The project has started in December 2010 and is funded under the Seventh Framework Programme (FP7) by the European Commission.
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Dear participants and colleagues,

On behalf of all of the investigators of the PRESTIGE consortium it is our great pleasure to welcome you to the final workshop of our project.

The last decades have seen tremendous progress in the field of cardiovascular medicine and we have been privileged to bear witness to how these advances have translated into improved outcomes and lower mortality for the patients that we care for. In particular advances in coronary angioplasty and stenting have improved the quality of life of millions of patients and prolonged life in countless more. Nevertheless, although it is infrequent, sudden stent failure due to stent thrombosis is an event that often has life changing consequences for our patients and its prevention and management remains an important challenge in interventional cardiology.

Against this background we are grateful to have had at our disposal a collaboration of clinician investigators, basic scientists, industry partners and small and medium sized enterprises to tackle the challenges provided by stent thrombosis. We divided our efforts into 4 scientific work packages, a summary of which you will see presented in the following pages. Over the course of the next day and a half we are delighted to present to you key scientific findings from each work package and to discuss with you the implications of these findings and the possibilities they open for future research and collaboration.

In the first part of our program we will share with you the central aims, the key structures and the principal achievements of our project work packages. In the oral and poster presentations that follow we will highlight some of the outstanding work that has already been realized as well as both the endeavours that are ongoing and the future projects that are planned. In addition we are delighted to welcome Prof. George Dangas from Mount Sinai School of Medicine, New York, who will deliver a state-of-the-art lecture on stent thrombosis and its risk stratification. Prof. Dangas and his group have been at the forefront of research into mechanisms of stent failure, new stent technology and adjuvant treatments for many years and we are honoured to add his unique insights and expertise to our symposium proceedings today.
We are particularly proud to welcome our partners from all over Europe. Meeting the challenges of stent thrombosis is best accomplished by a pan-European approach, drawing on the knowledge and interrelationship of a network of excellent researchers in the respective fields of medicine and industry. After all the field of interventional cardiology owes its origins to the pioneering efforts of European cardiologists from the first angioplasty procedures of Andreas Grüntzig performed around 300 km from here in Zürich through the early coronary stent procedures of Jacques Puel in Toulouse and Ulrich Sigwart in Lausanne. One of our strengths as Europeans lies in our cultural diversity and in this respect we have been fortunate to have had participation from experts from all across Europe – North, South, East and West. Moreover in view of the significant variation in rates and types of heart disease across Europe we feel that it is important to study patients and outcomes across the breadth of the European continent.

It is a particular pleasure to welcome you to the historic surroundings of the Weihenstephan Brewery in Freising. The Weihenstephan Brewery is the academic brewery of the Technische Universität München and the oldest brewery in the world. We hope that the surroundings provide a suitable backdrop to facilitate our scientific presentations and discussions and that they will leave a lasting impression on our participants and colleagues.

On an occasion such as this it is particularly important to acknowledge the central contribution of the European Commission in funding our collaboration. Without this financial support the work of our project would not have been possible. In particular we wish to express of sincere thanks to Dr. Virginija Dambrauskaite as well as to our Project Officer Miguel Lizaso for their interest in and support for our project.

Finally it is important to realize that the work of our consortium does not end here. Rather the true advantage of such collaboration is in the lasting network that remains, the infrastructure that facilitates ongoing scientific endeavours, exchange of researchers and ideas, and the achievement of future common research goals. Indeed we are very confident that once the scaffolding is removed the building will still stand strong!

Prof. Adnan Kastrati and Prof. Steffen Massberg
on behalf of the PRESTIGE consortium
Member of the PRESTIGE consortium at the Governing Board Meeting in Munich (January 2014)

Summary description of project context and objectives

The development of drug-eluting stent (DES) therapy is a significant milestone in the care of patients with obstructive coronary artery disease. In particular DES therapy represented a significant gain in the battle against coronary restenosis, the major limitation of percutaneous coronary intervention (PCI) in the bare metal stents (BMS) era. With the passage of time however it has become clear that this advancement has come at the cost of a small excess of thrombotic stent occlusion – stent thrombosis (ST) – particularly late after DES implantation.

The principal objectives of PRESTIGE may be considered under 3 interrelated categories. Firstly, the principal medical objective is to develop new strategies to prevent late ST at a cost of minimum bleeding risk. Secondly, the principal scientific objective is to dissect the mechanisms contributing to the occurrence of late ST. A better mechanistic understanding of late ST...
is a conditio sine qua non for the development of more specific anti-thrombotic regimens that have minimal effects on normal haemostasis. Thirdly, the major technological objective of PRESTIGE is to develop novel imaging technologies allowing for early diagnosis and a better risk prediction of late ST as well as to evaluate optimized stent designs that promote enhanced vascular healing.

The scientific work of PRESTIGE is divided into 4 interrelated work packages (WP) (Figure 1):

**WP1** is focused on better mechanistic understanding of the molecular and cellular events triggering late ST. In dissecting the basic pathophysiology leading to late ST, PRESTIGE has generated a collaborative platform integrating the expertise of European centres of excellence in platelet thrombosis, endothelial biology and coagulation aimed at unravelling the early steps initiating the ST cascade as well as characterizing the key similarities and differences of the processes involved in late ST as compared with normal haemostasis.

**WP2** is aimed at developing and validating novel strategies to reduce late ST: Using a multidisciplinary translational approach combining basic science, preclinical research and small- and medium-sized enterprises as well as the mechanistic insights gained in WP1, this WP is focused on the realisation of novel stent devices with reduced thrombotic risk and improved healing characteristics.

**WP3** is evaluating the role of novel imaging technologies for the assessment of vascular healing and dysfunction after coronary stenting: Utilizing a multidisciplinary approach bringing together imaging engineers basic researchers and clinical imaging specialists this WP is focused on twin imaging modalities of optical coherence tomography (OCT) and near-infrared fluorescence molecular imaging (NIRF). The aim is the thorough validation of imaging parameters mitigating an increased risk of ST via tissue characterization analysis, as well as applied histopathological correlation studies in preclinical models, autopsy specimens and in man. The ultimate clinical goal is the application of imaging technology towards the individualization of prolonged anti-platelet therapy and the consequent amelioration of global bleeding risk.
WP4 is performing multi-stranded characterisation of patients with late ST: The specific objective of PRESTIGE WP4 is the establishment of a pan-European stent thrombosis registry encompassing a collaborative network of centres from Central, Southern, Eastern and North-western Europe. Using a multi-centre case-control model with a recruitment phase of 3 years the PRESTIGE Registry is recruiting at least 500 patients presenting with late ST as well as matched control patients. All patients with ST recruited in PRESTIGE undergo a multi-stranded analysis, including an in-depth description of patient-demographic and procedure-related factors, analysis of genetic and bio-markers, platelet function testing, histopathological analyses of the thrombus retrieved from the involved coronary artery and intracoronary imaging of the involved segment of the coronary artery using OCT and intravascular ultrasound. An optimized identification of patients at risk for ST is prerequisite for the implementation of individualized anti-thrombotic therapies.

Summary of overall work flow of PRESTIGE
**Day 1, Friday 21 November 2014**

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<td>2.</td>
<td>Histological Features of Stent Thrombi – Results from the PRESTIGE Cohort</td>
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<td>3.</td>
<td>Mechanisms of coronary stent thrombosis assessed with Optical Coherence Tomography</td>
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<td>4.</td>
<td>Exploring genetic variations associated with a risk of stent thrombosis</td>
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<td>2.</td>
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## Abstract session
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<td><strong>2.</strong> A new dextran-graft-polybutylmethacrylate copolymer coated on 316L metallic stents enhances endothelial cell coverage</td>
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<tr>
<td><strong>3.</strong> Tissue Characterization after DES Implantation Utilizing Optical Coherence Tomography</td>
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<tr>
<td><strong>4.</strong> Intravascular near-infrared fluorescence imaging of endothelial cells</td>
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## Poster session
10:30 a.m. – 11:15 a.m.

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<th>Presentations</th>
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<td><strong>2.</strong> Oligonucleotide Coating of Co-Cr Stents for EPC Adhesion</td>
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<td><strong>4.</strong> <em>In vivo</em> detection of non-occlusive thrombi in drug-eluting stents by scintigraphy and radio-labelled Annexin V in a rabbit model</td>
</tr>
<tr>
<td><strong>5.</strong> A rabbit iliac model for testing coronary stents</td>
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Response to antiplatelet drugs 30 days after stent thrombosis


1 St. Antonius Hospital, Department of Cardiology, Nieuwegein, the Netherlands
Others: from the PRESTIGE study team from the UK2, Belgium3 and Germany4
(*) jurtenberg@gmail.com

Platelet function was measured 30 days after patients experienced a stent thrombosis. High platelet reactivity was observed in 28.3%, 9.0%, and 8.8% of the patients treated with respectively clopidogrel, prasugrel, or ticagrelor. HPR rates were different when measured with the VerifyNow or Multiplate.

Background
Stent thrombosis (ST) is a severe and feared complication of coronary stenting (PCI). Since blood platelets play a pivotal role in thrombus formation, antiplatelet drugs are an important cornerstone in the prevention of atherothrombotic events, including the high rate of recurrent ST.

Patients with high on-treatment platelet reactivity (HPR) have an insufficient response to antiplatelet drugs and are at higher risk for atherothrombotic events as compared to patients with a sufficient response to antiplatelet drugs (NPR: normal platelet reactivity) [1]. Many patients with ST seem to suffer from HPR [2]. Therefore, the aim of this study was to investigate the response to antiplatelet therapy in patients with ST treated with antiplatelet drugs 30 days after ST.

Methods
Consecutive patients presenting with definite ST, defined by the ARC criteria [3], were included in multiple European centers in the PRESTIGE study. Patients visited the hospital for blood sampling for platelet function testing 30 days after ST. Platelet reactivity was tested with the VerifyNow P2Y12 (VN-P2Y12) and/or Multiplate ADP test (MEA-ADP). HPR was defined as >235 P2Y12 Reaction Units (PRU) for VN-P2Y12 or >47 units (U) for MEA-ADP. When both tests were performed for one patient, HPR was defined as at least one test showed HPR.

Results
Platelet function data was available for 181 144, and 217 patients for the VN-P2Y12, MEA-ADP, pooled group VN-P2Y12/MEA-ADP, respectively. From the patients treated with clopidogrel HPR was observed in 28.3% (17/60), and only in 9.0% (9/100), and 8.8% (5/57) in patients treated prasugrel or ticagrelor, as shown in figure 1.

HPR in patients treated with ticagrelor was only observed when measured with the MEA-ADP. Comparison of the HPR rates measured with the two platelet function tests showed a significantly difference between the VN-P2Y12 and MEA-ADP (4.6% vs. 21.3%, p=0.007).

Early ST (0-30 days after stent implantation) was significantly associated with HPR when measured with MEA-ADP, and was also observed in the pooled group (MEA NPR vs. HPR: 20.5% vs. 48.1%, p=0.003; pooled group NPR vs. HPR 22.2% vs. 41.9%, p=0.019, table 1).

Conclusions
At 30 days after ST, most patients with HPR were treated with clopidogrel, and more often had suffered from an early ST. Prasugrel and ticagrelor may be an effective antiplatelet drug to combat HPR in patients who suffered from ST and to reduce the chance for recurrent ST.

HPR rates are different when measured with the VerifyNow and Multiplate, which makes it difficult to decide whether a patient possibly needs another antiplatelet drug.
References

Figure 1: Percentage of patients with NPR and HPR 30 days after stent thrombosis, maintenance dose with clopidogrel, prasugrel or ticagrelor. NPR: normal platelet reactivity; HPR: high platelet reactivity.

Table 1: Clinical characteristics at 30 days after stent thrombosis per measurement group

<table>
<thead>
<tr>
<th></th>
<th>VN-P2Y12</th>
<th>MEA-ADP</th>
<th>Pooled VN-P2Y12 / MEA-ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NPR</td>
<td>HPR</td>
<td>p-value</td>
</tr>
<tr>
<td>Number</td>
<td>173 (95.6)</td>
<td>8 (4.4)</td>
<td></td>
</tr>
<tr>
<td>Gender (male)</td>
<td>138 (79.8)</td>
<td>7 (87.5)</td>
<td>0.503</td>
</tr>
<tr>
<td>AGE at ST</td>
<td>60 ± 11</td>
<td>72 ± 12</td>
<td>0.004</td>
</tr>
<tr>
<td>BMI at ST</td>
<td>27.8 ± 5.0</td>
<td>26.8 ± 3.5</td>
<td>0.593</td>
</tr>
<tr>
<td>Smoking at ST</td>
<td>130 (76.9)</td>
<td>5 (62.5)</td>
<td>0.288</td>
</tr>
<tr>
<td>LVEF after ST</td>
<td>48 ± 11</td>
<td>42 ± 11</td>
<td>0.255</td>
</tr>
<tr>
<td>Aspirin at 30 days</td>
<td>169 (97.7)</td>
<td>8 (100)</td>
<td>0.833</td>
</tr>
<tr>
<td>Type of ST</td>
<td></td>
<td></td>
<td>0.276</td>
</tr>
<tr>
<td>Acute</td>
<td>13 (7.6)</td>
<td>2 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Subacute</td>
<td>29 (16.9)</td>
<td>1 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Late</td>
<td>10 (5.8)</td>
<td>1 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Very late</td>
<td>120 (69.8)</td>
<td>4 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Early (0-30 days)</td>
<td>42 (24.4)</td>
<td>3 (37.5)</td>
<td>0.320</td>
</tr>
<tr>
<td>Late (≥31 days)</td>
<td>130 (75.6)</td>
<td>5 (62.5)</td>
<td></td>
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</table>

NPR: normal platelet reactivity; HPR: high platelet reactivity; ST: stent thrombosis; LVEF: left ventricular ejection fraction. Statistical analyses are performed with a chi-square test ($\chi^2$) or unpaired Student's t-test. Categorical data are expressed as n (%), and continuous data as mean ± standard deviation.
Stentthrombosis (ST) is an unusual event with a low yearly incidence. Hitherto the published histological series are rather small. In an Europe-wide cooperation more than 200 ST could be harvested. Besides common components like platelets and fibrinogen large amounts of inflammatory cells and most important nuclear extracellular traps (NETs) known as promoter of immunothrombosis were found.

Background
Both bare-metal (BMS) and drug-eluting-stents (DES) can cause stent thrombosis (ST), an infrequent (0.5-1% incidence yearly) [1], but life-threatening complication after stent implantation. We have previously reported that innate immune cells together with coagulation and platelets operate thrombosis of naive arteries [2]. While histological analyses of thrombi indicated that inflammatory cells might also play an important role in ST in humans [3,4], previous studies have been too small to demonstrate their quantitative contribution. In our study, the histological findings from the so far largest series of human ST are presented.

Methods
From 2011-2014 237 ST and 98 native coronary thrombi (NT) were collected, the latter serving as controls. ST were formalin fixed and paraffin embedded. NT were stored at –80°C and embedded in tissue tec. Serial cross sections (5 µm) were cut and stained with Haematoxylin/Eosin to stain neutrophils as well as other blood constituents and LUNA-staining to identify eosinphils. In addition, we performed immunohistochemistry to detect neutrophil elastase, platelet CD41 and fibrinogen/fibrin. Neutrophil extracellular traps (NETs) were identified by Hoechst 33342 (Invitrogen) and neutrophil elastase co-staining. Images were acquired using a Leica DMRB epifluorescence microscope with a Zeiss AxioCam and processed with AxioVision 4.6 software (Zeiss).

Fig. 1: left: NETs in differend subsets of ST patients (classified according to ARC definition). Right, eosinophils ordered by age of ST compared with NT.
Results
Subacute (<30days) and very late ST (>12 months) was the major presentation of ST in DES- (32% and 55%, n=135) and BMS-treated patients (28% and 56%, n=68). The mean time frame between index procedure and late ST was 4.5 years in DES and for 8.2 years in BMS patients. Notably, leukocytes and neutrophils were found in similar amounts in all types of ST (acute ST 2182 ±410/1497±319 per mm² n=12, subacute ST 2234±232/1302±130 per mm² n=53, late ST 2894±533/1733±326 per mm² n=19, very late ST 2250±150/1325±89 per mm² n=111). Notably, eosinophils were also abundantly found in ST, both in BMS- and DES-treated patients (BMS 81±17 per mm²; n=64/DES 74±10 per mm²; n=128/native thrombi 83±13 per mm²; n=90). Patients with subacute ST presented with the highest numbers of eosinophils (120±27 per mm²; n=53). There was no significant difference between eosinophils in subacute ST and controls (p=0.977). The different drug-coatings did not influence the quantity of eosinophils in the thrombi. However, eosinophile counts differed among the different types of limus-eluting DES. Compared to ST, NT showed a significantly lower number of white blood cells (ST 2448±122 per mm² n=213; NT 1543±112 per mm² n=98; p<0.001). While eosinophil numbers were similar in ST and NT patients. In 23 % of ST and 20% of NT, NETs were found, negatively charged fibers of extracellular DNA released from neutrophils known to promote coagulation in vitro and in animal models in vivo [5,6]. NETs were detected in all types of ST (acute ST 16±5 per mm²; n=2/verylate ST 23±2 per mm²; n=28).

Conclusions
We show here that leukocyte, particularly neutrophil recruitment is a hallmark of human stent thrombosis. For the first time NETs, central effectors of immunothrombosis, could be detected in human ST, supporting their relevance in the pathophysiology of ST. Eosinophils are also recruited in ST, indicating that allergic reactions could contribute to this process.

References
Mechanisms of coronary stent thrombosis assessed with Optical Coherence Tomography

Adriaenssens T on behalf of PRESTIGE investigators¹

¹Department of cardiovascular Diseases, University Hospitals Leuven, Herestraat 49, Belgium, tom.adriaenssens@uzleuven.be

Using optical coherence tomography, the most detailed intracoronary imaging method currently available, we assessed the dominant cause of stent thrombosis, at various intervals after implantation. The prevailing dominant cause of stent thrombosis is the presence of uncovered struts, followed by neoatherosclerosis (with or without rupture), stent underexpansion, malapposition and severe stenosis with superimposed thrombus.

Background

Although its incidence has decreased since the introduction of next generation drug-eluting stents and more potent antiplatelet drugs, stent thrombosis remains an important clinical problem, associated with high morbidity and mortality rates. While clinical and procedural predictors of this event have been earlier described, the underlying mechanical causes of stent thrombosis have never been elucidated in the in vivo setting. Intracoronary optical coherence tomography (OCT), thanks to its superb resolution, allows for a reliable assessment of the causes of stent thrombosis the percutaneous coronary intervention at the time of stent thrombosis.

Methods

From January 2011 until October 2014, all cases of stent thrombosis presenting in one of the 9 participating centers of the Prestige consortium, or one of their satellite recruiting centers, were prospectively enrolled in the Prestige registry. When clinically and logistically possible, OCT acquisition at the time of presentation with stent thrombosis was performed. OCT pullbacks were reviewed in several expert panel review sessions where dominant and contributing factors to the occurrence of stent thrombosis were adjudicated. Factors analyzed were the presence of uncovered struts, malapposed struts, stent underexpansion, severe stenosis with thrombus formation, neoatherosclerosis (with or without rupture), extra stent cavity, edge dissection, atherosclerotic disease with or without plaque rupture in the edge segment and bifurcation lesion. When no predominant mechanism for stent thrombosis was found, this was reported as such and in some patients, the clinical/angiographic diagnosis of stent thrombosis had to be refuted based on OCT images.

Results

Between January 2011 and October 2014, 620 patients with stent thrombosis were included in the Prestige registry. In 250 of these cases, OCT acquisition during the angiographic procedure at the time of stent thrombosis was performed. In 27 of cases, the OCT acquisition was of insufficient quality to allow reasonable adjudication of stent thrombosis mechanism and these patients were excluded, leaving 223 pullbacks for further analysis. Dominant mechanisms, based on OCT expert panel analysis were the presence of uncovered struts (33%), neoatherosclerosis (19%) (12% with rupture, 7% without rupture), malapposed struts...
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Between January 2011 and October 2014, 620 patients with stent thrombosis were included in the Prestige registry. In 250 of these cases, OCT acquisition during the angiographic procedure at the time of stent thrombosis was performed. In 27 of cases, the OCT acquisition was of insufficient quality to allow reasonable adjudication of stent thrombosis mechanism and these patients were excluded, leaving 223 pullbacks for further analysis. Dominant mechanisms, based on OCT expert panel analysis were the presence of uncovered struts (33%), neoatherosclerosis (19%) (12% with rupture, 7% without rupture), malapposed struts (13%), underexpansion (9%), severe stenosis with superimposed thrombus (9%), extra stent cavity (1%), edge dissection (2%), edge segment disease (with or without plaque rupture) (4%), bifurcation (1%). In 7% of cases, no dominant cause was found and in 2% of cases, the problem seemed not to be located within the stented segment, based on OCT images.

Conclusion

The presence of uncovered struts, neoatherosclerosis, malapposition, stent underexpansion and severe stenosis are the predominant mechanisms of stent thrombosis.
Poster Session

Day 1
Friday 21 November 2014
Polymer-Free Sirolimus- and Probucol-Eluting vs. New Generation Zotarolimus Eluting Stents in Coronary Artery Disease

The Intracoronary Stenting and Angiographic Results: Test Efficacy of Sirolimus- and Probucol- and Zotarolimus-Eluting Stents (ISAR-TEST 5) Trial

Jonas Sorges¹ on behalf of Steffen Massberg, MD; Robert A. Byrne, MB BCh PhD; Adnan Kastrati, MD; Stefanie Schulz, MD; Jürgen Pache, MD; Jörg Hausleiter, MD; Tareq Ibrahim, MD; Massimiliano Fusaro, MD; Ilka Ott, MD; Albert Schömig, MD; Karl-Ludwig Laugwitz, MD; Julinda Mehilli, MD

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The main objective of the ISAR TEST 5 is to compare the efficacy of a sirolimus-probucol eluting polymer-free stent against the permanent polymer-based zotarolimus-eluting stent (Endeavor resolute). Regarding the results, out to 12 months polymer-free rapamycin/probucol-eluting stent is non-inferior to the permanent polymer-based zotarolimus-eluting stent in a large-scale study powered for clinical endpoints.

Background

Durable polymer coatings have been implicated in mid- and long-term adverse events following drug-eluting stent implantation. A polymer-free dual-drug sirolimus- and probucol-eluting stent and a new generation permanent polymer zotarolimus-eluting stent are recently developed technologies demonstrating encouraging results.

Methods and Results

In a clinical trial with minimal exclusion criteria, we randomly assigned 3002 patients to treatment with sirolimus- and probucol-eluting stents versus zotarolimus-eluting stents. The trial was designed to demonstrate non-inferiority of the sirolimus- and probucol-eluting stents. The primary endpoint was the combined incidence of cardiac death, target vessel-related myocardial infarction or target lesion revascularization at 1-year follow-up. Follow-up angiography was scheduled at 6-8 months.

The sirolimus- and probucol-eluting stent was non-inferior to the zotarolimus-eluting stent in terms of occurrence of the primary endpoint (Fig.1: 13.1% versus 13.5% respectively, \( P_{\text{non-inferiority}} = 0.006 \); hazard ratio = 0.97, 95% CI, 0.78-1.19; \( P_{\text{superiority}} = 0.74 \)). The incidence of definite/probable stent thrombosis was low in both groups (1.1% vs. 1.2% respectively; hazard ratio = 0.91 [95% CI, 0.45-1.84], \( P = 0.80 \)). Regarding angiographic efficacy, there were no differences between the sirolimus- and probucol-eluting stent and the zotarolimus-eluting stent in terms of either in-segment binary angiographic restenosis (13.3% versus 13.4% respectively; \( P = 0.95 \)) or in-stent late luminal loss (0.31±0.58 mm versus 0.29±0.56 mm respectively; \( P = 0.46 \)).
Conclusion

In this large-scale study powered for clinical endpoints, a polymer-free sirolimus- and probucol-eluting stent was non-inferior to a new generation durable polymer-based zotarolimus-eluting stent out to 12 months. Whether the hypothesized advantages of polymer-free DES coatings translate into improved patient outcomes remains subject to future studies of long-term outcomes.

References

**Contribution prepared for PRESTIGE – Final workshop**

Temporal course of neointimal maturity after implantation of biodegradable polymer sirolimus-eluting stents as assessed by optical coherence tomography gray scale signal intensity at 3, 6 and 9 months

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**Background** - We previously reported that in patients undergoing follow-up after coronary stenting, optical coherence tomography (OCT) gray-scale signal intensity (GSI) analysis can differentiate between stent coverage with mature (smooth muscle cell-rich) versus immature tissue (hypocellular fibrin-rich) using histopathologic data as gold standard [1]. However the temporal evolution of these changes in the months after stenting remains unknown.

**Methods** - We studied neointimal maturity as assessed by OCT-derived GSI analysis of tissue overlying stent struts in 3 separate cohorts of patients undergoing OCT follow-up at 3 months, 6 months and 9 months after coronary stenting with biodegradable polymer sirolimus-eluting stents (ORSIRO, Biotronik, Bülach, Switzerland). Offline analysis of contiguous cross-sections was performed at 1 mm longitudinal intervals within the stented segment. For each cross-section the neointimal region of interest (ROI) above each covered strut was manually delineated and 256-level GSI was measured for every pixel within the ROI (Fig. 1).

![Fig. 1: OCT image and gray level image with the regions of interest overlying the stent struts.](image-url)
Calibration was done with brightest spot GSI analysis using guide-wire at 3 and 6 months and stent strut at 9 months. Cut-off values for mature vs. immature tissue for each scale were derived from published data [1].

**Results** – OCT raw data were available for 8, 24 and 27 lesions at 3 months, 6 months and 9 months respectively. In patients undergoing follow-up at 3 months, 1602 ROIs were analyzed and median GSI scores were 90.7 [95%CI 52.9 – 134.7]; 28.2% of ROIs were categorized as mature. At 6 months, 5315 ROIs were analyzed and median GSI scores were 105.2 [62.0 – 148.4]; 38.2% of ROIs were categorized as mature. At 9 months, 3501 ROIs were analyzed and median GSI scores were 106.4 [63.3-149.5]; 58.8% of ROIs were categorized as mature (Fig. 2).

![Graphs showing GSI scores for mature and immature tissue at 3, 6, and 9 months](image)

**Fig. 2:** Percentage of struts covered by mature and immature tissue respectively at 3, 6 and 9 months after stent implantation.

**Conclusions** - In patients undergoing OCT follow-up after coronary stenting, GSI-derived scores of neointimal maturity show progressive change over time. While only a minority of tissue areas overlying stent struts were characterized as mature at 3 and 6 months, at 9 months after stent implantation the majority of covered struts could be classified as mature. Future studies should examine the clinical relevance of differences in tissue maturity over time.

**References**

Contribution prepared for PRESTIGE – Final workshop

Comparison of tissue coverage and neointimal maturity between sirolimus-eluting stents with biodegradable polymer versus everolimus-eluting stents with permanent polymer as assessed by optical coherence tomography: ISAR-ORSIRO randomized study

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Background - We sought to compare the strut coverage and neointimal maturity of sirolimus-eluting stents with biodegradable polymer (BP-SES, Orsiro) versus everolimus-eluting stents with permanent polymer (PP-EES, Xience Prime) using intravascular optical coherence tomography (OCT).

Methods - A total of 54 patients undergoing treatment of de novo coronary lesions were randomly assigned to receive BP-SES (n=29) or PP-EES (n=25). The rate of stent strut coverage by any tissue as well as neointimal maturity using OCT gray-scale signal intensity (GSI) was assessed at 6-8 months. Neointimal tissue above the stent struts was classified as mature or immature on a strut-by-strut basis, according to previous data obtained on animal models and autopsy specimens [1].

Results – OCT imaging was available for 24 lesions (5315 struts) in the BP-SES group and 18 lesions (4188 struts) in the PP-EES group. After adjustment for clustering, strut-level neointimal thickness was 67 μm [0 - 198 μm] in the BP-SES group and 102 μm [0 - 349 μm] in the PP-EES group (p=0.07).

Fig. 1: Percentage of strut coverage by any tissue (A) and by mature tissue (B) in the BP-SES and PP-EES groups respectively.
The rate of stent strut coverage with any tissue was 89.7% in BP-SES and 95.7% in PP-EES (P=0.14) (Fig. 1). Of 8089 struts covered with any tissue, OCT GSI value was 105.2 [95% CI 62.0 – 148.4] in BP-SES and 99.5 [95% CI 59.7 – 139.3] in PP-EES respectively (p = 0.12) (Fig. 2). The rate of stent strut coverage with mature tissue was similar between the two groups (33.7% in BP-SES versus 27.2% in PP-EES, p = 0.24) (Fig. 1).

Fig. 2: Distribution of gray scale intensity values in the BP-SES and PP-EES groups respectively.

There was no correlation between neointimal thickness and gray scale intensity values (Fig. 3).

Fig. 3: Correlation between neointimal thickness and gray scale intensity value.

Conclusions - New generation BP-SES as compared to PP-EES showed a tendency to be associated with a lower neointimal thickness and a lower rate of strut coverage with any tissue at 6-8 months. However, the rate of strut coverage with mature tissue was similar in the two groups.
References

Clinical profile of UK stent thrombosis patients

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As part of PRESTIGE we recruited patients presenting with coronary stent thrombosis (ST) at twelve UK centres. We collected demographic, clinical and angiographic data, as well as retaining intracoronary thrombus for histopathology and performing intracoronary imaging in a large number of cases. In addition, we undertook platelet function testing at three time points and collected blood for later DNA analysis. Control patients were also recruited, at a ratio of 1:2.56 according to a limited number of predefined criteria. The ST cases differed from the control group in a number of key clinical parameters including use of aspirin, severity of disease, complexity of previous stenting and prior non-cardiac surgery.

Background

Despite major advances in coronary stent technology, stent thrombosis (ST) remains as a significant complication of percutaneous coronary intervention (PCI). Although relatively rare, it is associated with a high rate of acute myocardial infarction and death. The exact mechanisms are thought to be multi-factorial and poorly understood. Previous studies have involved small sample sizes, with incomplete patient characterisation, a lack of intracoronary imaging or platelet function data.

Methods

As part of the European multi-centre PRESTIGE study, we enrolled and coordinated a total of twelve UK cardiac centres to recruit patients presenting with “definite” ST between April 2012 and October 2014. According to the protocol, we obtained demographic, clinical and angiographic data, whilst retaining intracoronary thrombus for histopathology and performing intracoronary imaging with intravascular ultrasound (IVUS) and/or optical coherence tomography (OCT) whenever possible. We also undertook platelet function testing, using the VerifyNow and/or Multiplate systems, acutely, at 24 hours and at 30-60 days post event. For the majority of patients, we have collected and stored blood samples for later DNA analysis. In addition we aimed to recruit five control patients for each ST case, matched according to timing (within 1 month of ST case’s initial PCI), indication (stable angina versus acute coronary syndrome) and location (PCI at the same treating centre).

Results

A total of 138 patients with “definite” ST were recruited in the UK, with 353 controls, a ratio of 1:2.56. Of the ST cases, 4.3% were acute (within 24 hours), 18.8% sub-acute (24 hours to 1 month), 8.7% late (1-12 months) and 68.1% very late (> 1 year) after the initial PCI. Platelet function testing was carried out in 74.6% and 86.4% of the ST patients and controls respectively, and 89.1% of the ST cases and 98.0% of the control patients had a blood sample stored for DNA analysis. Of the ST cases, 63.8% had thrombus retained, 38.4% had IVUS and 21.7% had OCT imaging.

The ST cases and control groups were well matched for gender, hypertension, heart failure, renal impairment and indication for PCI. The ST group
were younger (mean age 59.2±11.6 vs 63.2±10.5 years; p<0.01), had more smokers (39.1% vs 20.7%; P<0.01), previous MI (32.6% vs 21.4%; p<0.01), diabetes mellitus (22.5% vs 14.2%; p=0.03) and active malignancy (3.6% vs 0.6%; p=0.01). The angiographic features were well matched between the two groups, apart from less circumflex artery involvement in the ST group (13.0% vs 24.9%; p<0.01). Factors that differed between the ST cases and controls were use of aspirin on presentation in the ST group (84.1% vs 92.1%; p=0.01) non-cardiac surgery in the 90 days prior to randomisation (6.5% vs 2.8%; p=0.04), number of stents inserted (2.2 vs 1.7; p<0.01), a higher proportion of first generation drug eluting stents (31.9% vs 19.0%; p<0.01) and of two-stent bifurcation procedures (6.5% vs 2.8%; p=0.05).

Conclusions
Using a multi-centre approach the UK has contributed significantly to the collection of multi-source data from patients presenting with coronary ST, along with matched controls. Further analysis, including platelet function data will be conducted to identify important risk factors and to validate strategies to reduce the risk of ST.
Highly pro-Thrombogenic Properties of Stent cobalt Chromium Alloy

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The objective was to decipher in vitro, the molecular and cellular determinants leading to stent thrombosis with specific attention to bare metal alloy as regards to activation of platelets, leukocytes, coagulation and healing by endothelial cells. We show a major prothrombotic effect of CoCr alloy relying on adhesion and activation of platelets and leukocytes and activation of the coagulation. Despite healing of CoCr alloy was achievable by human coronary endothelial cell, protection against thrombosis is not secured since the phenotype of endothelial cells shifts from anticoagulant to procoagulant.

Background

The most effective treatment for obstructive coronary disease is percutaneous intervention with coronary stenting. However an important issue remains stent thrombosis due to a high mortality rate. The pathophysiological mechanism of stent thrombosis is not well defined but four predominant features are proposed, including enhanced platelet adhesion and aggregation, increased fibrin formation and deposition, defective endothelisation of the stent and local inflammatory response at the site of stent implantation. The objective of this study was to decipher in vitro the cellular and molecular mechanisms triggering the occurrence of stent thrombosis and in particular to evaluate the responsiveness of bare metal (CoCr used in stents), with respect to platelets, leukocytes, coagulation and healing.

Methods

Adhesion of platelets on CoCr devices was quantified and morphological changes observed using epifluorescence and Scanning Electron Microscopy (SEM). Thrombin generation at the surface of CoCr devices was performed using the Calibrated Automated Thrombogram (CAT) in platelet poor plasma (PPP) and platelet rich plasma (PRP). Neutrophil extracellular traps (NETs) formation was tracked using SEM, immunofluorescence labeling of histone H1 and quantification of cell free DNA after incubation of neutrophils and platelets alone or together on CoCr. Endothelisation of CoCr devices was studied using primary Human Coronary Artery Endothelial cells (HCAEC). The procoagulant versus the anticoagulant phenotype of HCAEC cultured on CoCr was characterized.

Results

CoCr devices promoted platelet adhesion and activation through β₁ and β₃ integrins and the GP Ib-IX complex. Activated platelets contributed to the amplification of thrombin generation observed in PRP at the surface of CoCr. Addition of Ticagrelor to PRP canceled the amplification of thrombin generation induced by CoCr indicating a contribution of the P2Y12 ADP receptor to the CoCr-triggered platelet procoagulant activity. CoCr-triggered thrombin generation involved activation of the coagulation contact pathway as demonstrated by its inhibition in PPP in the presence of the FXII inhibitor, corn trypsin inhibitor (CTI). Leukocytes sticked to CoCr and get activated with the formation of NETs observed by SEM and confirmed by the labeling of histone H1 and quantification of cell free DNA. Healing assays were performed and unexpectedly, HCAEC grown and formed a monolayer on CoCr. However HAEC grown onto CoCr acquired a pro-thrombotic phenotype as demonstrated by the increased rate and
extent of thrombin generation related to a significant increase in tissue factor (TF) activity. Furthermore, HCAEC grown on CoCr lost their anticoagulant properties as demonstrated by a 30% reduced level of protein C activation. Overall, CoCr induced a change in HCAEC phenotype with more potent procoagulant behavior of the cells.

Conclusions
Considering these data, careful evaluation of materials used to develop stents is important as we show here that CoCr alloy is prothrombotic since (i) it activates platelets and coagulation, (ii) it induces leukocytes adhesion and activation, and (iii) its endothelialisation is achievable but the shift in the phenotype of endothelial cells from anti-to pro-coagulant prevents securing the material against thrombosis.
A new dextran-graft-polybutylmethacrylate copolymer coated on 316L metallic stents enhances endothelial cell coverage

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Amphiphilic copolymers based on copolymerization of hydrophilic and hydrophobic moieties offer versatility in various applications in the field of biomedical materials. A new biocompatible copolymer of dextran-graft-polybutylmethacrylate was synthesized for the coating of metallic endovascular stents. Coating of metallic surfaces is performed and analyzed by XPS, ATR-FTIR, contact angle, atomic force microscopy and scanning electron microscopy before and after deformation corresponding to stent deployment by a balloon catheter. The resulting coating is smooth and uniform with neither cracks nor detachment after stent expansion. Interestingly, surfaces coated with the copolymer greatly improve in vitro adhesion and growth of endothelial cells and do not favor proliferation of smooth muscle cells. This copolymer provides new opportunities for implanted stents.

Background
The aim of this work was to investigate the feasibility of homogeneous coating on stainless steel SS316L. In a first step, the coating was performed on discs since their planar configuration allowed X-Ray Photoelectron Spectroscopy (XPS) and Atomic Force Microscopy (AFM) to be performed. Furthermore, coated discs were submitted to a deformation process leading to a 25% plastic deformation. This procedure is mimicking the deformation applied onto stents during the implantation procedure and allowed analyzing the coating stability. In a second step, stents were coated with the copolymer and, due to their 3D geometry, the surface topography was assessed by fluorescence and Scanning Electron Microscopy (SEM). The last step was performed to evaluate in vitro the biological properties of the dextran/n-butyl methacrylate copolymer coated on SS316L substrates by studying the proliferation of vascular smooth muscle cells and endothelial cells.

Methods
Materials: FITC-dextran (70 kg/mol), Dextran 70 kg/mol, Butyl methacrylate monomers, Ceric ammonium nitrate and nitric acid were commercially available.
Synthesis and characterization of the copolymer: dextran was dissolved in nitric acid and Ce(IV) dissolved in HNO\textsubscript{3} and BMA monomers were simultaneously added. The content of dextran in the copolymer was 11% (w/w). Attenuated Total Reflection Infra-Red Spectroscopy, Differential Scanning Calorimetry (DSC), Contact angles and Dynamic Mechanical Analyses were performed. The coated coupons were plastically deformed up to 25% using a custom-made small punch test device at a displacement rate of 0.05 mm.s\textsupers-1 and a load of 2200 N. The surface topography of coated samples were obtained using Atomic Force Microscopy by the tapping mode and by Scanning Electron Microscopy (SEM).
Cell proliferation on the coated surfaces: Endothelial cells (HUVEC-C from ATCC) and vascular smooth muscle cells (VSMC line Rb1) were used. Proliferation studies of ECs and SMCs were performed on discs coated with Dex-PBMA films. ECs were also directly seeded on the surface of stents.
Results

Synthesis and characterizations: Dextran-graft-polybutylmethacrylate copolymer, Dex-PBMA, was synthesized by radical polymerization using cerium in aqueous acidified medium. Copolymer films were prepared by solubilizing the copolymer in a precise mixture of THF and water (92:8 v:v). The presence of dextran increased the wettability of the surface, with a water contact angle decreasing from 98.9° for PBMA to 79.4° for Dex-PBMA. Creep tests showed a typical viscoelastic behavior and the propensity of the material to stretch. Indeed, more than 200% deformation was achieved before cracking by suspending a 200g load on a small film. The coating onto 316L metallic discs was characterized, before and after deformation. XPS analyses evidenced a similar chemical composition of the surface on samples, whatever the concentration used. Coatings obtained with PBMA or Dex-PBMA at 1 mg/mL were rough and less homogeneous compared to those obtained at 5 and 10 mg/mL. The Dex-PBMA coating on stent is uniform and smooth before and after dilatation by an angioplasty balloon. Microscopic analyses of the FITC-Dex-PBMA coated stent after expansion displayed a homogeneous coating, again, without cracks even in highly distorted areas.

In vitro vascular cell proliferation: Proliferation of endothelial cells as well as smooth muscle cells on non-coated metallic SS316L discs or disks coated with Dex-PBMA was studied in vitro for a period of 5 days. Proliferation analysis showed that the endothelial cells seeded on Dex-PBMA films exhibited a 14-fold increased proliferation between day 0 and day 5. In contrast, endothelial cells did not proliferate onto native SS316L discs. Interestingly, smooth muscle cell proliferation on Dex-PBMA was very limited as compared to endothelial cells. The copolymer Dex-PBMA showed an enhanced proliferation of SMCs and no proliferation of endothelial cells. Endothelial cells were then seeded directly on stents and, endothelial cells exhibited high coverage on the stent coated with the Dex-PBMA copolymer, in comparison with an uncoated stent.

Conclusions

The dextran-graft-butylmethacrylate (Dex-PBMA) copolymer is a new polymer with a balance of hydrophilic/hydrophobic properties. The Dex-PBMA copolymer exhibits elastic properties and therefore is used here as a coating for stents. First, the results evidence that Dex-PBMA coating is homogeneous and shows excellent mechanical properties after deformation both on SS316L discs and stents. Interestingly, our in vitro data of endothelialization of stents reveal a major difference between the polymer-coated and the bare metal stents. The Dex-PBMA copolymer thus displays the ability to stimulate endothelial cell proliferation while limiting vascular smooth muscle cell growth. Further biological assessments have to be performed to completely characterize the in vitro behavior of the copolymer. These tunable properties obtained with the copolymer could be of great interest for stent design, but also in various applications of polymeric biomaterials by surface modification of cardiovascular medical devices such as catheters.

Reference

Contribution prepared for PRESTIGE – Final workshop

Tissue Characterization after DES Implantation Utilizing Optical Coherence Tomography

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Background - To validate optical coherence tomography (OCT) data for assessment of vascular healing in a preclinical animal model and human autopsy cases, and to translate the findings to the assessment of vascular healing after drug eluting stent (DES) implantation in clinical practice.

Methods - DES and BMS were imaged after 28 and 42 days in atherosclerotic rabbits using OCT and evaluated by histology (Fig. 1). Following co-registration with histology, grey-scale signal intensity (GSI) was measured for identified mature or immature neointimal tissue. Autopsy specimens were imaged by OCT and GSI correlated with histology. Finally, prospective OCT imaging and GSI measurements were acquired in 10 patients undergoing 6 months follow-up (Fig. 2).

Fig. 1: Correlation of representative histopathology microscopy images of stented iliac arteries with optical coherence tomography frames. Top, Histological cross-sections stained by Movat pentachrome (A, C) at magnification x40. Bottom, The corresponding native OCT frames (B, D).
Results – Mature neointimal tissue consistently showed higher GSI values in animal and human autopsy specimens, respectively. ROC curve analysis displayed high sensitivity and specificity for detecting mature neointima (Fig. 3). Prospective GSI analysis revealed that a minimum of 27.7 % of areas above stent struts represented mature neointima in patients 6 months after DES implantation.

Fig. 2: Representative spread out vessel graphics demonstrating the spatial distribution of uncovered (red dots) and covered stent struts (green dots) along the entire stented vessel at (A) 28 days and (B) 42 days in the animal model, and (C) at 6 months in humans in zotarolimus-eluting stents.

Fig. 3: Representative example of matched histological and gray-scale OCT images and receiver operating characteristic curve analysis derived from animal and human autopsy data for the detection of mature neointimal tissue by gray-scale signal intensity (GSI) analysis. Regions of interest measured by GSI were coregistered with histology to identify mature and immature tissue.

Conclusions - Novel OCT GSI analysis allows distinction between mature and immature neointimal tissue in animal models, autopsy specimens and patients undergoing invasive surveillance in simple atherosclerotic lesions.
Intravascular near-infrared fluorescence imaging of endothelial cells

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The use of fluorescence targeting for assessing the condition of blood vessels \textit{in vivo} requires the use of specialized imaging equipment. In this work an intravascular imaging catheter was developed for near infrared fluorescence imaging. The system was tested for imaging endothelial cells in healthy and disease aortas in rabbit models.

Background

It has been well recognized that the diagnostic potential of imaging modalities may be significantly enhanced by the use of molecular markers that highlight specific biological processes at the cellular and molecular level [1]. In the case of coronary artery imaging, the small size and rapid movement of the coronary arteries poses a significant challenge for non-invasive imaging modalities, which may be overcome by adopting an intravascular approach, in which the arteries are imaged from within. One of the promising methods for intravascular molecular imaging is the use of fluorescence markers in conjugation with a 2D near-infrared fluorescence (NIRF) intravascular imaging catheter. This approach was used by Jaffer et al. for imaging inflammatory processes in New Zealand White Rabbits via an activatable fluorescent probe [2]. However, the method is general, and may be used to image almost arbitrary tissue constituents and biological processes for which appropriate fluorescent markers are available.

Methods

In this project we constructed a 2D NIRF catheter similar to the one developed in Ref. [2] to be used for imaging endothelial cells (ECs). An excitation laser was coupled to an optical fiber that was connected to a rotation/translation stage capable of 2D scans. The imaging catheter was connected to the other side of the stage and included a prism on its tip to guide the laser light to the blood vessel wall and collect the resulting fluorescence light. The light collected from the tissue was sent through optical bandpass filters that rejected the laser light and was measured by a photomultiplier tube (PMT). The signals were digitized and were used to form a 2D image. The system was designed for two fluorescence bands to enable versatility in the choice of the fluorescent molecule. Specifically, two excitation wavelengths were employed: one at 660 nm and the other at 780 nm. For each laser, appropriate optical bandpass filters were chosen to reject scattered laser light from the fluorescence channel.

The fluorescence labeling of ECs was performed using immunostaining. Specifically, wholemount immunostaining was performed using the primary antibody CD31 (dilution 1:20), which targets ECs conjugated to a secondary fluorescent antibody. Two secondary fluorescent antibodies were tested: one with Alexa Fluor 790 (Invitrogen) and one with CF660C (Sigma). In the first set of experiments, the staining procedure was tested on healthy rabbit aortas, where it was also compared to background staining without the primary antibody and to a native, completely unstained control. For each of the two fluorescent markers, the corresponding excitation laser was used, and the most promising marker was used in the second set of experiments, where we tested whether the staining procedure could distinguish between healthy and injured blood vessels. Accordingly, we repeated the
most promising staining procedure for a normal artery and a balloon-injured (denuded) artery. For both arteries, the results were compared to unstained arteries and to background staining without the primary antibody.

**Results**

In the first round of experiments, the system exhibited good sensitivity for the both fluorescent markers. However, in terms of specificity, conjugation to CF660C showed more promising results. Whereas labeling with untargeted Alexa Fluo 790 led to signal levels significantly higher than native imaging, the signals obtained from untargeted CF660C were almost indistinguishable from the signals obtained from the native arteries.

Typical results for the second set of experiments, which were performed with CF660C, are shown in Fig. 1 for the sets of arteries imaged. On the left is an illustration of the 6 different kinds of arteries which were imaged, where on the right are the resulting images obtained for two sets of arteries. Clearly, in both sets of arteries, only the arteries which were stained with the targeted fluorescent marker generated a significant signal. For the arteries stained with the targeted marker, a clear difference in the level of signal between the normal and denuded arteries was found.

**Conclusions**

The results show that the staining and imaging procedure developed in this work may be useful for imaging ECs intravascularly. This approach may be used to study the artery healing after interventional procedures in which stents are deployed. We are currently in the process of imaging additional rabbits to show the statistical validity of our initial results.
References
Poster Session

Day 2
Saturday 22 November 2014
Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice \textit{in vivo}

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Deep venous thrombosis (DVT) is a common clinical problem and due to pulmonary embolism one of the most frequent causes of cardiovascular mortality. However, the pathophysiology of DVT is mainly unknown. Stasis without endothelial injury is one of the most important triggers for DVT. Here, we report a novel murine model of stasis induced DVT, allowing the detailed analysis of the early stages of thrombogenesis and mimicking the dynamics and composition of human venous thrombi. Using intravital microscopy, we were able to visualize the early stages of thrombus formation, revealing a massive accumulation of leukocytes and platelets at the intact endothelial lining. In conclusion, we could show that interplay between these cell types is the pathophysiologic basis for activation of the coagulation system triggered by flow reduction resulting in venous thrombosis.
**Background**

Deep vein thrombosis (DVT) and pulmonary embolism (PE), collectively referred to as venous thromboembolism (VTE), constitute a leading cause of cardiovascular death. The incidence of VTE increases dramatically over 45 years of age and ranges from 300,000 to more than 2 million per year in the United States with similar rates in Europe. In light of an increasing life expectancy, strategies to prevent and treat VTE will become an even more important health care issue worldwide.

**Methods**

Thrombosis was induced in C57Bl6 mice by placing a narrowing ligature around the IVC, resulting in a reduction of blood flow velocity (n=16). NET formation in vivo was visualized by intravital epifluorescence microscopy. The IVC was harvested 48h after flow reduction, weighed and stained by immunofluorescence. Thrombogenesis and NET formation were quantified in DNase, anti-H2A-H2B-DNA antibody, and heparin treated animals.

![Fig. 1: (A) Immunohistological cross sections of the IVC 48 h after DVT induction display platelet accumulation (CD41+) within the thrombus. Nuclei are counterstained with DAPI; bar 200 µm. Representative of n=3 experiments. (B) Representative images of intravital video microscopy of blood cell recruitment taken at 6 h after DVT induction. Arrowheads: thrombi; arrows: single, adherent cells. Platelets, red (rhodamine B), leukocytes, green (Acridine Orange). Bar 100 µm (C) Time lapse images of the developing thrombus (arrowhead) visualized by 2-photon microscopy 6 h after DVT induction. Platelets (yellow) and neutrophils (green) are recruited from the bloodstream (blue) to the vessel wall (red).](image)

**Results**

The sequence of events that trigger large vein thrombosis in response to stasis has remained poorly defined. Employing a novel mouse model of flow restriction-induced DVT, we have uncovered the mechanisms underlying the previously proposed link between thrombosis and inflammation crucial for the pathogenesis of DVT. By 2-photon microscopy we show that flow restriction triggers the rapid accumulation of neutrophils and monocytes, which is indispensable for DVT development (Fig. 1). Innate immune cells initiate local fibrin formation predominantly through delivery of TF. In addition, neutrophils form NETs, triggering FXII-dependent coagulation. While TF appears to be required to initiate DVT, neutrophil-driven coagulation contributes to DVT propagation. Platelets foster leukocyte accumulation and support fibrin formation by enhancing neutrophil-dependent coagulation. Hence, by combining a novel DVT model with in vivo imaging we have uncovered different processes responsible for
the emergence of localized thrombosis in response to reduced venous blood flow, an important trigger of DVT in humans [1].

**Conclusions**

In conclusion, our study shows that DVT in response to perturbed blood flow is driven by a concerted interaction of monocytes, netting neutrophils and platelets and thus uncovers the mechanisms linking inflammation and venous thrombosis. Disruption of this cooperation and prevention of monocyte- and neutrophil-driven thrombogenesis paves the way for the development of novel therapeutic approaches, specifically targeting the cellular factors that initiate DVT development. Such a strategy might improve the benefit-to-risk profile of anticoagulant therapy in comparison with non-specific inhibition of the final common coagulation pathway that is characteristic of current therapies.

**References**

Oligonucleotide Coating of Co-Cr Stents for EPC Adhesion

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Aim of our work was the biofunctionalization of Co-Cr stents with a single-sequence oligonucleotide (ON), to bind endothelial progenitor cells (EPCs) and promote surface endothelialization. We were able to perform an easily reproducible method to fabricate biofunctional stents binding EPCs, combining basic treatment, silanization and ON-grafting. Our coating strategy was able to increase EPC adhesion to metal surfaces, with cell morphology and viability similar to that of tissue culture surfaces. These results potentially qualify the biofunctionalized surfaces for in vivo endothelialization, either by cell migration from adjacent native tissue, or by EPC capture and adhesion from the blood stream.

Background
Stent surface modification could overcome side effects due to the stent deployment. Moreover, the stent surface itself can result in hypersensitivity. As the native endothelium still represents the ideal surface, various endothelialization strategies have been attempted to improve the stent biocompatibility and non-thrombogenicity. Endothelial progenitor cells (EPCs) capture could accelerate endothelialization, preventing both thrombosis and restenosis [1, 2]. Aim of this work was the functionalization of Cobalt-Chromium (Co-Cr) stents with ON (aptamers), able to bind EPCs.

Methods
As a surface model, Co-Cr discs were used in addition to Co-Cr stents. Discs were aminosilanized; the reaction was assessed by attenuated total reflectance fourier transform infrared spectroscopy. Surfaces were then activated before deposition of a fluorescent endothelial-specific 73-b thiolated DNA ON. Immobilization of fluorescent ON was demonstrated by confocal laser scanning microscopy (CLSM). Porcine blood EPCs (10^4 cells/ml) were seeded on unmodified or ON-modified discs and cultured for 4 days before either MTT viability assay or staining (DAPI: nuclei; TRITC-phalloidin: F-Actin) and CLSM. Binding of fluorescent-ON to EPCs was assessed by flow cytometry. The same protocol was performed with stents. 2x10^4 EPCs/ml were seeded on unmodified or modified stents and cultured for 4 days, before staining and CLSM.

Results
Co-Cr surfaces resulted efficiently coated with aminosilane; the binding of fluorescent single-strand ON was confirmed by CLSM. Specific cell-capturing ON coating significantly increased EPC adhesion and viability (p<0.01), with an increased number of cell clones. Flow cytometry confirmed cell binding. Stents were successfully functionalized (Fig. 1); both ON and EPC binding were confirmed by CLSM.
Conclusions

Our study demonstrated the feasibility to fabricate biofunctionalized stents with capture specificity for EPCs. Such stents could be used as a strategy for in vivo endothelialization with patients’ autologous cells.

References

A humanized GPVI mouse model to assess the antithrombotic efficacy of anti-GPVI agents [1]

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The objective of this study was to develop a preclinical tool to evaluate the role of human GPVI in various models of thrombosis and to screen anti-GPVI compounds. A genetically modified mouse strain expressing human GPVI (hgp6) has been developed using a knock-in strategy. Platelets of hgp6 mice express about 3,700 copies of human GPVI and display normal responses to agonists. In vitro collagen-induced platelet responses as well as in vivo thrombosis in various models were reduced by the GPVI-blocking 9O12 Fab. In conclusion, we have developed a humanized mouse model which could be used in preclinical studies to evaluate the effects of anti-GPVI compounds.

Background

The treatment of acute coronary syndromes has been considerably improved in recent years with the introduction of highly efficient antiplatelet drugs, the current standard treatment being based on dual antiplatelet therapy with aspirin and a thienopyridine. However, this strategy still has significant limitations: the recurrence of adverse vascular events remains a problem and the improvement in efficacy is counterbalanced by an increased risk of bleeding. The search for antiplatelet drugs which efficiently prevent platelet thrombus formation while having a minimal effect on general hemostasis remains a competitive challenge.

GPVI has been proposed as a promising anti-platelet target since its blockade prevents experimental thrombosis without impairing hemostasis [2]. Despite the accumulating data in favor of GPVI as a major target, controversies subsist when considering the different mouse models of thrombosis. Mice are obviously not entirely satisfactory for preclinical studies, also due to sequence differences in several domains of GPVI, which could influence collagen-induced responses.

The objective of this study was to develop a preclinical tool to evaluate the role of human GPVI in various models of thrombosis and to screen anti-GPVI compounds.

Methods

A genetically modified mouse strain expressing human GPVI (hgp6) has been developed using a knock-in strategy. The gp6/KI mutant mouse line was established at the MCI/ICS (Mouse Clinical Institute, Illkirch, France; http://www-mci.u-strasbg.fr). Constructs were designed to knock-in the mouse gp6 gene by introducing the sequence of the human gp6 gene into exon 1 at ATG.

Platelet count and size, isolation, glycoprotein analysis, activation, in vitro thrombosis assays were performed according to standardized methods in mice as
well as the measurement of the bleeding time and loss and the realisation of in vivo thrombosis models.

**Results**

The hgp6 mice were viable and fertile and did not present any hematological defect. Platelet counts and size were normal. About 3,700 copies of human GPVI were detected at the platelet surface with a normal size and reactivity. Platelet aggregation, fibrinogen binding and P-selectin exposure were normal in response to various agonists. The 9O12.2 Fab fragment directed against human GPVI bound to hgp6 platelets *in vitro* and *ex vivo* and markedly reduced collagen- and CRP-induced responses. Injection of 9O12.2 into hgp6 animals did not prolong the tail bleeding time but provided protection against lethal thromboembolism induced by a collagen/adrenaline mixture. In addition, 9O12.2 reduced arterial thrombus growth by 44% after superficial laser injury, by 43% after deep laser injury in mice pretreated with hirudin and by 48% after mechanical injury.

**Conclusions**

We describe here a unique animal model, which permits the *in vivo* evaluation of agents targeting human GPVI in terms of efficacy and safety. In particular, this model can predict the response to anti-GPVI agents in man more accurately than conventional mouse models. This should enable one to determine doses and therapeutic combinations, thus providing an important preclinical tool, which may help, design future clinical studies.

**References**

List all references at the end of the paper. When referring to them in the text, type the corresponding reference number inside brackets [1] and the reference listed as in the following example:


**In vivo detection of non-occlusive thrombi in drug-eluting stents by scintigraphy and radiolabelled Annexin V in a rabbit model**

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**Background**

Non-occlusive thrombi are often in contact with non re-endothelialized stent struts of drug-eluting stent (DES) and could favor stent thrombosis [1-2]. Annexin V radio-labelled with 99mTc is a radiotracer with a high affinity for activated platelets [3]. Our objectives were: 1) to develop an animal model of non-occlusive thrombosis of stents, 2) to evaluate the ability of annexin V 99mTc for the detection of in-stent thrombi using scintigraphy.

**Methods**

Right carotid arteries of NZW rabbits (n = 14) fed a high cholesterol diet were implanted with overlapping DES. Nuclear imaging was performed 10 (n = 7) or 28 days (n = 7) after stent implantation. Rabbits underwent a first scintigraphy 2 hours after injection of 200 MBq of radiolabelled annexin V 99mTc. At the end of the first scintigraphy, a suture was placed surgically proximal to the injured carotid arteries in order to induce a thrombus-prone flow limiting stenosis. Four days later, a second scintigraphy was performed. After the second scintigraphy, stents were excised, imaged ex vivo and then fixed for histological examination and scanning electron microscopy (SEM).

![DES](image1)

![BMS](image2)

**Fig. 1**: DES and BMS stents explanted 10 days after implantation in the right carotid artery (left panel) and 15 days after implantation after surgical placement of a carotid suture proximal to the stents (right panel). Macroscopical and histological analysis of stents evidenced absence of thrombus before carotid stenosis and the presence of red aggregates in both DES and BMS stents (black arrows) only after creation of carotid stenosis.
Results

Activities measured *in vivo* in the stented carotid arteries after injection of annexin V $^{99m}$Tc increased on the second scintigraphy after creation of a surgical stenosis as compared to the first scintigraphy (1.2 vs. 1.0 respectively; $p < 0.05$). On the second scintigraphy, activities were higher at 14 vs. 32 days after stent implantation (22 vs. 11 counts/pixel/ MBq, respectively; $p < 0.05$). DES were re-endothelialized completely only 32 days but not 14 days after implantation on histological sections and SEM. High activities measured *ex vivo* in stents by scintigraphy were associated with the detection of thrombi on corresponding histological sections.

Conclusions

In this study, we developed a rabbit model of non-occlusive thrombosis of non re-endothelialized stents in carotid arteries. In this model, in-stent thrombi could be detected using annexin V $^{99m}$Tc scintigraphy.

References


A rabbit iliac model for testing coronary stents

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We describe a rabbit iliac model developed to investigate the acute thrombogenicity of coronary stents. We used balloon injury and flow reduction to induce a thrombotic response, measuring continuous flow post stenting and platelet accumulation using immunofluorescent labelling. Stented vessels were removed and fixed post mortem and further assessed using optical coherence tomography (OCT). A less invasive 28-day recovery model was used to assess the healing response of novel prototype stents developed as part of a pre-clinical work package of PRESTIGE.

Background

We developed a rabbit iliac model to investigate the acute thrombogenicity of newer generation coronary stents and scaffolds. A less invasive recovery model was used to assess the chronic biocompatibility of RGD peptide coated stents bio-engineered from ‘work package 2’ of the PRESTIGE study.

Methods

Male New Zealand White rabbits, weighing 3.0-4.0kg were used. For the acute model, animals had aspirin 1mg/kg and clopidogrel 1mg/kg administered in drinking water for at least 5 days pre-operatively. These doses were shown to produce a moderate anti-platelet response when tested by Multiplate impedance aggregometry. Following sedation, the central ear artery was cannulated with a 22G cannula and 18mls of blood was collected into 1/6 volume acid citrate dextrose anticoagulant. This was centrifuged at 200g for 15 minutes and 7mls of platelet rich plasma (PRP) was collected and incubated with apyrase 0.2 units/ml for 15 minutes. The remaining blood was centrifuged at 600g for 15 minutes to obtain platelet poor plasma (PPP) for later use. Prostacyclin 200ng/ml was then added to the PRP solution and centrifuged at 600G for 15 minutes. The supernatant was discarded and the platelet pellet re-suspended in pH 6 buffer, to which further apyrase was added. This solution was then incubated with MitoTracker Red fluorescent dye for 30 minutes to label the platelets. Finally, further prostacyclin was added before centrifuging at 600g for 15 minutes and re-suspending the labelled platelets in 4mls of PPP for re-injection pre-operatively.

General anaesthesia was induced and animals were appropriately prepared using strict asepsis. The femoral arteries were carefully exposed bilaterally and a midline abdominal incision was made to open the peritoneum. The abdominal aorta and common iliac arteries were exposed and freed of surrounding connective tissue and baseline blood flow was measured using perivascular flow probes placed around both common iliac arteries. An arteriotomy was made between two ligatures placed around the superficial femoral artery and a 3 x 12mm non-compliant angioplasty balloon was advanced under direct vision to the proximal common iliac artery and inflated to 12 atmospheres for 60 seconds. This was done three times, with a 2-minute recovery period between each inflation to induce deep arterial injury. Having removed the balloon catheter, a 3 x 12mm investigational stent was introduced in the
same way and deployed at the site of arterial injury at a nominal pressure for 30 seconds. The anatomical details are illustrated in figure 1 below.

Fig. 1: Schematic illustration of operative anatomy (reproduced from MD thesis by Rajesh Aggarwal).

Following stent deployment and removal of the balloon catheter, the superficial femoral artery was ligated at the arteriotomy site. The process of balloon injury and stenting was repeated on the contralateral side, using a control stent. Blood flow through the stented vessels was measured continuously for two hours, with flow probes placed immediately distal to the stents. Following this, animals were euthanised, the aorta cannulated and the stented vessels flushed with 0.9% sodium chloride. The vessels were removed and emersion fixed prior to measurement of immunofluorescence to compare platelet deposition between the two stented vessels. Finally, the vessels were assessed for thrombus formation using optical coherence tomography (OCT).
Results

We have so far performed 13 acute procedures as outlined above, initially involving unilateral implants with bare metal stents. 6 animals have received bilateral implants comparing the acute thrombogenicity of the ABSORB bioresorbable vascular scaffold to the Xience drug eluting stent. The process of washing and labelling rabbit platelets has been optimised, with minimal platelet activation and desensitisation confirmed by flow cytometry analysing binding of FITC–fibrinogen. Labelling efficiency of the rabbit platelets was >98%, as determined by flow cytometry.

In addition, we have undertaken 11 recovery procedures, involving limited groin dissections, bilateral direct stenting and a 28-day recovery period. Animals have been implanted with either bare metal or a novel prototype RGD-peptide coated stent. Following the 28 days recovery period, the stented vessels are perfusion fixed and explanted. The specimens will be shipped to the CVPath Institute in the USA for processing and assessment of injury, inflammation and endothelialisation.

Conclusions

Although many of the results are yet to be determined, the rabbit iliac model has proven to be a reproducible method of assessing both the acute thrombogenicity and more long-term biocompatibility of novel coronary stents and scaffolds.
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