Infection & Immunity

JOINT DOCTORAL RETREAT
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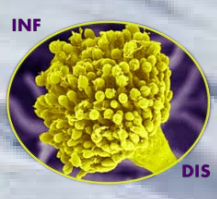
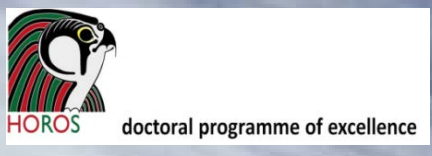
ABSTRACTS





1. A pilot trial to assess the efficacy of Argatroban (Argatra®) in critically ill patients with Heparin Resistance

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Background: Thromboembolism frequently occurs (incidence 40-80%) in critically ill patients. Therefore a sufficient anticoagulation is crucial. The standard therapy for a continuous intravenous administration of anticoagulants is unfractionated heparin (UFH). However, some critically ill patients develop a so called heparin resistance (HR). HR is present when, despite a high dose of heparin (1,200 IU/h), a sufficient anticoagulation, measured with the activated partial thromboplastin time (aPTT), cannot be achieved. There is no available anticoagulant registered for this indication. The aim of this study is to investigate, whether the administration of argatroban (Argatra®) is a superior antithrombotic therapy in comparison to a maximum dose of heparin regarding the elapsed time until sufficient anticoagulation.

Methods: Patients with heparin resistance are randomized into either treatment with the standard therapy, increasing heparin to a maximum dose (group H), or with argatroban (group A). The endpoints are achieving an aPTT-target range of 45 – 60 sec within 8 hours (primary endpoint) and 24 hours (secondary endpoint).

Results: 36 evaluable critically ill patients with heparin resistance treated at the General and Surgical Intensive Care Units of the Medical University Innsbruck, Austria, have been included so far. 16 patients were randomized to group H and 20 patients to group A. 56% of group H reached the target aPTT-level within 8 hours, whereas only 44% could maintain the target aPTT for 24 hours. 63% of group A achieved the target aPTT-range within 8 hours and after 24 hours 90% of the argatroban patients reached the target aPTT-level. The primary endpoint does not differ between groups (p=0.73, two-sided Fisher's exact test) whereas the secondary endpoint (p=0.004, two-sided Fisher's exact test) shows a significant advantage for group A.

Conclusion: Argatroban presents a significant advantage to heparin in providing an adequate anticoagulation within 24 hours. However, in the clinical setting 8 hours proves to be too short to demonstrate an advantage of argatroban to heparin in achieving the target aPTT-range. Nonetheless argatroban is clearly preferable for thromboprophylaxis in critically ill patients with heparin resistance who are in need of anticoagulation for more than 24 hours.

2. *Ex vivo* reversal of the effects of rivaroxaban evaluated using ROTEM thromboelastometry and thrombin generation assay

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Background: In major bleeding events, the new direct oral anticoagulants (DOACs) pose a great challenge for physicians. Aim of the study was to test for the effective *ex vivo* reversal of the DOAC rivaroxaban with various non-specific reversing agents: PCC (prothrombin complex concentrate), aPCC (activated prothrombin complex concentrate), rFVIIa (recombinant activated factor seven) and FI (human fibrinogen concentrate).

Patients/Methods: Blood was drawn from healthy volunteers and from patients treated with rivaroxaban. Blood samples from healthy volunteers were spiked with rivaroxaban to test the correlation between rivaroxaban level and coagulation tests. Patient blood samples were spiked with various concentrations of the above-mentioned agents and analysed using thromboelastography and thrombin generation.

Results: Rivaroxaban significantly affected thromboelastometry clotting time (CT) and thrombin generation (all parameters) when added *in vitro* and CT, lag time and peak thrombin generation (C_{max}) when measured *in vivo*. Regarding reversal of rivaroxaban, all tested agents significantly reduced EXTEM (extrinsic coagulation pathway) CT, but to different extents: rFVIIa and aPCC reduced EXTEM CT to a greater extent than did PCC and FI. Only rFVIIa reversed EXTEM CT to baseline values. Only PCC and aPCC altered overall thrombin generation (ETP) and increased C_{max}.

Conclusions: *Ex vivo* reversal of rivaroxaban with PCC, aPCC, rFVIIa and FI reduced EXTEM CT; only rFVIIa reached normal EXTEM CT values. PCC and aPCC increased ETP and C_{max}, but to above normal values.

3. The role of hospital surfaces in the transmission of nosocomial pathogens in wards and intensive care units - an overview of a PhD-project

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**Christian Doppler laboratory for Invasive Fungal Infections (modul 3 – self disinfecting surfaces)*

Background: Healthcare-associated infections (HCAIs) display a worldwide problem in hospital settings. Nosocomial infections (NI) predominantly evolve endogenously, however exogenous sources are increasingly being reported. It has been considered that contaminated surfaces play an important role in the transmission of pathogens. Hence, antimicrobial surfaces are an on-going hot-topic as an intervention of achieving clean hospitals. Nevertheless, their role in preventing NI in daily healthcare is unknown yet. The aim of this study is to clarify the necessity of "self-disinfecting" surfaces in hospital settings and therefore to evaluated the environmental burden in hospitals. Another issue is to investigate the technology of Egger™ Antimicrobial Surfaces (EAS) in relation to their antimicrobial potency against various pathogens under "real-life" conditions.

Material/Methods: Environmental burden was evaluated by taking patient-related surface samples at intensive care units by using contact plates. For investigating EAS the "dry-inoculation" method was operated. Therefore EAS, variable coated and uncoated (control), were contaminated each with clinical relevant pathogens such as *Staphylococcus aureus* (*S. aureus*), *Acinetobacter* spp., *Klebsiella* spp., *Escherichia coli, Enterococcus faecium* and *Pseudomonas aeruginosa.* After inoculation (~108 colony forming units) of the surfaces, samples were taken after defined contact times (0h, 1h, 3h) by use of contact plates. Incubation was done for 24h at 35 ± 2 °C. Reduction due to bactericidal activity was estimated by subtracting the CFU count on control versus test surfaces at different time points investigated. Antibacterial effects were determined as a twofold CFU log-reduction.

Results: Contamination of patient-related surfaces with nosocomial pathogens was monitored in the majority of intensive care units. Antimicrobial activity of EAS products was observed but further investigations on the technology have to be performed.

Conclusion: Environmental contamination with nosocomial pathogens could be detected in intensive care units, therefore antimicrobial surfaces could be considered as an infection prevention instrument. EAS products investigated showed antimicrobial efficacy against a broad range of bacteria. However, it's unknown yet whether EAS or other antimicrobial surfaces are able to influence the prevalence of nosocomial infections, a clinical evaluation is of major importance and under construction.

4. Non-molecular transduction of a biological signal using bacteria as a highly sensitive detection system — reproducing reported positive results is more difficult than expected.

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Background: The existence of a) so-called „memory-effects“ in liquids, and b) non-molecular transductions of biological signals eliciting a response it an appropriate biological system is highly controversial, but a small body of literature and several research groups claim to successfully demonstrate one or both of the above. Highly renowned but recently passed away Israeli researcher Eshel Ben-Jacob presented convincing data showing memory-effects and non-molecular signal transduction, utilizing a microbiological readout. The present work is aiming to pick up and reproduce Ben-Jacob´s results, i.e. the reduction of bacterial growth by only the “information“ of an antibiotic. Unfortunately, Ben-Jacob passed away before he had a chance to share the exact procedure of his setup.

Methods: Utilizing a simple tube-in-a-tube setup where a smaller tube is placed inside a bigger tube, sterile liquid broth in the inner tube is exposed to an antibiotic solution in the outer tube. After taking away the outer tube, gram-positive, swarming bacteria (Paenibacillus Dendritiformis, subtype T or C) are incubated in the liquid broth in the remaining (inner) tube, then brought centrally onto specially dried out, nutrient poor agar plates. Bacterial growth, i.e. the area covered by the bacteria, is assessed fully automatically using a custom-written R-package employing picture-recognition algorithms.

Results: Obtaining regular bacterial growth, i.e. at least ~80% plate-coverage within 3 to 5 days of plate incubation and growth-areas displaying a minimal variation within a group already proved to be more difficult than expected: naturally, the status of the bacteria prior to being used in the liquid culture, as well as the drying process of the plates prior to being used for growing the bacteria showed to be among the influential factors. So far, one single tube-in-a-tube experiment showed highly significant differences in bacterial growth area. For reasons that keep elusive, even the reproduction of these specific basic growth-properties, i.e. ~80% plate coverage within 3 days in P. Dendritiformis subtype C, failed so far. Other variations of the procedure showed good growth-area properties, but no consistent, meaningful differences between groups.

Conclusion: The available data do not prove the successful application of the tube-in-a-tube assay. Further fine-tuning of the procedure is needed, and the remaining time before pursuing different avenues will have to be discussed.

5. NK cells in immunity against Intracellular pathogens

U. Schleicher

Erlangen

6. Arginase 1 and inducible oxide: two key players in antimicrobial defence

Christian Bogdan
Erlangen

7. Antimicrobial function and intracellular localization of inducible nitric oxide synthase in Leishmania infections

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After transmission by sand flies the protozoan parasites Leishmania are rapidly taken up by phagocytes and transform to the intracellular so-called amastigote stage. Their iron-dependent survival within phagosomes is directly or indirectly counteracted by inducible nitric oxide (NO) synthase (NOS2). NOS2, which converts L-arginine into L-citrulline and NO, is expressed in phagocytes upon stimulation with cytokines (e.g. IFN-γ) or microbial ligands (e.g. LPS). Previously, NOS2 was detected in the cytosol, attached to the submembranous cytoskeleton and in vesicles provisionally termed “nitroxosomes”.

As NO is quickly oxidized to less toxic compounds after its release, direct effects on Leishmania require close vicinity between NOS2 and the pathogen. Using confocal and high resolution microscopy we confirmed that NOS2 is expressed in the cytosol and in vesicle-like structures. Infection with Leishmania did not alter the overall intracellular localization of NOS2. Interestingly, NOS2 staining in IFN-γ/LPS-simulated uninfected or promastigote-infected bone marrow-derived macrophages (BMM) co-localized with EEA1 (early endosomal antigen1) and calnexin (ER marker), but not with LAMP1 (lysosomal-associated membrane protein1). In contrast, in Leishmania amastigote-infected BMM NOS2 colocalized with LAMP1 and calnexin, but not with EEA1 indicating that NOS2 gets recruited to the Leishmania-containing phagolysosomes in infected host cells.

In order to test the hypothesis that the anti-leishmanial effect of NO not only relies on direct destruction of the parasites by damaging their DNA and proteins, but is also a result of the withdrawal of iron from the microenvironment of amastigotes, we performed killing assays with infected BMM that were stimulated for endogenous NO production or incubated with an exogenous NO donor in the presence or absence of exogenous Fe2+ (FeSO4) or Fe3+ (FeCl3). In both settings the Fe compounds were able to reverse the killing of intracellular Leishmania. To elucidate the underlying mechanism(s), we analyzed whether the expression of ferroportin-1 (Fpn1), the only known cellular export system for Fe2+/Fe3+, is increased in a NO-dependent manner. So far we could not detect any NO-dependent regulation of Fpn1 and of the iron storage protein ferritin.

In a reverse approach we could demonstrate that iron-overloading of L. major-infected mice caused an exacerbation of the infection. Whether iron-dependent regulation of NOS2 and/or modulation of other effector pathways are responsible for this effect, is currently under investigation.

8. Dopamine regulates iron homeostasis and innate immune responses of macrophages to salmonella infection

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Background: Siderophores are catechol based compounds which can bind iron. Iron is an essential growth factor for mammalian cells and microbes. Based on previous observations, showing increased bacterial growth in the presence of catechols, we asked whether this may be referred to hormone mediated alterations of iron homeostasis.

Methods: We studied the effects of the catecholamine dopamine on the regulation of iron in BMDM obtained from C57Bl/6wt mice and littermates knocked out for lipocalin-2, a mammalian siderophore binding peptide. The in vivo effects of dopamine were studied in wt mice infected with the Gram negative bacteria Salmonella typhimurium (S.tm.).

Results: Administration of dopamine to macrophages resulted in a dose dependent increase of heme oxygenase-1 and ferroportin expression, the latter being the major cellular iron exporter, which subsequently resulted in reduced intramacrophage iron concentrations. This effect could also be reproduced upon infection of macrophages with S.tm.. These effects were independent from the presence/ absense of the siderophore binding peptide lipocalin-2. The in vivo administration of dopamine to mice infected with S.tm. resulted in an increased bacterial burden in liver and spleen as compared mice receiving solvent. This is linked to an increased delivery of iron to bacteria in the presence of dopamine along with an impaired pro-inflammatory immune response of macrophages.

Conclusion: Our data demonstrate that dopamine may deteriorate the course of infection by promoting bacterial growth which can be a major concern from the treatment of patients with bacterial sepsis receiving catecholamines.

9. Effects of iron imbalances on mitochondrial activity *in vivo*

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Background: Mitochondria are dynamic organelles, involved in fundamental cell processes, including oxidative phosphorylation [1, 2]. Iron plays a decisive role in these processes because it is central part of mitochondrial enzyme complexes but also regulates citric acid cycle activity by modulating mitochondrial aconitase expression. Hence, imbalances of iron homeostasis impact on mitochondrial activity and, thus, on cell and organ functions [3, 4].

So far, little information is available on how to best measure tissue mitochondrial activity and its interaction with iron homeostasis *in vivo*; therefore we questioned whether determination of mitochondrial respiration in peripheral blood mononuclear cells (PBMCs) could be a good surrogate marker for that.

Methods: Human PBMCs were collected from buffy coats, purified cells (2x10^6 cells/ml) were resuspended in mitochondrial respiration medium (MiR05), and mitochondrial activity was assessed by high resolution respirometry (OROBOROS Instruments, Austria). Moreover, to access the impact of iron on mitochondrial respiration we studied mitochondrial function in livers of mice, receiving either iron deficient- or standard iron-diet two or four weeks before being sacrificed. The liver was collected and stored in Custadiol prior to homogenization in MiR05. Mitochondrial leak respiration, complex I and II maximal oxidative phosphorylation together with non-coupled respiration of the homogenates were assessed at a final concentration of 1 mg.

Results: Our ongoing experiments indicate that mitochondrial function testing can be successfully performed in human PBMCs as well as in mouse tissues. Analyses of liver samples from mice indicate that dietary iron supplementation triggers changes in oxidative phosphorylation, and has a direct impact on the activity of the electron transfer system complexes.

Conclusion: The use of high-resolution respirometry (OROBOROS Instruments, Austria) represents a powerful and reliable tool to investigate mitochondrial respiration in PBMCs, which might provide useful information on the tissue mitochondrial activity.

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[2] Horowitz MP & Greenamyre JT Mitochondrial iron metabolism and its role in neurodegeneration. *J Alzheimers Dis.* 20 Suppl 2:S551-68 (2010)

[3] Lakhal-Littleton S *et al.* Cardiac ferroportin regulates cellular iron homeostasis and is important for cardiac function. *PNAS* 112(10):3164-9 (2015)

[4] Martelli A *et al.*, Iron regulatory protein 1 sustains mitochondrial iron loading and function in frataxin deficiency. *Cell Metab* 21(2):311-22 (2015)

10. IFIH1-deficiency results in a cell-type specific alteration of autophagic activity in response to stimulation with S. typhimurium

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Background: Ulcerative colitis (UC) and Crohn's disease (CD) are the two mayor forms of inflammatory bowel diseases (IBD). To date, more than 160 genetic susceptibility loci for CD/UC have been identified with the majority conferring a modest risk. Recently, interferon induced with helicase C domain 1 (IFIH1) has been described as a susceptibility gene for IBD. IFIH1 represents an intracellular pattern recognition receptor (PRR) that has been implicated in the sensing of RNA and the subsequent induction of type I IFN-response. Furthermore, a physical interaction of IFIH1 with the autophagy-associated proteins Atg5 and Atg12 has been suggested. Therefore the aim of this study was to identify a potential functional relation between IFIH1 and autophagy.

Methods: We generated *Δ*Ifih1, *ΔRig and ΔIfih1/ΔRig-I* silenced epithelial cells and macrophages and subsequently stimulated autophagy through nutrient deprivation, *Salmonella enterica Serovar typhimurium* (*S. typhimurium*) or Rapamycin. The same experiments were performed in peritoneal macrophages (PM) from wild-type (WT) and IFIH1$^{-/-}$ mice. Additionally, WT and IFIH1$^{-/-}$ mice were challenged with *S. typhimurium* in vivo.

Results: We demonstrate that a deficiency of IFIH1 does indeed interfere in autophagic activity. However, this effect was highly cell-type specific. This coincides with a differential immunohistochemical distribution of IFIH1 and its related protein RIG-I on human colonic tissue sections with IFIH1 being predominantly expressed in intestinal epithelial cells (IECs). Accordingly, IFIH1-deficient Caco-2 colonic epithelial cells demonstrate a reduced autophagic flux compared to control-silenced cells. In vivo, we found low levels of autophagy in the epithelial scrapings of IFIH1$^{-/-}$ compared to wild-type animals after challenge with *S. typhimurium*. In contrast, IFIH1-deficient peritoneal macrophages (PM) showed significantly higher autophagic activity in response to *S. typhimurium* than wild-type control cells.

Conclusion: This work identifies a novel link between IFIH1 and autophagy. Interestingly, the observed effects are cell-type specific. We found reduced autophagy in IFIH1-deficient IECs in contrast to an increase of autophagy in IFIH1$^{-/-}$ peritoneal macrophages in response to *S. typhimurium.* These data lead to further studies to decipher the role of IFIH1 in autophagy and other related molecular pathways in vitro and in experimental models of IBD.

11. The role of prophylactic treatment with A.muciniphila in the acute alcoholic liver disease

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Background: Alcoholic liver disease (ALD) is a major cause for liver related deaths. ALD includes a clinical spectrum ranging from steatosis over alcoholic steatohepatitis (ASH) to liver fibrosis and hepatocellular carcinoma (HCC). Alcohol induces intestinal bacterial overgrowth and dysbiosis, these conditions are moreover associated with increased levels of lipopolysaccharides (LPS) and an altered gut barrier. Direct toxic impact of ethanol on the hepatocyte and translocation of LPS from the gut into the liver, play an important role in the pathogenesis of ALD. It was shown for A. muciniphila, a commensal bacterium, to restore gut barrier damage and thereby reducing systemic LPS levels.

Methods: Female wildtype mice were treated with 1,5x10^9 CFU of A. muciniphila on two days prior to alcohol administration (6g ethanol/ kg bodyweight). Eight hours after the gavage of ethanol, mice were sacrificed and samples were collected. To measure in-vivo gut permeability, FITC-Dextran was gavaged four hours after alcohol administration, followed by concentration measurements in the serum.

Results: Ethanol administration led to significant higher levels of ALT ($p<0,05$), whereas prophylactic treatment with A. muciniphila decreased ALT levels significantly ($p<0,01$). We further investigated the numbers of A. muciniphila in stool samples. Alcohol administration reduced the number of A. muciniphila significantly ($p<0,001$), independently if A. muciniphila was administered or not. Although we could see a reduction of FITC levels in probiotic treated mice, suggesting a restoration of the gut barrier, we couldn't see improved endotoxemia. Furthermore we could see a trend towards a reduced expression of pro-inflammatory cytokines, although just Tnf-alpha levels were significantly reduced by A. muciniphila treatment ($p<0,05$).

Conclusion: Our date indicate a potential role of A. muciniphila in the prophylactic treatment for ALD, but further experiments, in particularly for chronic alcohol consumption, should follow.

12. Single shot booster vaccination against diphtheria does not induce sufficient long-term protection, particularly in elderly people

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Immunization is one of the most successful health intervention against infectious diseases. However, the efficacy of vaccination is reduced in old age. Our study analyzed specific immune responses following a booster vaccination containing tetanus and diphtheria toxoid in healthy elderly (>60y; n=87) and young volunteers (25-40y; n=46). Long term protection was evaluated for 27 elderly and 17 young adults 5 years later. Tetanus- and diphtheria-specific antibodies were measured by ELISA and neutralization-assay. In addition, antigen-specific T-cells producing a panel of cytokines were quantified by stimulation of PBMCs with tetanus and diphtheria toxoids following intra cellular staining.
Before the vaccination 9% of the older and none of the young individuals had tetanus-specific antibody levels below the protective limit. The booster induced sufficient protection in both age-groups for the following 5 years. The protection against diphtheria was almost equal before the vaccination (52% for the elderly and 48% for the young donors). Antibody concentrations increased significantly 4 weeks after vaccination, but dropped substantially over 5 years leaving again 54% (elderly) and 24% (young) below protective antibody levels. Thus, compared to the elderly young adults have a significantly better, but still insufficient maintenance of diphtheria-specific antibodies. We found correlations between diphtheria-specific antibodies and diphtheria-specific T-cells producing different cytokines. Among those were GM-CSF-producing T-cells which we detected in a lower frequency in elderly compared to young adults.
In conclusion, our findings demonstrate that booster vaccinations induce long-lasting immunity against tetanus but not diphtheria, particularly in elderly people. GM-CSF might be useful as an adjuvant to improve diphtheria vaccination. We set up a mouse model to further investigate this question.

13. How fat influences the adaptive immune system in the bone marrow in old age

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Background: Multipotent haematopoietic stem cells differentiate to precursor cells and further to immature T-lymphocytes in the bone marrow, which then migrate into the thymus gland. The thymus is a primary lymphatic organ and responsible for the development of T-cells to mature naïve T-cells. With puberty the thymus starts to degenerate, also called involution. The functional part of the thymus tissue is reduced and replaced by fat with increasing age. A similar effect can be seen within the bone. There we find a reduction in bone formation and bone loss. A predominant property of age-related bone loss is the accumulation of bone marrow fat. Thus the mass of bone marrow is replaced by fat with the age. It is already known that due to the thymic involution a decrease in naïve T-cells occurs. But the effect in the bone has not yet been documented. The aim of the study is to investigate the infiltration of immune cells in subcutaneous fat in comparison to the infiltration in bone marrow fat from lean and obese donors in the context of aging. Therefore the polarization of macrophages (M1 or M2), of T-cells (Th1 or Th2) and the frequency of regulatory CD4+ T-cells (CD4+ Treg) in bone marrow fat compared to subcutaneous fat are examined. For a better understanding we examine the interaction of fat components on the adaptive immune system as well. According to histological sections and FACS-analyses the phenotype of adipocytes in subcutaneous and bone marrow fat will be determined. Additionally the influence of adipocyte molecules on T-cells and plasma cells will be analysed.

Methods: In cooperation with the Department of Orthopedics in Wels we receive human bone marrow and subcutaneous fat tissue. The donors are divided in four groups: young (< 65 years) and old (> 65 years), lean and obese. One part of subcutaneous and bone marrow fat tissue is used for paraffin sections to perform immunofluorescence staining. Immune cells and adipocytes are isolated from the remaining tissue and analysed with the FACS. Furthermore RNA will be isolated from the adipocytes from subcutaneous and bone marrow fat to generate comparative RNA profiles (micro-array). Appropriate results are further used for RT-PCR, western blotting and optional, additionally molecular biological methods.

14. Phenotypical and functional charac-terisation of immune cells in the human bone marrow and the impact of aging

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The various types of Immune cells begin their lives as hematopoietic stem cells in the bone marrow, from here, the presence of different transcriptional regulatory networks, transcription factors, proteins, and microenvironmental factors such as the exposure to antigen, can contribute to determining their fate as they differentiate into various cell types, and develop accordingly. Once activated, immune cells can proceed to the area of infection, circulate through the blood and lymph, or return to the peripheral lymphoid organs. It has been shown that many immune cells migrate back to the bone marrow, where they can remain in different activation states for an extended period of time, and the proteins they secrete, or express on their surface, can act as markers, suggesting where and what exposure they have experienced. The bone marrow has long been known for hematopoiesis and its role as a primary lymphoid organ, however the full extent of its role as a secondary lymphoid organ has not yet been explored, it is known to be involved with the regulation, function and survival of memory B and T cells in particular, as the number of CD4+ and CD8+ T cells in the bone marrow is maintained with aging, the composition changes, showing an increase in Memory cells and a decrease in Naïve cells (Herndler-Brandstetter, 2011). Just as there are many factors dictating the developmental fate of lymphocytes, many factors have been suggested to possibly contribute or control whether or not cells will migrate back to the bone marrow. The importance of understanding the mechanisms of long-lived cellular immune response generation is important in vaccine research and development, and particularly interesting in biomedical aging research to aid the safety and effectiveness of administered vaccines among a society with a rapidly growing elderly population.

Aim: To characterise the immune cells present in Bone Marrow Mononuclear cells (BMMC's) isolated from the Bone Marrow of patients undergoing hip replacement surgery, based on their phenotypes, functions, maturity and specificity.

Methods: Bone Marrow Mononuclear cells were isolated from bone marrow samples using gradient centrifugation. Cells were stained for various surface markers to identify and quantify them using FACS analysis. To identify the functionality, the cells were stimulated with PMA Ionomycin, along with Brefeldin-A for 6 hours, and intracellular staining performed to observe the antigen specific cytokine production using FACS analysis. ELISPOT was performed using the anti-IgG coating antibody, and antigen specific biotinylated detection antibodies to determine the presence and number of specific antibody producing cells.

Results: To follow.

15. GWAS as a toolbox to find new genetic associations and to support causality

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16. Precise determination of mitochondrial DNA copy number per cell

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Background: Human mitochondrial DNA (mtDNA) is a small maternally inherited DNA, present in hundreds of copies in a single human cell. The mitochondrial genome plays a crucial role in the metabolic homeostasis of the cell. Alterations of mitochondrial function, dynamics, and biogenesis have been observed in various metabolic disorders, including aging, cancer, diabetes, and obesity.
The aim of this project is to investigate if there is a variation in mtDNA copy number in the general population. We plan to analyze 5000 DNA samples from peripheral blood derived from CHRIS, a prospective epidemiological research study conducted in South Tyrol.

Methods: We established a duplex quantitative PCR assay that allows for targeting a single copy nuclear gene (ß2-microglobulin) and the mtDNA (t-RNA) simultaneously. Optimal qPCR conditions and primer concentrations were determined evaluating PCR efficiencies for both targets in serial dilutions. The relative mtDNA copy number was calculated as $2 \times E^{\Delta CT}$ where E equaled the averaged mean of the PCR efficiencies for both targets and ΔCT = nucDNA CT – mtDNA CT. The use of a plasmid containing both targets in a 1:1 ratio was used to normalize against differences of emission intensities of the fluorescent dyes VIC and FAM. The successful TA cloning of the two sequences into the pGEM vector was verified by sequencing. The distance between both inserts was chosen to be more than 500 bp to avoid the amplification of both targets as one amplicon.

Results: The validation of the duplex qPCR method revealed that the optimal primer concentrations were 300/600 nM (mt/n target) to reach equal PCR efficiencies for both targets. QPCR on the serial dilution of the calibrator plasmid revealed that the FAM dye emission signal exceeded over the VIC resulting in a ΔCT value of approximately one cycle corresponding to an apparent double amount of molecules.

Conclusion: We set up a universal and precise method to determinate the number of mitochondrial copy per diploid cell. The use of the dual insert calibrator plasmid allowed for correction against the overestimation of the mtDNA copy number resulted from unequal emission intensities of the differently fluorescence labeled targets. Furthermore, our calculation method takes into account the real PCR efficiency during the exponential phase of the amplification reaction.

17. Identification of genetic and clinical correlates of the complement activation system in the Cooperative Health Research in South Tyrol (CHRIS) study

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Background: The complement system is an important part of the innate immune response. It can be activated via three distinct pathways (classical, lectin and alternative) converging in the generation of C3 convertases and finally leading to the formation of the terminal complement complex (TCC). To maintain host defense while avoiding host damage, complement activation has to be controlled by several regulators, like factor H (FH). There are several reports on the association of complement protein deficiencies with various diseases, like FH deficiency and occurrence of chronic renal diseases or age-related macular degeneration or C7 deficiency and risk for Neisseria meningitides infections.

Aim: The aim of the present study is to identify gene polymorphisms within the human genome that are associated with elevated or decreased activation capability of the complement system via all three activation pathways. We will investigate whether carriers of risk alleles have a higher risk of infections, cardiovascular diseases and kidney diseases or whether they are subjected to a faster ageing process.

Study plan: Serum samples of 5000 study participants, for whom one million single nucleotide polymorphisms (SNPs) have been genotyped, will be analyzed for complement activation. We will determine functional activity of the classical, lectin and alternative complement pathways in addition to the terminal pathway by using a commercially available assay, the WIESLAB Complement system Screen. We will select 500 samples with regard to the clinical outcome(s) and we will measure levels of several complement proteins with an in-house-ELISA.

Methods: As a first step of our analysis, we will perform genome-wide association studes (GWAS) of the three pathways on all samples. In a second step, the identified genetic variant(s) will be tested for association with individual's information on infections, cardiovascular diseases, kidney diseases, autoimmune diseases, as well as neurological and psychiatric conditions collected in the CHRIS study (phenome-wide scan). In a third step, we will assess known polymorphisms of the respective complement proteins as well as their functional activity. Causal analysis might be investigated through Mendelian Randomization to establish causal pathways. All statistical analyses will be performed using linear mixed models to account for family structure and relatedness in general.

18. The relevance of novel missense mutations in the two newly described orthologues of lanosterol 14-α demethylase for azole resistance in clinically relevant Mucorales: from the phenotype towards the genotype.

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Background: Mucormycoses are potentially fatal infections, caused by members of the order Mucorales. Available treatment options for these diseases comprise amphotericin B (AMB) and posaconazole (PSC). Azole resistance among members of the order Mucorales was shown to be species-dependent. Therefore, an investigation of the possible molecular mechanisms involved in phenotypical PSC was studied. To this aim, Sanger sequence analysis of the two lanosterol 14-α demethylase orthologous genes were performed followed structural analyses of the protein model.

Methods: A strain collection of 131 Mucorales isolates was catalogued for a) species identification using direct sequencing analysis of the internal transcribed spacer region (ITS), and b) determination of PSC *in vitro* susceptibility testing by following the CLSI guidelines. Full gene sequences of two *CYP51* orthologues are available online for *Rhizopus arrhizus*, and *Mucor circinelloides* (www.broadinsitute.com). These were set as reference for amplification and sequencing analysis of the genes encoding for lanosterol 14-α demethylase, known as the target of azoles. All sequences of PSC-susceptible and -resistant strains were manually analysed for SNPs and translated into the amino acid sequence and screened for missense mutations. Furthermore, impact of missense mutations were *in silico* modelled based on the molecular 3D structure of the *Erg11* protein of *Saccharomyces cerevisae (Erg11p6xHis)*.

Results: Three species had subpopulations with reduced *in vitro* susceptibilities against PSC, namely *M. circinelloides* (Mc*CYP51A* and Mc*CYP51B*), *R. arrhizus* (Ra*CYP51A* and Ra*CYP51B*), and *R. microsporus* (Rm*CYP51A* and Rm*CYP51B*). These subpopulations plus the wild-type populations were genetically analysed. From the eight amino acid variations found for *R. arrhizus*, one in the orthologous protein of *CYP51B* was of particular interest ($I_{57}V$) as it might indirectly affect PSC binding. Furthermore, out of the 12 amino acid changes found for Rm*CYP51B*, the amino acid change $R_{Y}H$ might give rise to significant PSC resistance.

Conclusion: We provided for the first time, the full gene sequences of two orthologous *loci* of lanosterol 14-α demethylase in three clinically relevant Mucorales species. The amino acid substitution $R_{Y}H$ found in *R. microsporus* may confer reduced PSC susceptibility. However, experimental validation of this new amino acid change on its impact on PSC resistance is needed.

19. Establishment Of 3D Human Lung Tissue Model To Study Polymicrobial Infections

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Background: *Aspergillus fumigatus* is a saprophytic fungus which causes clinical syndromes ranging from colonization to deep-seated infections. Infection mainly begins in the lung tissue of individuals with a challenged immune system. Current studies primarily involve the use of animal models and cell lines to understand the fungal invasion process. However, it is essential to unfold mechanisms underlying the primary interactions and invasion process between the respiratory epithelium and the pathogen. Multiple predisposing factors such as role of immune cells, or cytokines released at the site of infection need to be studied additionally. Therefore there is need for a sophisticated *in vitro* lung model.

Methods: We employed Normal human bronchial epithelial (NHBE) cells in air-liquid interface culture (ALI) to set up the 3D lung model and further studied their development using immunofluorescence and live cell microscopy. We compared differentiated NHBE cells with ´ready-to-use´ respiratory epithelial cells (Epithelix). Preliminary interactions of *A.fumigatus* with MucilAir™ cells were studied using Scanning electron microscopy (SEM).

Results: Analysis over time by confocal microscopy showed that NHBE cells differentiated in ALI to form tight junctions, produced mucus and developed cilia. These findings were comparable with MucilAir™ airway cells. SEM studies of MucilAir™ infected with *Aspergillus* demonstrated that the host cell surface produced factors that inhibited the fungus from producing hyphae.

Conclusion: Our preliminary data will have future implications to use differentiated NHBE cells in combination with immune cells in a 3D setting reflecting *in vivo* conditions. This model will support better understanding of pathogenesis, detection, and treatment of polymicrobial infections. It may also have a broad impact in dissecting immune responses during co-infections and could additionally represent a valid alternative method to animal experimentation.

20. The impact of biologic therapies and immunosuppressants on the risk of opportunistic infections

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Background: Opportunistic infections became a serious health threat due to the high abundance of immunosuppressed patients nowadays. One of the major causes of immunosuppression is the administration of immunosuppressive drugs and biologics that increase the risk of infections with opportunistic bacteria and fungi. The opportunistic mold pathogen *Aspergillus fumigatus* is the most common cause of fungal lung infections in immunocompromised patients, thereby exhibiting high risk to people undergoing immunosuppressive therapy. In this study, we want to analyze how immunosuppressive therapy influences the disease development of *A. fumigat*us infections *in vitro* and *in vivo*.

Methods: In order to analyze the influence of immunosuppressive therapy we will expose cells and, in further instance, mice to the immunosuppressive agents Tacrolimus (FK506), Mycophenolate mofetil and Prednisolone alone and in combination thereof. In addition, the effects of the TNF-α Inhibitor Infliximab on fungal infection shall be determined. In order to do so, we will examine the immunological response to the fungus in the human cell lines A549 (lung epithelial cell) and THP-1 (Macrophage-like cell) during administration of those drugs. Furthermore, we will investigate the immune response in human and murine primary immune cells isolated from bone marrow (mice) and blood (human). Future experiments will include the implication of mouse models to study systemic pulmonary aspergillosis during immunosuppression *in vivo*. Obtained data can then be correlated to clinical outcomes in patients undergoing immunosuppressive therapy.

Results: First results suggest a partial decrease in cytokine expression in A549 lung epithelial cells during co-infection when exposed to different immunosuppressants. We are confident to validate those findings and, in further instance, reproduce these data in the macrophage-like cell line THP-1 and in murine primary macrophages.

Conclusion: Immunosuppression is one major risk factor for acquiring opportunistic infections, including infections with the fungal pathogen *A. fumigatus*. In this project, we will investigate the influence of biologic therapies and immunosuppressive drugs on the outcome of *A. fumigatus* infections *in vitro* and *in vivo*. We are certain that the results obtained during this PhD will reveal how the immune response alters due to immunosuppressants and how the outcome of infections with fungal pathogens in immunocompromised patients can be improved.

21. Tackling Aspergillus virulence from a nutritional angle

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The pathogenesis of mycoses relies on a plethora of factors and circumstances and is massively influenced by the intimate pathogen-host interplay. A common and fundamental theme is the quest for food and nutritional sources by the invading pathogen to support its growth, while the host restricts access to several elementary factors. The metabolic versatility of a fungal pathogen to circumvent this 'nutritional immunity' of the infected individual has to be considered as virulence determinant, and comprehensive knowledge about the pathways and regulatory circuits that are operative during infection is of importance given the pronounced specificity of fungal metabolism and the limited number of established antifungal targets.

The omnipresent mold *Aspergillus fumigatus* has emerged as leading cause of fungal infections in the presence of an underlying immunodeficiency. To assess the role of its metabolic versatility for virulence, we have addressed aspects of it primary metabolism, among them sulfur assimilation or biosynthesis of aromatic amino acids: The impact of the MetR transcriptional regulator on utilization of sulfur compounds and on virulence has been assessed to find a correlation between the capacity to assimilate distinct sulfur sources and virulence; furthermore, a regulatory crosstalk between sulfur metabolism and iron homeostasis became evident. Recent data emerging from mutant analyses indicate the source of sulfur during invasive pulmonary aspergillosis. In our aim to determine whether fungal growth in the host relies on endogenous biosynthesis of aromatic amino acids, we were able to demonstrate essentiality of the complete biosynthetic pathway, which could be corroborated by conditional gene expression in infected animals. Future studies aim at a comprehensive assessment of fungal primary metabolism by metabolic network modelling with the long-term goal to identify novel targets of antifungal therapy.

22. Leucine biosynthesis is crucial for adaptation to iron starvation and virulence of *Aspergillus fumigatus*

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Background: The mould *Aspergillus fumigatus* is the most common airborn fungal pathogen of humans. It can cause allergic reactions and severe invasive diseases, especially in immunocompromised patients. In order to characterize the fungal pathways involved in adaptation to the host niche and to identify potential novel targets for antifungal therapy, we investigated the mechanisms involved in biosynthesis and regulation of the amino acid leucine, which represents an essential amino acid for humans.

Methods: We generated three *A. fumigatus* mutant strains lacking either the leucine regulatory transcription factor LeuB (Afu2g03460) or the leucine biosynthetic enzymes LeuA (α-isopropylmalate isomerase, Afu2g11260) or LeuC (isopropylmalate synthase, Afu1g15000). The mutant strains were characterized by growth assays under different culture conditions and virulence assays in the *Galleria mellonella* infection model.

Results: Deficiency in either LeuA (strain *ΔleuA*) or LeuC (strain *ΔleuC)* resulted in leucine auxotrophy, whereby the *ΔleuC* mutant required significantly higher leucine supplementation for growth than the *ΔleuA* mutant. Deficiency in LeuB (strain *ΔleuB*) resulted in partial leucine auxotrophy, i.e. the mutant was able to grow without leucine supplementation but required leucine supplementation for full growth. Interestingly, the *ΔleuB* mutant displayed significantly decreased resistance to iron starvation. In the *Galleria mellonella* infection model, deficiency of LeuA, LeuB and particularly LeuC attenuated virulence of *A. fumigatus*.

Conclusion: These data demonstrate that leucine metabolism is a virulence determinant of *A. fumigatus* and reveal an unprecedented crosstalk between leucine and iron metabolism.

23. Identification and characterization of antifungal drug targets in *Aspergillus fumigatus*

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Background: *Aspergillus fumigatus* is the most prevalent airborne fungal pathogen causing invasive fungal infections in immunosuppressed individuals. Limitations in antifungal therapy arise from non-specific symptoms of infection, poor diagnostics and comparatively few options for treatment. The aim of this study is to explore the metabolism of *A. fumigatus* on a comprehensive scale as essential virulence determinant to generate a collection of *A. fumigatus* strains with a focus on primary metabolism to target fungal pathways that are absent in mammals.

Methods: Based on the annotated genome of *A. fumigatus*, metabolic network reconstruction served to identify fungal-specific pathways and key reactions. Predictions for unique enzymes resulted in a candidate list of genes, the inactivation of which is likely to result in an auxotrophic phenotype. The virulence potential of the generated auxotrophic mutant strains was then analysed in various host niches.

Results: We identified four essential biosynthetic pathways in *A. fumigatus*: (i) biosynthesis of the amino acid histidine, (ii) biosynthesis of the co-enzyme siroheme, which is essential for sulfate and nitrate assimilation, (iii) biosynthesis of the vitamin riboflavin and (iiii) biosynthesis of the vitamin pantothenic acid. The loss of histidine biosynthesis resulted in attenuation of pathogenicity in four virulence models: murine pulmonary infection, murine systemic infection, murine corneal infection, and wax moth larvae *Galleria mellonella*. Defective siroheme biosynthesis led to mild attenuation in the *Galleria mellonella* infection model but did not affect pathogenicity in murine infection models. Moreover, preliminary data indicate that inactivation of biosynthesis of riboflavin or pantothenic acid resulted in attenuated virulence of *A. fumigatus* in murine pulmonary infection models.

Conclusion: Via generation of auxotrophic mutant strains, genes encoding components of biosynthetic pathways for histidine, siroheme, riboflavin and pantothenic acid were identified and analysed by mutant strain phenotyping and virulence testing. The results characterize the host niche and reveal targets for development of novel antifungal therapeutic approaches.

24. The *Candida albicans* factor H binding molecule Hgt1p- in vitro and in vivo evidence that it functions as virulence factor

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Background: The complement system is tightly controlled by several regulators. In particular Factor H (FH) is preferentially acquired by pathogens conveying resistance to complement attack.

Methods: An *in vitro* phagocytosis study was performed to demonstrate the ability of *C. albicans* to bind FH and avoid the phagocytosis. The assay was performed using *C.albicans* wild type (SN-152) and knock-out strain (*hgt1Δ/Δ)*. Both strains were opsonized with human serum (HS) and stained with fluorescein isothiocyanate (FITC). Fresh PMNs cells were co-cultured with both strains and positive PMNs were detected using FACS analysis. This is complemented by an *in vivo* study employing the *Galleria mellonella* model.

Results: The wild type strain, able to bind FH, showed a reduced phagocytosis by PMNs in contrast to *hgt1Δ/Δ* knock-out strain unable to bind FH. *Galleria* larvae inoculated with *hgt1Δ/Δ* strain lived longer than those inoculated with the wild type strain.

Conclusion: CaHgt1p is not only a complement inhibitor, but also is a virulence factor, as corroborated by *in vitro* and *in vivo* data.

25. MBL is the main activator of complement on Aspergillus fumigatus conidia under immunocompromised conditions.

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Background: *Aspergillus fumigatus* is normally a non-pathogenic fungus. However, immunocompromised patients are in high risk of being fatally infected and fungal resistance to medicine is increasing. Valuable information for improved treatment could lie in the activation routes of complement on *A. fumigatus*, especially under immunocompromised conditions.

Methods: Flow cytometry experiments were performed with a clinical isolate of *A. fumigatus* using heat-inactivated resting conidia in normal human serum (NHS) under different conditions. We also applied various complement deficient sera and mimicked a compromised immune defense with umbilical cord serum (UCS).

Results: We found that Mg^{2+} amplified C3b deposition in NHS and that Ca^{2+} deprivation completely blocked the activation. Furthermore, C2 deficient serum was not functional without reconstitution of C2. Hence, AP provides a strong positive feedback loop, but classical or lectin pathway is a prerequisite for activation. Lectin pathway inhibitors in C1q deficient serum and a C1q inhibitor in MBL deficient serum decreased complement activation. The only pattern recognition molecule that affected activation in NHS was C1q. In UCS however, complement activation was dependent on MBL. An UCS pool with low MBL had a vastly lower C3b deposition than a corresponding NHS pool. Moreover we showed that inhibition of MBL in C1q deficient serum decreased phagocytosis of conidia by neutrophils.

Conclusion: C1q is the main activator of complement on *A. fumigatus* under normal immune conditions. However, this is not the profile for people getting severe infections. We propose that MBL is the main complement activator on *A. fumigatus* conidia in immunocompromised individuals.

26. Ficolins promote fungal clearance during the early stage of pulmonary infection with *Aspergillus fumigatus*.

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Background: *Aspergillus fumigatus* is a fungal pathogen causing severe and usually fatal invasive infections in immunocompromised patients. The Ficolins are pattern recognition molecules of the lectin pathway of complement and *In vitro* studies indicate a role for ficolins in the defence against *A.fumigatus.* However, little is known about their significance in fungal infections *in vivo*.

Thus, our aim of the current project was to establish a murine model of pulmonary *A. fumigatus* infection to study the relevance of ficolins in host protection against *A.fumigatus in vivo*.

Methods: Wildtype and ficolin knockout mice were infected intranasally with a sublethal dose of *A.fumigatus* conidia. Body weight and clinical signs of disease were monitored and the lungs were removed at different timepoints post-infection to assess the fungal load, expression of pro-inflammatory cytokines and neutrophil infiltration. In addition, the concentration of pro-inflammatory cytokines and PTX3 was measured in serum and bronchoalveolar lavage.

Results: After 24 hours of *A.fumigatus* infection, the pulmonary fungal load was significantly higher in ficolin knockout mice compared to wildtype mice. No statistically significant difference was observed in neutrophil infiltration and levels of PTX3 between WT and KO mice, however, the ficolin knockout mice showed impaired induction of proinflammatory cytokines in the lungs 24 hours post-infection.

Conclusion: These results demonstrate the importance of ficolins during the initial phase of *A. fumigatus* infections *in vivo*. Ongoing experiments will reveal the possible involvement of complement.

27. Antimicrobial and immunological competence of platelets

Cornelia Speth

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28. Relevance of platelets and complement for the pathogenesis of invasive fungal infections

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Background: *Aspergillus* and mucormycetes species are leading causes for invasive fungal infections. To better understand the antifungal immune reaction and relevant pathomechanisms we studied the interaction of the fungi with platelets and the complement system, two important elements of the innate immunity. We hypothesized that *Aspergillus fumigatus* secretes soluble galactosaminogalactan (GAG) which induces platelet activation and complement deposition on their surface. We expanded these studies to other *Aspergillus* species as well as to different mucormycetes.

Methods: Clinical isolates of different *Aspergillus* and mucormycete species were grown in RPMI medium for 48 hours followed by filtration to obtain the fungal supernatants (SN). Activation and complement deposition on platelets derived from healthy donors were investigated by FACS analysis using specific antibodies. GAG production was detected on the hyphae *of Aspergillus* and mucormycete spp. as stained with FITC-labeled SBA lectin.

Results: The culture supernatants of all tested clinical isolates of *Aspergillus fumigatus* and *Aspergillus flavus* triggered significant platelet activation as measured by quantification of the activation marker CD62P. In parallel, SN-induced deposition of complement factor C3 on the platelet surface as well as formation of the terminal complement complex (TCC) could be demonstrated. However, no or only weak induction of platelet activation or complement deposition on their surface could be achieved using the culture supernatants derived from *A. niger, A. versicolor* or *A*. *terreus* as well as from all mucormycetes species except one isolate of *Mucor racemosus*. These results correlated perfectly with the expression of GAG by the different fungi, as demonstrated by immunofluorescence/confocal microscopy using specific staining with SBA lectin.

Conclusion: The correlation between GAG expression by the *Aspergillus* and mucormycete species and isolates with the capacity of the fungal supernatants to stimulate platelet activation and opsonisation underlines our hypothesis that the polysaccharide GAG might be an important fungal immunomodulatory molecule. Putative consequences of its activity are platelet-mediated antifungal attack but also the formation of platelet thrombi.

29. The gut microbiome and its impact on inflammation

Alexander Moschen

Innsbruck

30. Microbial escape of innate immunity

Seppo Meri

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Infections pose a global threat because of spread of antibiotic resistance. Elucidation of microbial genomes provides new opportunities to tackle virulence mechanisms of pathogenic microbes and new immune-based interventions. We have been analyzing immune evasion mechanisms of important pathogens, like *Borrelia*. The presentation will describe mechanisms that pathogens use to escape innate immunity, particularly the complement system. This information will be important in developing new vaccines and ways to overcome microbial resistance to complement. The particular microbes we have been working on include *Borrelia burgdorferi*, meningococcus, pneumococcus, salmonella, yersinia, *Escherichia coli* and *Plasmodium falciparum*. The studies have been extended also to mosquitoes to find out why human blood does not destroy vectors for malaria and dengue fever. As an example of potential applications, new vaccines against group B meningococcus exploiting a complement factor H binding protein have been developed. Factor H (FH) is an important inhibitor of complement. Mutations in FH predispose to severe human diseases. An example is hemolytic uremic syndrome, where complement attacks self cells in contact with blood plasma (blood cells and endothelial cells). Thus, the same mechanisms we use for our own protection are used by microbes to escape immune attack.

31. FACIN, a novel complement inhibitor ….

Monika Jusko et al., *Lund University…..*

withdrawn.

32. Interaction of *Pseudomonas aeruginosa* with complement

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Background: According to the Infectious Disease Society of America, *Pseudomonas aeruginosa* is among the six top-priority dangerous drug-resistant microbes and accounts for 23% of nosocomial infections in intensive care units. Presently, no vaccination is available. Thus alternative approaches to combat this microbe are urgently needed. Among the numerous immune and inflammatory responses in response to acute infections of the lung, the complement system plays a critical role in the protection against *P. aeruginosa*. However this complement-mediated involvement in the immune response does not result in a complete elimination of the microbe, as *P. aeruginosa* acquires and binds fH, a regulator of complement activation (RCA) in fluid phase. By binding of fH on its surface, *P. aeruginosa* interferes with the activation pathways of the complement system and blocks the induction of the lytic pathway. Therefore, *P. aeruginosa* is protected against complement-mediated lysis (CML).

Methods: The objective of the PhD project is to inhibit the binding of fH to *P. aeruginosa* that should result in the efficient induction of CML, both *in vitro* and *in vivo*. For this, we will analyze the binding sites of fH to the microbe in competition assays by Western blot, ELISA and FACS utilizing fH-derived sequences. The bactericidal effect of these fH-derived peptides will be tested by in vitro lysis assays using normal human serum (NHS) and normal mouse serum (NMS) as a source of complement. To have a closer look at the *in vivo* situation C57BL/6 mice will be inoculated with *P.aeruginosa* pre-incubated with either fH-derived peptides or PBS. Mice will be sacrificed and bacterial load in the lungs will be estimated.

Conclusion: Experiments will elucidate if fH-derived peptides provide a therapeutic means to enhance the immune response against *P. aeruginosa*, which contributes to the clearance of the pathogen by the immune system in infected patients.

33. Specific acquisition of complement regulators by HCV

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Introduction: Preliminary studies indicated that HCV selectively incorporates CD59, a regulator of complement activation (RCA), while neither CD55 nor CD46 are associated with the virus. While blocking CD59 regulators, about half of the viral titer was reduced meanwhile the rest of HCV particles survived complement-mediated lysis (CML). This indicates that CD59 has an essential contribution to overcome CML, but it also suggest that other complement RCAs may be involved in the regulation process. Therefore, we want to determine whether HCV virions are also able to hijack soluble host RCAs, which could explain the remaining resistance to CML upon CD59 blockade. Furthermore our study should provide detailed information of the incorporation process of CD59 including the time point of acquisition and location of GPI-anchor. Thus our study will not only provide insight into potential evasion mechanisms but might also offer potential therapeutic options by interfering with CRPs on the virus.

Methods: To generate virus stocks the permissive Huh7.5.1 hepatoma cell line was infected with JFH1-derived cell culture HCV (ccHCV) using *RNA* electroporation. To analyze acquisition of soluble complement regulator proteins (CRP), ccHCV were opsonized with NHS followed by density gradient centrifugation. Virus containing fractions were determined by PCR and $TCID_{50}$ (infectious particles) and subsequently the presence of CRPs were analyzed via Western blot. To investigate potential involvement of E1-E2 surface glycoproteins of HCV in binding of CRPs, infectious HIV-1 based pseudo-particles expressing E1-E2 (HCV pseudo particles) were generated and investigated as described above. In addition a protein-specific capture assay, using magnetic beads to capture virus or complement proteins will be established. The location of GPI-anchored proteins, either within the budded ER-membrane or virus surrounding lipid droplets (ApoE), will be determined.

34. Complement and Toll's - searching for bottle-neck" molecules to treat inflammation

Tom Eirik Mollnes

Bodo/Oslo

35. The pig as laboratory animal in various relevant experimental *in vivo* models

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Pigs are closer anatomically and physiologically to humans, and the pig genome has higher sequence and structure homology with humans, as compared to rodents. Pigs are therefore suitable animals for medical research, and serve as an important biomedical model for human diseases and provide information that reaches far beyond what is achievable from *ex vivo* studies.

In a series of different experimental protocols, we have used landrace pigs, *sus scrofa domesticus,* of different weights (4-30 kgs), and been able to study clinical and inflammatory responses evoked by Gram negative-, Gram positive- and polymicrobial sepsis. These models induce hemodynamic alterations, capillary leak and inflammatory responses corresponding to human pathophysiology and mimic to large extent the initial phase of human septic shock. Additionally, we have studied endogenous induced inflammation in a piglet model of meconium aspiration syndrome and ischemia/reperfusion injury in an advanced and clinically relevant model of myocardial infarction. The size of the pigs allows the use of standard human monitoring equipment, including, ECG, central venous pressure, invasive blood pressure and not least pulmonary pressure. The latter is of great importance as pigs have resident macrophages in the lungs, thus extremely vulnerable to pulmonary vasoconstriction and may easily develop pulmonary hypertension. Invasive pressure monitoring and the use of thermodilution measurements provide important data on cardiac output and systemic vascular resistance, both vital hemodynamic parameters in sepsis. Large animal experiments with pigs enables repeated blood sampling for dynamic and comprehensive analysis. Furthermore, extensive organ tissue sampling during the experiments and after euthanasia is feasible, and provides detailed knowledge on how specific organs respond to systemic inflammation beyond the information gained by blood examination. The interpretation of data from these experimental studies can be integrated into a larger context were dynamic relations between physiology, inflammation, thrombogenicity and specific organs emerges. This is a strength and indeed of translational value.

36. The efficacy of upstream inhibition of complement and CD14 in sepsis and ischemia-reperfusion injury: Key results from porcine models

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Background: In the past 10 years our group has developed various experimental porcine models and studied local and general inflammation of endogenous and exogenous origin. Sepsis studies with Gram-negative and Gram-positive bacteria as well as polymicrobial induced sepsis by cecal ligation and puncture (CLP), have been conducted. In addition, a clinical relevant ischemia-reperfusion model of myocardial infarction (MI) has been developed. Interventional studies targeting complement and CD14 in these models are presented.

Methods: Landrace pigs, weighing from 4-30 kgs, were used in the protocols. In the sepsis experiments, incremental intravenous infusion of *Escherichia coli* or *Neisseria meningitides,* or CLP induced polymicrobial sepsis were studied. In the MI study, the left anterior descendant artery was ligated for 30 minutes. Inhibition of the complement component C5 and the TLR-co-receptor CD14, key upstream molecules of two main effector systems of innate immunity, were examined. A broad panel of inflammatory readouts in blood and tissues, clinical parameters, histopathology and Magnetic Resonance Imaging were utilized to evaluate the efficacy of the immunological intervention.

Results: CD14 inhibition significantly attenuated proinflammatory cytokines in blood and organs, reduced granulocyte activation and reversed the procoagulant state. C5 inhibition significantly reduced tumour necrosis factor and Interleukin-1β, as well as the procoagulant state. Combined inhibition of C5 and CD14 abolished a broad range of inflammatory mediators and reduced heart rate and pulmonary artery pressure. Combined inhibition of C5 and CD14 improved survival in polymicrobial sepsis and demonstrated a significant correlation between survival and plasma level of sC5b-9. In the MI study, C5-inhibition substantially reduced the size of infarction, improved cardiac ventricular function, and attenuated IL-1β production.

Conclusion: Using pigs as model animals, important information of inflammation driven by innate immune mechanisms were found. Intervention with inhibitors of the key innate immune effectors systems complement and CD14/TLR4 showed broad and significant reduction in inflammatory read-outs as compared to single inhibition. Taylor-made mediator targeted therapy is the future for human advanced biomedicine. We suggest an important role for combined inhibition of complement and CD14.

37. In vitro model of Ischemia/reperfusion (I/R) injuries

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Ischemia/reperfusion (I/R) injuries, tissue damage caused by restoration of the blood supply after a period of ischemia, are a serious clinical condition with a complex etiology relevant in draft survival and cardiovascular pathologies such as stroke and myocardial infarction. Recent evidence suggests that neo-epitopes exposed on the surface of the ischemic endothelium are recognized by the complement system, which in turn exacerbates the inflammatory reaction and tissue destruction.
We are interested in characterizing the interplay between the endothelium and the complement system in the pathophysiology of I/R injuries. We are currently working to mimic the conditions found in ischemic tissues using different models of the vasculature, and we will monitor changes in gene expression profiles, cell morphology and function. After setting up a robust in vitro model, we will characterize the complement system involvement in the development of I/R injuries, focusing on the recognition molecules of the lectin pathway of complement, and we will screen novel in-house produced inhibitors. Conclusions drawn from this research may be of remarkable relevance to the development of diagnostic tools and treatments.

38. The lectin complement pathway- recognition, function and regulation

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Pathogen-associated molecular pattern recognition by pattern- recognition molecules is a central hallmark of innate immunity to eliminate invading pathogens via immune defense mechanisms, such as complement activation and opsonophagocytosis. Collectins are members of the C-type lectin superfamily that recognize conserved structures exposed on pathogens, which facilitate opsonophagocytosis. In humans it comprises the classical C-type lectin superfamily consisting of mannose-binding lectin (MBL), surfactant proteins A and D, all of which possess two structural characteristics, a collagen-like domain and a carbohydrate-recognition domain, which are assembled into trimers and further organized into higher order oligomeric structures. Until recently MBL was the only human collectin known to be involved in lectin complement pathway activation sharing this feature with the ficolin protein family (ficolin-1, -2 and -3, respectively). However, recently, three novel human collectins, collectin-10 (collectin liver 1, CL-L1, CL10), collectin-11 (collectin kidney 1, CL-K1, CL-11) and collectin-12 (collectin placenta 1, CL-P1, CL-12) were identified. CL-10 and CL-11 as well as a hybrid between CL-10 and CL-11 have been detected in plasma and may function as recognition molecules in the lectin complement pathway. CL-10/-11 binds to a broad spectrum of microorganisms including bacteria, fungi and viruses in a Ca2+-dependent manner with particular specificity for carbohydrates. Thus, at present there are six initiator molecules in the lectin complement pathway. In addition CL-12 has been shown to be present in umbilical cord plasma in low concentrations, but is not a recognition molecule in the lectin pathway, but rather amplifies the alternative pathway. MBL, CL-10/-11 and the ficolins form complexes with associated serine proteases named MASP-1, MASP-2 and MASP-3 and two non-enzymatic proteins named sMAP (MAp19) and MAP-1(MAp44). MASP-1, MASP-3 and MAP-1 are all splice variants derived from the MASP1 gene, while MASP-2 and sMAP are splice variants derived from the MASP2 gene. MASP-2 has a defined role in the cleavage of C4 and C2 which leads to the formation of the C3-convertase enzyme complex, but MASP-2 needs MASP-1 to become activated in vivo. Further downstream activation leads to cleavage and deposition of C3b and culminates in formation of the terminal C5b-9 complement complex (TCC). On the other hand, MASP-1 cleaves C2, but not C4, and it has been shown that MASP-1 enhances complement activation triggered by MASP-2 complexes, but it cannot induce C3-convertase formation itself. Recent findings show that about the 75% of all C2 that becomes activated through lectin pathway activation is indeed mediated by MASP-1. MASP-1 is therefore, crucial for efficient activation of the lectin complement pathway. Regarding MASP-3 its functions and substrate specificities are still elusive, but it has been reported that MASP-3 triggers the activation of the alternative complement pathway by cleavage of pro-factor D to active D. MASP-1 appears also to have important activating roles in the coagulation and kallikrein-kinin systems. While the function of the non-enzymatic protein sMAP is unknown, both in vitro and in vivo data show that MAP-1 is a potent regulator of lectin complement

pathway activation along with C1-inhibitor, anti-thrombin and C4-binding protein. In general, genetically determined deficiency states in the lectin pathway lead to increased tendency for infections or to increased severity of certain conditions characterized by excessive inflammation. However, surprisingly mutations in the genes encoding CL-11 (COLEC11) as well as MASP-3 (the MASP1 gene) cause a severe rare human developmental syndrome, termed the 3MC syndrome. These evidences suggest a pivotal role of the lectin complement pathway not only in innate immune defense, but also in fundamental developmental processes.

39. Soluble collectin-12 (CL-12)/properdin crosstalk boosts innate immune recognition and complement activation

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Soluble defense collagens including the collectins play important roles in innate immunity. Recently, a new member of the collectin family named collectin-12 (CL-12 or CL-P1) has been identified. CL-12 is highly expressed in umbilical cord vascular endothelial cells as a transmembrane receptor and may recognize certain bacteria and fungi, leading to opsonophagocytosis. However, based on its structural and functional similarities with soluble collectins, we hypothesized the existence of a fluid-phase analog of CL-12 released from cells, which may function as a soluble pattern-recognition molecule. Immunoprecipitation and Western blot analysis of human umbilical cord plasma enabled identification of a natural soluble form of CL-12 having an electrophoretic mobility pattern close to that of shed soluble recombinant CL-12. Soluble CL-12 could recognize *Aspergillus fumigatus* (*A. fumigatus*) partially through the carbohydrate-recognition domain in a Ca^{2+}-independent manner. This led to activation of the alternative pathway of complement exclusively via association with properdin on *A. fumigatus* as validated by detection of C3b deposition and formation of the terminal complement complex. These results demonstrate the existence of CL-12 in a soluble form and indicate a novel mechanism by which the alternative pathway of complement may be triggered directly by a soluble pattern-recognition molecule.

40. MAP-1 defines a role of the lectin complement pathway as a trigger and amplifier of the coagulation system

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Cross talk between the coagulation and complement systems has been recognized, but the molecular mechanisms behind are not well established. Recently a novel regulator, MAP-1, of the lectin pathway of complement was discovered. Since the lectin pathway has been suggested to be important in the cross talk between the complement and coagulation pathways we hypothesized that MAP-1 is a key regulator in this cross talk. We established a number of different plasma and whole blood models to investigate the impact of MAP-1 at different levels in the coagulation cascade and performed in vivo experiments in a humanized mouse model of thrombosis. Our results showed that MAP-1 attenuated plasma fibrin formation, inhibited platelet activation and aggregation and decreased coagulation kinetics and amplitudes in whole blood. It reduced activation of the lectin pathway associated activation enzyme MASP-1 and FXII and generation of thrombin and kallikrein in plasma and whole blood coagulation models. Moreover, MAP-1 inhibited inhibited FeCl3 induced thrombosis in a humanized mouse. In conclusion, A hitherto novel complement and coagulation crosstalk mechanism regulated by MAP-1 was discovered.

41. Cholesterol crystals activate the lectin complement pathway via ficolin-2 and MBL – implications for the progression of atherosclerosis

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Cholesterol crystals (CC) play an essential role in the formation of atherosclerotic plaques by inducing inflammation and by functioning as an endogenous danger signal. CC activate the classical and the alternative complement pathways, but the role of the lectin pathway is unknown. In this study we hypothesized that pattern recognition molecules (PRM) from the lectin pathway bind CC and functions as an upstream innate inflammatory signal in the pathophysiology of atherosclerosis. We investigated the binding of the PRMs mannose-binding lectin (MBL), ficolin-1, ficolin-2, and ficolin-3, the associated serine proteases, and complement activation products to CC using recombinant proteins, specific inhibitors as well as deficient and normal sera. Binding was assessed by flow cytometry and microscopy. The results showed that the lectin pathway was activated on CC by binding of ficolin-2 and MBL, resulting in activation and deposition of complement activation products. MBL bound to CC in a calcium dependent manner while ficolin-2 binding was calcium independent. No binding was observed for ficolin-1 or ficolin-3. Moreover, we demonstrated that IgM, but not IgG bound to CC and that C1q binding was facilitated by IgM.

In conclusion our study demonstrates that PRMs from the lectin pathway recognize CC and provides evidence for an important role for this pathway in the inflammatory response induced by CC in the pathophysiology of atherosclerosis.

42. Fine-tuning of dendritic cell functions by complement-opsonized HIV

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43. Complement-opsonization of *A. fumigatus* modifies dendritic cell function

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Background: In this study, interactions of dendritic cells (DCs) with complement-opsonized and non-opsonized *Aspergillus fumigatus* strains and various mutants thereof were investigated. The opsonization pattern of the different strains and mutants, the binding and internalization by dendritic cells as well as the cytokine secretion and initial signaling pathways were investigated.

Methods: Fungi were opsonized using normal human serum as complement source. The opsonization pattern, binding of conidia to DCs and internalization were characterized by FACS analyses. Inhibition of fungal growth in presence of DCs and interactions with complement receptors were detected using confocal microscopy. Furthermore, phosphorylation of ERK1/2 and p38 were detected by immunoblot analysis.

Results: We could demonstrate in this study that melanin and β-1,3-glucan have high impact on the fungal virulence compared to the wildtype *Aspergillus* strains. With respect to dendritic cell binding and internalization complement-opsonization of conidia enhanced these processes compared to their non-opsonized counterparts independent on the fungal strain used.

Conclusion: These data revealed, that melanin and β-1,3- glucan are key effectors of masking complement deposition and binding of conidia by DCs. However opsonization of swollen conidia enhanced internalization in DCs as well as production of pro-inflammatory cytokines, thereby resulting in a favorable T_H1 immune response. These *in vitro* studies propose that the use of immune cells, like DCs or neutrophils, in combination with complement opsonins might act as potent vaccines against invasive aspergillosis.

44. An investigation of the expression and activation of intracellular complement in children with acute lymphoblastic T cell leukemia (T-ALL).

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The complement system plays a central role in the elimination of microbes and dying host cells, but recent literature illuminates that certain complement proteins are of importance in relation to cell growth and survival. Almost all immune cells - incl. T- and B-cells have receptors that can bind complement, and through complement receptors activate specific cellular processes. But complement proteins can also be activated intracellularly and it has been shown that particular complement proteins are of vital importance for the differentiation and function of specific lymphocyte populations such as T cells. Resting human CD4+ T cells contain intracellular stores of C3 and the protease cathepsin L (CTSL) in endosomal and lysosomal compartments, and CTSL cleaves C3 into C3a and C3b. Intracellular C3a can thus bind to the lysosomal-localized receptor, C3aR, and induce T-cell survival. This auto- and paracrine interaction between the complement system and the T cell will consequently determine T cell function and activity.

Since dysregulated cell growth is one of the hallmarks of lymphocytic leukemia, it is our hypothesis that overexpression and intracellular cleavage of C3 is a part of the pathophysiology of acute T cell leukemia and we will therefore examine whether differences in the presence and expression of intracellular C3 and CTSL in patients with acute lymphoblastic T cell leukemia is of pathophysiological and prognostic significance.

The study population consists of selected Danish T-ALL patients; mRNA from diagnostic bone marrow samples and frozen live cells. We will study the leukemic cells by means of flow cytometry, fluorescence microscopy and gene expression analysis.

45. The complement modulator SALSA in placenta

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Background: The salivary scavenger and agglutinin (SALSA) has known functions in the antimicrobial defense at the mucosal surfaces. We have previously shown that SALSA also modulates the activation of the complement system, both in solution and on surfaces. Furthermore, we recently described the presence of SALSA in amniotic fluid. Strict regulation of the maternal immune response to the fetus is essential for a healthy pregnancy, and complications are often accompanied by immune attack towards components of placenta or the fetus itself. In the placenta this involves activation of the complement system.

Methods: In this study we investigated the potential role of SALSA in pregnancy by analyzing its presence in amniotic fluid and placental tissue during healthy and complicated pregnancies. SALSA levels in amniotic fluid were investigated using ELISA, and the expression in placenta was analyzed by fluorescence immunohistochemistry.

Results: SALSA levels in amniotic fluid increased during pregnancy. Before 20 weeks of gestation the levels were slightly higher in patients who later developed pre-eclampsia than in gestation age-matched controls. In the placenta syncytial damage is often followed by the formation of fibrinoid structures. SALSA was found clustered into these fibrinoid structures in partial co-localization with complement C1q and fibronectin. *In vitro* analysis showed direct protein binding of SALSA to fibronectin.

Conclusion: In addition to antimicrobial defense, the data presented here suggest that SALSA, together with fibronectin and C1q, play a role in immunological regulation during pregnancy and may be involved in the containment of injured placental structures into fibrinoids.

46. The Viral Vector Vaccine VSV-GP as Vaccine Platform

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Background: Our group has recently shown that VSV pseudotyped with the glycoprotein (GP) of the lymphocytic choriomeningitis virus, VSV-GP, is a potent vaccine vector, overcoming limitations of wild type VSV.

Objective: Here, we evaluated the potential of VSV-GP as a vaccine vector for infectious disease such as HIV.

Methods: We incorporated antigens from pathogens, e.g. different variants of HIV envelope, or marker genes into the genome of VSV-GP and generated infectious viruses via reverse genetics. These viruses were analyzed *in vitro* for antigen expression, location and conformation. After mouse immunization studies distribution and kinetics of infected cells and antigen-specific as well as vector-specific immune responses were analyzed.

Results: Infectious viruses containing antigens from HIV or marker genes such as luciferase were generated. HIV envelope variants were expressed in VSV-GP infected cells and incorporated into VSV-GP particles. Crucial epitopes for the induction of neutralizing antibodies against HIV such as MPER, CD4 binding side and V1V2 loop were present on the surface of VSV-GP-env particles. The addition of an extra gene did not attenuate VSV-GP replication. After intramuscular immunization, viral replication was limited to injection side and the draining lymph nodes. No neutralizing antibodies against VSV-GP were induced even after seven boost immunizations. Therefore, homologous boost immunization was highly efficient and high titers of HIV-specific antibodies were induced.

Conclusion: Taken together, VSV-GP is non-neurotoxic, induces potent immune responses, enables boosting and thus is a promising novel vaccine vector platform.

47. Interaction of Shiga toxin and other EHEC virulence factors with the innate immune system

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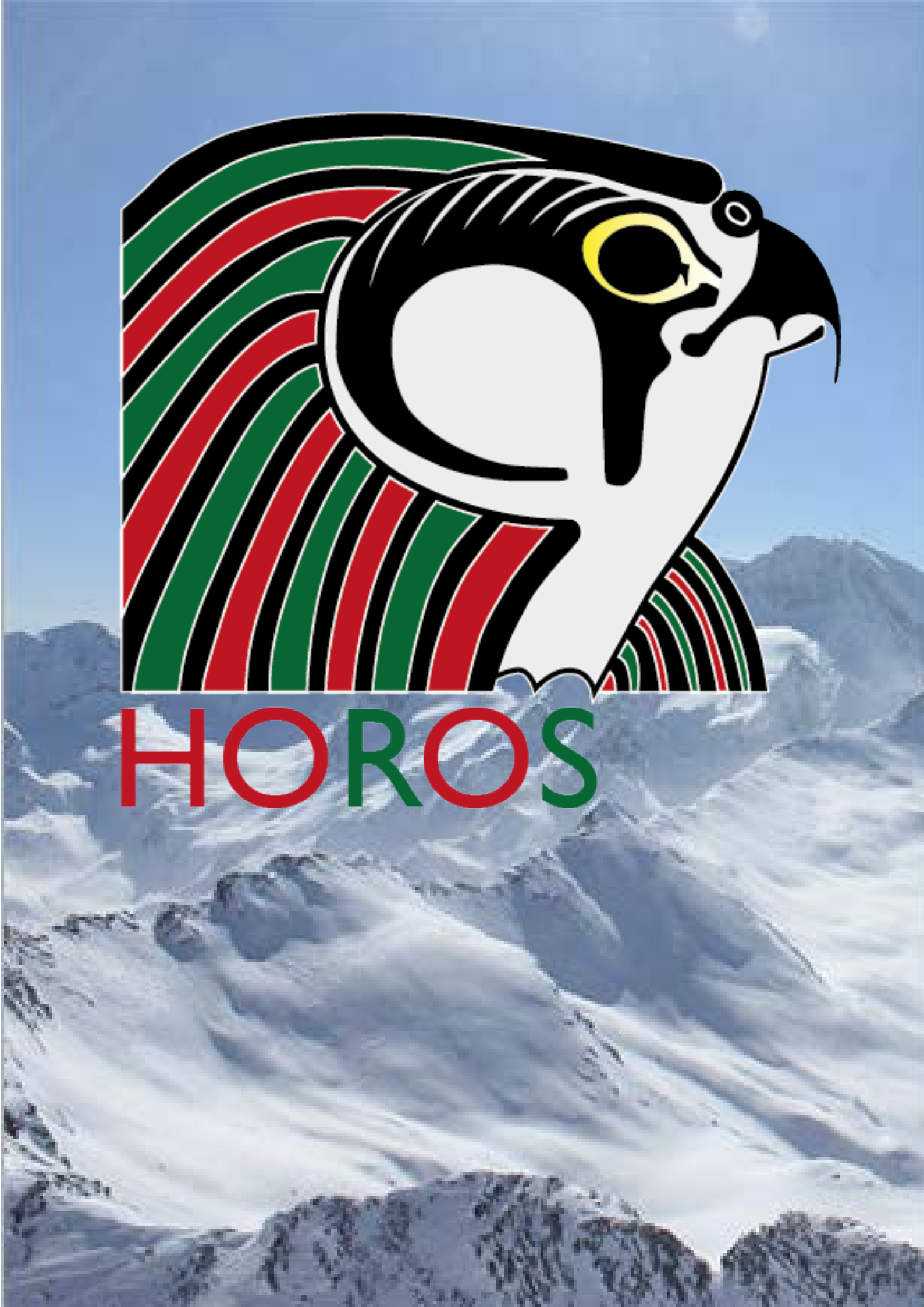
Hemolytic uremic syndrome (HUS) is defined by a characteristic set of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Shiga toxin produced by enterohemorrhagic *Escherichia coli* (EHEC) activates complement via the alternative pathway. It also binds to Factor H thus reducing its cofactor activity contributing as a major virulence factor involved in the pathogenesis of EHEC associated HUS (eHUS). Complement pathways unveil a high degree of amplification that necessitates the regulation to prevent attack of self. Membrane bound regulator CD59 inhibits complement activation on host cell surface by binding to C8 and C9 thus inhibiting terminal complement complex (TCC) assembly. Shiga Toxin 2 reduces CD59 expression on human renal tubular epithelial and glomerular endothelial cells (Ehrlenbach *et al.,*2013). Thus complement plays a crucial role in the execution of innate immunity which has been well characterized in the blood but requires attention for its evidence in gut.

Lipocalin 2 (LCN2) or Neutrophil Gelatinase Associated Lipocalin (NGAL) which is a dynamic 25 kDa protein with roles in innate immunity and in a variety of pathologies has been intensively used as a biomarker for acute kidney injury (AKI) and shows potential as an adjunctive tool to improve risk prediction in patients with eHUS.

Following ingestion, EHEC migrates through the gut attaching to the mucosal epithelial cells of the large intestine further translocating into the circulation, colonizing vulnerable target organs like kidneys and the brain. Although the mechanism has been widely investigated in the terminal zone of action, there remain ample possibilities to investigate its action in the gut which often leads to bloody diarrhea and thus destruction of gut mucosa.

The aim of this study is to inspect the interactions of shiga toxin and other associated EHEC virulence factors not just in the circulation but also in the gut. Particularly the prospect of a probable reciprocal action between shiga toxin and lipocalin is intriguing. This work also intends to explore the possible downregulation of CD59 in gut cells. Preliminary results will be presented in the symposium.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