Defibrotide in the human endotoxemia model - an exploratory trial investigating the effects and the mechanisms of Defibrotide

A randomized, double blind trial in healthy volunteers

Eudract-Nr.: **2016-001375-77**

Clinical Trials.gov: **NCT02876601**

Principal Investigator: Bernd Jilma, Ao.Univ.Prof.Dr.

Medical University of Vienna

Department of Clinical Pharmacology

Währinger Gürtel 18-20, 1090 Vienna

Sponsor: Medical University of Vienna

Department of Clinical Pharmacology

Währinger Gürtel 18-20, 1090 Vienna

Synopsis:

Title:	Defibrotide in the human endotoxemia model
Study Phase	IV

Background: Defibrotide (DF) is approved for the use in sinusoidal obstruction syndrome (SOS), which mainly occurs after chemotherapy and after hematopoietic stem cell transplantation. Furthermore it seems to reduce the incidence of graft versus host disease (GVHD). Although effective in clinical trials, the mode of action is still not fully understood. The manifold effects of DF may be summarized as anti-inflammatory, anti-coagulatory and profibrinolytic. However, *in vivo* data are scarce and no comprehensive data on the effects of DF in inflammatory states are available. This trial is designed to investigate the effects of DF in a human inflammation model using Lipopolysaccheride (LPS) as a stimulus.

Additionally, we will investigate the effects of DF on coagulation tests ex-vivo. Eight to 16 healthy volunteers will be included in this ex-vivo spiking study and blood sampling will be performed.

Trial hypothesis: DF reduces the activation of coagulation, the inflammatory response and the endothelial activation caused by infusion of LPS in healthy volunteers.

Trial objectives: The objectives of this trial are:

- To investigate the effects of DF on coagulation parameters and tests *ex-vivo*
- To investigate the anti-coagulant effects of DF
- To investigate the anti-inflammatory effects of DF
- To investigate the effects of DF on endothelial activation
- To investigate the safety of DF infusion in healthy volunteers
- To investigate the effects of DF on thrombeolastometry *ex vivo*
- *To investigate LPS pharmacokinetics in plasma of healthy volunteers*

Study design: This trial will be conducted as a randomized, double-blind, crossover trial in 20 healthy volunteers. Sixteen healthy volunteers will be treated with an LPS bolus (2ng/kg bodyweight) and an infusion of 6.25mg/kg bodyweight DF or placebo. A washout period of 6 weeks will be planned between two study periods.
Additionally, four healthy volunteers will receive a placebo instead of LPS and will be treated with DF or placebo to investigate the effects of DF in healthy volunteers without LPS as a stimulus.
Additionally we will investigate the effects of DF on thrombelastometry *ex vivo*.

Trial Products: Defibrotide (Defitelio, Jazz Pharmaceuticals), 6.25mg/kg bodyweight
LPS: 2ng/kg bodyweight US Standard Reference LPS (E.coli)

Paracetamol: 500mg Paracetamol Genericon (Genericon Pharma) on demand to alleviate flu-like symptoms

Placebo: 0,9% NaCl solution not distinguishable from the original products will be used

Primary variable: Prothrombin fragments F1+2 (F1+2) as a measure of thrombin generation and coagulation

Secondary variables:

- vWF antigen and activity
- microparticle associated tissue factor
- TNF-α levels
- tPA
- PAI-1
- D-Dimer
- Thrombin-Antithrombin-Complexes (TAT)
- Circulating endothelial cells
- E-Selectin
- Thrombelastometry (clotting time, clot formation time, maximum lysis, etc.)
- Proinflammatory exosome
- Differential blood counts
- Safety parameters
- LPS concentration

Risks/Benefits: There will be no benefits for participating subjects. However, by gaining knowledge about DF and better defining its effects in acute inflammatory states treatment of patients may be improved. Furthermore, our results may generate hypotheses about the use of DF in other diseases.

On the other hand use of DF in healthy volunteers may contain certain risks for participants. Exclusion criteria comprise pregnancy or breastfeeding, as well as intake of other medication beside contraceptives. Furthermore all participants will be required to perform effective contraception for the duration of the trial. However, these measures are precautions and due to a lack of information rather than known side effects. In studies, the side effect profile of DF was overall good. There were no reports of hypersensitivities or anaphylaxis in the marketing trials. However, in previously marketed DF formulations such reactions were reported and must therefore be considered. The most common side effects were hemorrhage, hypotension and coagulopathy. However, this may also be due to the selection of patients as they are typically severely thrombopenic, refractory to thrombocyte infusion and critically ill. Information about DF in healthy volunteers is scarce. Two studies are published investigating the effects of intravenous DF in healthy volunteers. No serious or severe side effects were reported.

In the ex-vivo spiking study 17 ml blood will be drawn which should not impose any significant risk on participating subjects.

1. Index

2. Introduction

2.1. Defibrotide

Defibrotide (DF) is a highly complex polydisperse mixture of single-stranded phosphodiester oligodeoxyribonucleotides derived from the controlled depolymerization of porcine intestinal mucosal DNA[1].

The entire mode of action of DF remains unknown and it seems to induce multiple anti-coagulatory, anti-inflammatory and profibrinolytic effects[2]. DF increases levels of prostaglandins[3] and reduced thromboxane levels[4]. Furthermore, DF significantly and dose-dependently increased tissue plasminogen activator (tPA) antigen and activity, and dose-dependently decreased PAI-1 antigen *in vitro* using endothelial cells and lipopolysaccheride (LPS) as a stimulus[5]. Interestingly, only in microvascular endothelial cell cultures, DF decreased the LPS induced tissue factor (TF) antigen expression[5]. Infusion of DF in healthy volunteers caused a significant decrease of PAI-1 and significantly increased prostacyclin and tissue factor pathway inhibitor[6]. In healthy volunteers, combined infusion of DF and heparin significantly enhanced the effect of heparin alone and prolonged the activated partial thromboplastin time and increased antithrombin III levels[7]. Moreover, in a model using endothelial cells and sera from patients undergoing autologous stem cell transplantation treatment with DF reduced expression of von Willebrand factor[8]. DF was effective in reducing thrombosis in a placebo controlled trial after general surgery [9]. In a malaria model using endothelial

cells incubated with plasmodium falciparum infected erythrocytes DF reduced factor X activation, which may be caused by reduced tissue factor expression of these cells[10].

DF is mainly used to treat veno-occlusive disease (VOD) of the liver after stem cell transplantation[2]. The current pathophysiological model of VOD suggests that high-dose chemotherapy causes endothelial injury with increased expression of TF and PAI-1 leading to a prothrombotic, hypofibrinolytic state with extracellular, subendothelial deposition of fibrinogen and factor VIII/vWF[11]. These aggregates may occlude the small pores in the liver endothelium leading to obstruction of the venous outflow and portal hypertension[12]. It was demonstrated in multiple trials that DF reduces mortality in VOD and that the use of DF significantly reduces the risk of developing a VOD and therefore may be used as prophylaxis[13].

2.2. The human endotoxemia model

The human endotoxemia model is well established and widely used to mimic systemic inflammatory disease states experimentally. The infusion of LPS (usually 1-4ng/kg bodyweight) leads to the release of proinflammatory and procoagulant mediators and consequently to increased parameters of inflammation, activation of coagulation, increased body temperature, hypotension and tachycardia[3]. LPS infusion leads to a systemic activation of inflammation and coagulation by increasing TF-mRNA, thrombin-anthithrombin complexes, D-Dimer, prothrombin fragments F1+2 (F1+2), TNF-α, IL-6, PAI-1, TPA, E-Selectin, P-Selectin, vWF-antigen, ICAM, VCAM and soluble thrombomodulin[14,15]. Thrombelastometry, a point-of-care whole blood assay that evaluates the viscoelastic properties of whole blood during clot formation and clot lysis is also affected by LPS and shows a significant activation of coagulation, correlating with

F1+2 (r=-0,83) and increased clot lysis, which correlated well with tPA levels (r=0,82). Infusion of LPS increases levels of circulating endothelial cells several fold in healthy volunteers[16]. This goes along with an increase in other endothelial activation markers such as E-Selectin, thrombomodulin and vWF[15].

2.3. Circulating endothelial cells

Circulating endothelial cells are a useful marker to monitor endothelial activation and/or damage[17]. Interestingly, levels of circulating endothelial cells increase after engraftment in patients with acute GVHD. On the contrary, in patients, who do not develop GVHD, after engraftment levels of circulating endothelial cells seem to decrease[18]. DF protects the endothelium from activation[19]. Moreover, pediatric patients after hematopoietic stem cell transplantation, who were treated with DF, had a lower incidence of developing acute GVHD than the control group[20]. However, the effect of DF on circulating endothelial cell levels as a marker of endothelial cell activation is not known[16].

2.4. Rationale of the trial

There is a lack of knowledge on the concise mode of action of DF. Additionally, available data mainly stems from cell based studies. However, human *in vivo* data is currently lacking. This project will investigate the anti-inflammatory effects, anti-coagulatory effects and effects on the endothelial activation of DF in a well-established human model of acute, transient systemic inflammation in healthy volunteers. The effects of many other anti-coagulatory and anti-inflammatory drugs on LPS infusion have been investigated similarly [14,21-25]. Thus, comparisons of DF with these substances may possibly be drawn and further hypotheses may be generated based on our data.

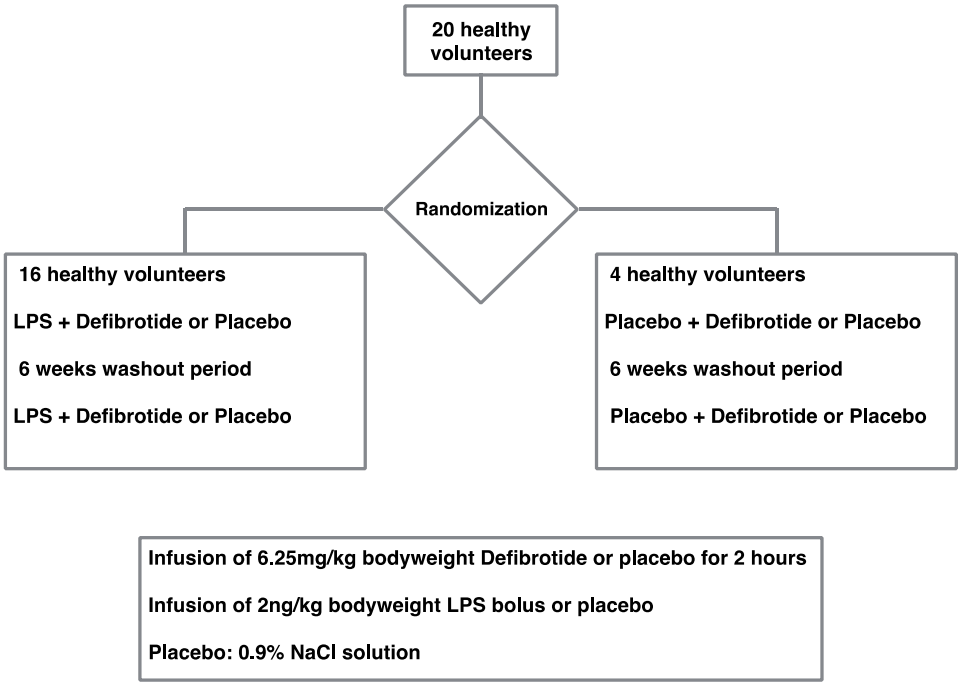
Additionally, the effects of DF on thrombelastography will be investigated and its potential use as a DF therapy-monitoring device will be examined. Therefore we will spike different doses of DF in whole blood of healthy volunteers ex-vivo.

2.5. Hypotheses and Objectives.

We hypothesize that DF infusion reduces the LPS induced activation of coagulation and the inflammatory response. Furthermore we hypothesize that DF infusion reduces the endothelial activation induced by LPS. Moreover we will investigate the influence of DF on thrombelastometry and the utility of this device for potential therapy monitoring of DF. Finally, the safety of DF infusion in healthy volunteers will be assessed.

3. Trial Design

3.1. Flow Chart



3.2. Trial design and rationale

Before initiation of the trial we will investigate the effects of DF on thrombelastometry ex vivo. In this part blood samples will be drawn from a minimum of eight healthy volunteers and after *ex-vivo* spiking of blood samples with increasing doses of DF thrombelastometry will be performed. The effects of activators (i.e. recombinant tissue factor) on DF in thrombelastometry will be investigated. The number of participants may be increased to 16 after analysis of the results of the first eight subjects.

This trial is designed as a randomized, double blind, crossover trial in 20 healthy volunteers. Sixteen healthy volunteers will be randomly assigned to receive LPS, whereas four healthy volunteers will be randomly assigned to the placebo group. In each group two treatment periods will be performed. In one treatment period all subjects will be treated with DF. In the other treatment period all subjects will be treated with placebo. A washout period of 6 weeks will be planned between both treatment periods to reduce the risk of any carry-over effects. Whether a subject starts with the placebo or the DF period will also be randomized.

3.3. Medication, dose rationale and dosing schedule

3.3.1. Defibrotide

Defibrotide is a highly complex polydisperse mixture of single-stranded phosphodiester oligodeoxyribonucleotides that exerts anti-inflammatory and anti-coagulatory effects[1]. It is mainly used for treatment of the sinusoidal obstruction syndrome, also called veno-occlusive disease (VOD)[2]. The approved dose of DF is 6.25mg/kg bodyweight every six hours. This sums up to daily doses of 25mg/kg bodyweight DF[26]. This dose is based on clinical data. In a trial investigating the effects of DF in patients with VOD treatment with DF was started at 10mg/kg bodyweight split into four daily doses and increased every

two to four days by 10mg/kg depending on tolerability and response. Most responses were observed at daily doses between 20 and 40mg/kg bodyweight. However, doses ranged between 10 and 60mg/kg bodyweight[27]. A phase II trial compared 25mg/kg bodyweight daily with 40mg/kg bodyweight daily and found no differences in clinical outcomes, except for a slightly higher bleeding rate in the higher dose group[28]. The FDA approved Defibrotide on March 30th [29].

3.3.2 LPS

In the human endotoxemia model doses of 2-4ng/kg bodyweight are regularly used [30]. WE will use an intravenous bolus of 2ng/kg bodyweight US Standard Reference *E.coli* LPS in this trial. This dose increases F1+2 reliably about eight- to tenfold[31,32]. Maximum effects in coagulatory parameters are usually detected two to six hours after infusion, whereas after 24 hours most levels have returned to the baseline. Moreover, in various performed trials this dose was demonstrated to be safe and well-tolerated[30]. In previous trials the dose of 2ng/kg bodyweight was better tolerated by subjects compared to 4ng/kg bodyweight, without eliminating the activation of the coagulation system.

3.3.3 Timing

For this trial we chose to infuse 6.25mg/kg bodyweight over a period of two hours, which is the approved dose of this drug. The LPS bolus will be infused 1h after the start of the defibrotide infusion. We chose this dosing regimen based on the T_{max} of DF of two hours, the $T_{1/2}$ of 0.7 hours and the effects of LPS with a maximum approximately four hours after infusion of the bolus.

3.3.4 Sodium Chloride solution

All subjects will receive an intravenous infusion of 0,9% sodium chloride at a rate of 100ml/h to maintain adequate hydration for healthy volunteers, as they will be kept in a

fasting state during the first four hours. This should prevent any interference of ingested food with any of the trial's endpoints or side effects such as nausea. A light, fat–reduced meal may be served after four hours, when nausea is no longer encountered.

3.3.5. Blinding of the medication

DF, after dissolution, is a colorless fluid not distinguishable from sodium chloride solution. A pharmacist not otherwise involved in the conduct of the trial will prepare weight adjusted medication of infusion labeled with Defibrotide/Placebo or LPS/Placebo.

3.4. Trial Schedule

Ex-vivo spiking study	April 2017
Planned inclusion of the first subject	May 2017
Planned recruitment period	6 weeks
Planned completion of the last subject	November 2017
Planned completion of the trial report	January 2018

4. Trial Population

4.1. Trial Population

The trial will be performed in 20 healthy volunteers. Sixteen healthy volunteers will be randomly assigned to the LPS group receiving two doses of 2ng/kg LPS once with a placebo and once with DF. Four healthy volunteers will be randomized to the control group and will not receive any LPS but a placebo, again once with DF and once with a placebo. In- and exclusion criteria will comprise:

Inclusion Criteria:

- >18 years of age
- <90kg body weight
- Normal findings in medical history and physical examination unless the investigator considers the abnormality to be clinically irrelevant
- Normal laboratory values unless the investigator considers abnormalities to be clinically irrelevant
- Ability to understand the purpose and nature of the study, as well as the associated risks

Exclusion Criteria:

- Intake of any drugs that may interfere with the trial's endpoints or drugs (i.e. platelet inhibitors, anticoagulants, etc.)
- Positive results of HIV or hepatitis virology
- Acute illness with systemic inflammatory reactions
- Known allergies, hypersensitivities or intolerances to any of the used substances
- Acute or recent bleeding episodes, increased risk of bleeding at the discretion of the investigator
- Participation in an LPS trial within 6 weeks of the first study day
- Pregnancy or breastfeeding

4.2. Withdrawal Criteria

The subject may be withdrawn from the trial at the discretion of the investigator if judged non-compliant with trial procedures or due to safety concern.

4.4. Subject Replacement

A subject will be considered evaluable if he or she has completed the first six hours of the trial's second period. Otherwise another subject may replace him. Based on previous trials, this is expected to occur in less than 10% of the cases.

5. Methods and Assessments

5.1. Visits and Procedures

5.1.1.Ex-vivo spiking Trial

Healthy volunteers will report to the study ward and before blood sampling is performed the following steps will be performed:

- Informed consent
- Medical History and physical examination
- Height and weight measurement
- Blood pressure and heart rate measurement
- Check for Ex- and inclusion criteria

Blood sampling will be performed thereafter. The needed blood volume will be 17ml.

5.1.2. Screening Visit

During the screening visit eligibility of the subjects for participation in the trial will be checked. This contains obtaining informed consent before any trial related activity is performed.

The following examinations will be performed at least one week before the first trial day:

- In-/Exclusion Criteria
- Physical examination and medical history
- Height and weight

- Blood pressure, heart rate, ECG evaluation
- Laboratory analysis incl. blood counts, blood chemistry, global coagulation assays and urinalysis
- Pregnancy test, if applicable
- Virology incl. HIV and Hepatitis C

5.1.3. Period 1 Trial Day 1

The subjects will report to the study ward at 08:00 at the Department of Clinical Pharmacology after an overnight fast. In- and exclusion criteria will be checked, a physical examination will be performed, intake of medication and other medical conditions since the screening visit will be noted. Vital parameters will be measured in a supine position (heart rate, blood pressure, oxygen saturation and body temperature) and urinalysis will be performed. If applicable, a pregnancy test will be performed.
Baseline blood sampling will be performed.
Two indwelling intravenous catheters will be placed in appropriate antecubital veins and infusion of the trial drugs will be started. In one of the catheter the LPS bolus will be injected. It will then be flushed using 0,9% sodium chloride and continuous intravenous infusion of sodium chloride at a rate 100ml/h will be started. The other catheter will be used for infusion of DF, which is initiated 1h before LPS bolus infusion.
Vital signs will be measured and documented every hour until discharge.
Blood sampling will be performed at the baseline, before any drug is infused, as well as one, two, four, six and 24 hours after infusion of the LPS bolus.
In three out of 20 subjects, 3 additional blood draws will be performed at 5, 10 and 20 minutes after LPS administration, respectively. These samples will serve for exploratory quantification of LPS. Each of the blood draws will be performed by single

venipunctures on the arm contralateral to LPS infusion. Participation to this part of the trial will be optional for each healthy volunteer.

Four hours after the LPS bolus subjects will be receiving a standardized fat-reduced meal. After six to eight hours subjects will be discharged from the ward depending on their medical condition.

5.1.4. Period 1 Trial Day 2

24 hours after infusion of the LPS bolus subjects will report to the Department of Clinical Pharmacology for further blood sampling, urinalysis and for a physical examination. Any signs of bleedings will be documented.

5.1.5. Period 2 Trial Day 1

Similar to Trial Day 1 of the period 1 the subjects will report to the study ward at 08:00 at the Department of Clinical Pharmacology after an overnight fast. In- and exclusion criteria will be checked, a physical examination will be performed, intake of medication and other medical conditions since first period will be noted. Vital parameters will be measured in a supine position (heart rate, blood pressure, oxygen saturation and body temperature) and urinalysis will be performed. If applicable, a pregnancy test will be performed. Baseline blood sampling will be performed.

Two indwelling intravenous catheters will be placed in appropriate antecubital veins and infusion of the trial drugs will be started. In one of the catheter the LPS bolus will be injected. It will then be flushed using 0,9% sodium chloride and continuous intravenous infusion of sodium chloride at a rate 100ml/h will be started. The other catheter will be used for infusion of DF.

Vital signs will be measured and documented every hour until discharge.

Blood sampling will be performed at the baseline, before any drugs are infused, as well as one, two, four, six and 24 hours after infusion of the LPS bolus.

Four hours after the LPS bolus subjects will be receiving a standardized fat-reduced meal. After eight hours subjects will be discharged from the ward depending on their condition.

5.1.6. Period 2 Trial Day 2

24 hours after infusion of the LPS bolus subjects will report to the Department of Clinical Pharmacology for further blood sampling, urinalysis and for a physical examination. Any signs of bleedings will be documented.

5.1.7. Final Visit

The final safety visit will take place 7-14 days after the second trial period. A urinalysis, physical examination, blood sampling for safety parameters, ECG evaluation and documentation of all other relevant information including concomitant medication and adverse events, will be performed.

This visit will be the final visit of the trial and all subjects will be discharged from the trial thereafter unless a medical reason exists for another follow-up visit.

5.2. Efficacy analysis

5.2.1. Primary endpoint

The primary endpoint of this trial will be levels of F1+2.

5.2.2. Secondary endpoints

Secondary endpoints will include the following:

- vWF antigen and activity
- microparticle associated tissue factor
- inflammatory biomarkers (TNF-α, etc.)
- Tissue-type plasminogen activator (t-PA)
- Plasminogen activator inhibitor-1 (PAI-1)
- D-Dimer
- Thrombin-Antithrombin-Complexes (TAT)
- Circulating endothelial cells
- E-Selectin
- Thrombelastometry parameters
- Proinflammatory exosome
- Differential blood counts
- Proinflammatory exosomes
- LPS concentration

5.2.3. Rationale of the chosen endpoints

F1+2 is a biomarker of thrombin generation and therefore coagulation activation. It has been repeatedly shown that infusion of a LPS bolus reliably increases F1+2 about 6-10fold[30-32]. We hypothesize that the tissue-factor driven activation of coagulation and therefore thrombin generation is reduced by treatment with DF. Levels of F1+2 will be measured by enzyme-linked immunoassay (ELISA) at predefined time-points.

Secondary endpoints include other markers of coagulation activation, such as TAT or microparticle associated tissue factor levels by enzyme-linked immunoassay or the clotting time or the clot formation time in thrombelastometry, which is a global

coagulation assay demonstrated to be sensitive to LPS induced coagulation activation[33]. However, it was demonstrated that DF treatment affects the fibrinolytic system and we will pay special interest to biomarkers of this system[2]. We will measure PAI-1, t-PA and D-Dimer levels by enzyme-linked immunoassays. Maximum lysis, a parameter of thrombelastometry also offers information on the fibrinolytic state of whole blood. Moreover, we will assess biomarkers of endothelial activation. Infusion of LPS is known to activate endothelial cells. DF was demonstrated to reduce endothelial activation [2]. Thus, vWF activity and antigen, E-Selectin (all by enzyme-linked immunoassay) and circulating endothelial cells (by flowcytometry analysis) will be measured. Moreover, anti-inflammatory effects of DF will be investigated and assessed by measuring inflammatory biomarkers such as TNF-a levels by enzyme-linked immunoassay. Finally differential blood counts will be performed repeatedly to assess effects of DF and LPS on cell counts.

The increase in tissue factor activity bearing microparticles is well known. However, the effects of endotoxemia on the release of proinflammatory exosomes, membrane vesicles smaller than microparticles, have not yet been investigated. Furthermore we want to assess the impact of DF on proinflammatory exosomes.

After administration, LPS is cleared very rapidly from the bloodstream. After intravenous bolus injection of 2ng/kg bodyweight, studies have reported absence of detectable endotoxin levels as soon as 30 minutes post-dose [34].

In a recently published clinical trial, colistin was shown to markedly reduce the inflammatory response in LPS-treated healthy volunteers [35]. This effect is thought to be due to binding and neutralization of circulating LPSby colistin. However, due to technical reasons this study did not include measurement of LPS plasma levels, and the ultimate evidence for the abovementioned hypothesis is still lacking.

Now, a collaboration with a research group from Uppsala, Sweden would provide us with the opportunity of measuring LPS by means of a chromogenic limulus amoebocyte lysate (LAL) assay.
The quantification of LPS kinetics as attempted in the present study is to be regarded as exploratory and will provide crucial information for potential future investigations on the topic.
The availability of a reliable assay for LPS quantification might trigger additional in vitro and in vivo experiments aiming at demonstrating the link between increase of colistin concentrations and decline of LPS levels in a more stringent way.

5.3. Safety Endpoints

Serum CRP, GPT levels and blood cell count at 24h, hemodynamic and febrile responses, as well as adverse events are considered safety endpoints.
We will specifically assess any signs of bleeding and perform a follow-up visit 1-2 weeks after the last study day.

5.4. Blood Sampling schedule, tubes and volume

Blood samples will be drawn at predefined time-points. Baseline measurements will be performed before infusion of any trial drug. Total blood volume will not exceed 240ml per period.

Table 1. Parameters, Samples and Schedule

Parameter	Sample	Time points
Coagulation biomarkers	Citrated plasma	Pre, 0h, 1h, 2h, 4h, 6h, 24h
Fibrinolysis biomarkers	Citrated plasma	Pre, 0h, 1h 2h, 4h, 6h, 24h
Endothelial biomarkers	EDTA-plasma/ Citrate plasma	Pre, 0h, 1h 2h, 4h, 6h, 24h
Inflammatory biomarkers	EDTA-plasma, Serum, Citrate-plasma	Pre, 0h, 1h 2h, 4h, 6h, 24h
FACS analysis	Citrated blood	Pre, 0h, 1h 2h, 4h, 6h, 24h,

MPTF	Citrated plasma	Pre, -0h, 2h, 4h, 6h, 24h
Chemistry	Serum	Pre, 24h
Differential blood counts	EDTA	Pre, 0h, 1h 2h, 4h, 6h, 24h
Global coagulation assays	Citrate	Pre, 0h, 1h 4h, 24h
LPS quantification	Citrate	0h, 5min ,10min, 20min

Pre= Before Defibrotide/Placebo infusion and before LPS, 0h=Before LPS injection, other time-points relate to the LPS injection

5.5. Preparations of samples

5.5.1. Thrombelastometry

Thrombelastometry (ROTEM; Pentapharm GmbH, Munich, Germany) will be performed using citrate anti-coagulated whole blood as previously described[33]. In short, thrombelastometry measures the viscoelastic properties of whole blood during clot formation and clot lysis. Thrombelastometry is sensitive to infusion of 2ng/kg bodyweight LPS[33].

5.5.2. Coagulation specific and fibrinolytic parameters

F1+2, TAT, t-PA, PAI-1, D-Dimer and MPTF activity will all be detected in citrate anti-coagulated plasma samples. Whole blood samples will be centrifuged at 2000g for 15 minutes at 4 degrees Celsius and supernatant plasma will be transferred to aliquots of approximately 500µl each. These aliquots will be frozen and stored until batch analysis. MPTF will be centrifuged twice to generate platelet free plasma. F1+2, TAT, t-PA, PAI-1 and D-Dimer will be assessed by commercially available enzyme-linked immunoassays. MPTF activity will be assessed as previously demonstrated[32].

5.5.3. Inflammatory biomarkers

EDTA-anticoagulated blood will be centrifuged at 2000g for 15 minutes at 4 degrees Celsius and supernatant plasma will be transferred to aliquots of approximately 500µl each. These aliquots will be frozen and stored until batch analysis. TNF-a will be measured by commercially available enzyme-linked immunoassays. We will assess changes in the proinflammatory exosome and the influence of DF on exosomes. Therefore, citrate anticoagulated blood will be centrifuged twice and isolated exosomes will be analyzed by mass spectrometry.

5.5.4. Endothelial biomarkers

vWF antigen levels and activity and E-Selectin levels will be assessed by commercially available enzyme linked immunoassays. EDTA- and citrate-anticoagulated blood samples will be centrifuged at 2000g for 15 minutes at 4 degrees Celsius and supernatant plasma will be transferred to aliquots of approximately 500µl each. These aliquots will be frozen and stored until batch analysis.

5.5.5. Differential blood counts

Differential blood counts will be performed at the central laboratory of the General Hospital of Vienna.

5.5.6. Blood Chemistry

Blood chemistry will be performed at the central laboratory of the General Hospital of Vienna before infusion of any trial drugs and 24 hours after infusion of LPS.

5.5.7 Global coagulation assays

Global coagulation assays will be performed at the central laboratory of the General Hospital of Vienna.

5.5.8. Circulating endothelial cells

Circulating endothelial cells will be assessed by flowcytometric analysis as previously described[36]. 1ml of freshly drawn EDTA-anticoagulated blood will be first mixed with 10ml of FACS lysing solution (10x, diluted 1:10) according to the manufacturers instructions. The white cells will then be blocked with 20µl of specific Fc-receptor antibodies (Octagam) and 200µl of mouse serum (Sigma, Gillingham, UK) for a minimum of 20min at room temperature. Next, the cells will be incubated with fluorochrome-labelled monoclonal anti-human mouse antibodies, namely FITC-CD45, PE-CD146 and PE-Cy5-CD34 (Becton Dickinson, Oxford, UK) for 20 min at room temperature, washed with cell buffer solution [PBS + bovine serum albumin (BSA) 1% + sodium azide 0.05%] and centrifuged at 500g to repellet the cells. The cells will be fixed with 200µl of 2% paraformaldehyde for 20min at 4°C and made up to a final volume of 1ml with cell buffer solution ready for analysis. Each sample will be analysed in a 3-colour FACScan flow cytometer (Becton Dickinson, Oxford, UK). Cells will be plotted according to forward scatter and side scatter profiles (a measure of size and granularity of an event, respectively) and gated to include only mononuclear cell events and excluding cell doublets, platelets, dead cells/debris, microparticles and high side scatter events (e.g. dead cells and polynuclear cells have higher side scatter). A second gate will be used to include only those cells negative for CD45 (FITC) (CD45 is a pan-leukocyte marker, and a 'dump' channel will be constructed to exclude all CD45 positive cells including CD45dim cells as included in these are endothelial progenitor cells) and are low to medium side

scatter singlets. A third gate will be used to analyse cells doubly positive for CD146 (PE) and CD34 (PE-Cy 5) expression, and only high intensity doubly fluorescent cells (i.e. CD146+++/CD34+++) will be defined as CECs. The sample will be analysed for a minimum of 500,000 mononuclear cellular events and a pre-set time period, corresponding to a known volume of sample (predetermined from previous validation studies, data not shown). Fluorochrome-matched isotype controls (FITC-IgG1, PE- IgG1, PE-Cy5– IgG1; Becton Dickinson, Oxford, UK) as well as non-stained samples will be used to set the appropriate gate parameters and served as negative controls.

5.5.9. LPS quantification

Blood for LPS quantification will be collected in citrate tubes and put on ice. Within 30 minutes from collection, samples will be centrifuged at 4°C and 2000 g for 10 minutes and resulting plasma transferred into 3 aliquots of approximately 500 µL each.
All aliquots will be snap frozen at -20°C and later stored at -80°C until analysis. LPS will be measured at the Department of Pharmaceutical Biosciences, Uppsala University, Sweden, by means of a chromogenic limulus amoebocyte lysate assay.
Optionally, a second method of LPS quantification (Haemotox rFC, Haemochrom Diagnostica, Germany) might be used and results compared with the LAL assay.

6. Trial Materials

6.1. Trial Products

LPS:	US Standard Reference Endotoxin, CC-RE Lot 2, vialed by MARP/NCI
Manufacturer:	FROC 2ng/ml
Dose:	2ng/kg administered at 0 min as an i.v. bolus over 1-2 min
Defibrotide:	Defitelio, 80mg/ml, Ampullae of 2,5ml
Manufacturer:	Jazz Pharmaceuticals

Dose: 6.25mg/kg bodyweight as a countinuous infusion over 2 hours

Physiologic Saline: Isotone Kochsalz-Lösung 0,9% Braun

Manufacturer: Braun

Dose: 100ml/h for six hours

Paracetamol: Paracetamol Genericon

Manufacturer: Genericon Pharma

Dose: Paracetamol 500mg p.o. on demand

6.2. Packaging and Labeling of trial products

Not applicable.

6.3. Storage and Drug Accountability of Trial Product(s)

All trial products should be stored in a secure place.

Defitelio should be stored at room temperature. After dissolution it should be kept refrigerated and used within 24 hours according to the manufacturer.

LPS should be kept refrigerated at 4-8 °C.

Saline and paracetamol should be stored at room temperature.

A record of all supplies dispatched to the investigator will be maintained by the Monitor. The investigator will keep accurate records of all used and unused sup-plies. No trial products may be dispensed to any person except to the subjects en-rolled in the trial.

6.4. Randomization and Blinding

All screened subjects will receive a screening number. This number will be the identifier of each subject within the trial. A subject identification list, which links the screening numbers with the names and birth dates of each participating subject will be kept in the trial master file and in the "study maker" (the computer system of the Department of Clinical Pharmacology). In future publications we will only refer to numbers of subjects, but not to names or birth dates.

A physician not performing other tasks in the trial will observe the subjects.

Block randomization will be performed using an open access randomization generator (www.randomization.com).

Three sets of sealed code/label with the randomization number containing information about the treatment allocation (LPS or placebo group, DF or placebo period) for the particular subject will be prepared for each subject.
The code for a particular subject can be broken in a medical emergency if knowing the identity of the treatment allocation would influence treatment of the subject. Whenever a code is broken, the person breaking the code must record the time, date and reason as well as their initials in the source documents.

All codes (whether broken or not) must be kept throughout the trial period.

Breaking of the code, however, does not necessarily mean that the plasma obtained from the subject is not eligible for analyses, which can still be performed in a blinded fashion.

6.5. Potential inconveniences and risks for the subjects

6.5.1. Defibrotide

Defibrotide is approved by the FDA and the EMA for treatment of the sinosoidal obstruction syndrome (also called veno-occlusive disease) after hematopoietic stem cell transplantation or chemotherapy. Only few studies exist investigating the effects of DF in healthy volunteers[7,37]. No serious or severe adverse events were reported in these trials.

The summary of product characteristics suggest that the most important adverse events of DF are hemorrhage, hypotension and coagulopathy[26]. Of note, compared to a historical control group these side effects did not occur more frequently in the DF group indicating that these side effects may be due to the selection of patients, who were all thrombopenic critically ill patients[38].

No hypersensitivities or anaphylactic reaction were reported in the marketing trials[20,38]. However, in previously marketed defibrotide products hypersensitivities and anaphylactic reactions have been reported [26]. Thus, all participant will be made aware of that risk and special attention will be paid to potential anaphylactic reactions.

We expect minor anti-coagulatory effects after infusion of DF. However, studies suggest that the effects of DF are only short termed and DF should be eliminated completely within 24 hours. All subjects will be made aware of an increased bleeding risk on the treatment days.

6.5.2. LPS

2-4ng/kg bodyweight are safe doses, which have already been used in clinical trials [30] and at the investigational site in over 1000 subjects. LPS produces flu-like symptoms consisting of fever, chills, myalgias, arthralgias, nausea and headache which are most

prominent 2-3h following endotoxin, and begin to resolve after 6 hours. Overall, infusion of endotoxin is generally well tolerated and trial subjects are virtually free of symptoms after 8 hrs.

6.5.3. Paracetamol

At therapeutic dosages (0.5 to 1g orally bid or tid) paracetamol is usually well tolerated and no common adverse effect has been established. Rarely allergic skin reactions may be seen. Chronic abuse or overdose can lead to disturbances in kidney and liver function.

6.5.4. Blood sampling

A total amount of approx. 500ml of blood will be collected during the duration of 8 weeks. This is about the volume of one blood donation. Between the two study periods a washout period of 6 weeks will apply. Thus, the blood volume should not impose any dangers on subjects. Care will be taken to avoid formation of subcutaneous haematomas after venipunctures.

6.5.5. Risk/benefit assessment

Endotoxin infusion in humans is a safe standard model, which induces a systemic inflammatory response and which has been safely used in more than 1000 volunteers at our institution. This inflammatory response is limited in duration and severity and can be used with restrictions as a model for the early phase of human sepsis.

Defibrotide has been used in clinical trials and clinical practice for over 30 years. Although many *in vitro* and animal studies have been published and effects in patients with SOS[38], with atherosclerosis obliterans[39] or peripheral arterial occlusive disease[40],

as a prophylaxis against deep venous thrombosis after surgery[9], and other diseases have been investigated, the mode of action is still not entirely understood. However, in all those studies the use of DF was safe and no serious or severe AEs occurred.

The aim of this trial is to better define the pharmacodynamic effects of DF in a well established model of acute, transient inflammation in healthy volunteers. Furthermore, this trial will allow comparison of DF and other drugs that have already been investigated in this model and will help to generate further hypotheses about new indications of DF. The main risk of DF is a potential allergic reaction or bleeding episodes. However, the anti-coagulatory effects of DF will be closely monitored and strong anticoagulatory effects are not expected. Allergic reactions are rare, but must be considered a potential risk.

In conclusion we consider the benefits to clearly outweigh the risks in this study.

6.6. Safety precautions

Throughout the trial-period the subjects will be observed by a physician. In addition, a physician with experience in emergency medicine will be at the unit during the first eight hours. The clinical trial center is equipped with monitoring facilities comparable to an intensive care unit.

The subjects will be supine with their chest tilted up throughout the trial period and their vital parameters monitored (ECG, heart rate and oxygen saturation continuously; blood pressure at 20 min intervals). Oral temperature will be checked every hour during the first 6 hours.

Frequent blood cell counts and monitoring of platelet function will be done.

Although our experience with more than 1000 LPS challenges showed no severe untoward effects, all adverse events will be managed by adequate medical measures according to standard practice. Evaluation of the safety parameters will be performed for each trial subject, which includes checks of C-reactive protein, GPT-levels blood cell count 24 hours after the endotoxin bolus.

7. Concomitant Illness and Medication

Concomitant illness: any illness that is present at the start of the trial

Concomitant medication: any medication other than the trial product that is taken during the trial, including screening and run-in periods

7.1. Concomitant Medication during the Trial

No concomitant medication is planned.

7.2. Precautions/Overdosage

As all drugs will be administered at the study site by a physician and controlled by an independent staff member according to SOP, no overdose are described in the Investigator's Brochure.

8. Adverse Events

8.1. Definitions

8.1.1. Adverse Events

An AE is any undesirable medical event occurring to a subject in a clinical trial, whether or not considered related to the trial product(s). This includes events not seen at baseline or worsened if present at baseline. The following should not be re-corded as AEs, if recorded at screening (on Screening Form or CRF):

Pre-planned procedure, unless the condition for which the procedure was planned has worsened since baseline

Pre-existing conditions found as a result of screening procedures.

8.1.2. Clinical Laboratory Adverse Events

A clinical laboratory AE is any clinical laboratory abnormality that suggests a dis-ease and/or organ toxicity and is of a severity which requires active management (i.e. changes of dose, discontinuation of drug, more frequent follow-up or diagnostic investigation).

8.1.3. Serious Adverse Events (SAE)

An SAE is any adverse drug experience that at any dose results in any of the follow-ing outcomes:

- death
- a life-threatening* experience
- in-patient hospitalization or prolongation of existing hospitalization
- a persistent or significant disability/incapacity
- a congenital anomaly/birth defect.

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon appropriate medical judgment, they may jeopardize the health of the subject or patient and may require medical or surgical intervention to prevent one of the out-comes listed

in this definition.

*The term life-threatening in the definition of serious adverse event refers to an event in which the subject was at risk of death at the time of the event. It does not refer to an event, which hypothetically might have caused death if it was more severe.

8.1.4. Non-Serious Adverse Event

A non-serious adverse event is any AE, which does not fulfill the definition of an SAE.

8.1.5. Severity Assessment Definitions

Mild: transient symptoms, no interference with the subject's daily activities.

Moderate: marked symptoms, moderate interference with the subject's daily activities.

Severe: considerable interference with the subject's daily activities unacceptable.

8.1.6. Relationship to Trial Drug Assessment Definitions:

Probably: Good reasons and sufficient documentation to assume a causal relationship

Possible: A causal relationship is conceivable and cannot be dismissed

Unlikely: The event is most likely related to an aetiology other than the trial treatment

Impossible to assess: Causality is not assessable, e.g. because of insufficient evidence, conflicting data or poor documentation.

8.1.7. Outcome Categories and Definitions:

Recovered

Stabilized: An AE is stabilized when, according to the Investigator, the subject is in a clinically stable condition. This term should only be used for chronic conditions and for a given subject only when he/she has completed the protocol

Recovered with sequelae: As a result of the SAE, the subject suffered persistent and significant disability/incapacity (e.g. became blind, deaf, paralyzed). Any AE re-covered with sequelae should be rated as an SAE.

Not yet recovered

Died

Unknown.

8.2. Collection, Recording and Reporting of Adverse Events

All events meeting the definition of an adverse event must be collected and reported from the first trial-related activity after the subject signs the informed consent and until the end of the protocol required post-treatment follow-up period.

At each contact to the site (visit or telephone, excluding safety visits, where the subject is not seeing the Investigator or his staff (e.g. visits to the laboratory)), the subject must be asked about adverse events. All adverse events, either observed by the Investigator or reported by the subject, must be recorded by the Investigator and evaluated.
The subjects will be asked a general question “How are you?” as well as a specific question “Have you experienced any problems since the last contact?”

The Investigator should record the diagnosis, if available. If no diagnosis is available the Investigator should record each sign and symptom as individual Adverse Events.

All adverse events must be recorded by the Investigator on the standard Adverse Event Form. One single Adverse Event Form/line must be used per adverse event from start to resolution. For serious adverse events, the Serious Adverse Event Supplementary pages 1-4 must also be completed.

The Investigator must inform the Health Authorities and IRBs/IECs in accordance with the local requirements in force and ICH guideline for GCP. The Monitor must be informed accordingly.

8.3. Follow-up of Adverse Events

During and following a subject's participation in a clinical trial, the Investigator/institution will ensure that adequate medical care is provided tot he subject for any adverse events, including clinically significant laboratory values related to the trial. The investigator/institution will inform the subject when medical care is needed for adverse event(s) of which the investigator becomes aware. The active post-treatment follow-up period will end withthe post-trial examination.

All adverse events classified as serious or severe or possibly/probably related to the trial product must be followed until the subject has recovered, stabilized, recovered with sequelae or died, and until all queries have been resolved.

All other adverse events must be followed until the subject has recovered or stabi-lized or until the end of the protocol required post-treatment follow-up whichever comes first, and until all adverse event related queries for the subject have been re-solved.

8.4. Case record forms

A case record form will be completed for each subject. The entries will be checked by trained personnel and any errors or inconsistencies will be corrected immediately. CRFs will be completed by trained personnel at the trial site.

8.5. Rules for completing CRFs

Print legibly using a ball-point pen. Ensure that all relevant questions are answered and that no empty data blocks exist.

If a test/assessment is not done and will not be available, indicate this by writing „N/D“ (Not done) in the respective answer field in the CRF. If the question is irrelevant (e.g. is not applicable) indicate this by writing „N/A“ (Not applicable) in the respective answer field.

The investigator or investigator's authorized staff must ensure that all information derived from source documents should be consistent with the source information. By signing the affirmation statement, the investigator confirms that the information is complete and correct.

8.6. Correction of CRFs

Corrections to the data on the CRFs must only be made by drawing a straight line through the incorrect data and by writing the correct value next to data that has been crossed out. Each correction must be initialed and dated and explained (if necessary) by the Investigator or the Investigator's authorized staff. If corrections are made by the Investigator's authorized staff after the date of the investigator's signature on the affirmation statement, the statement must be signed and dated again by the investigator.

Corrections necessary after the CRFs have been removed from the Investigator's site must be documented on a Query Resolution Form (QRF). If the affirmation statement for the subject has not been signed, corrections must be approved by the Investigators or her/his authorized staff. If the affirmation statement fort he subject has been signed, only the investigator can approve the correction.

8.7. CRF Flow

Paper CRFs will be used and stored at the trial side. Laboratory report of pre-and post-trial screening examinations will be attached as copies to the CRFs, in case of clinically relevant deviations from normal this will be protocolled in the CRF. The Investigator or

another authorized physician will sign analysis results (e.g. laboratory, ECG, etc.) as soon as they are available to verify that the results are reviewed.

9. Monitoring procedures

During the course of the trial, the Monitor will visit the investigational site approximately two times during each part and must be available for discussions by telephone. The purpose of these visits is to ensure that the CRFs are completed correctly, the protocol is adhered to, to monitor drug accountability, and to collect completed pages of the CRFs.

The Monitor must be given direct access to source documents (original documents, data and records). Direct access includes permission to examine, analyze, verify and reproduce any record(s) and report(s) that are important to evaluation of the clinical trial. Source data will be available for all data in the CRFs, including all laboratory results.

The Monitor will ensure that the CRFs are collected.

10. Data Management

The subjects will be identified by subject number, initials, site, and trial identification number. Subject initials will be retained in the database only during the period of trial data processing. Upon completion of the Integrated Clinical Trial Report, subject initials will be deleted or encrypted to ensure subject confidentiality.

Electronic data transfer of any CRF or subject related data must be approved by the responsible Data Management Unit(s). In cases where data is transferred via non-secure electronic networks, data must be encrypted at source.

11. Statistical Considerations

11.1. Sample Size Calculation

The sample size calculation is based on the experience with prior trials using anti-coagulants in the human endotoxemia model. Ten to 15 subjects in each group were sufficient to detect significant differences in the primary outcome parameter (F1+2 levels) between treatment groups in a parallel-group study[14,23,24]. Currently, we do not have a good estimate for the size of effect of defibrotide in the same model. In a prior LPS trial F1+2 levels of approximately 819±173 (Standard deviation) pmol/ml were measured [41]. This corresponds to an approximate 6fold increase in F1+2 levels and was similar in other endotoxemia trials[42]. The median intraindividual coefficient of variation was 15% in a recent study, the mean was 25%[32]. Thus, a sample size of 16 volunteers will be sufficient to detect a 25% decrease in the defibrotide period.

The placebo group consisting of four healthy volunteers will be an additional control group, which will also allow to assess the effects of DF in healthy volunteers without LPS. However, this analysis is exploratory and no sample size calculation for this group is performed.

11.2. Methods

Two-sided tests and a 5% critical value will be used throughout the trial. Test procedures to reduce multiple testing will be applied. Refer to the detailed, separate statistical analysis plan for further information.

11.2.1 Efficacy Analysis

The efficacy analysis will compare changes in the primary and secondary efficacy endpoints described above between the placebo and the DF periods.

The repeated measures analysis of variance (ANOVA) will be used for analysis of treatment and period effects (treatment=independent factor, period=independent factor, outcome variable=dependent factor). When significant, post hoc comparisons will be performed with nonparametric tests for reasons of robustness: The Friedman ANOVA and the Wilcoxon test for post hoc comparisons will be used.

Furthermore, graphs showing the time profiles at the individual subject and group mean levels will be presented.

Multiple parameters will be tested. However, to reduce the number of tests a stepwise statistical analysis plan will be applied. Moreover, this study is of exploratory nature and correcting for multiple testing is therefore not necessary.

11.3. Safety Analysis

All subjects who will receive trial drugs (including placebo) will be included in the safety analysis, which will compare the safety endpoints specified above between treatment periods. Tables of summary statistics will be presented. Detailed analysis specifications will be presented in a statistical analysis plan prior to analysis.

As LPS-infusion is associated with a number of adverse effects, particular interest will be focused on potential specific adverse effects of DF such as bleeding events or allergic reactions.

11.4. Missing, unused and spurious data

Missing or spurious data will not be intrapolated, but set missing. Statistical analyses will be performed for the intention to treat and the per-protocol population and compared. If results differ significantly it will be mentioned in any reports of trial results.

12. Ethics

The trial will be conducted in accordance with the Declaration of Helsinki for biomedical research involving human subjects.

12.1. Informed Consent Form of trial subjects (AMG §38)

In obtaining and documenting informed consent, the investigator must comply with the applicable regulatory requirement(s) and adhere to ICH guideline for GCP and the requirements in the Declaration of Helsinki.

Prior to any trial-related activity, the investigator or an authorized physician must give the subject oral and written information about the trial in a form that the subject can read and understand.

A voluntary, signed and dated informed consent form will be obtained from the subject prior to any trial-related activity. The subject must have consented to participate after the nature, scope and possible consequences of the trial have been explained in a form understandable to him or her.

The written informed consent must be signed by the person who conducted the informed consent.

If information becomes available that may be relevant to the subjects's willingness to continue participating in the trial, the investigator must inform the subject in a timely manner, and a revised written informed consent must be obtained.

12.2. Independent Ethics Committees (AMG §§ 40, 41)

Prior to commencement of the trial, the protocol, any amendments, Subject Informa-

tion/Informed Consent Form, any other written information to be provided to the subject, subject recruitment procedures (e.g. advertisements), if any, Investigator's Brochure [10], package insert (if marketed product), information about payments and compensation available to subjects if not mentioned in the subject information, the Investigator's current CV and/or other documentation evidencing qualifications, and other documents as required by the local Independent Ethics Committee (IEC) should be submitted. The submission letter should clearly identify (by including ver-sion no. and/or date of the document) which documents have been submitted to the IRB/IEC. Written approval/favorable opinion must be obtained from the IEC prior to commencement of the trial.

During the trial, the Investigator must promptly report the following to the IEC: Updates to IB, unexpected SAEs where a causal relationship cannot be ruled out, amendments to the protocol, notes of administrative changes, deviations to the protocol implemented to eliminate immediate hazards to the trial subjects, new information that may affect adversely the safety of the subjects or the conduct of the trial, annually written summaries of the trial status, and other documents as required by the local IEC.

Amendments must not be implemented before approval/favorable opinion, unless necessary to eliminate immediate hazards to the subjects.

12.3. Regulatory Authorities

The Regulatory Authorities will receive the protocol, amendments, reports on SAEs, and the Final Report according to Austrian regulations.

12.4. Insurance (AMG §32(2))

All subjects participating in this clinical trial will be insured through the Department of Clinical Pharmacology, Vienna University Hospital.

12.5. Confidentiality

All subject names will be kept secret in the investigator's files.

13. Audits and Inspections

Upon request, the Investigator will make all study-related source data and records available to a qualified quality assurance auditor mandated by the sponsor or to competent authority inspectors. The main purposes of an audit or inspection are to confirm that the rights and welfare of the subjects have been adequately protected, and that all data relevant for assessment of safety and efficacy of the investigational product have appropriately been reported to the sponsor.

All material used in clinical studies are subject to quality control.

14. Publication policy

The findings of this study will be published by the sponsor [43] in a scientific journal and presented at scientific meetings. The manuscript will be circulated to all co-Investigators before submission. Confidentiality of subjects in reports/publications will be guaranteed.

15. Premature Termination of the trial

The Investigator may decide to stop the trial or part of the trial at any time, but agreement on procedures to be followed must be obtained.

If a trial is prematurely terminated or suspended, the Investigator should promptly inform the subjects and assure appropriate therapy and follow-up. Furthermore, the Investigator should promptly inform the IRB/IEC and provide a detailed written explanation. The pertinent regulatory authorities should be informed according to national regulations.

16. Retention of Clinical Trial Documentation

Subject notes must be kept for the maximum time period as permitted by the hospital, institution or private practice. Other source documents and the Investigator's trial file must be retained for at least 10 years or longer in accordance with local regulation. However, the Subject Identification Codes must be kept for at least 15 years.

The Investigator must agree to archive the documentation pertaining to the trial in an archive after completion or discontinuation of the trial, if not otherwise notified.

17. References:

1. Palmer KJ, Goa KL. Defibrotide. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in vascular disorders. *Drugs.* 1993;45(2):259-294.
2. Keating GM. Defibrotide: a review of its use in severe hepatic veno-occlusive disease following haematopoietic stem cell transplantation. *Clin Drug Investig.* 2014;34(12):895-904.
3. Pegram AA, Kennedy LD. Prevention and treatment of veno-occlusive disease. *The Annals of pharmacotherapy.* 2001;35(7-8):935-942.
4. Ulutin ON, Balkuv-Ulutin S, Bezer-Goker B, et al. Effect of defibrotide on platelet function. *Semin Thromb Hemost.* 1996;22 Suppl 1:21-24.
5. Falanga A, Vignoli A, Marchetti M, Barbui T. Defibrotide reduces procoagulant activity and increases fibrinolytic properties of endothelial cells. *Leukemia.* 2003;17(8):1636-1642.
6. Cella G, Sbarai A, Mazzaro G, et al. Tissue factor pathway inhibitor release induced by defibrotide and heparins. *Clin Appl Thromb Hemost.* 2001;7(3):225-228.
7. Pogliani EM, Salvatore M, Fowst C, Girardello R, Marelli C. Effects of a defibrotide-heparin combination on some measures of haemostasis in healthy volunteers. *J Int Med Res.* 1989;17(1):36-40.
8. Palomo M, Diaz-Ricart M, Rovira M, Escolar G, Carreras E. Defibrotide prevents the activation of macrovascular and microvascular endothelia caused by soluble factors released to blood by autologous hematopoietic stem cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation.* 2011;17(4):497-506.
9. Mozzi E, Chiurazzi D, Germiniani R, Pacini F. Effectiveness of defibrotide for prophylaxis of deep venous thrombosis after general surgery: a double-blind, placebo-controlled clinical trial. *Haemostasis.* 1986;16 Suppl 1:36-38.
10. Francischetti IM, Oliveira CJ, Ostera GR, et al. Defibrotide interferes with several steps of the coagulation-inflammation cycle and exhibits therapeutic potential to treat severe malaria. *Arterioscler Thromb Vasc Biol.* 2012;32(3):786-798.
11. Kornblum N, Ayyanar K, Benimetskaya L, Richardson P, Iacobelli M, Stein CA. Defibrotide, a polydisperse mixture of single-stranded phosphodiester oligonucleotides with lifesaving activity in severe hepatic veno-occlusive disease: clinical outcomes and potential mechanisms of action. *Oligonucleotides.* 2006;16(1):105-114.
12. Kumar S, DeLeve LD, Kamath PS, Tefferi A. Hepatic veno-occlusive disease (sinusoidal obstruction syndrome) after hematopoietic stem cell transplantation. *Mayo Clinic proceedings.* 2003;78(5):589-598.
13. Richardson PG, Ho VT, Giralt S, et al. Safety and efficacy of defibrotide for the treatment of severe hepatic veno-occlusive disease. *Therapeutic advances in hematology.* 2012;3(4):253-265.
14. Derhaschnig U, Reiter R, Knobl P, Baumgartner M, Keen P, Jilma B. Recombinant human activated protein C (rhAPC; drotrecogin alfa [activated]) has minimal effect on markers of coagulation, fibrinolysis, and inflammation in acute human endotoxemia. *Blood.* 2003;102(6):2093-2098.
15. Jilma B, Blann A, Pernerstorfer T, et al. Regulation of adhesion molecules during human endotoxemia. No acute effects of aspirin. *American journal of respiratory and critical care medicine.* 1999;159(3):857-863.

16. Boos CJ, Mayr FB, Lip GY, Jilma B. Endotoxemia enhances circulating endothelial cells in humans. *Journal of thrombosis and haemostasis : JTH.* 2006;4(11):2509-2511.
17. Sabatier F, Camoin-Jau L, Anfosso F, Sampol J, Dignat-George F. Circulating endothelial cells, microparticles and progenitors: key players towards the definition of vascular competence. *J Cell Mol Med.* 2009;13(3):454-471.
18. Almici C, Skert C, Verardi R, et al. Changes in circulating endothelial cells count could become a valuable tool in the diagnostic definition of acute graft-versus-host disease. *Transplantation.* 2014;98(7):706-712.
19. Pescador R, Capuzzi L, Mantovani M, Fulgenzi A, Ferrero ME. Defibrotide: properties and clinical use of an old/new drug. *Vascular pharmacology.* 2013;59(1-2):1-10.
20. Corbacioglu S, Cesaro S, Faraci M, et al. Defibrotide for prophylaxis of hepatic veno-occlusive disease in paediatric haemopoietic stem-cell transplantation: an open-label, phase 3, randomised controlled trial. *Lancet.* 2012;379(9823):1301-1309.
21. Spiel AO, Derhaschnig U, Schwameis M, Bartko J, Siller-Matula JM, Jilma B. Effects of prasugrel on platelet inhibition during systemic endotoxaemia: a randomized controlled trial. *Clinical science.* 2012;123(10):591-600.
22. Leitner JM, Firbas C, Mayr FB, Reiter RA, Steinlechner B, Jilma B. Recombinant human antithrombin inhibits thrombin formation and interleukin 6 release in human endotoxemia. *Clinical pharmacology and therapeutics.* 2006;79(1):23-34.
23. Pernerstorfer T, Hollenstein U, Hansen JB, et al. Lepirudin blunts endotoxin-induced coagulation activation. *Blood.* 2000;95(5):1729-1734.
24. Pernerstorfer T, Hollenstein U, Hansen J, et al. Heparin blunts endotoxin-induced coagulation activation. *Circulation.* 1999;100(25):2485-2490.
25. Pernerstorfer T, Stohlawetz P, Hollenstein U, et al. Endotoxin-induced activation of the coagulation cascade in humans: effect of acetylsalicylic acid and acetaminophen. *Arteriosclerosis, thrombosis, and vascular biology.* 1999;19(10):2517-2523.
26. EMA. SmPC Defitelio.
27. Richardson PG, Murakami C, Jin Z, et al. Multi-institutional use of defibrotide in 88 patients after stem cell transplantation with severe veno-occlusive disease and multisystem organ failure: response without significant toxicity in a high-risk population and factors predictive of outcome. *Blood.* 2002;100(13):4337-4343.
28. Richardson PG, Soiffer RJ, Antin JH, et al. Defibrotide for the treatment of severe hepatic veno-occlusive disease and multiorgan failure after stem cell transplantation: a multicenter, randomized, dose-finding trial. *Biol Blood Marrow Transplant.* 2010;16(7):1005-1017.
29. Administration FaD. http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm493278.htm. 2016.
30. Mayr FB, Jilma B. Coagulation interventions in experimental human endotoxemia. *Transl Res.* 2006;148(5):263-271.
31. Derhaschnig U, Schweeger-Exeli I, Marsik C, Cardona F, Minuz P, Jilma B. Effects of aspirin and NO-aspirin (NCX 4016) on platelet function and coagulation in human endotoxemia. *Platelets.* 2010;21(5):320-328.
32. Schoergenhofer C, Schwameis M, Hobl EL, et al. Potent irreversible P2Y12 inhibition does not reduce LPS-induced coagulation activation in a randomized, double-blind, placebo-controlled trial. *Clinical science.* 2016;130(6):433-440.

33. Spiel AO, Mayr FB, Firbas C, Quehenberger P, Jilma B. Validation of rotation thrombelastography in a model of systemic activation of fibrinolysis and coagulation in humans. *Journal of thrombosis and haemostasis : JTH.* 2006;4(2):411-416.
34. van Deventer SJ, Buller HR, ten Cate JW, Aarden LA, Hack CE, Sturk A. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood.* 1990;76(12):2520-2526.
35. Matzneller P, Strommer S, Drucker C, et al. Colistin Reduces LPS-Triggered Inflammation in a Human Sepsis Model In Vivo: A Randomized Controlled Trial. *Clin Pharmacol Ther.* 2017;101(6):773-781.
36. Goon PK, Boos CJ, Stonelake PS, Blann AD, Lip GY. Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. *Thromb Haemost.* 2006;96(1):45-52.
37. Noseda G, Fragiacomo C, Ferrari D. Pharmacokinetics of defibrotide in healthy volunteers. *Haemostasis.* 1986;16 Suppl 1:26-30.
38. Richardson PG, Riches ML, Kernan NA, et al. Phase 3 trial of defibrotide for the treatment of severe veno-occlusive disease and multi-organ failure. *Blood.* 2016.
39. Avellone G, Mandala V, Pinto A, Martino A, Strano A. Clinical evaluation of short-term defibrotide treatment of patients with atherosclerosis obliterans of the lower limbs. *Haemostasis.* 1986;16 Suppl 1:55-58.
40. Sabba C, Zupo V, Dina F, Nazzari M, Albano O. A pilot evaluation of the effect of defibrotide in patients affected by peripheral arterial occlusive disease. *Int J Clin Pharmacol Ther Toxicol.* 1988;26(5):249-252.
41. Mayr FB, Spiel AO, Leitner JM, et al. Racial differences in endotoxin-induced tissue factor-triggered coagulation. *J Thromb Haemost.* 2009;7(4):634-640.
42. Schoergenhofer C, Schwameis M, Hobl EL, et al. Potent irreversible P2Y12 inhibition does not reduce LPS induced coagulation activation in a randomized, double-blind, placebo-controlled trial. *Clin Sci (Lond).* 2015.
43. Reilly PA, Lehr T, Haertter S, et al. The effect of dabigatran plasma concentrations and patient characteristics on the frequency of ischemic stroke and major bleeding in atrial fibrillation patients: the RE-LY Trial (Randomized Evaluation of Long-Term Anticoagulation Therapy). *J Am Coll Cardiol.* 2014;63(4):321-328.