders: the case of glycosylation type Ia in cultured human fibroblasts.** Nicola D'Amelio, Stefania Biffi, Chiara Garrovo, Giovanni Maria Severini

P6 **Exo- and endo-metabolome patterns in differentiating B lymphoma cells: optimizing sampling protocols.** Silvia Mari, Jose M. García Manteiga, Claudia Napoli, Anna Minoja, Simone Cenci, Roberto Sitia, Giovanna Musco

P7 **Global Spectral Deconvolution (GSD) and Its Applications to NMR-based Metabonomics Analysis.** Carlos Cobas, Chen Peng, Pablo Monje, Stanislav Sýkora

P8 **New methodology for single human embryo selection.** Maria Gómez-Mingot, Jesús Iniesta, Vicente Montiel, Juan Manuel Moreno, José Jesús López-Gálvez

POSTER PRESENTATION ABSTRACTS

P1

Metabolic profile differences in GBM's by HR-MAS and findings in histology and in vivo MRS

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Glioblastoma Multiforme (GBM) is the most aggressive and heterogenous glioma. Subtle biochemical changes can be detected in intact tissues or from chemical extracts by high-resolution MRS. These data have been used to reveal the status of tumour micro-heterogeneity, observing tumour metabolic alterations before they are morphologically detectable, and to correlate them to histopathological features and diagnosis. Proton magnetic resonance spectroscopy with high-resolution magic angle spinning (HR-MAS) was also demonstrated to preserve tissue histopathologic features while producing well-resolved spectra of cellular metabolites in the intact tissue specimens. Extensive metabolic identification and preliminary metabolic differences by HR-MAS were already shown in a set of 10 GBM[1].

The aim of this communication is to identify differences in GBM HR-MAS metabolic profiles by comparing ex vivo spectra of GBM and "non-affected" tissue (verified by histology). In vivo GBM pre-surgery spectra from these patients and a set of controls helped to stablish the representativity of HR-MAS spectra.

25 GBM tissue samples were studied by HR-MAS. 18/25 samples underwent histological examination after HRMAS study. 15/18 of the samples gave histological tumour result with variable portion of tumour and necrotic content. Remaining 3/18 samples were histologically classified as 'not tumour', being mainly CNS normal tissue or a small portion of infiltrated tumour edge, and were considered as 'reference' tissue. To increase the 'reference' group, 4 more glioma samples histologically classified as not tumour after HR-MAS were added to the 'reference' group. 7/25 samples, without histological assessment, were used as test for the group differences. In vivo spectroscopy was presurgically performed in 10 of the GBM patients (10/25). White matter spectra of 3 controls were used as in vivo metabolic profile reference.

In vivo ^{1}H MRS (1.5T) and ex vivo HR-MAS (11T) data were acquired according to eTUMOUR protocols [FP6-2002-LIFESCIHEALTH 503094]. The spectra were analyzed using home-made software with capability for spectra visualisation and statistical analysis.

High concentrations of NAA, Cr and other metabolites and almost null detection of macromolecules signals clearly differentiated "non-affected" and tumour tissues. Likewise, differences among GBM HR-MAS metabolic profiles were observed, mainly related to the intensity of myo-Inositol, Creatine, Proline, Glycero-phosphocholine, Posphoryl choline and other resonances without attending strictly to the amount of lipids, drawing two sets of GBM. When more samples of GBM without histological

information are added to the study set, they become part of the 'reference' group or part of the two sets of GBM, being preserved the initial splitting up of the samples assessed by histology. Moreover, an adequate correlation between in vivo and ex vivo spectra for 10 GBM was as well observed.

Clear differences have been detected in specific spectra regions between GBM and "non-affected" tissues by HR-MAS, in concordance with histology. Among GBM histologically proven tissues, metabolic profile differences have aroused and GBM tissues seem to be divided in two tumour pattern, which could be related to the tumour cellular condition, tumour behaviour or to the several microheterogeneous areas in GBM. These groups are preserved when more GBM samples are added to the set showing their robustness. The adequate agreement between ex vivo and in vivo metabolic characteristics, and the differences the GBM by HRMAS can provide relevant tissue state information for a better interpretation of in vivo spectra and for increasing their contribution to non-invasive brain tumour classification.

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The potential of HRMAS NMR for assessing the metabolic phenotype of lung cancer

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The ability to detect and follow metabolic changes in tumours is of paramount importance for investigating their altered cellular metabolism and finding new targets for therapy. In this work, we have applied High Resolution Magic Angle Spinning (HRMAS) NMR to human lung tumours and normal adjacent tissues in order to assess their composition and identify biochemical alterations related to tumour metabolism. The ^{1}H NMR profiles obtained showed great complexity and a high degree of signal overlap resulting from the many compounds present. Based on a range of one-dimensional (1D) and two-dimensional (2D) NMR experiments, about forty of these compounds, including amino acids, organic acids, choline metabolites, nucleosides and lipids, could be identified. As expected, this composition was found to differ between tumoral and non-tumoral tissue and, also, between tumours of different histological types. Metabolic variations suggest that glycolysis, gluconeogenesis and cell membrane turnover are affected in tumours. In the future, the analysis of an enlarged number of samples will be combined with multivariate statistics, aiming at determining specific compositional patterns that correlate to tumour diagnosis or grading.

Metabolomics in pre-diabetes research

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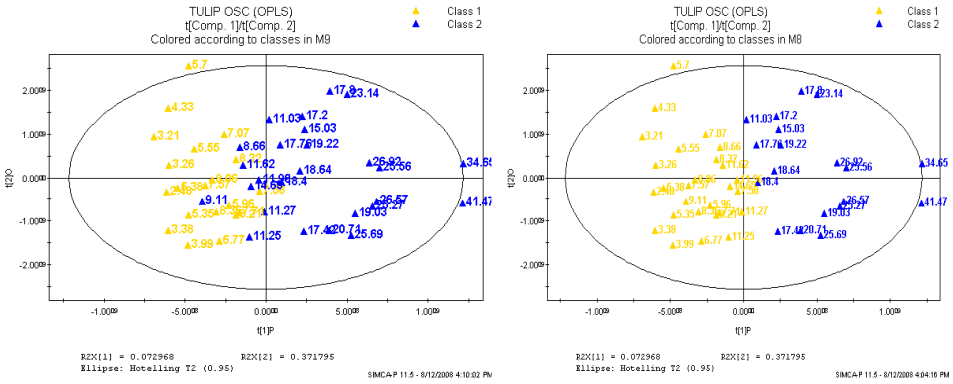
The metabolomics study applied to disease's evaluation has the main goal to diagnose health and identify factors that cause disease. These studies can lead to enhanced understanding of disease mechanisms and to new diagnostic markers as well as enhanced understanding of mechanisms for drug or xenobiotic effect and increased ability to predict individual variation in drug response phenotypes1 [Kaddurah-Daouk]. This approach combined with Ion Cyclotron Resonance Fourier Transform Mass Spectrometer (ICR-FT/MS) brings new information on the pre-diabetic state with its high resolving power, mass accuracy, sensitivity, and flexibility.

A non target approach is applied to group non-diabetic individuals with a high risk to develop type 2 diabetes. The major challenge in the post-genome era is to understand how interactions among the molecules in a cell determine its form and function. Information is achieved through multivariate display and statistical analysis tools, graph modeling and visualization and classify and store metabolites in database. The statistical tools start from unsupervised method to lead to supervised methods; such as principal component analysis, partial least square regression, discriminate analysis and a variety of clustering techniques; PCA and hierarchical cluster analysis are now widespread; and they provide an appropriate starting point for further analysis in the interpretation of metabolite profiling. Common to a great part of these methods is that they build up interdependencies between metabolites, relationships between the abundances of the metabolites as revealed by correlation, covariance or distance matrix.



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A NMR-based metabonomics approach to the study of GSPE intake in rats fed a hyperlipidic diet

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Procyanidins are phenolic compounds from the flavonoid group that are widely found in cereals, vegetables, and fruits such as grapes and apples, berries, cocoa and beverages such as red wine. They are receiving significant attention because of their potential importance to human health[1] and their wide range of biological activities[2]. They act as cardioprotectants, antioxidants, antigenotoxics, anti-inflammatories and anticarcenogenics[3]. Their protective action could extend to other metabolic anormalities such as insulin resistance, dyslipemia, arterial hypertension and obesity (collectivelt termed the metabolic syndrome). In spite of obesity being one of the main problems in industrialised societies, there have been few studies relating obesity and procyanidins[3]. There is a growing interest in the utilization of procyanidins for their dietary and pharmacological properties. In this context, Metabonomics, "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modifications"[4], provides a systems approach to understanding global metabolic regulations of organisms. This concept has arisen from the combination of NMR and pattern recognition to study the multicomponent metabolic composition of biological fluids, cells, and tissues. The generated metabolic profiles are processed by multivariate statistics to maximize the recovery of information to be correlated with well-determined stimuli such as dietary intervention or with any phenotypic data or diet habits[5]. Metabonomics approach is found to be ideally suited to nutrition research and it may provide an insight into in vivo mechanisms of action following nutritional intervention[5].

In the current work, a metabonomic approach has been applied to the study of the long-term biochemical consumption effect of GSPE (grape seed procyanidin extract) in rats under diet induced obesity conditions. Changes in ^{1}H-NMR spectral profile of serum collected from rats with three long term different dietary intervention studies were evaluated. Thus, chow-fed male were compared against cafeteria-fed male rats and against cafeteria-fed male rats orally administered with different GSPE concentration (either 25mg/kg or 50mg/kg). By applying various chemometric techniques to the NMR data, the biochemical effects of long-term GSPE intervention were determined. Some differences in the plasma lipoprotein and carbohydrate profiles were observed following GSPE intervention, suggesting a GSPE induced alteration in energy metabolism. This study has emphasized the potential of the metabonomic approach for studying bioactivity of polyphenols naturally occurring in many foods such as red wine.This study describes a novel application for ^{1}H-NMR analysis by determining subtle differences in biochemical profiles following dietary intervention and providing further insight into the mechanisms of action of procyanidines in vivo.

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NMR as a tool for investigating congenital disorders: the case of glycosylation type Ia in cultured human fibroblasts

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Congenital disorders of glycosylation (CDG) are a family of multisystem inherited disorders caused by defects in the biosynthesis of N- or O-glycans[1]. Although defects in phosphomannose mutase (PMM2) are among the most common biochemical proven causes of CDG (classified as CDGIa)[2], quantitative studies of the effects of PMM2 deficiency on mitochondrial metabolism and energetics have never been reported.

Herein, we report a biochemical investigation on fibroblasts derived from patients affected by CDGIa: enzymatic activity of PMM2 was quantitatively measured by ATP production. Furthermore, the metabolic profile of the cells was monitored by NMR spectroscopy providing ^{13}C labelled nutrients and observing their processing within the fibroblasts.

These experiments proved the ability of NMR to provide insight into the biochemical consequences of PMM2 deficiency.

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Exo- and endo-metabolome patterns in differentiating B lymphoma cells: optimizing sampling protocols

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A detailed description of the metabolome in mammalian cells and tissues, particularly in dynamic differentiation systems or in disease, is clearly a hot topic[1-3]; however, the collection of reliable data has so far been hampered by the limited robustness of quenching and extraction protocols.

When small B-lymphocytes bind their cognate antigens in the context of suitable signals, a dramatic differentiation program is activated that leads to the formation of professional antibody secreting plasma cells, with profound geno-proteomic changes[4-7]. Primary B cells and certain B lymphoma lines can be readily induced to undergo a real metamorphosis finalised to achieve massive antibody production. In this study, we used the murine I.29+ lymphoma to analyse the dynamic metabolome changes that underlie terminal B lymphocyte differentiation[7].

Since most published studies are based on bacteria or yeast[8-10], we first explored the introduction of quenching step(s) in the metabolome analysis, in order to properly stop metabolism and at the same time minimize metabolite leakage, which could occur during quenching procedures. As a complement of this study we have also analysed the exometabolome[11] of differentiating B cells at four different time points.

An NMR-1D approach has been used to analyse both endo- and exo-metabolome using Amix Sofware (Bruker Biospin) for the data processing and statistical analysis. The strategy of choice has been the construction of a Knowledge Base used for further analytical profiling and principal component analysis.

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Global Spectral Deconvolution (GSD) and Its Applications to NMR-based Metabonomics Analysis

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A typical experimental NMR spectrum comprises ***a)*** spectral peaks arising from the transitions of the studied spin system(s), ***b)*** satellite peaks from the very same spin system(s) isotopomers, ***c)*** solvent peaks due to the employed solvent, ***d)*** impurity peaks due to undesired chemical components of the sample, ***e)*** folded-over peaks from outside the spectral window, f) baseline distortions due to acquisition dead-time or undesirable broad solid-state signals, and g) spikes due to interfering RF signals from the instrument or from the broader environment. For both quantitative and qualitative analysis, it is desirable to decompose the spectrum into its individual components. In particular, one would like to reduce it automatically to an editable peak list which matches all the recognizable peaks in the experimental spectrum, leaving out any baseline drift and noise. The peaks in such a peak list, each described in a parametric form, can then be subjected to automatic and/or manual editing (filtering). For example, one can automatically recognize spikes (anomalously narrow peaks), solid impurities (very broad peaks), folded-over peaks (anomalous phase), and possibly even rotation sidebands and isotopomer satellites.

This task can be even more challenging when dealing with biofluids spectra for metabonomics analysis because such spectra are usually very complex and highly overlapped. While binning and bucketing are commonly used to de-resolve the spectra (in order to overcome peak misalignment) prior to multi-variate analysis,[1] resolving and deconvoluting overlapped peaks are very important operations in the later stage of such analysis, i.e., in the identification of altered molecules (potential biomarkers) and quantitative measurement of the changes to their concentrations.[2]

Several partial approaches to generate such peak lists have been developed in the past, including parametric Linear Prediction[3] and the Filter Diagonalization Method[4] (FDM). As opposed to those methods, based on the direct analysis of the FID, in this work we present a new approach which works directly on the frequency domain and makes use of novel methods for spectral resolution enhancement (Resolution Booster[3]) and automatic peak detection. This process can be carried out either by using a fast algorithm based on the knowledge of the first and second derivatives of the data and on limited, local fitting, or by employing a substantially slower massive fitting method to be used when highly accurate values are needed.

We will show several real-life examples of GSD as implemented in Mnova software.[5] Current limitations and restrictions, as well as the future potential and perspectives of this approach will be also presented.

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New methodology for single human embryo selection

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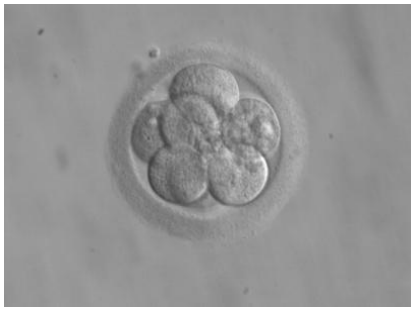
In human *in vitro* fertilisation (IVF) is impossible to determine *in situ* the implantation viability of embryos after retrieval. Embryonic morphology and development in an established nutritional culture medium are the sole tools to select the best embryos for further transfer to the female reproductive tract. However, embryos transferred classified as good or excellent quality do not reach implantation, and by contrast those scored as poorest quality do. The reason for this is that there are unreliable methods which might be able for the determination of potential parameters or biomarkers involved in implantation and pregnancy.

By using chromatographic methods (HPLC-UV), and chromatography coupled to advanced mass spectrometry (HPLC-MS), and NMR techniques, this study focuses on the determination of changes in the composition of a chemically defined embryo culture media on day 2-3 (involving analysis of organics acids, carbohydrates, amino acids and proteins). The assessment of the metabolic necessity of each embryo and the correlation of the above with its morphology and implantation rate will be addressed in this presentation.



Embryonic culture media are classified into three groups. Firstly, group I contains those media whose embryos have 100% of success and end up with pregnancy, Group II contains those media whose embryos do not reach implantation, and finally group III, where at least one or more embryos that are transferred, finally implant. The results obtained for 47 couples (around 130 embryo culture media analysed) have shown that embryos that finally implant behave differently to those that don't reach implantation. This means that some analytes present in the culture media are significantly important for embryo development. Application of Univariant and Multivariant statistical methods, such as ANOVA, Discriminant Analysis and Principal Component Analysis determine significant differences in metabolic capacities between distinct groups.

All this have important consequences in terms of searching and selecting a single embryo with the highest guarantees of implantation and result in a live baby. Moreover, benefits may be obtained from the replacement of multiple embryos in a treatment cycle, which results mostly in an unacceptably high multiple pregnancy rates, with increased obstetric, neonatal and economic costs to society.

DEMONSTRATIONS

11:30-13:00hrs, 23/10/08

Presenting Author

D1 **New NMR Data Processing Algorithms and their Potential Applications in Metabonomics studies.** Pablo Monje, Carlos Cobas, Chen Peng, Stanislav Sýkora' *Mestrelab Research S.L., Spain* and *Extra Byte, Italy*

D2 **NMR methodology as a powerful tool in epidemiology and disease screening.** M. Spraul, H. Schäfer, B. Schütz Bruker *BioSpin GmbH Germany*

DEMONSTRATION ABSTRACTS

D1

New NMR Data Processing Algorithms and their Potential Applications in Metabonomics studies

Pablo Monje[a],Carlos Cobas[a], Chen Peng[a], Stanislav Sýkora[b]

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The processing and analysis of metabonomics spectra present specific challenges in the sense that they require the efficient managing of large sets of data. For this reason, manual processing is no longer an option, making it necessary to take advantage of tools which can handle the processing of large groups of spectra in automation.

In addition, although many of the processing operations used with standard NMR spectra of small organic molecules are also directly applied to the spectra of biofluids or tissues, these pose some unique challenges, particularly in the context of phase and baseline correction.

In this talk we will present our solution to these issues, Mnova. This advanced, yet easy to use NMR software package allows the efficient processing of large sets of data in fully automated mode and includes new, powerful algorithms designed for the automatic phase and baseline correction of metabonomics-like NMR spectra, as well as several tools aimed at improving the quality of these spectra. In addition, we will introduce some of the results obtained with our newly developed GSD (Global Spectral Deconvolution) system, which we believe may introduce new opportunities for enhanced processing and analysis in the area of NMR based metabonomics.

NMR methodology as a powerful tool in epidemiology and disease screening

M.Spraul, H.Schäfer, B.Schütz.

Bruker BioSpin GmbH Germany

One of the fastest growing research areas currently is Metabonomics, the part of systems biology that deals with the composition and changes in concentration of small molecules in humans, animals, plants and cellular systems. NMR is a leading analytical tool due to its highest reproducibility, high dynamic range and quantitative properties. NMR is used in pharmaceutical applications like drug toxicity and efficacy screening, in food research to show effects of nutrition to health and in clinical research to find typical disease patterns or to investigate epidemiological studies. Also the environmental influences to the health conditions can be studied.

Clinical Research and epidemiological studies are areas for NMR to give new insight into disease processes, personalized metabolic profiles and ageing processes to name a few.

NMR can be hyphenated with LC-MS to increase the information content beyond the facts learned from the individual tools using for example covariance analysis. For human studies, urine, plasma, cerebrospinal fluid and tissue are most often investigated. Multiple examples are given to demonstrate the power of the analytical approach to Metabonomics described here.

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