

Free Communications

FC1 Cytokine and Cell Signaling

FC1-1

Signaling mechanism of extracellular RNA in endothelial cells

Fischer S¹, Nishio M¹, Gerriets T², Walberer M², Preissner K¹¹Justus-Liebig-Universität/ Biochemisches Institut, Giessen, Germany ²Justus-Liebig-Universität/ Neurologisches Institut, Giessen, Germany

Objectives: Extracellular RNA as well as the artificial analogue poly:IC have been shown to induce vascular endothelial growth factor (VEGF)- dependent hyperpermeability in endothelial cells, related to edema formation in vivo.

Methods: For permeability studies primary cultures of porcine brain-derived microvascular endothelial cells (BMEC) and for Western blot analysis and ELISA studies the human brain endothelial cell line HCEC/D3 were used.

Results: RNA, but not DNA, initiates cell activation by binding of VEGF to neuropilin-1 (NRP-1, a coreceptor of VEGF receptor 2, VEGF-R2), followed by VEGF-R2 phosphorylation and intracellular signaling involving an increase of intracellularly released Ca²⁺. Accordingly, RNA-induced phosphorylation of VEGF-R2 as well as changes of the permeability were abolished after pretreatment of cells with antisense oligonucleotides or neutralizing antibodies against NRP-1. Pretreatment of cells with heparinase (releasing glycosaminoglycans and VEGF) totally abrogated the RNA-induced permeability changes, whereas RNA together with VEGF completely restored VEGF-R2-mediated signal transduction. RNA-induced intracellular signaling also induced exocytosis of Weibel-Palade bodies leading to the release of RNase 1, which is partly stored in Weibel-Palade bodies. Furthermore, agents known to induce degranulation like thrombin or VEGF induced the release of RNase 1 immediately following stimulation.

Conclusion: These results indicate that extracellular RNA serves an important cofactor function to engage VEGF for VEGF-R2-dependent signal transduction, reminiscent of the coreceptor mechanism mediated by proteoglycans. Endothelial RNase 1 might serve as the natural antagonist of extracellular RNA in the vascular system.

FC1-2

Activated protein C regulates RhoA dependent podocyte survival via protease activated receptor-3

Thati M¹, Hongjie W, Kashif M, Ilya V, Khurram S, Nawroth P, Isermann B¹Department of Internal Medicine-1 and Clinical Chemistry, University of Heidelberg, Heidelberg, Germany

We have recently shown that APC protects against diabetic nephropathy by inhibiting high glucose induced endothelial and podocyte apoptosis. In endothelial cells APC inhibits apoptosis via protease activated receptor-1 (PAR-1) and endothelial protein C receptor (EPCR). However, it is not known how APC modulates podocyte function. Expression of coagulation regulators, receptors and signalling intermediates was determined by RT-PCR and immunoblotting in immortalized differentiated human podocytes. Apoptosis in podocytes was analyzed by TUNEL. EPCR-deficient mice (d/d EPCR) were made diabetic using streptozotocin and were analyzed after six months. In striking contrast to the enhanced diabetic nephropathy in mice with impaired PC-activation (TMPPro/Pro mice) albuminuria was not aggravated in diabetic d/dEPCR, indicating that APC mediates nephroprotection at least in part independent of EPCR. In vitro APC prevented high glucose or Puromycin induced apoptosis in podocytes at 2 and 20 nm. APC also inhibited in vitro correlates of podocyte effacement, such as PAN induced loss of actin stress fibres and podocyte migration. Of note, these cyto-protective effects of APC are mediated via PAR-3 and are independent of PAR-1 and EPCR. APC induced ERK1/2 and AKT phosphorylation. Inhibition of ERK1/2 phosphorylation, but not of AKT phosphorylation, abolished the anti-apoptotic effect of APC. Furthermore, inhibition of Rho kinase (Y27632) abolished the anti-apoptotic effect of APC in stressed podocytes. In conclusion, we demonstrate that APC mediates cyto-protective effect in podocytes via PAR-3/RhoA and ERK1/2. These data identify that PAR-3 mediates APC-dependent cellular signalling and establish a novel mechanism through which APC mediates cytoprotection.



FC1-3

Vasodilatory prostaglandins suppress protease-activated receptor-1 in human vascular smooth muscle cells via inhibition of nuclear factor of activated T-cells

Rosenkranz A¹, Rauch B¹, Freidel K¹, Schrör K¹

¹Institut für Pharmakologie & Klinische Pharmakologie, Universitätsklinikum Düsseldorf, Germany

Objective: Prostacyclin downregulates protease-activated receptor (PAR)-1 in human vascular smooth muscle cells (VSMC) via cyclic AMP and protein kinase A (PKA). We have investigated NFAT (nuclear factor of activated T-cells) as a potential mediator of this effect.

Methods: Human VSMC were serum-deprived for 48h prior to study. Protein and mRNA levels were quantified by western blotting and real-time PCR, promoter activity by luciferase reporter assay.

Results: NFAT inhibition with cyclosporin A (CsA) or siRNA suppresses PAR-1 mRNA and protein expression and impairs functional responsiveness (interleukin-6 induction; ERK1/2 phosphorylation) to thrombin or PAR-1 activating peptide (TFLLRN). CsA or mutation of the NFAT consensus-site blunts PAR-1 promoter activity, as does the prostacyclin analogue iloprost. CsA and iloprost induce NFAT translocation from the nucleus to the cytosol, indicating inactivation, and attenuate NFAT/PAR-1 promoter binding interactions in an electrophoretic mobility shift-assay. PKA inhibition reverses the effects of iloprost. PAR-1 mRNA and protein expression are also suppressed by the EP2 receptor agonist butaprost and phorbol-myristoyl-acetate which induces cyclooxygenase-2 and endogenous prostaglandin generation. Thus transcriptional regulation of PAR-1 is a general property of Gs-coupled prostaglandin receptors. Stimulation of the "effector protein activated by cyclic AMP" (EPAC) does not influence PAR-1 expression.

Conclusion: In this study, we provide first evidence that prostaglandins regulate PAR-1 thrombin receptors in human VSMC through PKA-dependent inhibition of NFAT transcriptional activity. This suggests an endogenous protective mechanism by which cyclooxygenase-2-derived prostaglandins, via modulation of downstream effectors such as NFAT and IL-6, may limit the atherothrombotic and inflammatory actions of thrombin after vascular injury.

FC1-4

Effects of G-CSF on systemic inflammation, coagulation and platelet activation in patients with acute myocardial infarction

Schulz S¹, von Wedel J¹, Schömig A¹, Kastrati A¹, Ott I¹

¹Deutsches Herzzentrum TU München, Germany

Background: In the prospective, randomised, double-blind, placebo-controlled Results of the Regenerate Vital Myocardium by Vigorous Activation of Bone Marrow Stem Cells (REVIVAL)-2 trial in patients with acute ST-elevation myocardial infarction and successful mechanical reperfusion stem cell mobilization with rh-G-CSF (10µg/kg s.c. for 5 days) was not associated with a reduction in infarct size or improvement in myocardial function. Aim of this substudy was to assess the impact of G-CSF on systemic inflammatory and procoagulant responses and platelet activation.

Methods: On day one and five after G-CSF or placebo systemic cytokines IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF were measured. For assessment of prothrombotic activity prothrombin fragment F1+2 and circulating tissue factor activity were analyzed. Platelet activation was characterized by cell surface expression of the activated fibrinogen receptor (PAC-1), P-selectin and CD40L in flow cytometry.

Results: Administration of G-CSF was associated with a significant increase in TNF, IL-6 and IL-12. In accordance the decrease in circulating C-reactive protein was diminished in the G-CSF group as compared to the placebo group indicating enhancement of systemic inflammatory responses by G-CSF. Moreover, circulating prothrombin fragments F1+2 were significantly elevated after G-CSF. In contrast circulating tissue factor activity was comparable in both groups. There was also no difference in platelet activation assessed by cell surface expression of PAC-1, CD40L and P-selectin.

Conclusion: Treatment with G-CSF in patients with acute myocardial infarction induced a release of proinflammatory cytokines and an enhanced thrombin formation. This was not caused by an increase in circulating tissue factor or platelet activation.

FC1-5

High glucose upregulates protease-activated receptor-4 (PAR-4) in human vascular smooth muscle cells

Dangwal S¹, Rauch B¹, Schrör K¹, Rosenkranz A¹

¹Institut für Pharmakologie & Klinische Pharmakologie, Universitätsklinikum Düsseldorf, Germany

Objective: Diabetes promotes thrombosis, inflammation and vascular remodeling. Thrombin stimulates smooth muscle cell (SMC) mitogenesis via protease-activated receptors (PAR-1, PAR-3, PAR-4). We investigated if high glucose transcriptionally regulates PARs in human vascular SMC.

Methods: Human saphenous vein SMC maintained in normal glucose (5.5 mm) were stimulated with high glucose (25 mm) ± study drugs. PAR mRNA, protein expression and promoter activity were determined by real-time PCR, western blotting/immunofluorescence and luciferase reporter assay.

Results: PAR-1 and PAR-3 were not regulated by high glucose. PAR-4 mRNA was induced 3-fold within 1.5h (n=7, P<0.05); Significant upregulation was seen at 96h (n=7-10, P>0.05), accompanied by increased protein expression (n=4, P>0.05). PAR-4 promoter activity was also induced at 6-24h (n=4, P<0.05), indicating regulation at transcriptional level. Accordingly, stimulatory effect of PAR-4 activating peptide (GYPGQV, 200µM) on TNFα expression was enhanced in SMC pretreated (48h) with high glucose. Since diabetes is reported to induce cyclooxygenase-2, we investigated if prostaglandins could regulate PAR-4. Exogenous PGE2 (1µM) or the stable prostacyclin analogue cicaprost (10nM) suppressed PAR-4 mRNA at 6-24h (n=5, P<0.05). Similar downregulation by the adenylate cyclase activator forskolin (10µM) and the presence of a CREB site in the PAR-4 promoter implicates cyclic AMP as a possible counterregulatory signal to transcriptionally control PAR-4.

Conclusion: High glucose induces a rapid and sustained induction of PAR-4 in human vascular SMC, while vasodilatory prostaglandins have the opposite effect. Since PAR-4 contributes to the mitogenic actions of thrombin, such transcriptional regulation might be relevant for the enhanced vascular remodeling in diabetes.

FC1-6

Circulating microparticles express oxidation-specific epitopes that are recognized by natural IgM antibodies

Perkmann T^{1,2}, Hörkkö S³, Wagner O¹, Witztum J⁴, Binder C^{1,2}

¹Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Austria,

²CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria,

³Department of Internal Medicine, Biocenter Oulu, University of Oulu and Clinical Research Center of Oulu University Hospital, Finland, ⁴Department of Medicine, University of California, San Diego, USA

Cell derived microparticles (MP) have been shown to have pro-coagulatory and pro-inflammatory properties in vitro, and increased levels of circulating MPs have been found to be associated with higher risk for cardiovascular disease. We hypothesize that circulating MPs mediate some of their pro-inflammatory properties via the expression of oxidized lipid moieties implicated in atherogenesis, and that atheroprotective natural IgM antibodies (Abs) bind these epitopes on MPs. Using flow cytometry we could first demonstrate that in vitro generated microparticles isolated from culture supernatants of stimulated endothelial cells and monocytes express oxidation epitopes recognized by specific monoclonal Abs. Accordingly, we then characterized isolated circulating MPs from the plasma of 18 healthy volunteers using monoclonal natural IgM Abs against oxidized phosphatidylcholine (EO6), malondialdehyde (LRO4), oxidized cardiolipin (LRO1), and a control IgM Ab. While the control IgM bound less than 1.0±0.7% of circulating MPs, 8.4±3.7%, 37.9±9.7%, and 11.3±5.3% were recognized by EO6, LRO4, and LRO1, respectively. Moreover, consistent with our hypothesis, we could show that 20.2±8.2% of circulating MPs carry endogenous IgM Abs bound to their surface, and subsequent characterization of these MP-bound IgM demonstrated their specificity for oxidation epitopes, such as MDA. In summary, we found that a relevant percentage of circulating MPs in healthy humans carry various oxidized lipid moieties on their surface and that these same MPs have IgM Abs bound to their surface. Our findings suggest a novel functional role for oxidation-epitope specific IgM Abs, namely in neutralizing pro-inflammatory effects of oxidized lipids on circulating MPs.

FC2 Coagulation Factors – New Insights from the Benchmark

FC2-1

Structural analysis of six novel coagulation Factor XIII subunit A missense mutations

Ivaskевичius V¹, Schroeder V², Kohler H³, Rott H⁴, Petrides P⁵, Biswas A¹, Knoefler R⁶, Oldenburg J¹

¹Institute of experimental Haematology and Transfusion Medicine, Bonn, Germany, ²University of Leeds, Leeds, UK, ³University Clinic Bern, Bern, Switzerland, ⁴Haemostaseology Ambulance, Duisburg, Germany, ⁵University of Munich, Munich, Germany, ⁶University Clinic Dresden, Dresden, Germany

Objectives: In previous studies we identified six novel Factor XIII subunit A gene (F13A) mutations (Tyr167Cys, Pro289Arg, Arg540Gln, Gly592Ser, Arg611His, Asp668Gly) resulting in Factor XIII Deficiency. The aim of this study was to characterize these missense mutations by structural analysis.

Methods: Structural analysis was based on the crystal structure of the FXIII A2-homodimer obtained from the Protein Data Bank (ref. no. 1F13) and was performed with the Deep View/Swiss-Pdb Viewer Software, version 3.7.

Results and Conclusions: The Tyr167 residue is located at the edge of the beta-sandwich domain. Substitution with the mutant Cys167 residue (which has a smaller side chain) might hinder side chain interactions and therefore may destabilize the protein molecule. The replacement of the Pro289 (situated in a tightly packed region

of the core domain) with the bulkier Arg289 residue might cause clashes with the surrounding residues leading to conformational changes. Arg540 is situated in the outer part of barrel 1 where its positively charged side chain may be important for interactions with the outer hydrophilic environment. The mutant Gln residue on the other hand has no charged side chain for the possible hydrophilic interactions and this may have a destabilizing influence on the molecule. Gly592Ser mutation may disturb the loop (Gln590-Gln601) of the two beta-strands thereby possibly destabilizing the barrel 1 domain. The Arg611His and Asp668Gly mutations predict the absence of hydrogen bonds with neighbouring residues (Glu356 and Thr293 respectively). This may lead to destabilization and conformational changes of FXIII A subunit protein.

FC2-2

mRNA-expression of genes involved in the Vitamin K-cycle in mice

Watzka M¹, Czogalla K¹, Westhofen P¹, Marinova M¹, Müller J¹, Oldenburg J¹
¹Inst. f. Exp. Hämatologie und Transfusionsmedizin, Universitätsklinik Bonn, Germany

Background: The vitamin-K-cycle is necessary for posttranslational modification of vitamin-K-dependent peptides. These proteins are involved in coagulation as well as in calcium metabolism. Dietary vitamin-K-quinone needs reduction to vitamin-K-hydroquinone, which serves as cofactor in gamma-carboxylation, leading to biologically active Gla-domains in the previously mentioned proteins. This process is performed by the enzyme GGCX, which in turn generates vitamin-K-epoxide as a by-product. Subsequent reduction of vitamin-K-epoxide to the quinone form and further to the hydroquinone form by VKORC1 completes a recycling mechanism known as the vitamin K cycle. To gain deeper insight into this important physiological pathway, we analysed the tissue specific expression of GGCX, VKORC1 and the isozyme VKORC1L1 in mice.

Methods: Twenty-eight different tissue were prepared from each 5 female and 5 male mice. Samples were snap frozen and RNA isolated using Qiagen RNeasy-kit. RNA was reverse transcribed into cDNA to act as template for real-time-PCR. Standards were prepared from plasmids containing VKORC1, GGCX, and VKORC1L1-cDNA, respectively. The housekeeping gene hydroxymethylbilane synthase was used for equilibration.

Results and Discussion: The genes tested were expressed in almost all tissue. The highest expression of VKORC1 and GGCX was found in liver where coagulation factor synthesis takes place. Intermediate expression of both genes can be observed in kidney, brain, and lung. Most interestingly, the isozyme VKORC1L1 shows highest expression in brain and intermediate expression in lung tissue. This protein is thought to play a role in cellular antioxidation and the expression pattern may suggest brain as the main target for VKORC1L1 action.

FC2-3

Force dependent structural changes of the von Willebrand Factor A2 domain are required for VWF cleavage by ADAMTS13

Schneppenheim R¹, Baldauf C², Budde U³, Obser T¹, Pieconka A³, Schneppenheim S³, Graeter F²
¹Universitäts-Klinikum Hamburg-Eppendorf, Hamburg, Germany, ²CAS-MPG Partner Institute for Computational Biology, SIBS, Shanghai, China, ³Aesculab Labor Hamburg, Coagulation Lab, Hamburg, Germany

Background: Function of the multimeric, shear-flow sensitive von Willebrand factor (VWF) in primary haemostasis is regulated by its protease ADAMTS13. However, the specific proteolytic site located in the VWFA2 domain is predicted to be buried (Sutherland et al. 2004 J Mol Model p259), suggesting requirement of prior VWFA2 unfolding for ADAMTS13 cleavage. VWFA1 and VWFA3 flanking VWFA2 each possess a disulfide-bonded loop that apparently locks them in the folded state. In contrast, lack of an analogous disulfide-bond in the A2 domain, presumably enables unfolding of A2 by shear stress in blood vessels. The artificial mutation N1493C in VWFA2 should generate a disulfide-bonded loop analogous to those in VWFA1 and VWFA3 thereby inhibiting VWFA2 unfolding and ADAMTS13 proteolysis.

Methods: A homology model of the wt and mutant VWFA2 domain (N1493C) was validated by molecular dynamics (MD) simulations. Force-probe MD simulations were applied to wtVWFA2 to investigate unfolding. Recombinant wt and mutant full length VWF were digested by rADAMTS13, and analyzed by electrophoresis.

Results and Discussion: Subjecting the termini of the A2 domain to a pulling force mimicking the tension in stretched VWF leads to stepwise unfolding of VWFA2 in force probe MD simulations. The cleavage site gets exposed and accessible to ADAMTS13. In contrast mutant VWFA2N1493C is locked in the folded state and resistant to ADAMTS13 which was confirmed by our experimental studies.

Conclusion: Our theoretical and experimental approach suggests that locking of VWFA2 prevents a shear response and emphasizes the requirement of a force sensitive VWF structure for ADAMTS13 proteolysis.

FC2-4

Four novel KLKB1 mutations in severe prekallikrein deficiency

Geisen C¹, Farac J¹, Delev D¹, Kadar J², Siegemund A³, Zieger B⁴, Seiffried E¹, Oldenburg J⁵
¹DRK Blutspendedienst BW-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt, Germany, ²Praxis-Labor für Transfusionsmedizin, Köln, Germany, ³Institut für Klinische Chemie, Gerinnungslabor, Leipzig, Germany, ⁴Zentrums für Kinder- und Jugendmedizin, Freiburg, Germany, ⁵Institut für Experimentelle Hämatologie und Transfusionsmedizin, Bonn, Germany

Objectives: Hereditary prekallikrein (PK) deficiency is an extremely rare entity. Despite a marked aPTT-prolongation PK deficiency does not lead to a haemorrhagic disorder. Since contact system factor activation initiates fibrinolysis, PK deficiency might however result in a hypercoagulable state. Here we describe the clinical phenotype and the genetic findings in seven patients with severe PK deficiency. **Methods:** Mutation analysis was performed by direct sequencing of the KLKB1 gene on an automated sequencing system (ABI Prism 3100). The genomic DNA of 200 healthy blood donors was analysed as a control group. Molecular modelling was performed using the PyMOL software.

Results: In three homozygous and four compound heterozygous patients sequence analysis of the KLKB1 gene revealed six different mutations, two missense mutations, one nonsense mutation, two small deletions and one small insertion. Four of these have not been reported before (c.451dupT, c.717_719delCTT, c.689T>A, c.1165delA). One of the novel variants (c.689T>A, p.Ile230Asn) was also detected in the control DNA of one blood donor. As this amino acid is highly conserved and as the mutation generates a novel n-glycosylation motif in the protein, it is predicted that this mutation affects protein function. None of the patients displayed any bleeding tendency. In one patient a history of myocardial infarction was reported.

Conclusion: So far five different mutations of the KLKB1 gene have been described. Consequently, with the results of this study the number of the known mutations nearly doubles. However it remains uncertain whether total PK deficiency may lead to a prothrombotic phenotype in man.

FC2-5

Recombinant human coagulation Factor IX (rFIX): influence of serine phosphorylation and tyrosine sulfation on pharmacokinetic properties in FIX-knock-out mice

Böhm E¹, Dockal M¹, Hasslacher M¹, Konetschny C¹, Mitterer A¹, Muchitsch E¹, Reiter M¹, Scheiflinger F¹
¹Baxter Innovations GmbH, Wien, Austria

Objectives: Recombinant Factor IX (rFIX) from Chinese hamster ovary (CHO) cells differs in pharmacokinetics from plasma-derived FIX (pdFIX). Although rFIX is close to pdFIX in structure and function, differences in post-translational modifications exist. The lower degrees of phosphorylation of serine 155, and of sulfation of tyrosine 158 have been hypothesized in literature to be causative for the 30-50 % lower in-vivo recovery of CHO-rFIX observed in clinical studies. However, mean residence time and terminal half-life were similar. To prove the assumption that a higher degree of phosphorylation and sulfation improves rFIX in-vivo recovery, we used HEK293 cells for rFIX production.

Design and Methods: A rFIX-producing clone was generated by stable transfection of HEK293 cells. rFIX was produced and purified from one fermentation run using two different down-stream processes: the first to enrich high-phosphorylated and -sulfated rFIX, the second to purify total rFIX at high yield. For pharmacokinetic comparison in FIX-knock-out mice, both HEK293-rFIX materials, CHO-rFIX, and pdFIX were administered in the same buffer.

Results: In-vivo recovery and area under the curve were statistically significantly higher for high phosphorylated and sulfated rFIX than for total rFIX derived from HEK293 cells. However, both parameters were lower for both HEK293-rFIX preparations than for CHO-rFIX, and lower for CHO-rFIX than for pdFIX. This may be due to glycosylation differences observed between preparations. Mean residence times and terminal half-lives were similar for all.

Conclusions: In summary, these findings emphasize that the degree of sulfation and -phosphorylation influences the pharmacokinetic properties of rFIX.

FC2-6

Experimental validation of computational predicted branchpoints in human prothrombin intron 13

Algner M¹, Andag R¹, Oellerich M¹, von Ahnen N¹
¹Abt. Klinische Chemie/Zentrallabor, Göttingen, Germany

Objective: The prothrombin (F2) intron 13 mutation 19911A>G affects splicing and causes elevated prothrombin levels and eventually thrombosis. We studied further intronic sequence features, specifically whether predicted branchpoints could be validated experimentally.

Method: For prediction of the branchpoints we entered the sequence of prothrombin Intron 13 into the EBI-ASD Database (www.ebi.ac.uk). The top-ranking



predicted branchpoints BP1-BP5 were inactivated by site directed mutagenesis. Following this we measured the splicing efficiency as luc β -gal activity by double reporter assay (1). Experiments were repeated 5 times in duplicates.

Results: Predicted branchpoint scores were 7.2 (BP1), 6.7 (BP2), 4.4 (BP3), 4.3 (BP4) and 4.2 (BP5). Corresponding reporter gene activities (setting F2 19911A to 100) were: BP1 99 (13) [mean(SD)], BP2 1 (1), BP3 103 (15), BP4 89 (10), BP5 84 (15). This revealed that BP2 is the putative branchpoint.

Conclusion: We find that EBI-ASB predicted branchpoints in intron 13 did not agree well with experimental data. BP1 had the highest hit with a score of 7.2 but did not influence splicing. BP2 had a score of 6.7 and is the putative branchpoint and only identified branchpoint in intron 13. Branchpoints BP3 to BP5 had scores between 4.8 and 3.4 but did not influence splicing. It is advisable to validate the predictions from the EBI-ASD-database by double reporter assay or RT-PCR. The EBI database is a good starting point for finding branchpoints but results are rather unspecific. 1. von Ahsen N., Oelerich M., Blood 2004;103:588

SY/FC5 Pediatric Session

SY/FC5-2

Influence of ABO(H) blood group on Factor VIII and von Willebrand Factor levels in children

Klarmann D^{1,3}, Eggert C², Geisen C¹, Becker S³, Kreuz W³, Seifried E¹, Klingebiel T³

¹Institut für Transfusionsmedizin und Immunhämatologie, DRK Baden Württemberg-Hessen, Germany, ²MDK Hessen, Oberursel, ³Klinikum der J.W. Goethe-Universität, Frankfurt, Germany

Background: The modulation of factor FVIII:C, VWF:Ag as well as VWF:RCO by the ABO(H) blood group is well established in adults. The conversion of the straight-chain I to the branched-chain I structure is regulated to occur after birth.

Objective: Given the reduced activity of β -1,6-N-acetylglucosaminyltransferase involved in the branching of ABO(H) bearing structures during the first 18 months of life we reasoned that if the relationship between ABO blood group and VWF levels were causal, no such difference should be observed in the first months of life, and the difference might be attenuated throughout childhood.

Patients and Methods: We undertook to quantitatively assess FVIII:C and vWF parameters in 574 presumably healthy children aged 1 month – 18 years and correlated the observed values with ABO(H) blood type.

Results: Differences between blood group O vs. non-O values of FVIII:C, VWF:Ag and VWF:RCO were not observed in infancy, became apparent during childhood, and reached the magnitude described for adults in adolescence.

Conclusion: We confirm the fundamental difference in the regulation of the hemostatic system between adults and children particularly in the first year of life, and provide pediatric reference intervals which are specific for the testing system used.

SY/FC5-3

Blockade of maternal anti-HPA-1a-mediated platelet clearance by an HPA-1a-epitope-specific F(ab')₂ in an in vivo mouse model of alloimmune thrombocytopenia

Bakchoul T^{1,2}, Boylan B², Sachs U¹, Bein G¹, Ruan C³, Santoso S¹, Newman P¹

¹Institute for Clinical Immunology and Transfusion Medicine, Justus-Liebig University, Giessen, Germany, ²Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, USA, ³Jiangsu Institute of Hematology, the First Affiliated Hospital of Soochow University, Suzhou, China

Objective: Neonatal alloimmune thrombocytopenia (NAIT) is most commonly caused by transplacental passage of maternal HPA-1a antibodies that bind to fetal platelets and mediate their clearance. SZ21, a monoclonal antibody directed against platelet glycoprotein IIIa, competitively inhibits the binding of anti-HPA-1a alloantibodies to platelets in vitro. The purpose of this investigation was to determine whether SZ21 F(ab')₂ fragments might be therapeutically effective in inhibiting or displacing maternal HPA-1a antibodies from the fetal platelet surface and preventing their clearance from circulation.

Design and Methods: Resting human platelets from HPA-1ab heterozygous donors were injected into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Purified F(ab')₂ fragments of SZ21 or control IgG were injected intraperitoneally 30 minutes prior to introduction of HPA-1a antibodies. Blood samples were taken periodically and analyzed by flow cytometry to determine the percentage of circulating human platelets.

Results: Anti-HPA-1a IgG from NAIT-cases were able to efficiently clear HPA-1a-positive platelets from murine circulation. Administration of SZ21 F(ab')₂ fragments not only inhibited binding of HPA-1a antibodies to circulating human platelets, preventing their clearance, they also displaced bound HPA-1a antibodies from the platelet surface.

Conclusion: F(ab')₂ fragments of HPA-1a-selective mAb SZ21 effectively inhibit anti-HPA-1a-mediated clearance of human platelet circulating in an in vivo NOD/SCID mouse model. These results suggest that agents that inhibit binding of anti-HPA-1a to platelets may have therapeutic potential in the treatment of NAIT.

SY/FC5-4

A novel Antithrombin Mutation (g.6566A>G:Lys222ARG) causing hereditary antithrombin deficiency in a 16-year-old girl

Fischer R¹, Heidering K¹, Blüters Sawatzki R², Pavlova A³, Oldenburg J³, Kemkes-Matthes B¹

¹Haemostasis Center, University Hospitals Giessen and Marburg GmbH, Germany, ²Department of Children, University Hospitals Giessen and Marburg GmbH, Germany, ³Experimental Haematology, University Hospital Bonn, Germany

Background: Antithrombin (AT) is the major inhibitor of blood coagulation, inactivating thrombin and factor Xa. Individuals with inherited AT deficiency have an extremely increased risk for thromboembolic events. The prevalence of inherited AT deficiency is about 0.2%. Sometimes functional AT tests give normal results despite of antithrombin defects – thus, AT deficiency won't be detected in those cases.

Case report: 16 year old girl, body weight 80kg, with spontaneous severe pulmonary embolism. Thrombophilia screening was normal concerning: Factor V Leiden Mutation, Prothrombin (G20210) Polymorphism, MTHFR (C677T) Polymorphism, PT, aPTT, fibrinogen, antithrombin, aPC ratio, protein C, protein S, factor VIII:c, factor XII and antiphospholipid antibodies. Lipoprotein(a) was 116 mg/dl. Antithrombin was measured using Berichrome AT III (A) from Dade Behring. Antithrombin level was normal (79 % of n.). Due to extremely high dosage of LMWH Dalteparin needed (48000 IE/d) to reach therapeutic anti factor Xa levels, we performed sequencing of the AT gene. Therewith, a new missense Mutation in exon 4 (g.6566A>G:Lys222Arg) of the AT gene was detected. Moreover, we were able to demonstrate a 50 % reduced binding capacity of antithrombin to LMW-Heparin in vitro.

Conclusions: 1. The new Antithrombin Mutation described (g.6566A>G:Lys222Arg) is clinically important due to lack of LMWH effect. 2. Patients with need of extremely high heparin dosage should be considered as AT deficient, even when AT levels are normal. 3. A gap in the diagnostics of Antithrombin deficiency was shown. 4. Prevalence of inherited AT deficiency might be much higher as actually estimated.

SY/FC5-5

Thrombin generation in pediatric patients with Crohn's disease and ulcerative colitis

Bernhard H¹, Deutschmann A¹, Novak M¹, Heidl H¹, Rosenkranz A¹, Leschnik B¹, Muntean U¹

¹Department of Pediatrics, Medical University of Graz, Austria

Background and Aim: In adults, inflammatory bowel disease (IBD) is associated with an increased risk of thromboembolic complications. This might be caused by increased levels of hemostatic parameters like factor V, VII, VIII and fibrinogen, caused by the chronic inflammatory process. Also the pathogenesis of IBD is not really clear and a high thrombin activity might contribute to disease progression. We measured thrombin generation by means of Calibrated automated Thrombography (CAT) to detect this hypercoagulable condition in children with IBD. Results of CAT shows a high interindividual variability, but measures rather constant over time in one person. We wanted to see whether children with IBD have a higher thrombin generation.

Methods: Plasma samples were collected of twenty-two pediatric patients with IBD and of sixty healthy controls. Age ranged from 10 to 19 years. Thrombin generation was measured by means of CAT. The disease activity was estimated, using the Pediatric Crohn's Disease Activity Index (PCDAI).

Results: There was a significant increase of endogenous thrombin potential (ETP), lag time and time to peak (TTP) in patients with IBD, while peak showed no difference to healthy controls. ETP and F1+F2 in children with IBD also showed a significant correlation with PCDAI and fibrinogen.

Conclusion: Our study shows that IBD in children is associated with high thrombin generation, but this seems to be caused mainly by the inflammatory process than by an individual disposition.

FC3 Epidemiology of Thrombosis

FC3-1

The long-term clinical course of patients with thrombosis involving the inferior vena cava

Linnemann B¹, Kraft C¹, Hecking C¹, Lehmeier S¹, Schwonberg J¹, Zgouras D¹, Schindewolf M¹, Lindhoff-Last E¹

¹J.W.Goethe University Hospital, Division of Vascular Medicine, Frankfurt am Main, Germany

Background: Inferior vena cava thrombosis (IVCT) is rare and data about its long-term clinical course are scarce.

Methods: We used the prospective MAISTHRO registry to identify IVCT patients and enrolled 60 patients (36 females, 24 males), treated in our University Hospital since March 2000. Median age at IVCT manifestation was 36.5 years [9–83]. The

clinical presentation and complications during long-term follow-up were registered, and patients' outcome was compared to that of patients with isolated lower extremity deep vein thrombosis (LE-DVT) matched for gender and age.

Results: IVC thrombosis was the initial VTE event in 47 patients (78.3%). Concomitant symptomatic pulmonary embolism was observed in 16 (27%) IVCT patients and 7 (12%) patients with isolated LE-DVT ($p=0.064$). Clinical outcome data were obtained from 59 patients (98%). During a median observation time of 32 months [3 months to 36 years], 6 IVCT patients (10%) and 4 (7%) LE-DVT patients died (n.s.). Of those who survived, 12/53 (23%) IVCT patients and 16/53 (30%) LE-DVT patients presented with clinical symptoms indicating chronic venous insufficiency at follow-up (n.s.), using the revised CEAP criteria. Digital photoplethysmography (D-PPG) revealed pathologic results in 70% (30/43) of IVCT patients and 73% (38/52) of those with LE-DVT (n.s.). Cumulative VTE recurrence rates were 20% (12/60) and 21.1% (12/57) in IVCT and LE-DVT patients, respectively (n.s.).

Conclusions: Thus, we finally conclude that the long-term clinical course of patients with previous thrombosis involving the inferior vena cava is not worse when compared to patients with isolated lower extremity DVT.

FC3-2

Thrombophilic risk factors and recurrent deep venous thromboembolism (DVT): Concept for long-term anticoagulation based on the individual risk of lethal pulmonary embolism (PE) and lethal bleeding using oral anticoagulants

Zolt R¹, Sucker C², Araba F³, Bux I³, Gerhardt A⁴

¹Praxis fuer Haemostaseologie und Transfusionsmedizin, Düsseldorf, Germany, ²LaboMed - Gerinnungszentrum Berlin, Germany, ³MVZ für Blutgerinnungsstörungen und Transfusionsmedizin, ⁴Blutgerinnung Ulm, Germany

Objectives: Indications for an indefinite oral anticoagulation are a matter of debate in patients with a first unprovoked proximal DVT.

Methods: An individual risk-benefit analysis is made using published prospective studies by determining the patient-specific lethal risk of bleeding under oral anticoagulation compared with the estimation of lethal PE-risk by type of initial thrombosis (spontaneous vs. secondarily caused, with or without PE).

Results: According to this risk-benefit analysis, long-term oral anticoagulation is indicated to prevent lethal PE in all patients with low risk of bleeding (1% per year, 0.1% lethal bleeding per year) in the risk group with lethal PE > 0.2% per year. This risk group includes patients with idiopathic proximal thrombosis and PE in the initial event (also without thrombophilic risk factors) and patients with an idiopathic initial event without PE, who have relevant thrombophilic risk factors with a relative risk > 2, such as antithrombin deficiency, homozygous Factor V Leiden or a combined heterozygous Factor V Leiden and prothrombin G20210A mutation. In case of a higher bleeding tendency (0.3% lethal bleeding per year in a patient group with 1-2 bleeding risk factors like age >65 or diabetes) other risk-benefit estimations are present.

Conclusions: Our individual risk stratification is in contrast to current therapy recommendations, which generally consider long-term oral anticoagulation for patients with an idiopathic initial proximal DVT with a low bleeding risk, but do not specify these in individual cases.

FC3-3

Factor XIII Val34Leu polymorphism in patients surviving or not surviving ischemic stroke

Shemirani A^{1,2}, Antalfy B^{1,3}, Pongrácz E⁴, Muszbek L^{1,5}

¹Clinical Research Center, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary, ²Erzsébet Hospital, Central Laboratory, Sátoraljaújhely, Hungary, ³Department of Pathology, Diósgyőri Vasgyári Hospital, Miskolc, Hungary, ⁴Department of Neurology, State Health Center, Budapest, Hungary, ⁵Haemostasis, Thrombosis and Vascular Biology Research Group of the Hungarian Academy of Sciences at the Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

Objectives: A few reports have been published concerning the effect of FXIII-A Val34Leu polymorphism on the risk of ischemic stroke, however the results were inconclusive. In these studies stroke survivors and non-survivors were not analyzed separately. Here, we investigated the effect of the polymorphism on the occurrence and the lethal outcome of ischemic stroke.

Design and methods: FXIII-A Val34Leu genotype of 508 patients (male/female: 157/351) who survived ischemic stroke (mean age: 53) and 508 age and sex matched controls was determined using melting point analysis and fluorescence resonance energy transfer detection. DNA samples for genotyping were also isolated from tissue sections of 461 patients (male/female: 223/238) died of ischemic stroke (mean age: 73) and the results were compared to a sex matched population control group (mean age: 64).

Results: Neither homozygous nor heterozygous Val34Leu polymorphism changed the risk of the occurrence of non-lethal stroke significantly. Leu34 carriership was

also without significant effect. Similarly, heterozygous Val34Leu polymorphism and Leu34 carriership did not confer a significant risk of stroke with lethal outcome. However, homozygosity for Val34Leu polymorphism represented a significant risk of lethal stroke with an OR: 1.467 (CI: 1.106-1.945; $p=0.006$). The risk was higher in females (OR: 1.707, CI: 1.150-2.533; $p=0.008$); while in males the increase of the risk was not statistically significant (OR: 1.267, CI: 0.834-1.925; $p=0.267$)

Conclusions: Only the homozygous form of FXIII-A Val34Leu polymorphism had a significant effect on the risk of ischemic stroke, and even that influenced only the severity (lethal outcome) of stroke in females.

FC3-4

D-Dimer and prothrombin fragment 1+2 predict venous thromboembolism in cancer patients – results from the Vienna Cancer and Thrombosis Study (CATS)

Ay C¹, Vormittag R¹, Dunkler D², Quehenberger P³, Wagner O³, Zielinski C⁴, Pabinger I¹

¹Clinical Division Of Haematology And Haemostaseology, Department Of Medicine I, Medical University Of Vienna, Austria, ²Core Unit for Medical Statistics and Informatics, Section of Clinical Biometrics, Medical University Of Vienna, Austria, ³Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Austria, ⁴Clinical Division of Oncology, Department of Medicine I, Medical University of Vienna, Austria

Objectives: Venous thromboembolism (VTE) is a well-recognized complication of cancer. Laboratory parameters might be useful to assess VTE risk in cancer patients. The aim of this study was to investigate D-Dimer and prothrombin fragment 1+2 (F1+2), reflecting activation of blood coagulation and fibrinolysis, for prediction of cancer-associated VTE.

Design and Methods: CATS is a prospective observational cohort study of patients with newly diagnosed cancer or progression of disease who did not recently receive chemotherapy, radiotherapy or surgery. The study endpoint is occurrence of objectively confirmed symptomatic VTE. D-Dimer and F1+2 plasma levels were measured with standardized assays. Kaplan-Meier and Cox-regression analyses were applied for statistical calculation.

Results: Eight-hundred-and-twenty-one patients with malignancies of the breast (n=132), lung (n=119), stomach (n=35), colorectal (n=106), pancreatic (n=46), kidney (n=22) and prostate (n=101) and high-grade glioma (n=102), lymphoma (n=94), multiple myeloma (n=17) and other tumour types (n=47) were followed for a median [IQR] observation period of 501 [255-731] days. VTE occurred in 62 (7.6%) patients. The cut-off level for elevated D-Dimer and elevated F1+2 was set at the 75th percentile of the total study population. In multivariable analysis the hazard ratio (95% CI) of VTE in patients with elevated D-Dimer (1.8 [1.0-3.2], $p=0.048$) and elevated F1+2 (2.0 [1.2-3.6], $p=0.015$) was statistically significantly increased including age, sex, surgery, chemotherapy and radiotherapy. The cumulative probability of developing VTE after 6 months was highest in patients with both elevated D-Dimer and F1+2 (15.2%) compared to patients with non-elevated D-Dimer and F1+2 (5.0%) ($p<0.001$).

Conclusions: High D-Dimer and F1+2 levels independently predict occurrence of VTE in cancer patients.

FC3-5

Substantial increase of mortality risk in primary care patients with diabetes mellitus or peripheral arterial disease: epidemiological study

Diehm C¹, Darius H², Habert R³, Pittrow D⁴, Mahn M⁵, Allenberg J⁶, Tepohl G⁷, Trampisch H⁸

¹Innere Medizin, SRH-Klinikum, Karlsbad-Langensteinbach, Germany, ²Vivantes Klinikum, Berlin Neukölln, Germany, ³Neurologische Klinik, Städt. Klinikum München GmbH, Germany, ⁴Institut für Klin. Pharmakologie, TU Dresden, Germany, ⁵Medizinische Abteilung, Sanofi-Aventis Berlin, Germany, ⁶Universitätsklinikum Heidelberg, Germany, ⁷Angiologische Praxis, München, Germany, ⁸Inst. für Medizinische Informatik, Biometrie und Epidemiologie, Ruhr-Univ. Bochum, Germany

We aimed to assess the 5-year risk of death associated with peripheral arterial disease (PAD) and/or diabetes mellitus (DM) in elderly patients in primary care. In a monitored prospective cohort study (German epidemiological study on ankle brachial index, getABI), 6,880 unselected patients ≥ 65 years are followed up by 344 representative primary care physicians in Germany since November 2001. PAD was defined as ankle brachial index (ABI) <0.9 determined with standard Doppler sonography. DM was defined as physician diagnosis, use of antidiabetic medication, or HbA1c >7%. At baseline, mean age was 72.5 yrs, 58% were females, 46% (ever) smoker, 65% had hypertension, 52% lipid disorders, 25% DM, and 20.8% PAD. Compared to patients without PAD or DM (8.5%), 5-year mortality in patients with DM alone (12.6%), PAD alone (17.0%) or both diseases (28.1%) was substantially increased. On multivariate analysis in the Cox model, with adjustment for age, gender and the known cardiovascular risk factors the hazard ratio (HR) for mortality, compared to patients without PAD and DM, was 1.6 [95% confidence interval 1.3-2.0] for patients with PAD alone, 1.6 [1.3-1.9] for patients with DM alone, and 3.0 [2.5-3.7] for patient with PAD+DM. Patients with PAD (as indicator disease for generalized atherosclerosis) or DM, and particularly those with the two diseases concomitantly, carry a substantially increased risk for all-cause mortality.



Our data confirm the necessity to screen for these high-risk patients in primary care to ensure timely treatment of the atherosclerotic risk factors and the metabolic condition.

FC3-6

Reduced endothelial progenitor cells in type 2 diabetic patients with microalbuminuria and macroalbuminuria

Hoellerl F¹, Brix J², Feder A², Koppensteiner R¹, Scherthaner G², Scherthaner G²
¹Medical University of Vienna, Internal Medicine II, Angiology, Vienna Austria, ²Rudolfstiftung Hospital, Medicine I, Vienna, Austria

Objectives: Type 2 diabetic (T2D) patients presenting with microalbuminuria (Mi-A) or macroalbuminuria (Ma-A) have an increased risk for cardiovascular morbidity and mortality. Endothelial progenitor cells (EPC) are bone marrow derived and predict macrovascular disease and mortality. Thus, it was of interest to investigate the potential role of EPC in T2D patients presenting with Mi-A or Ma-A in comparison with normoalbuminuric (No-A) patients.

Design and Methods: 138 patients with T2D were included: 72 No-A, 42 Mi-A and 24 Ma-A. The patients in the three groups did not differ for age, diabetes duration, HbA1c, BMI, systolic and diastolic blood pressure, total cholesterol, LDL-cholesterol, triglyceride as well as serum creatinine. EPC (CD34+/133+/309+) were enumerated by flow cytometry in peripheral blood.

Results: EPC were decreased in Mi-A vs. No-A: 102 ± 54 vs. 144 ± 84 , $p=0.01$. In Ma-A, EPC were even more decreased (53 ± 29 vs. 144 ± 84 ; $p<0.001$). Mi-A or Ma-A were also significantly different for EPC numbers (102 ± 54 vs. 53 ± 29 , $p<0.001$). Multivariate regression revealed that EPC were independently associated with diabetes duration (Beta=-0.165, $p=0.036$) and history of cardiovascular disease (Beta=-0.203, $p=0.01$) but strongest with status of albuminuria (Beta=-0.380, $p<0.001$).

Conclusions: In conclusion, this is the first study demonstrating decreased numbers of endothelial progenitor cells in T2D patients with microalbuminuria or macroalbuminuria. Since low EPC are important predictors of future cardiovascular morbidity and mortality in nondiabetic high risk patients, these new findings could be relevant for the understanding of the high cardiovascular risk of T2D patients with microalbuminuria or macroalbuminuria.

FC4 Clinical Research in Hemorrhagic Disorders

FC4-1

Frequency of functional polymorphisms of the Factor VII gene and its relevance for FVII deficiency (FVIID) subjects and blood donors

Wulff K¹, Members of the International study group FVII deficiency
¹Institut für Humangenetik, Greifswald, Germany

More than 1000 FVII deficiency patients (FVIID) were investigated in the „Greifswald registry FVII“ (GR FVII). In these investigations 135 different causative FVII gene mutations were detected. In FVIID family studies the influence of functional FVII polymorphisms of the FVII:C levels was found. FVIID individuals without causative FVII mutation very often carry exclusively FVII-reduced polymorphisms. In the control group of 199 German blood donors the frequencies of FVII polymorphisms and their haplotypes were analysed. FVII mutations and polymorphisms of patients of „Greifswald registry FVII“ were detected by sequencing (ABI sequencing systems ABI 377) according to previously described methods. The polymorphisms g.-402G>A, g.-401G>T, g.-324_g.-325ins10bp, g.-122T>C, g.G>A, g.7880C>T, VNTR (37bp) intron 7, g.10976G>A and g.11293_94insAA of 199 blood donors were analysed by previously reported PCR based methods. In 399 FVII alleles of the controls, 6 different haplotypes (combination of polymorphisms) were analysed. 98% of all FVII alleles carry the three main haplotypes: type I in 63% of all with common forms, haplotype II in 24% with the FVII increasing polymorphic form g.-402A and haplotype III in 11% containing the rare polymorphic forms g.-324ins10bp, g.-122C, g.73A, g.7880T, p.Q353 and 3' UTR insAA. Haplotype III represents the FVII-reducing haplotype. We demonstrate that heterozygous FVII carriers of a causative FVII mutation and „FVII increased FVII haplotype II“ have FVII:C > 50% and were very often not identified. Whereas heterozygous individuals with the genotype causative FVII mutation and FVII-reduced polymorphisms (haplotype III) preferential were identified in the clinical practice.

FC4-2

Survival analysis of patients with haemophilia at the adult haemophilia care center in Vienna, Austria

Reitter S¹, Waldhör T², Vutuc C², Lechner K¹, Pabinger I¹
¹Medizinische Universität Wien, Universitätsklinik für Innere Medizin I, Austria, ²Medizinische Universität Wien, Zentrum für Public Health, Austria

Survival of patients with haemophilia is still a matter of particular interest. We performed a survival analysis in 226 patients with haemophilia A and B (128 severe haemophiliacs), who were treated at the adult haemophilia care center in Vienna. We requested information from the Austrian Death Register, concerning mortality in our patient cohort. The findings showed that 96/226 patients (42.5%) had died between 1983 and 2006. 37 (38.5%) died from HIV-infections, 15 from HCV-infections, 15 from bleedings (15.6% respectively) and 29 (30.2%) from various other causes. Regarding co-infections, the mortality of HIV-positive patients was 74.3% (n=55) and that of HCV-positive patients was 40.4% (n=55) for the analysed period. The patient mortality rates were compared with those of the general Austrian male population after adjustment for age and calendar period. We found that the cumulative relative survival of all patients was decreased to 0.694 (95% CI 0.614–0.767). In patients with severe haemophilia (F VIII or IX level < 1%) the cumulative relative survival was decreased to 0.489 (0.394–0.579), but normal (0.986; 95% CI 0.858–1.082) in patients with mild or moderate haemophilia (F VIII or IX level 2–50%). The survival rate was lowest in HIV-positive patients (0.287; 95% CI 0.186–0.398), but was also decreased to 0.874 (0.776–0.951) in HIV-negative patients. We conclude that survival of patients with severe haemophilia is still decreased compared to those with non-severe haemophilia and the general male population, regardless of HIV-infection.

FC4-3

Genetic and clinical variability in 40 families with dysfibrinogenemia

Sittinger K¹, Miesbach W², Heller C³, Weiss D⁴, Bidlingmaier C⁵, Seifried E¹, Oldenburg J⁶, Geisen C¹
¹DRK Blood Donor Service, University Hospital Frankfurt, Germany, ²Haemophilia Center, University Hospital Frankfurt, Germany, ³Paediatrics, University Hospital Frankfurt, Germany, ⁴University Hospital Erlangen, Germany, ⁵University Hospital München, Germany, ⁶University Hospital Bonn, Germany

Objectives: Congenital dysfibrinogenemia is highly heterogeneous in respect to mutational spectrum as well as clinical presentation ranging from asymptomatic to bleeding, abortion or thrombosis. Rare variations like amyloidosis or hepatic storage disease are also reported. Here we present the results of a genotype-phenotype correlation in 40 families with dysfibrinogenemia.

Design and Methods: FGA, FGB and FGG genes of dysfibrinogenemia patients were analysed by direct sequencing on an automated sequencing system (ABI Prism 3100).

Results: Genetic testing revealed the most common variation at amino acid residue 16 (Arg16Cys, Arg16His, Arg16Gly) of the FGA gene in approximately half of dysfibrinogenemia patients. 30% of them demonstrated severe bleeding symptoms (mostly prolonged bleeding after trauma), 25% suffered from severe thrombotic events like apoplex and pulmonary embolism whereas the major part did not present any clinical symptom. Patients with some mutations in the FGB (Arg14Cys and Arg44Cys) and in the FGG gene (Asn319_Lys320del and Ala 327Thr) showed an increased tendency towards severe thrombotic events at a very young age (mostly sinus vein thrombosis). In one asymptomatic patient the missense mutation Arg-375Trp in the FGG gene was detected causing hepatic endoplasmatic reticulum storage disease. Additional analysis of the patients' family revealed elevated transaminase levels.

Conclusions: Our data suggest that there is apparent correlation between certain mutations and the clinical phenotype. Additionally a good characterization of clinical history may help to identify modifier genes. Thus, molecular diagnosis may predict clinical manifestation and improve the treatment of dysfibrinogenemia patients.

FC4-4

Secondary prophylactic versus on-demand treatment with sucrose-formulated recombinant Factor VIII in adults with severe haemophilia A: results from a 13-month study

Collins P¹, Faradji A², Morfini M³, Enriquez M⁴, Gorina E⁵, Schwartz L⁶, Lemm G⁵
¹University Hospital, Cardiff, UK, ²Hautepierre Hospital, Strasbourg, France, ³University Hospital, Firenze, Italy, ⁴Bayer HealthCare AG, Wuppertal, Germany, ⁵Bayer HealthCare Pharmaceuticals, Berkeley, CA, USA, ⁶Bayer HealthCare Pharmaceuticals, Montville, NJ, USA

Objectives: In severe haemophilia A on-demand therapy cannot prevent chronic haemophilic arthropathy and guidelines thus recommend primary prophylaxis in children and adolescents. This prospective clinical trial in adults compared the

effect of late secondary prophylaxis with sucrose-formulated recombinant Factor VIII (rFVIII-FS) versus on-demand treatment on the number of joint bleeds.

Design and Methods: In this 13-month study, men (30–45 years) with severe haemophilia A and frequent bleeding, treated on-demand (>100 exposure days; no inhibitors), received rFVIII-FS on-demand for 6 months. Patients then switched to prophylactic rFVIII-FS (20–40 IU/kg t.i.w.) for 7 months with the first month a stabilization run-in period. Bleeds and health-economics parameters were assessed throughout the study. Gilbert score and Haemo-QoL questionnaire at baseline, after 6 and 13 months.

Results: 20 patients received mean doses of 31.3 IU/kg/wk and 87.3 IU/kg/wk during on-demand and prophylaxis periods, respectively. With prophylaxis the mean numbers of joint and total bleeds decreased significantly (1.5 ± 2.1 and 1.9 ± 3.3 , respectively) compared with on-demand treatment (18.5 ± 11.6 and 23.7 ± 13.3 ; $P < 0.001$) and >50 % of patients were bleeding free. Mean total Gilbert scores indicated better joint function at the end of prophylaxis. There were no statistically significant differences between treatments in the pharmacoeconomic variables, mean total Haemo-QoL score, or number of patients reporting adverse events during on-demand and prophylactic treatment. No de-novo inhibitor was observed.

Conclusions: Prophylaxis with rFVIII-FS reduced the frequency of joint and other bleeds compared with on-demand treatment in previously treated adults with severe haemophilia A.

FC4-5

Assessment of the between-laboratory and the long-term within-laboratory analytical variation of Factor VIII testing

Meijer P¹, De Maat M¹, Verbruggen B¹

¹ECAT Foundation, Leiden, The Netherlands

Introduction: The measurement of Factor VIII clotting activity is used for both the diagnosis of haemophilia A and the monitoring of treatment and therefore requires precise laboratory measurement. Therefore we assessed the between-laboratory variation and the long-term analytical imprecision of individual laboratories for Factor VIII testing.

Methods: Test results of Factor VIII of 187 laboratories from the external quality assessment programme of the ECAT Foundation were evaluated for the period 2005 – 2007. The between-laboratory variation (BCV) was assessed at different Factor VIII levels. The long-term analytical coefficient of variation (LCVa) was assessed using a linear regression model (P. Meijer et al. Clin Chem 2002;48: 1011–15). The LCVa is a measure for the long-term analytical performance of a laboratory.

Results: The BCV (%) for > 25 % for Factor VIII levels < 20 U/dL and $10 - 20$ % for levels > 20 U/dL. The mean LCVa was 15.7 % (95 % range: 5.1 – 39.9 %). A significant effect on the LCVa was observed for the activator ($p=0.002$), deficient plasma ($p=0.001$), calibrator ($p=0.009$) and equipment ($p=0.003$) used in the test. This was the first time such an evaluation was performed for Factor VIII testing.

Conclusion: A considerable variation was observed for both the BCV and the LCVa. Application of the long-term linear regression model allows the investigation of factors that play a role in the imprecision of laboratory testing.

FC4-6

Contribution of polymorphisms in the IL-10, TNF- α , CTLA-4 genes and HLA class II molecules to the risk of inhibitor development: A case-control study on 260 patients with severe haemophilia A

Pavlova A¹, Delev D¹, Oldenburg J¹

¹Institute for Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Bonn

Background: Development of Factor VIII inhibitors is the most severe and challenging complication of haemophilia treatment. In this case-control study we characterized HLA class II type and several polymorphisms in the IL-10, TNF- α , and CTLA-4 genes in 260 severe haemophilia A patients with and without inhibitors.

Materials and Methods: All polymorphisms were investigated by direct sequencing. The HLA typing was performed by SSO and SSP.

Results and Discussion: Two polymorphisms in the IL-10 gene showed association with inhibitor development. A predisposing association was found for the A>G polymorphism at -1082 (OR of 1.5, 95%CI 1.0–2.1, $p=0.012$). A week protective tendency was registered for the G12 allele in the CA repeat polymorphism (OR=0.5). In the TNF- α gene only the -308 G>A polymorphism showed association. Homozygosity for the -308A allele was described in 12 patients. Ten (83.3 %) were with inhibitors compared to 2 (16.6 %) without inhibitors (OR of 4.6 95%CI 0.9–21.4, $p=0.033$). No polymorphism in the CTLA-4 gene was found to be associated with inhibitor development. The DRB1*15 allele was more frequently presented in inhibitor patients corresponding to an OR of 1.9 (95%CI 1.1–3.1, $p=0.012$) compared to non-inhibitor patients. Furthermore, the DQB*06 allele was found more frequently in inhibitor than non-inhibitors (OR- 1.7; 95%CI 1.1–2.6, $p=0.012$).

Conclusions: In conclusion these findings suggest that inhibitor formation in patients with haemophilia is a complex process, involving not only the Factor VIII gene mutations but also polymorphisms in immunoresponse genes including those coding for IL-10, TNF- α and MHC class II molecules.

FC5 Understanding Platelet Structure and Biology

FC5-1

Identification of G protein-coupled receptors (GPCRs) in human platelets by mRNA expression profiling

Hansen A¹, Spath B², Bokemeyer C², Eifrig B², Langer F²

¹Universitätsklinikum Hamburg-Eppendorf, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Germany, ²Universitätsklinikum Hamburg-Eppendorf, Onkologisches Zentrum, II. Medizinische Klinik und Poliklinik, Germany

Background: GPCRs play critical roles in hemostasis, thrombosis, and vascular development. However, our knowledge of the distributions and functions of GPCRs in cells and tissues of the body, including megakaryocytes and platelets, is incomplete. In part, this is because they are encoded by non-abundant mRNAs and are fully functional at levels of $< 1 \times 10^4$ proteins per cell.

Methods and Design: We use multiplex (50 primer pairs) one-step RT-PCR to amplify GPCR transcripts, and dye label and hybridize the amplicons to an array of 55-base oligonucleotides. This technique is more sensitive and specific than conventional cDNA or oligonucleotide microarrays. In addition, the comparison of GPCR profiles generated from 100 ng and 10 ng of total RNA allows for discrimination between relatively abundant and rare receptor transcripts. Using this novel technology, we studied the GPCR mRNA expression profile of human platelets isolated from leukocyte-free platelet concentrates.

Results: In total, we detected 119 GPCRs, and this list included all transcripts of GPCRs previously described in platelets. In addition, we detected an array of GPCRs previously unrecognized on human platelets, including receptors for various short- and long-chain fatty acids, chemokines (e.g. CXCL9–11,13 and XCL1,2), and (neuro)hormones (e.g. head activator, neurotensin, galanin, relaxin-3, α -melanotropin, thyrotropin, and prokineticins 1,2). For 39 GPCRs the natural ligand has not yet been identified (orphan receptors).

Conclusions: The obtained GPCR mRNA expression profile may provide a valuable platform for further research into the role of this pharmacologically highly relevant group of receptors in megakaryopoiesis and platelet biology.

FC5-2

Fractalkine desensitizes platelets to platelet inhibition by the endogenous platelet inhibitor prostacyclin

Flierl U¹, Bauersachs J¹, Schäfer A¹

¹Medizinische Universitätsklinik Würzburg, Germany

Background: Fractalkine plays a pivotal role in atherogenesis. Platelet activation is a central factor in atherosclerosis progression. We previously reported that fractalkine induces platelet degranulation and P-selectin expression facilitating platelet-leukocyte adhesion. Fractalkine attenuates the bioavailability of the important endogenous platelet inhibitor nitric oxide.

Methods: The influence of preincubation of platelets with fractalkine (1 μ g/ml) on platelet inhibition by the platelet inhibitor prostaglandin E1 (PGE1) was assessed by two assays: first, the ability to prevent PGE1-induced attenuation of ADP-induced platelet aggregation, and second, the capability to decrease PGE1-induced vasodilator-stimulated phosphoprotein (VASP)-phosphorylation determined by flow-cytometry. Samples were preincubated with fractalkine and stimulated with different concentrations of PGE1.

Results: PGE1 significantly inhibited ADP-induced platelet aggregation. In samples pre-treated with fractalkine, prevention of ADP-induced platelet aggregation by PGE1 was abrogated. While secondary platelet aggregation was prevented by PGE1 (1 μ M), preincubation with fractalkine for 10 minutes reestablished secondary platelet aggregation. These results indicate that fractalkine desensitized platelets to PGE1. PGE1-induced VASP-phosphorylation was significantly attenuated by preincubation with fractalkine in a time- and concentration-dependent pattern, whereby a maximum effect was achieved after preincubation of platelets with fractalkine for 10 minutes, which reduced PGE1 (1 μ M)-induced VASP-phosphorylation by ~25 % ($p < 0.01$ vs. PGE1 without fractalkine).

Conclusion: Fractalkine desensitizes platelets to PGE1 suggesting that platelet inhibition by the endogenous platelet inhibitor prostacyclin is affected by fractalkine. These results indicate a new pathomechanism by which this atherogenic chemokine facilitates relevant steps leading to platelet activation and leukocyte recruitment, namely rendering platelets less responsive to endogenous platelet inhibition.



FC5-3

Complex formation of platelet Factor IV with extracellular RNA results in alteration of PF4 properties

Jaax M¹, Krauel K¹, Füllr B¹, Appelt B², Müller S², Fischer S³, Preissner K³, Greinacher A¹
¹Institut für Immunologie und Transfusionsmedizin, Greifswald, Germany, ²Institut für Biochemie, Greifswald, ³Institut für Biochemie, Giessen, Germany

Objectives: Extracellular RNA has recently been shown to activate the intrinsic clotting system and enhance thrombus formation (Kannemeier et al., PNAS 2007). Under certain conditions extracellular RNA may locally reach high blood concentrations. As a polyanionic compound, RNA can directly interact with basic proteins such as heparin-binding cytokines, serving as an essential cofactor for signal transduction (Fischer et al., Blood 2007). Based on these findings we assessed the interaction between RNA and the basic polypeptide PF4 and functional consequences thereof.

Design and Methods: PF4 was purified from human platelets, natural RNA was isolated from human smooth muscle cells and RNA constructs of different length and structure were synthesized. Binding of biotinylated RNA to PF4 was assessed in solid phase assays, and PF4-binding to platelets was determined by flow cytometry in the presence of various RNA-constructs or the synthetic RNA analogue poly-IC.

Results: RNA-binding to isolated PF4 was specific and followed a saturable characteristic. Other polyanionic compounds such as poly-IC, heparin or DNA competed for RNA-binding. PF4-binding to gel-filtrated platelets in the presence of different concentrations of RNA (particularly high molecular weight RNA species and poly-IC) followed a bell-shaped curve, reminiscent of the influence of heparin on PF4-platelet interaction. RNase abrogated the RNA-dependent enhancement of PF4-binding to platelets.

Conclusions: Extracellular RNA was identified as a new ligand for the chemokine PF4, and complex formation resulted in modification of PF4 interaction with platelets. Further studies will clarify the respective cell-binding mechanism and other consequences for the functional properties of PF4.

FC5-4

Application of the „Human PlateletWeb“: profiling of EVH1-domain interactors and functional characterization of new interacting partners

Dittrich M², Straßberger V¹, Jarchau T¹, Tas P³, Lewandrowski U⁴, Walter U¹, Dandekar T², Birschmann I¹

¹Institut für Klinische Biochemie und Pathobiochemie, Würzburg, Germany, ²Lehrstuhl für Bioinformatik, ³Klinik und Poliklinik für Anästhesiologie, ⁴Protein Mass Spectrometry and Functional Proteomics Group

Platelets are both central players in haemostasis and also involved in the pathophysiology of several cardiovascular diseases, inflammatory processes and metastasis. Recently we established a comprehensive database of the platelet transcriptome and proteome and derived a first model of the platelet interactome, which effectively allows the analysis of platelet proteins in their cellular interaction context (Dittrich et al., ATVB, 2008). The entire data is available as a centralized and easy-to-use internet platform ‚PlateletWeb‘ knowledgebase (<http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de>). Here we demonstrate an application and extension of this work integrating in silico and in vitro techniques. First we used sequence analysis methods to predict domain-specific interaction partners of the EVH1 domain which is known to bind target proteins with a characteristic polyproline rich region (FP4 motif). Scanning the sequences of the entire platelet proteome using a profile-based motif search we found established as well as novel potential interactors. Subsequent affinity purification using the VASP EVH1 domain and combination with mass spectrometry validated some of these predicted interaction partners. Using Y2H mutational studies we further characterized in detail the polyproline rich binding motif of one candidate protein as has been predicted by bioinformatics. Immunofluorescence staining of unstimulated and stimulated platelets gave a first hint at the function of the interaction. In conclusion, we have demonstrated the value of a comprehensive platelet proteome database using a combination of in silico and in vitro approaches. This is particularly exemplified by the detection and characterization of a novel interaction partner of the VASP EVH1 domain.

FC5-5

Functional expression of the nicotinic acetylcholine receptor alpha-7 in human platelets

Schedel A¹, Schloss P², Klüter H¹, Bugert P¹

¹Institut für Transfusion Medicine und Immunologie, Mannheim, Germany, ²Central Institute of Mental Health, Mannheim, Germany

Objective: Nicotinic acetylcholine receptor $\alpha 7$ subunits (nAChRa7) are expressed on a variety of non-neuronal cells, including endothelial cells and various blood cells. Recently, we could extend this list to human blood platelets. The function of

nAChRa7 in non-neuronal cells reaches from its essential role in inflammation to the involvement in calcium signalling. However, the expression in megakaryocytic cells and its role in platelet function has not been investigated so far. In this study, we present evidence that megakaryoblasts (MEG-01 cells) and megakaryocytes (MKs) also express nAChRa7 subunits and investigated its potential role in platelet activation.

Design and Methods: The expression of nAChRa7 subunits was investigated at the RNA level by RealTime-PCR and protein level by Westernblot and flow cytometry. Multiple electrode platelet aggregometry (MEA) in whole blood (WB) and flow cytometry in platelet rich plasma (PRP) was applied, to investigate the involvement of $\alpha 7$ channels in platelet activation.

Results: RealTime-PCR analysis revealed gene transcripts for CHRNA7 and the expression of the corresponding nAChRa7 protein could be demonstrated by Westernblot analysis and flow cytometry in MEG-01 cells, MKs and platelets. Treatment of platelets with the $\alpha 7$ selective antagonists α -bungarotoxin (α -BGT) and methyllycatonitine (MLA) caused a significant inhibition of platelet aggregation and degradation induced by the thromboxan analog U46619, whereas inhibition of 2-methylthio-ADP and TRAP-6 induced activation was only moderate.

Conclusions: The nAChRa7 protein is expressed throughout the entire megakaryopoiesis. In platelets the function may be related to calcium signalling, potentially by transient Ca^{2+} flux through Ca^{2+} channels formed by nAChRa7 homopentamers.

FC5-6

Beneficial effect of clopidogrel in a mouse model of polymicrobial sepsis

Seidel M¹, Dahlke K, Winning J, Claus R, Bauer M, Lösche W

¹Universitätsklinikum Jena, Germany

Background: Systemic inflammation is associated with activation of blood platelets and coagulation, and there is some evidence that inhibition of platelet activation may reduce sepsis-associated organ failure.

Methods: Clopidogrel was added to drinking water (0.14g/l) and given 5 days before induction of a sublethal sepsis by intraperitoneally injection of 0.2ml of a human faeces suspension. Venous blood samples were taken using a 1.2ml lithium heparin Monovette[®]. Haematological and biochemical parameter were measured using POCH-100iV and FUJI DRI-CHEM instruments (Sysmex), respectively. Data are given as means \pm sem.

Results: Subsequent to induction of peritonitis mice rapidly developed sepsis symptoms. Plasma LDH activity and bilirubin level increased within 6h from 321 ± 43 to 707 ± 83 U/l and 0.50 ± 0.02 to 1.28 ± 0.27 mg/dl, respectively, and both tended to normalise at t=12h. No changes were seen in plasma gamma GT activity and creatinin level. Clopidogrel significantly inhibited the increase in LDH and bilirubin by about 70% and 80%, respectively. Haematocrit and haemoglobin level increased within 6 h after sepsis induction from 45.7 ± 0.6 to $53.3 \pm 1.7\%$ and from 16.0 ± 0.2 to 18.6 ± 0.6 g/dl, respectively, and these changes were not affected by clopidogrel. Platelet count decreased from $984 \pm 85 \times 10^3/\mu\text{l}$ to $483 \pm 155 \times 10^3/\mu\text{l}$ and $615 \pm 190 \times 10^3/\mu\text{l}$ after 6 and 12h, respectively. Clopidogrel prevented the initial drop in platelet count after 6h but not the later platelet consumption after 12h.

Conclusion: In our mouse model faecal induced sepsis cell and tissue injury was indicated by an increase of LDH activity and bilirubin level. Clopidogrel did not only prevent initial platelet consumption but also inhibit organ injury.

FC6 Basic Research in Hemorrhagic Disorders

FC6-1

A new hemophilic mouse model that is immunologically tolerant to native human Factor VIII

van Helden P¹, Sasgary M¹, Schuster M¹, Antoine G¹, Zimmermann K¹, Ahmad R¹, Schwarz H¹, Reipert B¹

¹Baxter Bioscience, Vienna, Austria

Objectives: Current efforts to improve products for hemophilia therapy focus on the extension of half-life by chemical and/or molecular modifications of FVIII. However, any modification of the FVIII protein poses the risk of creating neo-antigens that might cause the induction of FVIII inhibitors in patients. Our aim is to develop a new model for hemophilia A that discriminates between native human FVIII and human FVIII that carries neo-antigens.

Design and Methods: FVIII transgenic mice were created by random integration of full-length human FVIII under the control of an albumin promoter. Transgenic founder mice were crossed with hemophilic mice and bred to homozygosity, giving rise to different sublines. FVIII gene expression could be shown in the liver of all sublines at different levels. No FVIII antigen was detected in the circulation ($< 1\text{ng/ml}$). We treated the mice intravenously with eight weekly doses of human FVIII (Advate) and analyzed the potential development of anti-FVIII antibodies.

Results: Transgenic mice of sublines E and I are immunologically tolerant to therapeutic doses (200 ng) of native human FVIII. In contrast, mice of subline g developed high titers of anti-FVIII antibodies. Preliminary data suggest that the degree of immunological tolerance against human FVIII correlates to a certain extent with the expression levels of the human FVIII transgen in liver and/or thymus.

Conclusions: We conclude that transgenic expression of human FVIII under the control of an albumin promoter is able to induce immune tolerance to human native FVIII in hemophilic mice.

FC6-2

Co-stimulatory requirements for T-cell independent re-stimulation of FVIII-specific memory B cells in the presence of activated dendritic cells

Pordes A¹, Hausl C¹, Allacher P², Ahmad R², Muchitsch E², Ehrlich H², Schwarz H², Reipert B^{1,2}

¹BMT Research, Vienna, Austria, ²Baxter BioScience, Vienna, Austria

Background and Objectives: Previously we showed that ligands for toll-like receptors (TLR) 7 and 9 are able to re-stimulate FVIII-specific memory B cells in the absence of T-cell help. However, alternative "helper cells" such as dendritic cells (DCs) are essential for providing help to memory B cells under such conditions. Based on these findings, we asked which co-stimulatory interactions are required for the T-cell independent re-stimulation of memory B cells in the presence of DCs and ligands for TLR.

Methods: We generated highly purified memory B cells, CD4+ t cells and DCs by a combination of magnetic bead separation and fluorescence-activated cell sorting. Memory B cells were cultured in the presence of FVIII and different combinations of CD4+ t cells, ligands for TLR 7 and 9 and DCs. Blocking antibodies and competitor proteins were added to specify the co-stimulatory interactions required.

Results: Our results demonstrate that the blockade of B7-1 and B7-2 as well as the blockade of CD40L inhibit the re-stimulation of FVIII-specific memory B cells and their differentiation into anti-FVIII antibody-producing plasma cells not only in the presence of T-cell help but also in the presence of DCs and ligands for TLR 7 or 9.

Conclusions: Based on these results we conclude that B7-CD28 interactions and CD40-CD40L interactions are important co-stimulatory interactions for T-cell dependent and T-cell independent re-stimulation of FVIII-specific memory B cells.

FC6-3

The manufacturing process of human cell line recombinant Factor VIII, (Human-cl rhFVIII)

Agerkvist J¹, Martinelle K¹, Winge S¹, Åslund T¹, Sandberg H¹

¹Octapharma AB, Stockholm, Sweden

A recombinant human Factor VIII for therapeutic use was produced in the human embryonic cell line HEK 293F. The advantage of using a human cell line for expression is based on the fact that a human post-translational pattern of modifications such as glycosylation is obtained. This provides the basis for an improved function and a reduced immunogenicity of the product compared to the presently used recombinant Factor VIII products that have a murine glycosylation pattern as those are produced in hamster cell lines. The manufacturing process of the product, named Human cell line recombinant Factor VIII (Human-cl rhFVIII), comprises the cultivation of the HEK 293F cells with the Factor VIII vector system in a defined completely serum-free medium devoid of any animal sourced compounds. The harvested product is concentrated and purified through a series of chromatography steps that is completely free of animal sourced compounds and does not require mouse hybridoma cell produced monoclonal antibodies. In addition, a solvent/detergent treatment and nanofiltration step have been introduced to further improve viral safety. The production cell-line HEK293F has been thoroughly characterized and safety tested in an extensive viral test programme demonstrating that the cell-line is free of any signs of viral infections or the presence of retrovirus like particles. The high capacity of this process to efficiently produce large quantities of the product with significant and reproducible removal of host cell- and process-derived impurities results in a safe highly pure Factor FVIII for the treatment of patients with hemophilia A.

FC6-4

Factor IX variants for the treatment of hemophilia A

Schüttertrumpf J^{1,2}, Milanov P¹, Abriss D¹, Tonn T¹, Seifried E¹

¹DRK-Blutspendedienst Baden-Württemberg Hessen, Frankfurt, Germany, ²Biomedizinisches Forschungsinstitut Georg-Speyer-Haus, Frankfurt, Germany

Objectives: Treatment of patients with inhibitory antibodies remains a major challenge in hemophilia therapy. Currently, infusion of activated proteases is the therapy of choice. Unfortunately, available substances only have short half lives and long term administration of activated proteases raises safety concerns.

Design and Methods: Here, we describe the generation of FIX variants which do not require FVIII and confirm their therapeutic potential in patient samples and in the hemophilia A mouse model.

Results: In the initial screening one single mutation was identified which showed 6.6 % clotting activity at physiological levels (FIX antigen 100 %) in FVIII deficient plasma and 191 % clotting activity in presence of FVIII. This mutation was further combined with other candidate mutations which led to the generation of FIX variants with up to 22 % clotting activity in absence of FVIII (~100,000-fold increase compared to wild type FIX). FVIII inhibitor bypassing activity was confirmed in plasma of patients with high titers of inhibitory antibodies. Further, three different variants were expressed in FVIII knockout mice using non-viral gene transfer. At FIX expression levels ranging from 7500 to 19000ng/ml partial normalization of the aPTT and of blood loss following tail clip assay were observed in all three variant groups (n=3-6 mice/group), while wild type FIX expressing mice did not differ from untreated animals.

Conclusions: The described FIX variants offer a new FVIII inhibitor bypassing strategy. The use of these not previously activated proteases could presumably allow prophylactic treatment or even gene therapy in inhibitor patients.

FC6-5

The anti von Willebrand Factor aptamer ARC1779 prevents the desmopressin-induced thrombocytopenia in VWD type 2B

Paulinska P¹, Schaub R², Gilbert J², Firdas C¹, Jilma B¹, Knoebl P³

¹Dept. of Clinical Pharmacology, Medical University of Vienna, Austria, ²Archemix Corp., Cambridge, MA, ³Dept. of Internal Medicine, Div. of Hematology, Medical University of Vienna, Austria

Introduction: Von Willebrand Disease (VWD) Type 2B is characterized by increased affinity of VWF for the platelet glycoprotein receptor Ib (GPIb). Consequently, infusion of desmopressin, a standard of treatment for other forms of VWD, causes transient thrombocytopenia in patients with VWD-2B. The aim of the study was to test whether the novel anti-VWF aptamer ARC1779, which inhibits the interaction of VWF with GPIb, could abrogate the transient desmopressin-induced thrombocytopenia in VWD-2B.

Patients and Methods: This was part of a clinical trial using a double-blind, randomized, placebo-controlled, crossover design. A patient with VWD 2B received desmopressin (0.4 mcg/kg) alone, or desmopressin after pre-treatment with ARC1779 targeted to a plasma concentration of 10 mcg/mL. Multiple blood samples were obtained over 24 hours to measure von Willebrand Factor Antigen, Ristocetin Cofactor (RiCo), FVIII activity and multimers.

Results: Desmopressin alone (open circles) increased VWF:Ag by 38 %, and VWF:RiCo 3.4-fold within 30 min, and profoundly decreased platelet counts by 87 %. In striking contrast, ARC1779 (solid triangles) completely prevented the desmopressin induced fall in platelet counts although the increase in vWF:Ag and vWF:RiCo was greater (2.7-fold and 12-fold, respectively).

Conclusions: This randomized, double-blind, placebo-controlled experiment shows that ARC1779 effectively prevents consumption of VWF and platelets in response to desmopressin in VWD Type 2B. It provides in vivo proof of concept that ARC1779 is a potent inhibitor of the VWF A1 domain interaction with GPIb, which may be used therapeutically in disorders where vWF is causative.

FC6-6

The kinetics of recombinant VWF cleavage by human ADAMTS13 is similar to that of plasma-derived VWF

Rottensteiner H¹, Varadi K¹, Vejda S¹, Schreiner J¹, Gritsch H¹, Turecek P¹, Ehrlich H¹, Schwarz H¹

¹Baxter BioScience, Vienna, Austria

Objectives: A recombinant human CHO-expressed von Willebrand Factor (rVWF) consisting of ultra-high molecular weight (UHMW) multimers resembles the VWF that is stored in Weibel-Palade bodies of endothelial cells. Once secreted into plasma, UHMW multimers are rapidly cleaved by ADAMTS13 and are usually missing in plasma-derived VWF. We analyzed in vitro whether ADAMTS13 cleavage of rVWF is similar to that of plasma VWF.

Design and Methods: The kinetics of ADAMTS13-mediated proteolysis of rVWF was explored under denaturing conditions (1.5 M urea) to expose the ADAMTS13 cleavage site of VWF. 1 VWF:Ag IU/ml of rVWF was exposed to various concentrations of recombinant and plasma-derived purified ADAMTS13, ranging from 4 mU/ml to 1 U/ml (corresponding to 0.4 to 100 % of the normal human plasma concentration), and to ADAMTS13 present in normal human plasma and VWF-deficient plasma. The multimeric structure and function of VWF were analyzed by multiple assays.

Results: Recombinant VWF was cleaved with the same efficiency using recombinant or plasma-derived ADAMTS13. UHMW multimers disappeared within seconds at physiological concentrations of ADAMTS13. Using lower concentrations of ADAMTS13 (10-30 mU/ml), UHMW were cleaved within 30 minutes. The typical satellite bands appeared very early in an ADAMTS13 dose-dependent manner.



Although plasma-derived VWF differs substantially from rVWF in its multimeric structure, the decrease in activity was similar for the recombinant and plasma-derived VWF.

Conclusions: ADAMTS13 is able to readily cleave human rVWF even at low concentrations and the UHMW multimeric fraction of human rVWF is removed within minutes by ADAMTS13 in vitro.

FC7 Thrombosis and Atherosclerosis

FC7-1

Local complement activation triggers leukocyte recruitment to the site of thrombus formation in acute myocardial infarction

Kubicek M¹, Distelmaier K², Dunkler D³, Winkler S², Jakowitsch J², Gerner C⁴, Lang I², Wagner O¹

¹Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Austria, ²Institute of Internal Medicine II, Department of Cardiology, Medical University of Vienna, Austria,

³Section of Clinical Biometrics, Core Unit for Medical Statistics and Informatics, Medical University of Vienna, Austria, ⁴Institute of Internal Medicine I, Department of Cancer Research, Medical University of Vienna, Austria

Objectives: Atherosclerotic plaque rupture with subsequent mural thrombus formation is considered the main event compromising epicardial flow in acute myocardial infarction (AMI). Although it has been shown that the number of white blood cells aspirated from AMI target vessels correlates with the magnitude of ischemia, the precise mechanisms underlying acute coronary occlusion are unknown.

Design and Methods: We compared the proteomic profiles of systemic plasma and plasma derived from fresh coronary thrombus aspirates of 34 patients with ST-elevation myocardial infarction by two-dimensional gel electrophoresis, nano-LC "shotgun" mass spectrometry (MS) and ELISA.

Results: We identified a local activation of the complement system, with a selective accumulation of the complement activator C-reactive protein (CRP) and the downstream complement effectors C3a and C5a. CRP in coronary thrombus colocalized with C1q and C3 immunoreactivities, suggesting classical complement activation. In vitro, coronary thrombus derived plasma enhanced leukocyte chemotaxis in a C3 dependent fashion.

Conclusions: We present the first direct evidence for localized complement activation at AMI culprit lesion sites. By enhanced leukocyte recruitment, local complement effectors may amplify the vascular occlusion process in AMI.

FC7-2

TRAF-1 promotes atherosclerosis in mice by enhanced monocyte recruitment to the vessel wall

Missiou A¹, Köstlin N¹, Pfeifer D¹, Münkler C¹, Ernst S¹, Bode C¹, Libby P², Zirkil A¹

¹Universitätsklinikum Freiburg, SGBM Universität Freiburg, Freiburg, Germany, ²Brigham and Women's Hospital and Harvard Medical School, Boston, USA

Members of the tumor necrosis factor (TNF) superfamily such as CD40L, TNF α , and IL-1 β potentially promote atherosclerosis in mice and likely also in humans. TNF receptor associated factors (TRAFs) mediate signal transduction for this group of cytokines. We recently reported over-expression of TRAFs in murine and human atheromata. Here we test the hypothesis that TRAF-1 modulates atherosclerosis in vivo. TRAF-1/LDLR- mice fed a high cholesterol diet for 18 weeks developed significantly smaller atherosclerotic lesions compared with LDLR- controls. Intimal lesion size decreased by up to 56 \pm 6% and 33 \pm 5% in sections of the aortic arch and aortic root, respectively ($n > 10$ per group, $P < 0.01$ each). Plaques of TRAF-1-deficient animals contained up to 46 \pm 9% and 55 \pm 4% fewer macrophages while smooth muscle cell content increased by up to 32 \pm 6 and 36 \pm 7%, characteristics associated with non disrupted plaques in humans. Lipid content, collagen content, and lymphocyte content remained unchanged. In vitro, gene expression profiling revealed reduced expression of adhesion molecules (VCAM-1, ICAM-1), chemokines (CXCL2), and growth factors (M-CSF) in TRAF-1-deficient endothelial cells, verified in vitro in mice and by siRNA studies in human cells. Finally, both deficiency of TRAF-1 in endothelial cells and monocytes reduced adhesion of inflammatory cells to the endothelium in static and dynamic adhesion assays. We present the novel finding that TRAF 1 deficiency attenuates atherosclerosis in mice, an effect most likely mediated by impaired monocyte recruitment to the vessel wall. These data identify TRAF-1 as potential treatment target for chronic inflammatory diseases such as atherosclerosis.

FC7-3

Telmisartan inhibits biglycan expression in atherosclerotic plaques in apoE-deficient mice

Nagy N, Melchior-Becker A, Fischer J

¹Institut für Pharmakologie, UK Essen, Germany, Universität Duisburg-Essen, Germany

Objectives: Biglycan, a small leucine-rich proteoglycan (SLRP), is secreted by vascular smooth muscle cells (SMC) into the extracellular matrix (ECM) during remodeling of the artery wall. Biglycan seems to be a pro-atherogenic proteoglycan that might play a key-role in the "response to retention hypothesis" of atherogenesis. Aim of the study was to examine the effects of telmisartan on biglycan accumulation and ECM composition in a murine model of accelerated atherosclerosis.

Design and Methods: Male apoE-deficient mice on Western-Diet were treated for 12 weeks with telmisartan (10 mg/kg), hydralazine (500 mg/dl), simvastatin (50 mg/kg) and simvastatin plus telmisartan. Animals were sacrificed and the extend of atherosclerosis, plaque morphology and ECM composition of the aorta and the aortic root were analysed by immunohistochemistry and immunoblotting.

Results: Biglycan accumulation in the aorta and the aortic root was significantly inhibited by telmisartan. Furthermore, telmisartan inhibited aortic plaque score and plaque size at the aortic root. SMC, macrophage and collagen content of plaques was not affected by either treatment as determined by α SM-actin-, mac2- and Sirius red staining. Interestingly, the combination of telmisartan plus simvastatin increased the mRNA expression of tissue transglutaminase-1 and procollagen lysylhydroxylase-1 suggesting increased matrix crosslinking.

Conclusion: The study shows that biglycan accumulation in aortic lesions is responsive to telmisartan treatment which might contribute to the anti-atherogenic effects of telmisartan.

FC7-4

Hypercoagulability promotes plaque stability during atherogenesis in hyperlipidemic mice

Shahzad K¹, Herzog S¹, Vinikov I¹, Kashif M¹, Thati M¹, Wang H¹, Nawroth P¹, Isermann B¹

¹University Of Heidelberg, Germany

Hypercoagulability is an established risk factor for venous thrombosis, but not for arteriosclerotic disease. To address the role of coagulation activation during atherogenesis, e.g. de novo development of atherosclerotic disease, we employed two mouse models with genetically increased coagulation activation and hyperlipidemia (FVLq/q ApoE^{-/-} and TMPPro/Pro ApoE^{-/-} mice). In both mouse models hypercoagulability resulted in larger plaques without increasing vascular stenosis secondary to positive vascular remodelling. Based on morphological characteristics plaque stability was enhanced in hypercoagulable mice. This was associated with more smooth muscle cells and less macrophages within the plaques. Long term anticoagulation using LMWH (enoxaparin) reversed these changes. In vitro thrombin (1 or 10 nm) inhibited migration of THP-1 cells through an endothelial cell layer. Pre-incubation of THP-1 cells, but not of endothelial cells, with thrombin or TRAP-1 likewise inhibited THP-1 cell migration. Identical results were obtained when using the murine cells (J-774 and SVEC4-10, which lack TM-expression). Thrombin's inhibitory effect on THP-1 cell migration was PAR-1 and PLC-beta / PI3K and NO dependent. These in vivo and in vitro data provide a rationale for the paucity of epidemiological data showing an increased frequency of atherosclerotic disease in individuals with risk factors for hypercoagulability. Besides the inhibitory effect of thrombin on monocytic cell migration thrombin likely enhances plaque stability through enhanced SMC proliferation and extracellular matrix formation. These data identify a new pathway through which the coagulation system modulates atherogenesis and establish that hypercoagulability conveys a protective effect during de novo atherogenesis.

FC7-5

Thrombogenic function of circulating microparticles does not correlate with microparticle number

Vetr H¹, Perkmann T², Geiter S¹, Graf T¹, Mager J¹, Kaufmann V¹, Binder C², Binder B³

¹Technodone GmbH, Vienna, Austria ²Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Austria, ³Department of Vascular Biology and Thrombosis Research, Medical University, Vienna, Austria

In this study we established a functional assay for circulating thrombogenic microparticles and correlated the results to already available assays and to microparticle counts. This assay is based on measuring the difference in thrombin generation between platelet-poor-plasma (PPP) and microparticle-free-plasma (MFP) from the same sample (expressed as "MP-related peak thrombin"). PPP was prepared by centrifugation for 15min at 2,500xg of citrated whole blood obtained from normal donors. MFP was generated by filtration of PPP under standardized conditions through a 0.2 μ m filter (Ceveron[®] MFU-500). Thrombin generation



was measured using the Technothrombin®TGA assay. MPs were also measured using the Annexin-V based Zymuphen-MP assay, and the number of microparticles in PPP was analyzed by flow-cytometry. In filtered samples, peak thrombin ($129\text{ nM} \pm 50$) was significantly lower than in non-filtered samples ($207\text{ nM} \pm 71$; $p < 0.05$). MP-related peak thrombin correlated with values for Annexin-V positive MPs ($R^2 > 0.6$) as measured by the Zymuphen assay. While the analysis of purified HUVEC-MP diluted in MP free plasma showed that peak thrombin correlates to the number of microparticles, MP counts in normal plasma neither correlated with MP-related peak thrombin nor with values obtained in the Zymuphen assay ($R^2 < 0.2$). These data indicate that thrombin generation by MPs is significantly related to their Annexin V binding capacity and likely due to their phosphatidylserine and other negatively charged phospholipid content but not due to tissue factor. Furthermore, total number of microparticles in PPP as measured by FACS analysis contain a significant population that does neither possess Annexin V binding capacity nor thrombin generating activity.

FC8 Research in Platelet-associated Prothrombotic Disorders

FC8-2

Characterization of five homozygous ADAMTS13 mutations in hereditary thrombotic thrombocytopenic purpura (TTP) – towards a phenotype-genotype correlation?

Meyer S¹, Jin S², Cao W², Zheng X², Lämmle B¹, Kremer Hovinga J¹

¹Department of Hematology and Central Hematology Laboratory, Bern University Hospital and University of Bern, Bern, Switzerland, ²Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia and The University of Pennsylvania, Philadelphia, USA

Objectives: The clinical presentation of hereditary TTP varies considerably. We studied five ADAMTS13 mutations found in homozygous state in six patients with hereditary TTP to identify a potential phenotype-genotype correlation.

Methods: We analyzed the patients' histories, ADAMTS13 activity and antigen. We studied recombinant mutants for synthesis, intracellular localization, secretion and specific activity under static and flow conditions.

Results: Patients homozygous for ADAMTS13 mutations W688X, R692C or C804R presented with TTP as neonates, relapsed repeatedly and had undetectable plasma ADAMTS13 activity and antigen. Homozygotes for R1060W or G1239V showed a later onset of TTP after strong triggers. In patients with R1060W, residual plasmatic ADAMTS13 was detectable. Recombinant mutants R692C, C804R, R1060W and G1239V migrated at 190kDa like wild-type, were impaired in secretion and retained in the endoplasmic reticulum. This defect was less pronounced for R1060W, whereas G1239V additionally reduced the specific activity. The W688X mutant migrated at 97kDa and was efficiently secreted. Its enzymatic activity was preserved under static conditions, but substantially reduced under fluid shear stress.

Conclusions: All five mutations are causative for hereditary TTP. The missense mutations disrupt ADAMTS13 secretion while the truncating mutation affects activity under fluid shear stress. This study of homozygous patients demonstrated severe courses of TTP because of mutations in the middle carboxyl-terminal domains, but later onset of TTP after triggers due to mutations in the distal carboxyl-terminal domains. Homozygotes for R1060W showed residual plasmatic ADAMTS13 consistent with the in vitro studies and the clinical course. These findings suggest a phenotype-genotype correlation in hereditary TTP.

FC8-3

The temporal profile of the anti-PF4/heparin immune response

Greinacher A¹, Kohlmann T², Strobel U¹, Sheppard J³, Warkentin T³

¹Institut für Immunologie und Transfusionsmedizin, Ernst-Moritz-Arndt Universität Greifswald, Germany, ²Institut für Community Medicine, Ernst-Moritz-Arndt Universität Greifswald, Germany, ³Department of Pathology and Molecular Medicine, Michael G. DeGroote School of Medicine, McMaster University, Hamilton, Ontario, Canada

Background: The immune response in heparin-induced thrombocytopenia (HIT) is puzzling: heparin-naïve patients can develop IgG antibodies and clinical HIT as early as day 5, and evidence for an even more rapid ('anamnestic') response upon heparin reexposure is lacking.

Methods: We assessed daily serum samples by anti-PF4/heparin enzyme-immunoassay (EIA) in patients receiving heparin thromboprophylaxis, and followed patients during prolonged outpatient low-molecular-weight heparin (LMWH) thromboprophylaxis.

Results: Of 435 patients, 56.1% showed an increase in EIA optical density (OD) of $\geq 15\%$, with $>90\%$ starting between days 4-14. After reaching maximum reactivity by days 10-12, ODs declined despite heparin continuation, including in two patients with clinical HIT. Individual IgG/A/M classes showed identical time of onset (median, day 6). Most (58.7%) antibody-positive patients developed all three

Ig classes; only 11.3% lacked IgG response. IgG/A/M increase usually occurred simultaneously ($\pm 1\text{d}$) with no general tendency for IgM precedence. Consistent with the transient immune response, none of the IgG-EIA-positive ($\text{OD} > 0.5$) patients at discharge developed clinically-evident thrombosis during extended LMWH-thromboprophylaxis.

Conclusion: The rapid onset of the anti-PF4/heparin immune response, its transience, and the simultaneous appearance of antibodies of different classes with no IgM precedence suggest short-term activation of B-cells that have previously undergone Ig class switching even without previous pharmacologic heparin exposure.

FC8-4

Platelet response to clopidogrel assessed with the multiplate analyzer and drug-eluting stent thrombosis

Sibbing D¹, Braun S¹, Morath T¹, Mehilli J¹, Vogt W¹, Schömig A¹, Kastrati A¹, von Beckerath N¹
¹Deutsches Herzzentrum München, Germany

Objectives: Studies employing light transmission aggregometry (LTA) have shown that insufficient suppression of platelet reactivity to adenosine diphosphate (ADP) following clopidogrel treatment is associated with an increased risk of adverse cardiovascular events after percutaneous coronary intervention (PCI). However, LTA is time- and labor-intensive and inconvenient for the routine. A point-of-care assay with similar predictive power would be of great value. The aim of this prospective trial was to assess whether platelet response to clopidogrel assessed with multiple electrode platelet aggregometry (MEA), a newly developed point-of-care assay, correlates with the risk of drug-eluting stent (DES) thrombosis (ST).

Methods: A total of 1608 consecutive patients with coronary artery disease and planned DES implantation were enrolled. Before PCI all patients received 600 mg clopidogrel. Blood was obtained directly before PCI. ADP-induced platelet aggregation was assessed in whole blood with MEA on the Multiplate analyzer. The primary endpoint was definite ST at 30 days.

Results: The upper quintile of patients according to MEA measurements ($n=323$) were defined as clopidogrel low-responders. Compared with normal-responders ($n=1285$), low-responders had a significantly higher risk of definite ST within 30 days [2.2% vs. 0.2%; OR 9.4; 95% CI, 3.1-28.4; $P < 0.0001$]. The composite of death or ST was higher in low- vs. normal-responders [3.1% vs. 0.6%; OR 5.1; 95% CI, 2.2-11.6; $P < 0.001$].

Conclusions: Low-response to clopidogrel assessed with MEA is significantly associated with an increased risk of ST. MEA is likely to be helpful for tailoring anti-platelet treatment to the needs of individual patients treated with PCI.

FC8-5

Pronounced platelet hyper-function in patients with cardiac arrest achieving restoration of spontaneous circulation

Spiel A¹, Frossard M², Mayr F³, Kliegel A², Janata A², Uray T², Sterz F², Jilma B¹

¹Department of Clinical Pharmacology, Medical University of Vienna, Austria, ²Department of Emergency Medicine, Medical University of Vienna, Austria, ³CRISMA Laboratory, Department of Critical Care Medicine, University of Pittsburgh Medical Center, Pittsburgh, PA

Objective: Markers of platelet activation are increased in patients undergoing cardiopulmonary resuscitation (CPR). Hyper-functional platelets may contribute to impairment of microcirculatory function and overall poor outcome despite restoration of spontaneous circulation (ROSC). Patients with myocardial infarction have hyper-functional platelets, which predict the degree of myocardial necrosis. Thus, we hypothesized that platelets may be even more activated in patients whose myocardial infarction leads to cardiac arrest and compared them to patients whose cardiac arrest was due to a non-cardiac origin.

Design: Prospective observational study. Setting: Emergency Department of a tertiary care hospital. Patients: 104 patients with witnessed cardiac arrest who achieved ROSC. Interventions: Blood sampling.

Measurements and Main Results: We assessed collagen adenosine diphosphate closure time (CADP-CT) with the platelet function analyzer (PFA-100), and measured plasma levels of von Willebrand factor (vWf:RiCo) levels by turbidometry. Independent physicians diagnosed the origin of cardiac arrest. The majority of cardiac arrests were caused by myocardial ischemia. Invariably, CADP-CT values (55s; 95%CI: 52-58s) were much shorter in these patients compared to patients with other causes of cardiac arrest (110s; 95%CI: 84-135s, $p < 0.001$). vWf:RiCo plasma levels were more than 3-fold above normal values in both groups.

Conclusions: Patients with myocardial ischemia triggered cardiac arrest had the highest degree of platelet hyper-function under high shear rates, which was not solely due to increased vWF. Future trials are necessary to clarify whether rapid, more aggressive anti-platelet therapy improves outcome after cardiac arrest.



FC8-6

Classification of platelet aggregation defects by characterization of dysfunctional integrin α Ib β 3

Scharf R¹, Kirchoff E¹, Hoffmann T¹

¹Dept. of Hemostasis, Hemotherapy, and Transfusion Medicine, Heinrich Heine University Medical Center, and Biological Medical Research Center, Heinrich Heine University Düsseldorf, Germany

Objectives: Normal platelet aggregation requires (i) agonist-induced activation of α Ib β 3, (ii) binding of fibrinogen (Fg), and (iii) postoccupancy events following ligand binding. To assess aggregation defects in these terms, we used flow cytometry and specific antibodies that can distinguish between resting, activated, and ligand-occupied forms of α Ib β 3.

Design and Methods: We studied 30 patients. PAC1 (directed to activated α Ib β 3) and anti-LIBS-1 (recognizing a ligand-induced binding site on β 3) were used. LIBS is either expressed upon receptor occupancy by Fg, a process which requires activation, or by Fg-mimetic RGD peptides, which bind to α Ib β 3 by an activation-independent manner. This approach offers a strategy to distinguish defects in activation, ligand binding, or postoccupancy events.

Results: Among the patients, 25 had aggregation defects in response to epinephrine (EPI) and/or ADP despite normal expression of α Ib β 3. Of these 25 patients, 15 failed to bind PAC1 upon stimulation with EPI or ADP, but 11 of them bound PAC1 in response to PMA, a signal mimetic which circumvents agonist-induced platelet activation by directly activating protein kinase C. In 11 of the 15 patients, binding of anti-LIBS-1 was concomitantly absent in response to EPI or ADP, but intact upon incubation with RGD. In the other 4 patients, RGD failed to induce binding of anti-LIBS1. The remaining 10 patients showed intact binding of PAC1 and anti-LIBS-1 in response to EPI, ADP, or PMA, indicative of postoccupancy dysfunction.

Conclusion: Platelet aggregation abnormalities can be classified with regard to defects of activation, ligand binding, or postoccupancy dysfunction of α Ib β 3.

FC9 Pharmacological Modulation of the Platelet and Coagulation System

FC9-1

Actively anticoagulant surfaces targeting Protein C

Maitz M¹, de Moraes Schmittgens L¹, Sperling C¹, Werner C^{1,2}

¹Leibniz Institut Für Polymerforschung Dresden, Germany, ²Institute of Biomaterials and Biomedical Engineering, University of Toronto

Background: Surface modifications are frequently applied to provide anticoagulant properties biomedical devices in contact with streaming blood. They mainly consist of surface passivation or are direct or indirect inhibitors of the coagulation factors thrombin or FXa. The present study intends to impart the properties of the anticoagulant enzyme activated protein C to a surface.

Method: Recombinant human activated protein C (Drotrecogin Alpha) and a direct Protein C activator from snake venom (Protac) were immobilized covalently via spacer molecules to model surfaces. The activity of the immobilized enzymes was probed by a chromogenic substrate. The efficiency as anticoagulant surface was tested by incubation with plasma and whole blood.

Results: Both enzymes could be immobilized in active form to the surface. For drotrecogin about 8% of the theoretical maximal activity of a monolayer was obtained. The efficiency of Protac immobilization was reduced due to the immobilization of bulking proteins in the applied formulation. In whole blood incubation, immobilized drotrecogin exhibited anti-hemostatic effects on the coagulation cascade and on blood platelets. It further reduced the inflammatory response of leukocytes. Despite the reported rapid inhibition of the enzyme, the effects still could be observed after two hours incubation. Beneficial effects of Protac apparently were superposed by the co-immobilized bulking proteins.

Conclusion: Targeting the physiological anticoagulant protein C system is an innovative and promising approach to form actively anticoagulant surfaces.

FC9-2

Exosite-specific targeting of coagulation enzymes by ssDNA-aptamers

Müller J¹, Isermann B³, Oldenburg J¹, Mayer G², Potzsch B¹

¹Universität Bonn / Institut für Exp. Hämatologie und Transfusionsmedizin, Germany, ²Universität Bonn / Kekulé-Institut für Organische Chemie und Biochemie, Germany, ³Universität Heidelberg / Abteilung Innere Medizin I und Klinische Chemie, Germany

Objectives: Specific targeting of activated coagulation factors is a prerequisite for the development of sensitive diagnostic assays and enzyme-modulating drugs. We generated ssDNA-aptamers that target the serine proteases thrombin and activated protein C (APC) and evaluated their binding characteristics, specificity, and inhibitory potential.

Design and Methods: The thrombin-binding bivalent aptamer HD1-22 was assembled by connecting previously described ssDNA-aptamers targeting exosites I and II, respectively. A class of APC-binding ssDNA-aptamers (HS02) was identified by SELEX. Binding characteristics were assessed by filter binding experiments and SPR-analysis. The inhibitory potential and the binding regions of the aptamers were determined by functional and competitive assays.

Results: Both classes of aptamers bind to their corresponding target with subnanomolar affinities while binding to the corresponding zymogens was found to be on an at least 100-fold lower level. The bivalent thrombin aptamer HD1-22 simultaneously binds to both exosites of thrombin and showed strong anticoagulant characteristics that could be rapidly antagonized by the addition of antisense-oligodeoxynucleotides. The HS02-aptamers selectively target the basic exosite of APC and thereby modulate the protease activity in a way that the anticoagulant functions of APC are inhibited and its reactivity towards the protein C inhibitor is augmented in a glycoaminoglycan-like fashion whereas APC's anti-apoptotic and cytoprotective functions remain unaffected.

Conclusions: Exosite-specific targeting of thrombin and APC by the described ssDNA-aptamers allowed selective modulation of enzyme functions. This is an important advantage over active site blocking inhibitors and makes the described aptamers to promising candidates for the development of therapeutic and diagnostic strategies.

FC9-3

Prevention of thrombotic events by Factor XIIa inhibitors

Schmidbauer S¹, Nerlich C¹, Weimer T¹, Kronthaler U¹, Metzner H¹, Schulte S¹

¹CSL Behring GmbH, Marburg, Germany

Activated Factor XII (FXIIa) is able to activate zymogens that are involved in different physiological pathways. Nevertheless, the main in-vivo function of FXIIa is yet unknown. Interestingly, it recently was described that FXII^{-/-} mice are protected against pathological thrombus formation (Renné et al.; JEM; 2005) without showing an increased bleeding tendency. Therefore, derivatives of the previously described (Campos et al.; FEBS Lett; 2004) specific FXIIa inhibitor Infestin domain 4 (Inf-4) were investigated here as protective agents against thrombosis without affecting haemostasis. Different Inf-4 derivatives were expressed in HEK 293 cells, purified by chromatography and characterized by SDS-PAGE and Western blotting. In-vitro functionality was examined by a dose-dependent prolongation of the clotting time of SHP in an aPTT assay (Siemens Healthcare). For a selected derivative, in-vivo functionality was tested in FXII^{+/+} mice in a Fe(III)Cl₃-induced model of arterial thrombosis. The observation period following the lesion was 1h. To investigate effects on hemostasis tail tip bleeding experiments were performed. His-tagged Inf-4 and an albumin-Inf-4 fusion protein (rHA-Inf-4) were obtained with a purity of > 95%. The Inf-4 derivatives prolonged the aPTT by a factor of up to > 3 when applied in a 20-fold molar excess over FXII whereas the PT remained unaffected. Dose-dependent protection from arterial thrombosis reached 100% of treated mice and even the fully protective dose did not interfere with hemostasis. In summary, it is concluded that FXII may have a significant role in thrombus formation and, therefore, FXII/FXIIa may be an appropriate target for thrombosis prevention.

FC9-4

Dabigatran inhibits arterial thrombosis in a porcine model of cyclic flow reduction

van Ryn J¹, Priepeke H¹, Huel N¹, Waldmann L¹, Wiene W¹

¹Boehringer Ingelheim Pharma GmbH & Co kg, Biberach, Germany

Objective: To investigate the antithrombotic and anticoagulant effects of dabigatran in an arterial thrombosis model of cyclic flow in pigs.

Design and Methods: Domestic pigs (n=4-6) were anesthetized and ventilated. Thrombus formation from acute compression damage and stenosis of the carotid artery was measured using Doppler flow. Mechanical embolization of the clot restored flow through the vessel until a new thrombus reoccluded the vessel minutes later. Dabigatran (0.1-3 mg/kg i.v. bolus) was given before (prevention) or after (treatment) injury. Antithrombotic effectiveness was measured as reduction in closure frequency during each interval. Anticoagulant effects were monitored as aPTT.

Results: Placebo-treated animals had ~10 closures per interval (9.81 ± 0.92). Prevention: Dabigatran, given prior to vessel damage, resulted in a dose-dependent cyclic closure inhibition, 94% with 1 mg/kg i.v. in the first 20 min. There was still a 66% reduction in cyclic closures 1.5-2 hrs after injury. Baseline aPTTs of 14.7 ± 0.5 s were elevated in a dose-dependent manner. Treatment: Dosing dabigatran post injury resulted in a dose-dependent inhibition of cyclic closure. Dabigatran, 3 mg/kg i.v., resulted in an 84% reduction in the first 20 min. The reduction in cyclic closure frequency was maintained over the entire 2 hr period. These antithrombotic effects were associated with a dose-dependent elevation in the aPTT.



Conclusions: Dabigatran is an effective inhibitor of arterial thrombosis in this model. A single bolus was sufficient to attenuate the thrombogenic response of the damaged arterial wall for 2 hrs post injury, suggesting the potential benefit from clot-bound thrombin inhibition.

FC9-5

The fungal metabolite gliotoxin inhibits platelet function in vitro

Bertling A¹, Niemann S¹, Uekötter A², Lass-Flörl C³, von Eiff C², Kehrel B¹

¹University Hospital Münster, Department of Anesthesiology and Intensive Care, Germany, ²University Hospital Münster, Department of Medical Microbiology, Germany, ³Innsbruck Medical University, Department of Hygiene, Microbiology, and Social Medicine, Austria

Objectives: Gliotoxin (GT), which possesses immunosuppressive properties, is produced by diverse fungi, including the opportunistic pathogens *Aspergillus fumigatus* and *Candida albicans*. The mode of action involves the formation of mixed disulfides with host proteins. Disulfide exchanges play an important role in platelet activation. Therefore, we investigated whether GT affects platelet function in pathophysiologically relevant concentrations.

Design and methods: The effects of GT on the binding of FITC-coupled soluble fibrinogen and FITC-coupled coagulation Factor VIII to platelets and expression of CD62P and CD40L on the platelet surface after agonist (ADP, collagen, thrombin, TRAP)-induced platelet activation were measured via flow cytometry. The influence of GT on ADP-induced platelet aggregation was determined by light transmission.

Results: GT (0,05–0,5 µm) inhibited binding of fibrinogen and of Factor VIII to platelets activated with ADP, collagen, thrombin or TRAP in a dose dependent manner. At the same concentrations GT also inhibited ADP-induced platelet aggregation. Alpha granule release, measured via CD62P surface expression, and CD40L surface expression were less or not affected.

Conclusions: The fungal metabolite GT possesses in addition to its immunosuppressive function antithrombotic properties. GT production by pathogens could contribute to their survival in the blood stream during vascular infections. The knowledge of the underlying mechanisms of the antithrombotic properties might help to fight against fungal infections as well as thrombosis.