X-HiDE presents

Inflammation in focus

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Abstract collection

Exposure to environmental contaminants associated with alteration of metabolite profiles in immune-mediated diseases

Bagavathy Shanmugam Karthikeyan[1),2)], Tuulia Hyötyläinen[1)], Tannaz Ghaffarzadegan[1)], Eric Triplett[3)], Matej Orešič[2),4)], Johnny Ludvigsson[5)]

1)School of Science and Technology, Örebro University, Sweden.
2)School of Medical Sciences, Örebro University, Sweden.
3)Department of Microbiology and Cell Science, Institute of Food and Agricultural Sciences University of Florida, USA.
4)Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, Finland.
5)Crown Princess Victoria's Children's Hospital, Region Östergötland, Division of Pediatrics, Linköping University, Sweden.

Background/Objective: Growing evidence suggests that prenatal exposures to environmental contaminants interfere with metabolism, leading to several adverse health effects in children`s early life and later. Recent studies show genetic predisposition, environmental factors and their interactions play a significant role in the etiology of autoimmune diseases. Herein, we hypothesized that exposure to environmental contaminants might interfere with cord serum metabolomes of a general population cohort (All Babies In Southeast Sweden, ABIS), thus may contribute to the development of autoimmune diseases.

Method: ABIS cohort comprises children who later progressed to one or more autoimmune diseases such as Type 1 diabetes, Crohn's disease, Celiac disease, Hypothyroidism and Juvenile Idiopathic Arthritis (cases, N = 62) along with matched controls (N = 268). Cord serum levels of metabolites and contaminants were determined by ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) followed by statistics and data analysis.

Result: Statistical analysis showed significant differences in the concentration levels of contaminants between control and cases. Subsequent analysis showed alteration of cord serum metabolomic signatures associated with levels of contaminants exposure. Specifically, Triglycerides of lipidome, amino acids and high polar compounds of polar metabolites showed significant fold changes between cases and controls at the specific disease groups. Further we developed penalized (ridge) regression models and pathway analysis helps to identify top linear predictors and common perturbed metabolic pathways, respectively.

Conclusion: Our study suggests that cord serum metabolome is altered by exposure to environmental contaminants in the ABIS cohort.

Cardioprotective, antithrombotic and anti-inflammatory piperazinyl-purine analogue is a bifunctional drugs with promising therapeutic potential

Magnus Grenegård[1)], Theano Fotopoulou[2)], Safia Ansari[1)], Maria Zervou[2)], Josef Jakobsson[1)], Dimitra Pournara[2)], Eleni Ifanti[2)], Eftichia Kritsi[2)], Madelene Lindkvist[1)], Sofia Ramström[1)], Kristofer Nilsson[1)], Maria Koufaki[2)*], Karin H Fransén[1)*]

School of Medical Sciences, Cardiovascular Research Centre Örebro University, Sweden[1)]
Institute of Chemical Biology, National, Hellenic Research Foundation, Athens, Greece[2)]

Background/Objective: Nitrate ester bearing 6-piperazinyl-purine analogues (denoted MK drugs) are cardioprotective in infarction animal models and act as inhibitors of Janus kinase (JAK) and Rho-associated kinase (ROCK). Despite the presence of a nitrate ester moiety, the MK drugs do not release nitric oxide (NO). To further improve therapeutic potential, we synthesized a derivative with two nitrate ester moieties and characterized this drug (MK177) in a number of experimental models.

Method: This translational research project convers number of methods in medical chemistry, biomedicine, and medicine. More specifically we utilize organic chemistry platforms to synthesis, purify and characterize MK177, *in silico* analysis of binding interactions to molecular targets, cell-free and cellular assays to elucidate anti-thrombotic and anti-inflammatory activities, tissue models to analyze vasodilation, and animal models to evaluate drug activities *in vivo*.

Result: In anesthetized pigs, intravenous infusion of the novel dinitrate ester MK177 produced "nitroglycerin-like" effects on vital parameters (*e.g.* reduction of arterial blood pressure). Analysis of exhaled air confirmed release of NO. MK177 caused dose-dependent relaxation of iliac arteries and this effect was mediated by activation of the NO/cyclic GMP signaling pathway in vascular smooth muscle cells. It is noteworthy that other members of the MK drug family (which are mononitrate esters) did not cause NO-induced vasodilation. In cellular model systems, MK 177 evoked anti-thrombotic effects by targeting ROCK, and this molecular mechanism was not dependent on NO-release.

Conclusion: We have successfully developed a bifunctional drug molecule that act by NO-dependent and NO-independent mechanisms. MK 177, in common with nitroglycerin, induced NO effects *in vivo* and relaxed vessels *in vitro*. Opposite to nitroglycerine, MK177 also prevented blood platelet activation via ROCK inhibition. Blood platelets are key players in initiating arterial thrombosis and highly sensitive to NO. However, it is well known that platelets cannot metabolize clinically used nitrate esters, like nitroglycerin, to yield free NO molecules. The bifunctional nature of MK177 caused NO-independent suppression of platelet reactive and that activity can be of main importance in thrombotic disease. Collectively, the novel cardioprotective and bifunctional drug MK177 has promising therapeutic potential.

Inflammatory markers as a measure of hazardous occupational particle exposure – Examples from the iron foundry environment

Alexander Hedbrant

1) School of Medical Sciences/Faculty of Medicine and Health, Örebro University

2) Inflammatory Response and Infection Susceptibility Centre (iRiSC)

Background/Objective: Particulate matter (PM) exposure is a serious health issue, with ambient PM exposure estimated to cause millions of deaths per year worldwide. Although PM exposure can be much higher in occupational settings compared to ambient exposures, the health hazards associated with occupational exposure is generally not as clearly defined. Therefore, we have examined the relationship between particle exposure and different inflammatory readouts in iron foundry workers, to determine if current exposure levels trigger low-grade inflammation, and possibly could contribute to the development of disease. The diseases associated with PM exposure, such as cardiovascular diseases and chronic obstructive pulmonary disease, have a clear inflammatory aetiology, and biomarkers of inflammation could therefore be a useful tool to assess the lowest observed adverse effects level (LOAEL) of PM exposure, and to help elucidate the inflammatory mechanisms triggered by different PM.

Method: We have measured personal PM exposures, including respirable dust (< 5µm aerodynamic diameter) and quartz, in two Swedish iron foundry cohort, including 40 and 85 foundry workers, respectively, and determined the workers inflammatory status with different methods, including plasma levels of inflammatory mediators, white blood cell counts and NLRP3 activation in monocytes.

Result: Out of all inflammatory markers measured, the NLRP3 inflammasome measures were consistently correlated with exposure in both studies. NLRP3 inflammasome markers correlated with quartz and/or respirable dust included IL-18 and IL-1Ra in plasma, and caspase-1 activation in monocytes.

Conclusion: Quartz is a known activator of the NLRP3 inflammasome, and our results indicate that current quartz-containing dust exposures in the iron foundry environment is sufficient to affect NLRP3 inflammasome activity in exposed workers.

Immunopsychiatry from a transdiagnostic perspective - the immunometabolic interplay

Ulrika Hylén[1,2,3], Samira Salihovic[2,3], Tuulia Hyötyläinen[4], Mats Humble[2], Susanne Bejerot[1,2], Daniel Eklund[2,3], and Eva Särndahl[2,3]

1) University Health Care Research Center, Faculty of Medicine and Health, Örebro University, Sweden

2) School of Medical Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden.

3) Inflammatory Response and Infection Susceptibility Centre, (iRiSC), Faculty of Medicine and Health, Örebro University, Örebro, Sweden.

4) Man-Technology-Environment Research Center, School of Science and Technology, Örebro University, Sweden

Background/Objective: Mental disorders are common and significantly impact the quality of life. Inflammatory processes are proposed to contribute to the emergence of mental disorders. In addition to inflammation, disturbances in metabolic pathways have been seen in individuals with different mental disorders. At the crossroad between inflammation and metabolism stands the Nod-like receptor 3 (NLRP3) inflammasome, which is an intracellular protein complex responsible for cleaving members of the IL-1 family to their active form. The overall aim of the project is to understand the interplay between metabolism and inflammation in a transdiagnostic cohort of individuals with severe mental disorders.

Method: Patients with severe mental disorder (n=39) and age and sex-matched healthy controls (n=39) were included in the studies. Psychiatric diagnoses, comorbidities, severity, and functioning was measured using a number of validated assessment scales. Biological parameters, such as circulating immune markers, gene expression, and metabolites were analyzed using electrochemiluminescent immunoassay, qPCR, and UHPLC-MSMS, respectively.

Result: The results reveal that in individuals with mental disorders, immune cells were primed in regard to the NLRP3 inflammasome with elevated inflammasome-related cytokine levels, regardless of diagnosis. In addition, positive metabolic inflammasome regulators, such as lactic acid, serine, and glutamine were significantly higher in the patients and the main metabolic pathways that were affected included arginine and proline metabolism and tryptophan metabolism. A number of these parameters could also correlate with the disease severity of the patients. Lastly, the patients as a group displayed transdiagnostic changes in immune-lipid pathways. In particular, strong associations could be observed between two triglyceride ether, with the inflammatory markers OPN and IL-1Ra.

Conclusion: Severe mental disorders are associated with changes in the inflammasome system and its corresponding cytokines as well as metabolic dysregulation. The data indicate that, while these systems are known to be associated, their interplay seems limited to relatively few inflammatory mediators and metabolites in patient group. Lastly, while large overlaps were seen between different primary diagnoses, identification of unifying, transdiagnostic patterns of inflammatory and metabolic dysregulation were weak and needs further studies in a larger cohort.

Serum from hibernating brown bears enhances human fibroblast metabolism and proliferation *in vitro*

Ylva Falck[1], Helena Isaksson[1,2], Jon Arnemo[3,4], Ole Fröbert[1,5,6,7], Mikael Ivarsson[1]

[1]Faculty of Medicine and Health, Örebro University, Sweden
[2]Laboratory Medicine, Dept. of Pathology and Genetics, Örebro University Hospital, Sweden.
[3]Department of Forestry and Wildlife Management, Inland Norway University of Applied Sciences, Koppang, Norway
[4]Department of Wildlife, Fish and Environmental Studies, Swedish University of Agricultural Sciences, Umeå, Sweden
[5]Department of Clinical Medicine, Aarhus University Hospital, Aarhus, Denmark
[6]Department of Clinical Pharmacology, Aarhus University Hospital, Denmark
[7]Steno Diabetes Center Aarhus, Aarhus University Hospital, Denmark

Background/Objective:

Impaired wound healing is a major clinical problem for which there are limited treatment options and novel therapeutics are warranted. Hibernating bears endure physical inactivity under hypothermic conditions for half a year without compromising organ function, including wound healing capacity. We hypothesized that serum from hibernating brown bears (*Ursus arctos*) may contain factors that alter or mirror wound healing parameters that could be tested *in vitro*.

Methods:

Serum from four hibernating bears was added to fibroblasts in monolayer cultures or collagen gels. Serum from the same bears in summer active state served as controls. Metabolism of cells grown on plastic was analysed by monitoring reduction of resazurin to resorufin (CellTiter-Blue® assay). Proliferation was measured by trypsinizing and counting cells. Migration was analysed by introducing a scratch in monolayers of fibroblasts, and monitoring repopulation of denuded areas. Contraction of fibroblast-populated collagen gels was analysed by measuring gel diameter.

Results:

10% serum from hibernating bears increased fibroblast metabolism with 46+/-34% after 24 h, 50+/-26% after 48h and 168+/-67% after 6 days compared to summer serum. The same serum concentration from hibernating bears increased cell proliferation with a later onset: 9,3+/-16% after 24h, 11+/-13% after 48h, and 211+/-33% after 6 days. Addition of 10% winter serum did not alter migration or collagen gel contraction significantly compared to summer serum.

Conclusions:

The results suggests that serum from hibernating bears contains factors that stimulate metabolism and proliferation in fibroblasts, which may contribute to altered tissue dynamics during physical inactivity. Further studies are warranted to examine the exact factor(s) contributing to this. Since fibroblasts are important regulators of innate immunity and inflammation, it is also of interest to elucidate how hibernating bear serum may alter pathways in these processes. Such data may also provide new insights in tissue resilience that could be translated to humans.

Developing a novel tick-borne encephalitis vaccine against av virus on the rise

Anna Valkó[1)], Naveed Asghar[1)], Karl Ljunberg[2)], Wessam Melik[1)], Magnus Johansson[1)], Developvaccines@oru [1, 2, 3)]

1) School of Medical Science, Örebro University, Örebro, Sweden
2) Eurocine Vaccines AB, Solna, Sweden
3) Academy of Quality in Pharm Science AB (AQPS), Södertälje, Sweden; Adlego Biomedical AB, Solna, Sweden; GU Ventures AB, Göteborg, Sweden; Karolinska Cell Therapy Center (KCC/ Vecura), Huddinge, Sweden; Karolinska Institutet, Department of Laboratory Medicine, Huddinge, Sweden; MIVAC Development AB, Göteborg, Sweden; Nordic BioAnalysis AB (NBAB), Södertälje, Sweden; Probi AB, Lund, Sweden; Svenska Vaccinfabriken produktion AB (SVF), Sollentuna, Sweden; Valneva SE, Solna, Sweden

Tick-borne encephalitis (TBE) is one of the most important tick-transmitted diseases in Europe and Asia. Most infections with the TBE virus (TBEV) are asymptomatic or cause mild flu-like symptoms, but they may induce severe neurological disorders with permanent sequelae. The incidence of TBE cases showed a remarkable elevation in recent years probably due to the geographic expansion of TBEV and its vectors, which is concerning in the absence of a specific antiviral treatment. Vaccination remains the best protective measure against TBE. However, currently available vaccines have a burdensome immunization schedule, and poor immunogenicity in the elderly, which may contribute to observed vaccine failures, i.e. TBE occurrence in vaccinated people.

One aim within the Developvaccines@oru project is to develop a novel TBE vaccine that could provide improved immunogenicity using fewer doses. Our strategy is to induce an immune response already at possible virus infection sites by either a modified live attenuated vaccine based on Langat virus, or a subunit vaccine derived from the TBEV strain Torö-2003 identified in Sweden.

We have successfully created infectious clones of Langat virus based on the strain TP21. The strategies to create these infectious clones can be used to construct the modified live attenuated vaccine candidates, which will be compared to the “original” strain using cell based and animal models. The subunit vaccine components were selected and cloned for propagation and maintenance in a standard *E. coli* strain. After sequence confirmation, pure plasmids from these bacteria will be used to transform another *E. coli* strain for protein expression under the T7 promoter. To establish a baseline for animal experiments with our vaccine candidates, we planned a pilot study using the “original” Langat virus. First, we conducted a pre-pilot experiment to optimize the study design and evaluation methods.

Preliminary data on the establishment of vaccine candidates *in vitro* and the development for *in vivo* models are presented.

Building the knowledge base to understand cellular signal transduction in different inflammatory phenotypes

Marcus Krantz, Robert Kruse, Eva Särndahl, Katarina Persson, on behalf of the X-HiDe consortium

School of Medical Sciences and Inflammatory Response and Infection Susceptibility Centre (iRiSC), Faculty of Medicine and Health, Örebro University, Örebro, Sweden

Background/Objective: Within the X-HiDE project, we aim to understand the establishment and resolution of inflammation, and how different states of the underlying signal transduction network results in different inflammatory phenotypes. However, the biochemistry of this signal transduction network is notoriously complex: Each component may be regulated by multiple modifications and interaction partners, which can be combined in a large number of different configurations. Furthermore, single inputs trigger multiple downstream signalling processes, which each may be triggered or antagonised by multiple inputs. Finally, the function of the signal transduction system differs between individuals and cell types, depending on genetic variation and gene expression differences. Consequently, a useful knowledge base must be comprehensive, to account for all those interacting processes, as well as mechanistically detailed, to account for allele and expression differences as well as the impact of drug treatments.

Method: Using the rxncon modelling language and toolbox, we collect and encode empirical knowledge on the mechanisms and causalities in signal transduction, and assemble this information into a knowledge base that defines an executable network model.

Result: Here, we present a literature based mechanistic model of the network recognising infection, from the recognition of pathogen-associated molecular patterns by the toll-like receptors to activation of NF-kappa-B and IRF3/IRF7 mediated transcription. By using rxncon, the reaction-contingency language, we avoid the combinatorial complexity associated with microstate-based formalisms, and hence we can – in contrast to previous efforts – integrate all processes into a single network that defines a unique logical model that can be executed without further parametrisation. While limited to qualitative predictions, it provides a powerful tool for network validation and genotype-to-phenotype analysis.

Conclusion: Taken together, we present an approach that reconciles mechanistic detail and scalability in signal transduction modelling, opening the door to comprehensive – in scope and detail – models of the regulatory network in health and disease.

POSTER 8

Distinct gene dysregulation patterns along with multilevel omics integration unravel key interferon, STAT1, PLK1, B and plasma cell signatures in systemic lupus erythematosus, and invigorate their druggability potential to ameliorate disease

Julius Lindblom[1)], Daniel Toro-Domínguez[2)], Elena Carnero-Montoro[2)], Maria Orietta Borghi[3)], Jessica Castillo[4)], Yvonne Enman[1)], PRECISESADS Clinical Consortium, Chandra Mohan[4)], Marta E. Alarcón-Riquelme[2,5)], Guillermo Barturen[2)], Ioannis Parodis[1,6)]

1) Division of Rheumatology, Department of Medicine Solna, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden

2) GENYO, Centre for Genomics and Oncological Research: Pfizer, University of Granada / Andalusian Regional Government, Granada, Spain, Medical Genomics, Granada, Spain

3) Department of Clinical Sciences and Community Health, Università degli Studi di Milano and Istituto Auxologico Italiano, Cusano Milanino Mi, Italy

4) Department of Biomedical Engineering, University of Houston, Houston, TX, USA

5) Institute of Environmental Medicine, Karolinska Institute, Stockholm, Sweden

6) Department of Rheumatology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

Background/Objective: We aimed at investigating whole-blood transcriptome, expression quantitative trait loci (eQTLs), and levels of selected serological markers in patients with systemic lupus erythematosus (SLE) versus healthy controls (HC) and other autoimmune diseases to gain insights into pathogenesis and identify drug targets.

Method: We analysed differentially expressed genes (DEGs) and dysregulated gene modules in a cohort of 350 SLE patients and 497 HC from the European PRECISESADS project (NTC02890121), split into a discovery (60%) and a replication (40%) set. Replicated DEGs qualified for eQTL, pathway enrichment, regulatory network, and druggability analysis.

Result: Analysis of 521 validated DEGs identified multiple interferon signalling pathways among enriched Reactome pathways. Three distinct gene module clusters were defined i.e., “interferon/plasma cells”, “inflammation”, and “lymphocyte signalling”; predominant upregulation of the interferon/plasma cells cluster denoted articular, haematological, and serological activity. Druggability analysis revealed several potential drugs interfering with dysregulated genes within the “interferon” and “PLK1 signalling events” modules. STAT1 was identified as the chief regulator in the most enriched signalling molecule network. Drugs annotated to 15 DEGs associated with cis-eQTLs included bortezomib for its ability to modulate CTSL activity. Belimumab was among drugs annotated to three replicated DEGs whose upregulation overlapped with elevated serum levels of their encoded proteins in SLE patients compared with HC. Daratumumab was annotated to CD38 among the remaining replicated DEGs.

Conclusion: Modulation of interferon, STAT1, PLK1, B and plasma cell signatures showed promise as viable approaches to treat SLE, pointing to their importance in SLE pathogenesis.

Human Stem Cell-Derived Organoids: A Platform for the Study of Inflammaging and Beyond

Aidan McGlinchey[1]

1) Institute for Medical Research, Örebro University, Örebro, Sweden

Overview:

Chronic, low-level inflammation is one of the key drivers and distinctive hallmarks of the ageing process in humans and in models thereof. Inflammaging, from the ever-increasing prevalence of pro-inflammatory senescent cells, to dysregulated and damaging oxidative metabolism, disrupted insulin signalling, ever-decreasing activity and efficiency of intracellular micro and macro-autophagic (recycling) machinery, destructive immune dysfunction, to overall progressive loss of homeostatic capability and repair/regenerative mechanisms, inflammation plays numerous interconnected roles in the degenerative processes of ageing.

A key issue facing the basic science of ageing / healthy ageing / ageing reversal fields of biological science is the availability of suitable models in which to study these key inflammatory processes which are proving to be of critical importance. Performing studies in humans clearly faces issues regarding the complexity of biological sample acquisition, ethical boundaries, reporting time, as well as a plethora of confounding factors (diet, lifestyle, compliance, medication, survey reporting, to name only a few). Animal models and standard 2D culture methods face their own range of disadvantages when attempting to perform larger-throughput basic science / manipulation and screening studies. Organoids, however, answer the needs of increased physiological relevance, repeatability, standardisation and scalability necessary for this type of study.

Recently, approval and funding has been awarded from the X-HiDE group for the startup of an organoid cell culture lab here at the Institute for Medical Research at Örebro University, to build a platform for construction and interrogation of human organoid models of inflammaging. This laboratory will use human, induced pluripotent stem cell-derived material for the generation of organoids, starting with liver organoids. This will be carried out in conjunction with the expertise and materials of two commercial entities, specialising in metabolic measurement instrumentation and in stem cell derivation and maintenance.

However, the organoid platform is not intended to be used solely for the singular case of the study of inflammaging, and, once the initial liver organoids are established and best practices formulated (after approximately 1-1.5 years), the platform will go on to act as a hub and base for collaborative efforts concerning other studies in other diseases and cell types, already in conjunction with several other groups at Örebro.

This presentation will detail the ideas behind, current practical progress of, and future milestones / direction of the new organoid platform, both in the context of the inflammaging model already proposed, as well as in the wider context of its future use within X-HiDE, but also with other interests and groups such as systems medicine, metabolomics, immune/cancer biology and brain-gut-diet disease research.

Mathematical modeling of cytokine interplay in human monocytes during LPS stimulation

Niloofar Nikaein* [1)], Kedeye Tuerxun* [1,2)], Daniel Eklund† [1,2)], Alexander Persson†[1,2)], Robert Kruse [2)], Eva Särndahl [1,2)], Eewa Nånberg [1,2)], Antje Thonig [1,2)], Gunnar Cedersund† [3,4)], Elin Nyman† [3)], Dirk Repsilber†[1)], on behalf of the X-HiDE Consortium

1) School of Medical Sciences, Faculty of Medicine and Health, Örebro University, SE-701 82 Örebro, Sweden 2) Inflammatory Response and Infection Susceptibility Centre (iRiSC), Faculty of Medicine and Health, Örebro University, SE-701 82 Örebro, Sweden 3) Department of Biomedical engineering, Linköping University, Linköping, Sweden 4) Center for Medical Image Science and Visualization (CMIV), Linköping University, Linköping, Sweden

* = Equally shared authorship †= Equally shared supervision

Background/Objective: Inflammation is one of the vital mechanisms through which the immune system responds to harmful stimuli. During the course of inflammation, both anti- and pro- inflammatory signaling pathways are activated to regulate proper responses. The consequent immune responses are finely tuned by the interplay between the expressed anti- and pro-inflammatory cytokines. Imbalance in this interplay may result in immune disorders. However, this complex interplay is not yet fully understood. Here, we use a mathematical modeling approach to study the interplay between two prominent pro- and anti- inflammatory cytokines i.e., tumor necrosis factor (TNF) and interleukin 10 (IL-10), during the course of inflammation ex vivo, in human monocytes. These two cytokines are involved in the NFκB signaling pathway, a central pathway driving inflammation, which is used as the basis for our mathematical model.

Method: The system of non-linear Ordinary Differential Equations (ODEs) was trained and evaluated based on several biologically relevant scenarios, generated by a primary human monocyte cell culture model derived ex-vivo from several donors. The two scenarios, which were designed to generate training data sets, involved stimulating the human cell culture model with: 1) 10ng/mL of lipopolysaccharides (LPS), and 2) with both 10ng/mL of LPS and 100ng/mL of IL-10, at time point 0h. The validation data set was generated by stimulating the cell culture model with 10ng/mL of LPS at time point zero and 100ng/mL of IL-10, 4 hours after the administration of LPS. In each scenario, four cytokines (TNF, IL-10, IL1Ra, and IL1β) were measured at five timepoints.

Result: The proposed mathematical model successfully regenerates experimental data in all different scenarios and explains dynamics of TNF and IL-10 cytokines.

Conclusion: The in-silico model is a step towards understanding mechanisms governing inflammatory responses and can be used for designing test experiments to further expand the knowledge in the area. Future work will involve developing the model to a Non-Linear Mixed Effects (NLME) model to account for individual variations between different donors.

Inflammatory endotypes and its impact on allergen immunotherapy (AIT)

M. Berge[1], O. Hultgren[2], S. Hugosson[3], A. Saber[3]

[1] Department of Otolaryngology, Örebro University Hospital, Örebro, Sweden

[2] Department Clinical Immunology and Transfusion Medicine, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

[3]Department of Otolaryngology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

Background/Objective: Allergic rhinitis (AR) is a chronic upper airway disease that affects up to 30% of the population. As a Th2-driven inflammatory disease, the immune response in AR is modulated by cytokines such as interleukin 4, 5 and 13 (Scadding, 2014). Another group of molecules, called immunologic checkpoint molecules, also plays a role in regulating the T-cell response. For example, programmed cell death protein 1 (PD-1) and it's ligands have been shown to play a role in allergic diseases.
Most patients suffering from AR use strictly symptomatic treatment such as antihistamines or corticosteroids. The only currently known potential cure for allergic disease is allergen immunotherapy (AIT), which seeks to induce an immune tolerance to otherwise non-harmful targets such as pollens (Jutel et al., 2016). However, AIT has been shown to have small to no effect in up to 30% of patient and the reason for this is largely unknown.
Earlier studies have examined allergen specific immunoglobulins as a potential biomarker for AIT-success, but no such connection between AIT effect and allergen specific immunoglobulins have been found.
The aim of this project is to study the potential connection between AIT effect and profiles of immune-regulatory proteins such as cytokines and immunologic checkpoint molecules.
Method: Within the ALLVAC-project, at the Otorhinolaryngology department in Örebro, 128 patients who have undergone AIT have been included. These subjects have been asked on a questionnaire about allergic symptoms before and after treatment and has thus been stratified into responders and non-responders to AIT. Furthermore, the subjects have all given blood sampled collected before the start of AIT.
From the total 128 subjects, 30 non-responders have been matched based on age and gender to 30 responders. These 60 subjects' blood samples have then been analyzed for a total of 92 different cytokines (Target 96 Inflammation, Olink) and 14 different checkpoint molecules (Immuno-Oncology Checkpoint 14-Plex Human ProcartaPlex™ Panel 1, ThermoFisher). These results will be further analyzed to find any potential correlation between AIT-effect and one or more profiles of cytokines and checkpoint molecules.
Result: The results are yet to be analyzed.
Conclusion: There is, to the authors' knowledge, no earlier studies examining the correlation between AIT and cytokine/checkpoint molecule profiles. If this study would find signs of such a connection, it could be the groundwork to further, more extensive research to see if measurement of cytokines and/or checkpoint molecules could prove to be a viable means to select patients most suited to be offered AIT.

The interplay of environmental exposure, bile acids and lipids in inflammatory bowel disease

Samira Salihovic[1,2)*], Frida Fart[1], Matej Oresic[1,3)*], Jonas Halfvarson[1,4)†], Ida Schoultz[1)†], Tuulia Hyötyläinen[2)†]

1) School of Medical Sciences, Örebro University, 702 81 Örebro, Sweden 2) School of Science and Technology, Örebro University, 702 82, Örebro, Sweden 3) Turku Bioscience Centre, University of Turku and Åbo akademi University, 20520 Turku, Finland 4) Department of Gastroenterology, Faculty of Medicine and Health, Örebro University, 702 81 Örebro, Sweden

* = Equally shared authorship †= Equally shared supervision

Background/Objective: Environmental factors have been implicated in inflammatory-bowel disease (IBD), particularly late-onset disease. However, it is not fully understood how exposure to environmental pollutants may impact the development of IBD and the interplay between bile acids and lipids. To investigate the association of pollutants, we measured serum levels of persistent organic pollutants in late-onset IBD and related these with serum bile acids and lipidomics.

Method: Serum samples were collected from patients diagnosed with ulcerative colitis (n=20) and Crohn's disease (n=20) at the age of ≥ 55 years. Blood donors (n=20) were used as healthy age-matched controls. Persistent organic pollutants were measured by atmospheric pressure gas chromatography (APGC) coupled to tandem mass spectrometry (MS/MS), while perfluoroalkyls (PFAS) and bile acids (BA) by ultra-performance liquid chromatography UHPLC coupled to a triple quadrupole mass spectrometer and lastly non-targeted lipidomics with UHPLC coupled with quadrupole-time of flight mass spectrometry (QTOFMS).

Result: Our results indicate a higher concentration of persistent organic pollutants in late-onset UC compared to CD and healthy controls. In addition, there was a significant difference in bile acids and lipid profiles between UC, CD and healthy controls.

Conclusion: Our data indicate that the interplay between bile acids and lipids is disturbed in CD and UC, and the environmental pollutants may contribute to the disease by altering the bile acid and lipid profiles.

Metabolomics and lipidomics profiles in gestational diabetes mellitius (GDM)

Salihovic S1,2, Sinoja T2, Knip M3, 4, 5, Oresic M1, 6, Hyötyläinen T2.

1School of Medical Sciences, Örebro Univeristy, Örebro, Sweden

2School of Science and Technology, Örebro Univeristy, Sweden

3Pediatric Research Center, Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

4Faculty of Medicine, University of Helsinki, Helsinki, Finland

5Tampere Center for Child Health Research, Tampere University Hospital, Tampere, Finland

6Turku Bioscience Centre, University of Turku, Turku, Finland

Gestational diabetes mellitus (GDM) is a glucose intolerance that is first identified during pregnancy. GDM occurs in approximately 7%–20% of pregnant women depending on the diagnostic criteria. The etiology of GDM is complex, with genetic and environmental factors implicated in both mechanistic and observational studies. From this perspective, metabolomics approaches may enhance understanding of GDM etiology and contribute to the refinement of diagnostic criteria. Thus, the main goal of the present work is to identify metabolomics and lipidomics profiles predicting GDM. 254 pregnant women from the Early Dietary Intervention and Later Signs of Beta-Cell Autoimmunity (EDIA) cohort were included and comprehensive lipidomic and metabolomic profiles were determined using liquid and gas chromatography combined with quadrupole-time of flight mass spectrometry. 48 (19%) of the 254 women, developed GDM and 206 were used as controls.

Logistic regression and least absolute shrinkage and selection operator (lasso) logistic regression with a split sample approach (split 75/25 with 10-fold cross-validation) was used to identify metabolomic and lipidomic predictors of GDM. Several metabolites (polar metabolites and lipids) were found to be significantly increased among pregnant women with GDM vs. controls following adjustment for age and pre-pregnancy BMI. Our results encourage additional studies investigating the underlying mechanisms and the potential clinical utility of our findings.

Proteome characterization of extracellular vesicles from human breast milk

Emelie Ahlberg[1], Anders Karlsson[3], Roger Karlsson[3,4], Maria C Jenmalm, Lina Tingö[1,2]

[1] Department of Biomedical and Clinical Sciences, Division of Inflammation and Infection, Linköping University

[2] School of Medical Sciences, Nutrition-Gut-Brain Interaction Research Center, Örebro University

[3] Nanoxis Consulting AB, SE-40016 Gothenburg, Sweden

[4] Department of Clinical Microbiology, Sahlgrenska University Hospital, SE-41346 Gothenburg, Sweden

Background/Objective: Extracellular vesicles (EVs) are cell-derived membrane micro-vesicles secreted from most mammalian cell types into the extracellular space. EVs, also referred to as exosomes, carry a diverse set of bioactive molecules between cells, such as micro-RNA and proteins. Human breast milk is particularly rich in EVs and it has been hypothesized that they are important messengers delivered from mother to baby. Here, we have performed a pilot study focused on characterizing the proteome of breast milk EVs using a novel lipid-based protein immobilization (LPI) technique followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

Method: Breast milk EVs were isolated from four lactating women, three month post-delivery. The isolation was carried out by several consecutive centrifugation steps, followed by ultra-centrifugation to collect the milk EVs. Subsequently, the milk EVs were characterized by transmission electron microscopy, flow-cytometry and western blot. For proteomic characterization, the EVs were loaded onto LPI® FlowCells and subsequently subjected to digestion by trypsin in either phosphate buffered saline (PBS) or ammonium bicarbonate (ambic), for 15 min or 45 min. The resulting peptides were eluted from the LPI® FlowCell and analyzed using LC-MS/MS. Under optimal circumstances this novel method allows for characterization exclusively of the surfaceome, *i.e.* detecting only surface expressed and membrane bound proteins. As comparison, the EVs were also digested by trypsin in-solution digestion in 1% sodium deoxycholate (SDC) overnight, allowing for a complete proteome analysis of the entire vesicle and its content.

Result: The digestion by trypsin in ambic produced better results (more unique peptides for each protein detected) than trypsin in PBS, as did the 45 min digestion as compared to the 15 min one. LC-MS/MS on the trypsin in ambic digestions identified a total of 596 proteins identified by >1 peptide. Out of the 596 proteins, 312 were detected in more than one individual. Among the detected proteins, most of the non-tissue specific EV markers suggested in the Minimal information for studies of extracellular vesicles from 2018 was represented, except for CD63. A number of immune-regulating molecules, such as CD53 and CD45, as well as CD14 receptor and Toll-like receptor 2, were also detected.

Conclusion: This pilot study is the first to perform proteome characterization on breast milk EVs by LPI followed by LC-MS/MS. Digestion by trypsin in ambic was superior to that of trypsin in PBS and the shorter (15 and 45 min) and longer (overnight) digestions produced different protein profiles, indicating partial or complete digestion of the vesicle. By putting more samples through this novel analysis, we hope to be able to target and enrich the results towards the EV surface-bound proteins as opposed to intra-vesicular cargo, providing a comprehensive proteome characterization of human breast milk EVs.

Immune-related microRNAs in breast milk and their effect on regulatory T cells in breastfed children: a randomized placebo-controlled trial of pre- and postnatal supplementation with Lactobacillus reuteri and ω-3 fatty acids

Emelie Ahlberg[1], Magalí Martí Generó[1], Maria Jenmalm[1], Lina Tingö[1,2]

[1] Department of Biomedical and Clinical Sciences, Division of Inflammation and Infection, Linköping University

[2] School of Medical Sciences, Nutrition-Gut-Brain Interaction Research Center, Örebro University

Background/Objective: Breast milk (BM) is one of the first source of immune stimuli that the infant encounters. MicroRNAs (miRNA) are small RNA molecules that modulate gene expression on a post-transcriptional level. Human BM is particularly rich in miRNA; since several miRNAs are involved in immunological pathways, we hypothesize that milk miRNAs are important in infant immune regulation. The aim of this study was to evaluate the expression of 24 immune-related miRNAs in BM after pre- and postnatal supplementation with *Lactobacillus (L) reuteri* and ω-3 polyunsaturated fatty acids. Expression of the miRNAs was also related to proportion of regulatory T cells (Treg) in the breastfed infants.

Method: One-hundred twenty women included in a double-blind, randomized, placebo-controlled trial, received *L reuteri* and/or ω-3 daily from gestational week 20. *L. reuteri* supplementation stopped at delivery, while ω-3 supplementation continued until 3 months post-delivery. miRNA was quantified using Taqman qPCR array cards from BM obtained at birth and after three months of lactation. The proportion of regulatory T cells ($CD4^{dim}CD25^{high}$) activated Treg ($CD45RA^{-}FoxP3^{++}$) and resting Treg ($CD45RA^{+}FoxP3^{+}$) cells were analyzed using flow cytometry in whole blood at 6 (n=43), 12 (n=52) and 24 (n=61) months from the children.

Result: Relative expression changed significantly over the lactation period for all miRNAs, except miR-24-3p and miR-145-5p; ten miRNAs decreased and ten increased. Modest effects of the intervention could be seen for miR-181c-3p and miR-16-5p in the mature milk. Also, let-7e-3p tended to be lower in colostrum from mothers with atopy in comparison to healthy mothers (p adj = 0.068). Concerning Treg proportions in the breastfed infants, *colostrum* miR-181a-3p correlated with *resting* Treg cells at 6 months, while no correlations were observed at 12 months for any of the parameters. However, at 24 months *colostrum* miR-148a-3p and let-7d-3p correlated with proportion of *activated* Treg cells, as did *mature milk* miR-181a-3p and miR-181c-3p. In addition, *mature milk* miR-181c-3p tended to correlate with *resting* Treg cells (p adj=0.053).

Conclusion: Expression changed significantly for 22 out of the 24 investigated miRNAs over the first three month of lactation. However, maternal supplementation with probiotics and ω-3 during pregnancy and post-delivery only showed modest effects on miR-181c-3p and miR-16-5p in *mature milk*. Interestingly, some of the investigated miRNAs correlate with the different Treg subpopulations in the breastfed children, supporting the hypothesis that the milk miRNAs are important in infant immune regulation.

Cytokine responses to LPS in reprogrammed monocytes are associated with transcription factors PU.1, EPAS1 and SIRT1

Kedeye Tuerxun[1,2] *, Kristine Midtbö[1,2] *, Eva Särndahl[1,2] Egor Vorontsov[3], Roger Karlsson[4,5,6], Alexander Persson[1,2], Robert Kruse[1,2,7], Daniel Eklund[1,2], and the X-HiDE Consortium

1. School of Medical Sciences, Faculty of Medicine and Health, Örebro University, SE-701 82 Örebro, Sweden.
2. Inflammatory Response and Infection Susceptibility Centre (iRiSC), Faculty of Medicine and Health, Örebro University, SE-701 82 Örebro, Sweden.
3. Proteomics Core Facility, Sahlgrenska Academy, University of Gothenburg, Sweden.
4. Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy of the University of Gothenburg, Sweden
5. Department of Clinical Microbiology, Sahlgrenska University Hospital, Region Västra Götaland, Sweden
6. Nanoxis Consulting AB, Gothenburg, Sweden
7. Department of Clinical Research Laboratory, Faculty of Medicine and Health, Örebro University, SE-701 82 Örebro, Sweden.
* Equally shared first authorship

Background/Objective: Myeloid-derived suppressor cells (MDSCs) are functionally immunosuppressive cells that arise and expand during highly inflammatory conditions by increased hematopoietic output or reprogramming of normal immune cells. In sepsis, an increase of circulating MDSCs is associated with adverse outcomes, but there is still a lack of unique traits that can be used to identify increased activity of MDSCs. By using endotoxin-tolerance as a model of sepsis-induced MDSC-like cells, the aim of this study was to identify novel markers of MDSC activity.

Method: Primary monocytes were isolated from blood collected from anonymous healthy donors. Cells were then cultured and stimulated with 10ng/ml LPS to induce MDSC-like cells. After the second challenge with LPS, the supernatants were collected. A panel of 181 inflammatory markers were screened using proximity extension assay (PEA) and 16 of which were further validated by an electrochemiluminescence assy. Gene expression of five candidate transcription factors tracked from the validated markers using software application IPA (ingenuity pathway analysis) were analyzed by real-time PCR.

Result: A cytokine profile of 12 markers in total was discovered, including unique response markers of naïve monocytes (CXCL-10, IL-12p40 and CCL2) and MDSC-like cells (HGF and CXCL5), and shared response markers in both phenotypes (TNF, IL-10, CCL8, CCL4, TGF-alpha, CXCL6 and IL1alpha). Transcription factor *EPAS1* was downregulated, while *SPI1* was upregulated in MDSC-like cells.

Conclusion: The study identified unique markers of MDSC-like cells activity after threat recognition (LPS response) and two potential transcription factors with differential expression in naïve and MDSC-like cells. The present study may contribute to the development of novel early phase markers for MDSCs activity in sepsis. Moreover, the ex vivo MDSC-like cell model established here can be used as an approach to further study the role of MDSCs in inflammation.

VPS34 inhibitor SB02024 activates cGAS-STING signaling and sensitizes tumors to STING agonist

Yasmin Yu[1)2)*], Muhammad Zaeem Noman[3)†], Santiago Parpal[1)2)], Simone Caroline Kleinendorst[4)], Kristine Bilgrav Saether[5)], Andrey Alexeyenko[6)7)], Jenny Viklund[2)], Martin Andersson[2)], Jessica Martinsson[2)], Katja Pokrovskaja Tamm[1)], Angelo De Milito[1)2)†], Bassam Janji[3)†]

1) Karolinska Institutet, Stockholm, Sweden [Department of Oncology-Pathology]

2) Sprint Bioscience, Huddinge, Sweden

3) Luxembourg Institute of Health, Luxembourg City, Luxembourg [Tumor Immunotherapy and Microenvironment (TIME) group, Department of Oncology]

4) Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands [Department of Medical Imaging, Nuclear Medicine]

5) Karolinska Institutet, Stockholm, Sweden [Department of Molecular Medicine and Surgery]

6) Karolinska Institutet, Stockholm, Sweden [Department of Microbiology, Tumor and Cell Biology]

7) Evi-networks, www.evistat.se, Huddinge, Sweden

* = Equally shared authorship †= Equally shared supervision

Background/Objective: Novel approaches to reduce tumor immunosuppression and improve responses to anti-cancer immunotherapies based on immune-checkpoint inhibitors are needed. Emerging evidence demonstrates that autophagy inhibition enhances anti-tumor immunity by tumor cell-intrinsic and extrinsic mechanisms. Recently, we reported that pharmacological inhibition of VPS34 (PIK3C3), a lipid kinase regulating autophagy initiation, decreases tumor growth and improve the efficacy of anti-PD-1/PD-L1 therapy in melanoma and colorectal cancer mouse models. This effect was dependent on increasing T and NK cell infiltration as well as the expression of CCL5 and CXCL10 chemokines in the tumor microenvironment. Here, we explored the signaling mechanisms underlying the chemokine release following treatment with VPS34 inhibitor SB02024.

Result: We found that both pharmacological and RNAi-mediated inhibition of VPS34 activated a cGAS-STING-dependent type I interferon response in renal cell carcinoma (RCC) and melanoma cell lines. Furthermore, combination treatment of VPS34 inhibitor SB02024 with STING agonist ADU-S100 or cGAMP increased the mRNA expression and release of proinflammatory cytokines in human and murine RCC and melanoma cancer cell lines. Oral administration of SB02024 in combination with intratumoral injections of ADU-S100 significantly decreased tumor growth and weight and improved mice survival of B16-F10 tumor-bearing mice.

Conclusion: Taken together, our data demonstrates that targeting of VPS34 results in a cGAS/STING-mediated increase of pro-inflammatory cytokine secretion and synergizes with a STING agonist. We believe that systemic VPS34 inhibition using SB02024 would be of major interest in combination or as an alternative to STING agonists to improve anti-tumor immune responses.

FCGR2A overexpression and immunothrombosis signaling in the platelet proteome of myeloproliferative neoplasms

Maria Åström[1)], Ellen Ljungberg[2)], Sofia Ramström[3)], Erik Ahlstrand[1)]

1) Department of Medicine, Faculty of Medicine and Health, Örebro University, Örebro, Sweden
2) Cardiovascular Research Centre, School of Medical Sciences, Örebro University, Örebro, Sweden
3) Cardiovascular Research Centre, School of Medical Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

Background/Objectives: The myeloproliferative neoplasms (MPNs) polycythemia vera, essential thrombocythemia, and primary myelofibrosis are characterized by myeloid proliferation and increased blood cell counts. Patients with MPNs have increased risks of arterial and venous thromboses. Platelets, anucleate cells produced by bone marrow megakaryocytes, play a major role in blood clotting and in MPN-related thrombosis. Transcriptomics on platelets from MPN patients recently pointed out inflammatory gene networks promoting thrombosis, but similar proteomic studies have been lacking. Based on our results of novel platelet mass spectrometry proteomics in MPN patients and controls, we used bioinformatics to search for dysregulated proteins and biofunctions contributing to mechanisms of thrombosis. A complementary aim became to validate upregulation of a proposed immunothrombotic protein by flow cytometry, in a separate cohort.

Methods: The platelet proteomics study by quantitative mass spectrometry compared 5 MPN patients with thromboses, 6 MPN patients without thromboses and 11 healthy controls. We analyzed the proteomics data by Ingenuity Pathway Analysis (IPA) regarding diseases and biofunctions. Upregulation of the immunoglobulin receptor FCGR2A was chosen for validation by flow cytometry in another cohort of 6 MPN patients, who all had a history of arterial thromboses, in comparison to 6 age matched controls.

Results: The platelet proteomics identified 3544 proteins in total. Immune cell responses and inflammation were activated biofunctions in the thrombotic MPN platelets. The immune receptor FCGR2A was upregulated in both non-thrombotic and especially thrombotic MPN, and contributed to activation of the “thrombus” disease / biofunction. However, “thrombus” inhibitory protein dysregulations dominated in non-thrombotic MPNs. Flow cytometry confirmed elevated FCGR2A in MPN platelets, with fold change 1.89 ($p=0.002$).

Conclusion: A major difference between thrombotic and non-thrombotic MPNs in the platelet proteomics study was that several immune and inflammatory biofunctions were more activated in thrombotic MPNs. For the first time we found and validated upregulated FCGR2A immune receptor protein in MPN platelets. Further studies of FCGR2A and related aspects of immunothrombosis in MPN seem warranted, ultimately aiming for improved therapeutic options.