

Other Biological Fields

OB1

CHARACTERIZATION OF EPITHELIAL SODIUM CHANNEL ALPHA SUBUNIT TRANSCRIPTS AND THEIR CORRESPONDING MRNA EXPRESSION LEVELS IN DAHL S VERSUS R RATS' KIDNEY CORTEX.

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AIMS/HYPOTHESIS: The α -subunit of the amiloride-sensitive epithelial sodium channel (ENaC) is critical for the expression of functional channels. In humans and rats, non functional alternatively spliced forms of α ENaC have been proposed to act as negative regulatory components for ENaC. The purpose of this study was to investigate the mRNA expression levels of alternatively spliced forms of α ENaC in kidney cortex of Dahl salt resistant (R) versus salt sensitive (S) on normal and four-week high salt diet. **METHODS:** Using quantitative RT-PCR strategy, we examined the mRNA expression levels of previously reported α ENaC a and b alternatively spliced forms in kidney cortex of Dahl S and R rats on normal and four-week high salt diet and compared their corresponding abundance to the wildtype α ENaC mRNA levels. Using polymerase chain reaction, we identified, cloned and sequenced 2 novel non coding C-terminus spliced forms and examined their mRNA expression in Dahl S versus R rats. We also tested the presence of five previously reported lung-specific α ENaC spliced forms in Dahl rats' kidney cortex (CK479583, CK475461, CK364785, CK475819, and CB690980). **RESULTS:** Previously reported ENaC a and b variants were significantly higher in Dahl R versus S rats ($P < 0.05$). Four-week high salt diet significantly increased α ENaC b ($P < 0.05$), but not α ENaC a transcript abundance in Dahl R, but not S rats. The expression levels for the two newly identified non coding ENaC c and d variants were comparable in Dahl S versus R rats. Compared to α ENaC wt, α ENaC a, c and d were low abundance transcripts, in contrast to α ENaC b transcript abundance that was 32 +/- 3 folds higher than α ENaC wt. We could not identify any of the five previously reported lung-specific α ENaC spliced forms (CK479583, CK475461, CK364785, CK475819, and CB690980) in Dahl rats' kidney cortex. **CONCLUSIONS/INTERPRETATION:** α ENaC spliced forms, particularly, α ENaC b, might likely act as dominant negative proteins for ENaC activity, thereby rescuing Dahl R rats from developing salt-sensitive hypertension on high salt diet.

OB2

OVEREXPRESSION OF HUMAN PROEGF CYTOPLASMIC DOMAIN CAUSES REDUCTION IN BODY AND ORGAN WEIGHT IN TRANSGENIC MICE.

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The cytoplasmic domains of proTGF- α -cyt and proHB-EGF-cyt precursors were shown to encode protein binding motifs for Naked2 (NKD2) and promyelocytic leukemia zinc finger protein (PLZF) nuclear transcriptional repressor, respectively. The cytoplasmic domains of proTGF- α and proAR were demonstrated to contain basolateral sorting information. The soluble cytoplasmic domain of neuregulin1 (NRG1-cyt) was shown to be a nuclear transcriptional suppressor for regulators of apoptosis and to associate with LIM-kinase implicating NRG1-cyt as a regulator of cytoskeletal processes. Here we show that transgenic mice overexpressing human proEGFcyt, but not a proEGFcyt splice form proEGFdel23, display significantly reduced body weight. The differences in body and organ weights were significant and more pronounced in female than in male transgenic animals. Detailed analysis revealed significantly reduced weights for kidney and brain tissues in proEGFcyt mice. A new polyclonal antiserum specific for proEGFcyt demonstrated the basolateral presence of proEGFcyt protein in distal tubules and collecting ducts in transgenic kidneys. Growth retardation had previously been shown in transgenic mice overexpressing total human proEGF. Here we show that over-expression of human cytoplasmic proEGF domain alone is sufficient to cause reduction in body weight. Further histological and molecular studies are in progress to determine the effects of proEGFcyt on specific organ functions in these proEGFcyt mice.

OB3

ENVIRONMENTAL TOXINS INDUCE METABOLIC AND STRUCTURAL CHANGES IN IMMORTALIZED HUMAN ENDOMETRIAL CELLS.

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Environmental pollutants and Arylhydrocarbon/Dioxin Receptor (AhR-) agonists are xenobiotics and have been implicated in reduced female fertility and in the pathogenesis of endometriosis. The molecular mechanisms elicited by dioxin-type toxins in the human endometrium are not understood. We established a telomerase-immortalized human endometrial epithelial cell line (hTERT-EEC) as a novel in-vitro tool to investigate molecular actions of these AhR agonists. We show that hTERT-EEC express functional arylhydrocarbon-receptor (AhR). Exposure to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and coplanar polychlorinated biphenyls (PCB) results in a time- and dose-dependent transcriptional activation of AhR as indicated by xenobiotic response element (XRE-)luciferase assay and the induction of AhR target genes of p450 microsomal enzymes CYP1A1 and CYP1B1. TCDD did not change estrogen receptor- (ER-) alpha protein levels suggesting that dioxin does not induce proteasomal degradation of ER in hTERT-EEC. However, TCDD induced marked alterations in membrane surface morphology as determined by scanning electron microscopy (SEM) suggesting a novel role of TCDD in the organization of sub-membranous cytoskeletal structures.

OB4

ESTROGEN ENHANCES THE INVASIVE POTENTIAL IN HUMAN THYROID CARCINOMA CELLS.

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The incidence of human thyroid carcinoma is three fold higher in women than in men suggesting gender-specific factors to be involved in thyroid carcinoma induction and progression. The thyroid gland is considered to be a non-classical target organ for sex steroids and estrogen-receptors

have been detected in thyrocytes of the normal thyroid gland and in benign and neoplastic thyroid tissues. Several human thyroid carcinoma cell lines express estrogen receptor alpha (ERα) on the transcript and protein level. The aim of this study is to detect changes in the protease profile in human thyroid carcinoma cells induced by exposure to estrogen. Methods: the human follicular thyroid carcinoma cell line FTC-133 was treated with 17-beta-estradiol (E2) at 10 nM and 100 nM. RT-PCR, Western analysis and immunofluorescence were applied to detect quantitative changes and determine alterations in the cellular distribution of the lysosomal proteases Cathepsin-D, -L and -B, the extracellular matrix degrading proteases MMP-2/9 and the serine protease urokinase plasminogen activator (uPA). Filter migration assays were used to examine alterations of in-vitro invasiveness. FTC-133 exposed to estrogen responded with elevated ERα levels indicating the ability of this steroid hormone to enforce it's own actions within this follicular thyroid carcinoma cell line. Upon culture with estrogen, FTC-133 revealed an increase in expression of the acidic lysosomal proteases cathepsin-B, -C and -L and also showed changes in their cellular distribution. Furthermore, estrogen induced MMP9 and MMP2, as well as the proform and processed uro-plasminogen activator (uPA). Estrogen-treated thyroid carcinoma cells revealed increased cellular motility. We conclude that estrogen is an important tumor promoter in follicular thyroid carcinoma. We are currently investigating the molecular pathways elicited by estrogen in thyroid carcinoma cells and their functional consequences for tumor cell invasiveness.

OB5

DETECTION AND CHARACTERIZATION OF SURFACTANT PROTEINS IN EXHALED HUMAN BREATH.

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Pulmonary surfactant (PS) is a complex lipoprotein mix that lies at the most peripheral areas of the lungs. It functions to generate surface tension within the gas exchange regions thus preventing lung collapse at minimum diameter. The importance of PS is underscored by many studies which have shown that premature infants born prior to the onset of full PS production develop Respiratory Distress Syndrome which is a leading cause of mortality in this group. Within the PS four characteristic proteins are present, SPA, SPB, SPC and SPD. These proteins have important functions related to the surface tension lowering properties of PS. Changes in protein composition or

function have been documented in disease states such as asthma, COPD or BPD. In addition there is some preliminary evidence that exposure to mold toxins or tobacco smoke alters protein profiles or functional status. In these cases surfactant proteins have been analyzed following bronchoalveolar lavage. Such approaches have some degree of invasiveness. Exhaled breath represents air transferred from the most peripheral regions of the pulmonary tree. Thus it may be expected that components of exhaled breath might provide some indications as to the state of health of the peripheral airways. Nevertheless it has not been determined if components of the PS are even present in exhaled breath. To this end, we collected exhaled breath as a condensate (EBC) to determine if components characteristic of the PS, specifically the pulmonary surfactant proteins, were detectable and to establish a profile for their presence in EBC. Using a new amplification system and antibodies to the PS proteins, we established that SPA and SPB were detectable in PS although SPB levels were higher. SPB levels in EBC of five subjects were similar, suggesting this protein may be a useful marker and provide some degree of uniformity for assessing EBC in health and disease.

OB6

ROLE OF PHD2 IN THE INTERFERENCE OF AHR AND HIF-1ALPHA SIGNALLING.

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The PAS family of transcription factors acts as sensors for various environmental stimuli. Members of this family are the aryl hydrocarbon receptor (AhR) and hypoxia-inducible factor-1alpha (HIF-1 α). Ligands of AhR, such as the lipophilic environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are known to accumulate in mammary gland tissue. HIF-1 α is activated by hypoxia caused by insufficient vascularization as a typical feature of many tumors, e.g. breast cancer. Since both transcription factors share ARNT (aryl hydrocarbon receptor nuclear translocator) as a common dimerisation partner, we analysed the interference between the AhR and HIF-1 α pathway in the human breast carcinoma cell line MCF-7 following exposure to 10nM TCDD and hypoxia (5% O₂). Specific attention was paid to prolyl hydroxylase domain containing protein 2 (PHD2), the main regulator of HIF-1 α stability. Combined treatment with TCDD and hypoxia reduced the stabilization of HIF-1 α and decreased the hypoxia response element-mediated promoter activity if compared to exposure to hypoxia alone. AhR inhibition by alpha-naphthoflavone prevented these effects. Lowered HIF-1 α amounts were not caused by transcriptional down-regulation. Both, a distinct hypoxic stress or TCDD treatment increased PHD2 promoter activity,

transcript and protein amounts. However, no such changes were found under simultaneous exposure to TCDD and hypoxia. After a 6 hour exposure, neither a TCDD mediated increase nor a TCDD/hypoxia mediated decrease in PHD2 amounts could be correlated with HIF-1 α stabilisation, indicating that PHD2 is not the target for the TCDD mediated decrease in HIF-1 α stabilisation.

OB7

INTERACTIONS OF PEPTIDES WITH SINGLE-WALLED CARBON NANOTUBES AND ITS APPLICATIONS.

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Peptides selected from phage displayed libraries have been found to exhibit high-affinity binding to carbon nanotubes including single-walled carbon nanotubes (SWNTs), multi-walled carbon nanotubes (MWNTs) and single-walled carbon nanohorns (SWNHs). One unique feature of these peptides is that their amino acid sequences are rich in tryptophan and histidine residues. The aim of this study was to investigate the importance of tryptophan residue in a newly identified SWNT-binding peptide, UW-1, which contains the motif, XTHXXPWTX, where X is any amino acid. Tryptophan was altered in the following ways: mutation to alanine or substitution with three unnatural tryptophan analogues, i.e. 5-fluorotryptophan (5-FW), 5-hydroxytryptophan (5-OHW) and 7-azatryptophan (7-AZ). Analysis of experimental and computational data suggest that the highest occupied molecular orbital (HOMO) of the tryptophan residue in the peptide interacts with the lowest unoccupied molecular orbital (LUMO) from the SWNT. The application of the current results to cell imaging and protein-protein interaction assays will be also demonstrated.

Nanomedicine

N01

E. COLI AS INTELLIGENT DRUG DELIVERY MACHINES: POSSIBILITIES AND PERSPECTIVES.

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Engineered organisms need to interact with their environment in a programmable fashion in order to perform useful functions. We are interested in developing a “designer E. coli” strain that has the ability to bind to non-native target surfaces, sense the binding response and respond by emitting fluorescence. Our primary objective is to modify the extracellular domains of two selected E. coli proteins to enable the adhesion of transgenic cells to synthetic surfaces. The subsequent part of this study is aimed at identifying genes that are differentially regulated in response to adhesion and design reporters based on this data. FimH is an adhesin found at the distal tip of Type 1 fimbriae (long thread-like surface organelles which extend from the outer membrane) and mediates adhesion to mannosylated residues found on the surface of target epithelial cells. E. coli strains bearing a deletion of the regulatory fimE gene are preferably found in hyper-fimbriated state while only 5 % of cells in a wild-type heterogeneous population are fimbriated. We inserted HIS tags consisting of six or twelve histidine residues at the amino terminus, I52, Y137 or Q224 residues of the fimH gene. When expression was induced from these constructs in a fimE null background, the cells robustly bound to Nickel-NTA agarose beads. Similar constructs bearing purported streptavidin-tags in the same locations did not, however, confer binding to streptavidin-agarose beads. The outer membrane protein (OmpA) is present upto 500,000 copies throughout the outer membrane of the cell. We inserted HIS tags, similar to those above, at E49 of OmpA which conferred adhesion under ambient conditions to transformed cells. However, we were not able to replicate previously reported binding of cells containing OmpA modified with a streptavidin-binding tag. We then used microarray analysis to compare changes in expression levels of genes whose expression is dependent primarily on their adhesion to an external substrate. Analysis demonstrated a number of genes consistently upregulated by a factor of more than two in the adherent cells as compared to the non-adherent ones. These were found to be mainly present in stress response pathways, members of the multiple antibiotic resistance operon and several genes with unknown function. Based on these findings, we have designed Green Fluorescent Protein (GFP) reporters to test their functionality as synthetic adhesion sensors and will discuss their effectiveness. Substitution of GFP with a pharmaceutically relevant protein could result in E.coli capable

of delivering these molecules upon binding to cells in desired tissue and prove to be an intelligent way of targeted drug delivery.

N02

FLUORESCENT NANOPARTICLES FOR NEUROIMAGING.

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Nanoparticles have been extensively investigated for biomedical purposes in the past decade, and are promising as drug delivery systems and neuroimaging tools. Highly luminescent nanoparticles, such as quantum dots (QDs), have excellent physicochemical properties and are potential alternatives to traditional fluorescent dyes for live single cell and whole animal imaging. Neuroimaging using multifunctional QDs is especially attractive; however, their wide use has been limited by questionable QD stability and potential hazards due to their tissue sequestration. It has also been well established that noxious QDs induce cell death by activating oxidative stress pathways. The aim of this study is to investigate how different types of QD surface modifications, with antioxidants (e.g. N-acetylcysteine, NAC; lipoic acid) and biopolymers (e.g. polyethylene glycol), can improve QD biocompatibility in neural cultures. We show that QD toxicity was prevented by surface coating with biopolymers. We also show that antioxidants provide bimodal protection to counter QD cytotoxicity. Firstly, QD surface conjugation or capping with NAC enhances stability by altering QD physical properties. Secondly, cell preconditioning with NAC can reduce cell susceptibility to QD-insult. In conclusion, QDs coated with biopolymers or cytoprotective drugs and functionalized for targeted delivery, are potentially the leading choice as neuro-diagnostic tools, especially suited for repeated neuroimaging in real-time.

N03

QUANTUM DOT DISTRIBUTION IN BLOOD VESSELS OF THE CHICKEN EMBRYO CHORIOALLANTOIC MEMBRANE.

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Quantum Dot (QD) nanoparticles are currently being applied in a variety of biological environments, mostly for optical imaging. Their unique properties (size-tunable, narrow emission spectra; broad absorption spectra; capacity for surface functionalization, and high photostability compared to organic fluorophores) make them valuable

biological probes; QDs continue to be developed as diagnostic and imaging tools despite insufficient understanding of their physical interactions with biological tissue. The focus of our study is vascular tissue, since nanoparticles are commonly administered by intravenous injection for biological studies. We investigated QD behavior in blood vessels using the chorioallantoic membrane (CAM) of the chicken embryo as a model system. The femtolitre excitation volume achieved by two-photon excitation (TPE) fluorescence correlation spectroscopy (FCS) can be used to measure the diffusion and concentration of QDs within blood vessels as small as 10 μm in diameter. Streptavidin, biotin, aminoPEG, carboxylate and methoxy functionalized QDs were all examined in this work. We have determined that these functionalized nanoparticles do not bind to blood serum proteins. Moreover, using TPE-FCS we have observed localization of QDs to blood vessel walls and measured uptake rates from the blood.

N04

EFFECT OF THE NANOSCALE LIPID ARRANGEMENTS ON THE FUNCTION OF LUNG SURFACTANT.

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Scanning probe microscopy is used to image surfaces at the nanoscale and single molecule level, as well as to investigate physical properties of the sample surface using a technique known as force spectroscopy. We review our recent collaborative efforts studying structure and functions of molecular films of pulmonary surfactant BLES. We visualized the structural changes produced by lateral compression of the films and incorporation of cholesterol excess with atomic forces microscopy (AFM). Cholesterol at elevated concentrations produces changes in molecular arrangements of the surfactant films observed by AFM. These changes correlate with changes in adhesion and surface potential, observed with atomic force measurements and via imaging of potential distribution map with novel frequency modulated Kelvin probe force microscopy (FM-KPFM). Therefore the molecular mechanism of cholesterol induced surfactant failure includes distinguished changes in the film molecular architecture and physical properties of the lipid nanostructure within the film.

N05

RGD ROSETTE NANOTUBES INDUCES MAPKINASE SIGNALING CASCADE IN HUMAN AIRWAY EPITHELIAL CELLS.

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Integrin-targeted nanoparticles for site-specific delivery of a therapeutic drug open up an entire new era in nanotechnology. The Arg-Gly-Asp (RGD) peptide sequence is known to specifically bind to the $\alpha\text{v}\beta\text{3}$ integrin and induce apoptosis. In the present investigation, Calu-3, a human airway epithelial cell line was used to study the effect of RGD-rosette nanotubes (RGD-RN) to induce apoptosis and to understand if MAPKinase cascade is triggered by RGD-RN. Calu3 cells were grown in 25 cm^2 flasks and incubated at 37°C and 5% CO_2 . Seventy per cent confluent cultures were trypsinized and cells were transferred to 2 ml sterile tubes for treatments. Cells (1×10^7 cells / ml) were incubated for 2 h at 37°C and 5% CO_2 prior to RGD-RN (100 $\mu\text{g}/\text{ml}$) exposure for 2 to 60 minutes. Western blot analysis showed rapid increase in the phosphorylation of P38 and P42/44 kinases. Levels of phosphorylated P38 and P42/44 kinases were highest in 15 and 10 minutes, respectively. Extra cellular regulated kinases (ERK) may regulate apoptosis and cell survival at multiple points including caspase 3 regulation. Experiments are in progress to understand if RGD-RN are able to induce apoptosis in Calu3 cells and to understand if MAPKinase cascade is involved in regulation of signal for RGD-RN – induced apoptosis.

N06

BIOCHEMISTRY IN BACTERIOFERRITIN.

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Bacterioferritin (MW24 = 444) is an iron storage protein that consists of 24 subunits forming a spherical cage-like structure. The C-terminus of each subunit points towards the inside of the cavity, while the N-terminus is exposed on the surface of the protein. Bacterioferritin was used as a model for the study of host-guest interactions and guest encapsulation. A hexahistidine-affinity tag fused to the C-terminus of each bacterioferritin subunit (host) was constructed. This affinity tag was able to form strong interactions with a nickel-nitrilotriacetic acid (Ni^{2+} -NTA)

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linked dye molecule (guest). This interaction was used to develop conditions for the control of guest molecule encapsulation within the spherical cavity of the 24-subunit bacterioferritin protein molecule. Successful encapsulation was examined by laser light scattering, gel filtration chromatography and fluorescent measurements. These designed nanosized biomaterials should find future application in the areas of targeted nano delivery systems and imaging materials.

N07

EVALUATION OF NANOPARTICLE TOXICITY USING RAINBOW TROUT CELLS IN CULTURE.

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The emerging and increasing use of nanoscale materials are causing growing concerns about their unintentional impact on human and animal health as well as on overall environmental health. The objective of the present study was to assess the toxicity profile of sample nanoparticles used in many industrial and biomedical applications on fish cell lines derived from tissues that are environmentally susceptible such as gills, liver, brain, spleen, representing epithelial barrier, metabolically active organs, neural tissue and immune cells, respectively. Nanoparticles of various sizes, shapes and composition, and at a range of relevant concentrations were tested using the trout cell lines derived from the previously indicated tissues. Cellular morphology, general metabolic activity, membrane integrity and lysosomal function were assessed under control (vehicle) and exposed conditions (2 to 72 h of exposure) using phase contrast microscopy and fluorescence assays. Results of the acute exposures and their relevance to fish and the environment will be discussed.

N08

SENSITIVITY MODULATION OF CARBON-NANOTUBE CHEMICAL SENSORS VIA QUANTUM DOT HETEROSTRUCTURES.

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Carbon nanotube-quantum dot heterostructures were formed using multiwalled carbon nanotubes and CdSe quantum dots, which were trapped as networks between electrodes via dielectrophoresis. Chemical exposure to 10ppm CuSO₄ displayed a size-dependent modulation of chemical sensitivity, with the heterostructures using 2.32nm quantum dots displaying a 12% resistance change, while those using 4.57nm quantum dots displayed a 20% change. Both represent a

decrease in sensitivity from amine-functionalized carbon nanotubes without quantum dots, which show a 47% resistance change. This drop is partly attributed to charge transfer between the nanotubes and the CdSe quantum dots. The demonstrated modulation of nanotube properties has potential applications in multiplexed chemical sensors.

N09

SCANNED PROBE MICROSCOPY CHARACTERIZATION OF FIBRONECTIN BINDING CONFORMATIONS ON SELF-ASSEMBLED MONOLAYER SURFACES.

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Scanned Probe Microscopy (SPM) is being developed to analyze the different conformations that fibronectin (FN) assumes upon adsorption onto hydrophilic and hydrophobic self-assembled monolayer (SAM) surfaces. Novel SPM methods being developed include single molecule force spectroscopy, immuno-AFM, and apertureless near-field scanning infrared microscopy (ANSIM). Single molecule force spectroscopy uses AFM as a force probe to study the mechanical properties of FN. Immuno-AFM uses monoclonal antibodies (Ab) and AFM to study FN binding conformations by observing the changes in height distribution of the sample due to the binding of Ab to FN in a desired conformation on the surface. ANSIM provides a means to investigate FN binding conformations in ambient conditions at a spatial resolution (ca 10 nm) far superior than that attainable by conventional infrared microscopy. In order to further improve the resolution of the instrument, new tips are being developed that utilize polarizable dielectric materials such as boron carbide and boron nitride in order to increase the tip polarizability. The high spatial resolution of the ANSIM technique makes it possible to locally characterize the secondary structure of FN deposited on surfaces. Our long term goal is to characterize how these conformations influence the protein's interaction with endothelial cells.

Metabolomics

M01

EFFECT OF HATHA YOGA ON DIURNAL CHANGES OF SALIVARY ADRENAL AND GONADAL HORMONES.

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Purpose: The purpose of this study was to evaluate the effect of hatha yoga on diurnal changes of adrenal and gonadal hormones. **Method:** 16 volunteers (over 40 year old females) who have menstruation period were collected and divided into two groups as control and experimental groups. The subjects for this study participated in a 16 week program of hatha yoga exercise three times per week for 60 mins with the collection of four saliva samples (20-30 minutes after waking, before noon (10-11 AM), before evening (3-4 PM) and before bed) from each group prior to practice, at 8 weeks, and at 16 weeks of practice. The cortisol, DHEA, progesterone and estradiol levels in the saliva were measured using radioimmunoassay. A paired t-test was performed for data analysis at the 0.05 level of significance. Statistical techniques for data analysis were 2*3*4 repeated and ANOVA was used to determine the difference between the hatha yoga exercise group and the control group. The 5% level of significance was utilized as the critical level for acceptance of the hypotheses for the study.

Results: The cortisol levels were decreased to within normal ranges for individuals with high ranges after 16 weeks of yoga practice. The abnormal diurnal pattern of DHEA was changed to a normal pattern after 16 weeks of hatha yoga practice. The ovarian steroid diurnal rhythm was also changed after 16 weeks of yoga practice. Some volunteers showed abnormal diurnal progesterone and estradiol rhythm prior to practice but post-awakening steroid levels were maintained higher than the 11AM levels after 16 weeks of yoga practice. **Conclusions:** Taken together, hatha yoga practice could modulate the sympathetic nervous system and be effective in the mitigation of acute stress but not chronic stress. The long-term practice of hatha yoga affects modulation of adrenal function on hyper or mildly hypo-adapted individuals. The modulation of the sympathetic nervous system by hatha yoga practice could have indirect effects on ovarian function to maintain a normal diurnal rhythm.

M02

MICROSOME-BASED METABOLITE IDENTIFICATION FOR EXPANDING THE HUMAN METABOLOME MS/MS DATABASE.

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One of the challenges associated with metabolome profiling in complex biological samples, such as human urine, is to identify new and unknown compounds. Typically, standards are used to help identify compounds in these complex mixtures, yet standards cannot be purchased or synthesized for the unknown. In this work we present the use of human liver microsomes (HLM) to metabolize known endogenous human metabolites (test compounds), producing potentially new endogenous human metabolites that have yet to be documented. The metabolites produced by HLM can be identified based on the associated test compounds, known metabolic processes, MS/MS fragmentation patterns and, if necessary, accurate mass measurements. Once identified, these metabolites can be used as reference for positive identification of these same compounds in complex biological samples. Currently a total of 18 potentially new human metabolites have been identified from HLM incubations using 12 different test compounds. Each of the 18 metabolites were treated as new previously undocumented endogenous human metabolites and used as standards, resulting in three different matches to compounds in a single human urine sample. Identification of these compounds showed that two have previously been documented as endogenous human metabolites and that the third compound has not been previously reported. This new compound can now be documented and added to the Human Metabolome Database (<http://www.hmdb.ca>), a publicly available database of endogenous human metabolites containing chemical and biological information on each compound.

M03

TEXT ANALYSIS IN THE HUMAN METABOLOME PROJECT.

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The Human Metabolome Project (HMP) attempts to identify, quantify, annotate and catalog all of the metabolites that can potentially be found in human tissues

and biofluids at concentrations greater than one micromolar. This data is freely accessible in an electronic format to all researchers through the Human Metabolome Database (www.hmdb.ca). To facilitate these processes, the HMP uses several Data Mining tools, including Biospider, Polysearch, and Metabolite Classification. Biospider allows users to type in almost any kind of biological or chemical identifier, including metabolite names, and it returns an in-depth synoptic report (~3-30 pages in length) about that biomolecule and any other biomolecule it may interact with. This information is gathered from many sources throughout the web. Biospider can be used in tandem with hand-curation to aid in the annotation of metabolites in the database. PolySearch allows users to perform multiple types of data/text mining searches, with a special emphasis on human disease, gene, drug, metabolite and mutation. PolySearch identifies and extracts metabolite information in biomedical text to aid the annotation process. In particular, this helps with the annotation of metabolites with respect to genes and proteins associated with them. Given a partial description of a metabolite, the Metabolite Classification system predicts the value of some of the certain unspecified attributes (such as chemical class), using classifiers that were trained on other better-described (typically hard curated) metabolites. Knowing the chemical categories for each metabolite makes the database more complete, and can in turn aid in further annotation of these compounds.

M04

PREDICTION OF DIETARY-INDUCED INSULIN RESISTANCE IN MICE BY TARGETED PROFILING OF MAGNETIC RESONANCE SPECTRA.

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Due in part to complex etiologies and the heterogeneous nature of the populations sampled, nuclear magnetic resonance (NMR) studies of diseases such as Type 2 diabetes have had mixed results¹. A proof of concept study was undertaken to elucidate potential biochemical factors in the development of insulin resistance and evaluate the validity of metabolic analysis on a small set of animals under highly controlled experimental conditions. ¹H nuclear magnetic resonance (NMR) spectra of serum from male, C57BL/6J mice on a control (n=10) or high fat diet (n=10) were acquired. Targeted profiling of fifty metabolites was performed on each. Supervised Partial Least Squares (OPLS-DA) analysis of the concentration matrix yielded significant one and two dimensional models, the loadings for which were a weighted list of metabolites changing with each diet. The most significant compounds were used to refine and investigate the involved segments of various pathways, including lipid

metabolism, branched chain amino acid (BCAA) signaling and energy regulation. Non-residual variability (R²) and cross validation predictive power (Q²) of the model were 85% and 65% respectively, sufficient to inspire confidence in the method on relatively small numbers of animals. In addition to the biochemical interpretation the model, metabolite concentrations were used in an investigation of different methods for quantifying spectra, and the effects on resulting models. Potential differences were established between significance and utility of models based on full, partial, binned, and profiled NMR spectra. Many of the unsuccessful studies of spectra made use of binning to quantify spectra, which our results show may have significantly mitigated the power of the models.

M05

METABOLOMICS AND THE HUMAN METABOLOME PROJECT.

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The primary objective of the Human Metabolome Project (HMP) is to identify and quantify all major (greater than 1 μ M) endogenous and exogenous metabolites found in the human body. The secondary objectives of the HMP are: 1) to develop a publicly available database, the Human Metabolome Database (HMDB), containing clinically relevant human metabolome data and 2) to develop a publicly available library, the Human Metabolome Library (HML), a repository containing physical samples of many human metabolites. Just as GenBank has served as a foundation to modern genomic research, the intent of the HMDB and HML is to serve as a foundation to future research in metabolomics, systems biology, and clinical chemistry. The determination of the human metabolome and the assembly of the HMDB involve both "backfilling" via computer-aided text mining and experimental measurements of appropriate clinical samples (CSF, blood, urine, and liver microsomes) using NMR, MS-MS, GC-MS, FT-MS, and HPLC techniques.

M06

FLOWING JUICES: AN INVESTIGATION OF URINE AND PLASMA METABOLITES.

David Duong Hau and David Wishart, Department of Computing Science, University of Alberta. **Source of Research Funds:** Genome Alberta, Genome Canada, CFI, ISRIP, University of Alberta, AICML, Chenomx Inc.

As the Human Metabolome Project draws to completion, the metabolite information generated by the project is now being harnessed by a new generation of endeavors. One such project involves the analysis of urine samples collected from a variety of cancer patients. Cancer patients often acquire cachexia, a condition characterized by extreme emaciation. By determining the metabolite levels within the urine samples, the goal is to determine biomarkers which correlate with observed changes in patient mass over time. Another project involves the metabolite analysis of plasma samples from heart transplant patients. Similar to the cachexia project, it is hoped that a biomarker(s) will be identified that will allow one to predict whether or not a patient will reject a transplanted heart.

M07

IDENTIFICATION AND QUANTIFICATION OF THE HUMAN CSF METABOLOME.

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Metabolomics involves the characterization of the small molecule metabolites found in an organism. Recent advances in the field have been spurred largely by the wide variety of potential applications, which range from toxicology to food research to disease screening. In particular, metabolomics projects relating to human health have generated a lot of interest, but the field is limited by the fact that the human metabolome has not been thoroughly characterized. The goal of the present research is to catalogue the metabolites found in human cerebral spinal fluid (CSF), thus providing a baseline for which compounds can normally be detected using standard techniques, as well as their concentration ranges. To this end we are applying both nuclear magnetic resonance (NMR) and gas chromatography/mass spectrometry (GC-MS) to identify and quantify endogenous metabolites. The data will be entered into the Human Metabolome Database (www.hmdb.ca) as a reference for future research.

M08

OPTIMIZATION OF SOLID PHASE EXTRACTION FOR METABOLOMICS APPLICATION.

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Solid phase extraction (SPE) has been proven a valuable tool for sample preparation by removing matrix interferences and concentrating analytes of interest. It is also effective at

reducing ion suppression in sample analysis by liquid chromatography/mass spectrometry (LC/MS). However, setbacks when using solid phase extraction are selecting the best type of sorbent and method development. This work focuses on the optimization of solid phase extraction for cleanup and isolation of several substrates and their metabolites. The analytes of interest were a range of acidic, basic and neutral compounds. Optimized methods were performed on Waters Oasis® HLB, MCX and MAX cartridges. Endogenous substrates were incubated using human liver microsomes for 0.5, 1, 3, 6 and 24 hours. The incubation mixtures were loaded onto the cartridges and the analytes were eluted with 100% methanol, water/methanol mixture, acidic and basic solutions. After evaporation of the eluate and reconstitution in LC mobile phase A (4% acetonitrile, 0.1% formic acid in water), LC/MS and LC/MS/MS analyses were done in positive and negative modes. Reproducible recoveries of greater than 80% were obtained using representative compounds at concentrations of 50 µM spiked into a pooled control incubation mixture. An increased number of metabolites was observed in the extracts of SPE than in the samples analyzed without SPE, enabling analysis of low concentrations of metabolites. A modified method using two-dimensional polar/non-polar and ion exchange cartridges was also performed, which was sensitive in extracting low level metabolites from the incubation mixture.

M09

QUANTITATIVE ANALYSIS OF METABOLITE CONCENTRATIONS IN HUMAN URINE SAMPLES USING ¹³C{¹H} NMR SPECTROSCOPY.

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Quantification of metabolite concentrations in metabolomics studies using targeted profiling of ¹H NMR spectra sometimes can be hampered due to the enormous complexity of ¹H NMR spectra of biofluids, such as urine. A number of the issues relating to ¹H NMR spectroscopy can be overcome using ¹³C{¹H} NMR spectroscopy, which produces simplified spectra. A ¹³C{¹H} NMR database was created using Chenomx NMR Suite®. The ¹³C{¹H} NMR database was standardized through the analysis of a series of metabolite solutions containing varying concentrations of 20 distinct metabolites, where the metabolite concentrations were maintained at the range of values to more accurately simulate biological conditions. The measured concentrations have a good linear correlation to the actual concentrations and all 20 metabolites demonstrate reasonable average percent errors with some being slightly under estimated

(negative values). NMR spectra of urine samples were collected using $^{13}\text{C}\{^1\text{H}\}$ NMR spectroscopy and profiled using the Chenomx NMR Suite and our $^{13}\text{C}\{^1\text{H}\}$ NMR library. More than 40 metabolites were conclusively identified and quantified in those urine samples using $^{13}\text{C}\{^1\text{H}\}$ NMR targeted profiling. Quantification of metabolite concentrations was compared with ^1H NMR targeted profiling results. The proton decoupling and larger spectral window provided easier identification and more accurate quantification for a number of metabolites, including sugars and amino acids with a high degree of overlap in their ^1H NMR spectra. As a result, we propose that $^{13}\text{C}\{^1\text{H}\}$ NMR targeted profiling can be complementary to ^1H NMR targeted profiling for specific applications.

M10

THE HUMAN METABOLOME LIBRARY.

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The Human Metabolome Library (HML) is a one-stop chemical resource to order/acquire one or more compounds to confirm, validate or quantify suspected metabolites in tissues or biofluids. The Human Metabolome Library has been developed through the efforts of the Human Metabolome Project. Users may order or request any reasonable quantity (1 mg – 10 g) for as many as 100 compounds at a time. Currently the HML has more than 800 compounds listed on the HML website, including purchased, synthesized, and purified compounds. Through the HML website, collaborators and commercial labs are able to request compounds through the HML's electronic order forms. Researchers may obtain metabolites from the HML in one of two ways. Scientists wishing to use these compounds for basic research purposes or in collaborative efforts with the HML are given access to the compounds as long as they acknowledge the Human Metabolome Project (and Genome Alberta/Genome Canada) through co-authorship or acknowledgements. Alternatively, metabolites may be purchased for a nominal fee.

M11

AUTOMATIC METABOLITE IDENTIFICATION IN BIOFLUIDS USING 2D-TOCSY SPECTROSCOPY.

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One of the greatest challenges facing metabolomics is to quickly and accurately identify and quantify metabolites in biological samples. Although one-dimensional proton nuclear magnetic resonance (NMR) spectra can be acquired quickly and analyzed using a variety of peak-fitting software tools, this process is difficult to automate due to severe peak overlap. Here we report our effort toward automatic metabolite identification from two-dimensional total correlation spectroscopy (TOCSY) experiments. Our results show that the performance of our automated method is comparable to human analysis. TOCSY data were collected from three databases: the Human Metabolome Database, the Biological Magnetic Resonance Data Bank, and the Magnetic Resonance Metabolomics Database. These data were manually curated to remove redundancy and inconsistency. In total, we have 249 compounds that cover the majority of known endogenous metabolites in human biofluids. This library was further classified into cerebral spinal fluid (CSF) metabolites (81), plasma metabolites (136), and urine metabolites (96), with concentration information from our experimental data and the literature. Since most metabolites are present at very low concentrations in biofluids, many coupled peaks will not be detectable. To address this problem, we developed a heuristic scoring algorithm based on the uniqueness of the peak, its relative height/intensity, and the abundance of the metabolite. This information is combined to create a "minimal signature peaks" for each compound. A software tool written in Java called TOCSYscape was implemented to enable peak processing, searching and annotation. Visual inspection and direct editing are supported at each step. The final result can be overlaid on the real TOCSY image for further refinement. Users can also easily build their own TOCSY library or edit our library to fit their experimental conditions. We tested our program using automatically picked peaks from TOCSY experiments on CSF, plasma and urine samples, both recall and precision were around 90% .

M12

METABOLOMIC PROFILE OF HUMAN URINE GENERATED BY FTICR-MS.

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Metabolic fingerprints reflect immediate physiological responses to the environmental or pathological changes in a living organism, thus providing a dynamic biological state of the organism, which could be used for many applications including disease diagnosis. Mass spectrometry can potentially be used for rapid metabolic fingerprinting due to high sensitivity and selectivity. The objective of our research is to generate a comprehensive metabolomic profile of

human urine that includes all metabolites identified, LC retention index, and MS and MS/MS spectra. In our experiment, a urine sample from a healthy person was collected and concentrated to 6 fold using a rotavapor. After centrifugation, the supernatant was filtered, aliquoted, and stored at -20°C until further analysis. Both reversed phase C18 (RP) and hydrophilic interaction chromatography (HILIC) column were used on HPLC and UPLC systems, which were combined with FTICR-MS. LCQ ion trap was also used for LC-MS/MS analysis. In a single LC-MS run, more than 100 chromatographic peaks were observed in the LC chromatogram. Many metabolites co-elute resulting in multiple metabolite detection within a single chromatographic peak. Most of the metabolites were found to be very polar and could be separated with an HILIC column better than with a C18 column. Negative ionization mode generated more peaks than the positive mode. The mass measurement errors, determined from the known metabolites in human urine, were found to be typically less than 2 ppm and in most case less than 1 ppm after recalibration with external standards. Accurate MS data and MS/MS data were used to search against the Human Metabolome Database (HMDB) for metabolite identification. In total, more than 200 entries were identified by combining the accurate mass measurement of the precursor ions and their MS/MS spectra. Some of previously unidentified metabolites include indoleacrylic acid, pantothenic acid, thymine, tropic acid, salicylic acid, etc. To conclude, more than 200 metabolites have been identified in human urine by LC-FTICR-MS and MS/MS.

M13

IMPROVING METHODOLOGY FOR MICROSOME-BASED METABOLITE IDENTIFICATION.

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L-Carnitine is capable of forming ester derivatives with fatty acids and is responsible for the transportation of several compounds through the mitochondrial membrane, therefore L-Carnitine and its acylcarnitine derivatives have a crucial role in maintaining cell viability. Due to its biological importance, this family of compounds was chosen as a model for the improvement of microsome-based metabolite identification. The incubation process of these endogenous compounds was optimized in order to maximize the number of metabolites formed. Acylcarnitines were incubated with either pooled human liver microsomes or a mixture of individual cDNA-expressed enzymes prepared using a baculovirus expression system, both purchased from BD Biosciences. Solid Phase Extraction was performed on these samples, followed by analysis using high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography

(UPLC) mass spectrometry, respectively. Mass spectrometry and tandem mass spectrometry were carried out using both ion trap and Fourier Transform mass spectrometers. Numerous metabolites of the different acylcarnitines were identified and confirmed by the methods described. Many of the possible structural isomers of these metabolites were able to be resolved and identified.

M14

HUMAN SERUM METABOLOMICS STUDIED BY ¹H-NMR.

Jun Peng, David Duong Hau, Jeff Xia, Mike Lewis, David Wishart, Department of Computing Science, University of Alberta. **Source of Research Funds:** Genome Alberta, Genome Canada, CFI, ISRIP, University of Alberta, AICML, Chenomx Inc.

Proton nuclear magnetic resonance spectroscopy (¹H-NMR) was employed for human serum metabolomics because it can readily provide quantitative information as well as qualitative information about small molecule metabolites. However, in order to get the reliable quantification results, it is desirable to remove the serum proteins. We developed the protocols for ultrafiltration (centrifugal filter molecular weight cut off 3K Da) and also for protein precipitation (methanol and acetonitrile). These protocols enabled the metabolic profiling of serum samples by ¹H-NMR and the quantification results using CHENOMX software are pretty consistent. The serum samples of heart-transplant patients and multiple sclerosis patients were analyzed by ¹H-NMR using ultrafiltration protocol and the quantitative results of serum metabolites will be very potentially useful for metabolic biomarker discovery of the diseases.

M15

MetaboLIMS: A GENERAL LABORATORY INFORMATION MANAGEMENT SYSTEM FOR METABOLOMICS.

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MetaboLIMS (<http://www.hmdb.ca/labm/>) is a web-based portable, robust, and scalable laboratory information management system designed to meet the production and clinical needs of laboratories involved in metabolomics research. It offers a wide variety of functions such as Compound and Sample Tracking, Electronic Notebook Entry, GANTT Charting, Instrument and Meeting Scheduling, Text Data Entry, Spectral Trace or Image Entry, Automated Analysis and Report Generation, Audit Trail Tracking, Relational Query Searches as well as NMR and

MS Spectral Searches. MetaboLIMS is also supplemented with a large database of ~1400 metabolites and unique set of search tools (called BioSpider and PolySearch) to facilitate metabolite annotation and identification. MetaboLIMS employs a customizable modular design, allowing users or administrators the ability to add or remove LIMS GUI components and lists as needed. MetaboLIMS uses a number of advanced web-building technologies, including HTML, CSS, XML, Javascript, Java Servlet and JSP, as well as MYSQL for its backend database engine. MetaboLIMS is freely available and can be easily installed as a local web server on any Windows and Unix operating platform, making it ideal for labs with limited budget.

M16

THE EFFECT OF VITAMIN E I.M INJECTIONS ON PFK ACTIVITY IN SLOW AND FAST-TWITCH MUSCLES OF CRITICALLY ILL RATS.

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Reactive oxygen species (ROS) and free radicals generated in oxidative stress have been implicated in tissue injury during critical illness. Free radicals are known to negatively affect the glycolytic cycle in vitro. It was hypothesized that intramuscular (i.m) injections of the antioxidant vitamin E would minimize oxidative damage to the rate-limiting enzyme of glycolysis, phospho-fructokinase (PFK) in the slow-twitch soleus but will offer no visible protection to the fast-twitch extensor digitorum longus(EDL) muscles of metabolically-stressed and malnourished rats. Forty-eight male Wistar rats were assigned to three groups (control, zymosan-stressed and pair-fed), fed a liquid diet (Osmolite-HN) and sacrificed on days 3 and 7. PFK activity was measured in EDL and soleus muscles using a fluorometric assay. **Results:** Rats lost 10-20% body weight over 3 days. PFK activity expressed as mmol of product/mg protein/sec was significantly lower in the soleus of zymosan-stressed rats compared to controls and pair-fed rats on day-3. The PFK activity in the soleus of vitamin-E treated rats was comparable to pair-fed rats and significantly higher than zymosan-stressed rats on day-3. In the EDL, PFK activity remained normal throughout the acute critical illness phase lasting 2 days in both malnourished pair-fed and zymosan-stressed rats. **Conclusions:** The findings suggest that glycolysis in the slow-twitch but not in fast-twitch muscle of metabolically stressed rats is adversely affected by oxidative stress and malnutrition, and that i.m vitamin E injections given over 3 days can provide some protection to slow-twitch muscles against oxidative damage to glycolytic integrity.

M17

THE INFLUENCE OF NICOTINE ON DMSO-INDUCED HUMAN LEUKEMIC PROMYELOCYTES AND SUBSEQUENT EFFECTOR FUNCTION.

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Neutrophils leave the bone marrow as terminally differentiated cells yet little is known of the influence of tobacco smoke on neutrophil differentiation and subsequent effector function. In this study, the effects of nicotine (10-7M to 10-4M), a specific component of tobacco smoke, on DMSO-induced neutrophilic differentiation of HL-60 cell was examined by assessing (a) cell growth kinetics, (b) cellular morphology and ultrastructure, (c) the expression of CD11b, (d) the ability to mount an oxidative burst, and (e) growth phase and apoptosis. The expression of nicotinic acetylcholine receptors (nAChRs) in HL-60 cells was identified using western blot and immunofluorescence. The influence of nicotine on effector functions of DMSO-stimulated HL-60 cells were also examined, i.e. respiratory burst and matrix metalloproteinase (MMP) secretion. The results showed that both promyelocytic and neutrophil-differentiated HL-60 cells expressed the $\alpha 7$ -nAChR subunit (55kDa). Expression of the $\alpha 7$ -nAChRs was up-regulated upon the addition of DMSO, irrespective of nicotine exposure. Nicotine exposure during differentiation suppressed the oxidative burst in HL-60 cells; inhibited bacterial killing; and increased the release of MMP-9, but not MMP-2, in an $\alpha 7$ -nAChR-dependent manner. Nicotine did not significantly elevate the percentage of DMSO-undifferentiated or -differentiated HL-60 cells committed to apoptosis. Nicotine increased the percentage of cells in late differentiation phases (metamyelocytes, band cells and segmented neutrophils) compared to DMSO alone ($p < 0.05$). Furthermore, there were no statistically significant differences in any other markers of neutrophil differentiation between DMSO-treated and control HL-60 cells (all $p > 0.05$). Thus, pharmacological doses of nicotine do not affect gross markers of neutrophil differentiation or induce commitment to apoptosis in the biochemical profiles of $\alpha 7$ -nAChR-expressing HL-60 cells. Exposure to nicotine during cellular differentiation alters effector function in neutrophils which may partially explain the increase in susceptibility of tobacco smokers to bacterial infection.