Histopathological evaluation of thrombus in patients presenting with stent thrombosis. A multicenter European study: a report of the prevention of late stent thrombosis by an interdisciplinary global European effort consortium†

Julia Riegger1,2‡, Robert A. Byrne2,3‡, Michael Joner3,4, Sue Chandraratne1,2, Anthony H. Gershlick5, Jurrien M. ten Berg6, Tom Adriaenssens7,8, Giulio Guagliumi9, Thea C. Godschalk6, Franz-Josef Neumann10, Dietmar Trenk10, Laurent J. Feldman11,12,13, Philippe Gabriel Steg11,12,13,14, Walter Desmet7,8, Fernando Alfonso15, Alison H. Goodall5, Roman Wojdyla16, Dariusz Dudek16, Vanessa Philippi1,2, Sheryl Opinoldo1,2, Anna Titova1,2, Nikesh Malik3,5, James Cotton17, Darshni A. Jagroop6, Antonius A.C.M. Heesters18, Peter Sinaeve7,8, Paul Vermeersch19, Christian Valina10, Christian Schulz1,2, Adnan Kastrati2,3*, and Steffen Massberg1,2*, On Behalf of the Prevention of Late Stent Thrombosis by an Interdisciplinary Global European Effort (PRESTIGE) Investigators

1Medizinische Klinik und Poliklinik I, Ludwig-Maximilians-Universit"at, Marchioninistrasse 15, Munich 81377, Germany; 2DZHK (German Centre for Cardiovascular Research), Partner Site Munich Heart Alliance, Munich, Germany; 3Deutsches Herzzentrum Muenchen, Klinik an der Technische, Universit"at Muenchen, Lazaretstrasse 36, Munich 80636, Germany; 4CVPath Institute, Gaithersburg, USA; 5Department of Cardiovascular Sciences, University of Leicester and Leicester NIHR Cardiovascular Biomedical Research Unit, Glenfield Hospital, Leicester LE3 9QP, UK; 6Department of Cardiology, St. Antonius Hospital, Nieuwegein, The Netherlands; 7Department of Cardiology, University Hospitals Leuven, Leuven, Belgium; 8Department of Cardiovascular Sciences, KU Leuven, Leuven, Belgium; 9Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy; 10Universit"ats-Herzzentrum Freiburg Bad Krozingen, Germany; 11INSERM, U-1148, Paris, France; 12DHU FIRE, H"opital Bichat, AP-HP, Paris, France; 13Universit"e Paris-Diderot, Sorbonne Paris-Cit"e, Paris, France; 14NHLL, Royal Brompton Hospital, Imperial College, London, UK; 15Hospital Universitario de La Princesa, Madrid, Spain; 16Samodzielny Publiczny Zaklad Opieki Zdrowotnej Szpital Uniwersytecki w Krakowie, Krakow, Poland; 17The Royal Wolverhampton Hospitals NHS Trust, Heart and Lung Centre, New Cross Hospital, Wolverhampton WV10 9QP, UK; 18Department of Cardiology, Medical Center Alkmaar, Alkmaar, The Netherlands; and 19Antwerp Cardiovascular Institute, ZNA Middelheim, Lindendreef 1, Antwerpen B-2020, Belgium

Received 7 July 2015; revised 27 July 2015; accepted 6 August 2015; online publish-ahead-of-print 30 August 2015

See page 1550 for the editorial comment on this article (doi:10.1093/eurheartj/ehv036)

Background

Stent thrombosis (ST) is a rare but serious complication following percutaneous coronary intervention. Analysis of thrombus composition from patients undergoing catheter thrombectomy may provide important insights into the pathological processes leading to thrombus formation. We performed a large-scale multicentre study to evaluate thrombus specimens in patients with ST across Europe.

†An abstract of this work was presented as a late breaking translation science presentation at the European Society of Cardiology Congress 2015 in London, UK.
‡These authors contributed equally to this work.
*Corresponding author. Tel: +49 89 44007 2371, Fax: +49 89 44007 8870, Email: steffen.massberg@med.uni-muenchen.de (S.M.); Tel: +49 89 1218 4577, Fax: +49 89 1218 4083; Email: kastati@dhm.mhn.de (A.K.)

© The Author 2015. Published by Oxford University Press on behalf of the European Society of Cardiology.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
Histological characterization of coronary stent thrombosis

Methods

Patients presenting with ST and undergoing thrombus aspiration were eligible for inclusion. Thrombus collection was performed according to a standardized protocol and specimens were analysed histologically at a core laboratory. Serial tissue cross sections were stained with haematoxylin–eosin (H&E), Carstairs and Luna. Immunohistochemistry was performed to identify leukocyte subsets, prothrombotic neutrophil extracellular traps (NETs), erythrocytes, platelets, and fibrinogen.

Results

Overall 253 thrombus specimens were analysed; 79 (31.2%) from patients presenting with early ST, 174 (68.8%) from late ST; 79 (31.2%) were from bare metal stents, 166 (65.6%) from drug-eluting stents, 8 (3.2%) were from stents of unknown type. Thrombus specimens displayed heterogeneous morphology with platelet-rich thrombus and fibrin/fibrinogen fragments most abundant; mean platelet coverage was 57% of thrombus area. Leukocyte infiltrations were hallmarks of both early and late ST (early: 2260 ± 1550 per mm² vs. late: 2485 ± 1778 per mm²; P = 0.44); neutrophils represented the most prominent subset (early: 1364 ± 923 per mm² vs. late: 1428 ± 1023 per mm²; P = 0.81). Leukocyte counts were significantly higher compared with a control group of patients with thrombus aspiration in spontaneous myocardial infarction. Neutrophil extracellular traps were observed in 23% of samples. Eosinophils were present in all stent types, with higher numbers in patients with late ST in sirolimus-and everolimus-eluting stents.

Conclusion

In a large-scale study of histological thrombus analysis from patients presenting with ST, thrombus specimens displayed heterogeneous morphology. Recruitment of leukocytes, particularly neutrophils, appears to be a hallmark of ST. The presence of NETs supports their pathophysiological relevance. Eosinophil recruitment suggests an allergic component to the process of ST.

Keywords

Eosinophils • Histopathology • Neutrophils • Neutrophil extracellular traps • Platelets • Stent thrombosis • Thrombus aspiration

Translational perspective

Stent thrombosis is a rare but life-threatening complication of percutaneous coronary intervention. A detailed analysis of thrombus aspirates from patients during percutaneous revascularization may provide a deeper understanding of the pathological processes involved. Here, we report the results of the largest analysis of thrombus samples from patients with stent thrombosis performed so far. Besides the presence of platelets and fibrin, we found a significant number of immune cells, mainly neutrophils, some of which released prothrombotic fibres of DNA, and also eosinophil granulocytes. Our findings suggest that immune cells could represent a novel target for the prevention of stent thrombosis.

Introduction

Stent thrombosis (ST) is a life-threatening complication of percutaneous coronary intervention. Recent large-scale clinical registries reported an incidence of up to 0.4–0.6% per year though rates appear to be lower with newer-generation drug-eluting stents (DES) devices.1,2 The majority of ST patients present with acute myocardial infarction and rates of mortality following presentation are as high as 20–40%.3 In addition, patients treated with DES—the dominant devices used in contemporary practice—have been shown to be at higher risk of late ST and although this risk appears to be ameliorated with newer-generation DES devices, clinical practice guidelines continue to recommend a more prolonged duration of dual antiplatelet therapy after stenting with DES when compared with after bare metal stents.4

Analysis of thrombus specimens from patients presenting with ST can provide useful information regarding the pathophysiological process leading to thrombotic stent occlusion. Prior studies in small numbers of patients demonstrated that thrombus aspirates from patients with ST were comprised of fragments of fibrin and platelet-rich thrombus as well as trapped red blood cells.5 Moreover, the presence of leukocyte populations in significant numbers suggested an important role for inflammation in the pathogenesis of ST events. This latter observation is in keeping with increasing recognition of the importance of inflammation and immune response in atherothrombosis in general,6 as well as the known contribution of hypersensitivity reactions particularly following stenting with durable polymer DES.7–10 Although some pathological processes associated with ST have been identified, the triggering mechanisms remain incompletely understood, and the influence of factors such as timing of ST after the procedure, stent type or polymer coating is poorly characterized. Moreover existing studies did not include detailed characterization of immune cells and related extracellular components. In addition large-scale, multicentre studies with systematic analysis of thrombus from patients presenting with ST remains a notable scientific gap.

The Prevention of Late Stent Thrombosis by an Interdisciplinary Global European Effort (PRESTIGE) consortium was established to investigate ST across Europe.11 A general description of the project is provided in Supplementary material online, Appendix. The central work of the clinical work package was a prospective multicentre registry of patients presenting with ST to participating centres across Europe. As part of this study, thrombus specimens retrieved from catheter thrombectomy were systematically collected and analysed in a central core laboratory. The present report details the main findings from the histopathological evaluation of thrombus specimens from these patients.
Methods

Study population and patient treatment

Patients presenting with stent thrombosis and undergoing catheter thrombectomy at participating centres were eligible for inclusion. Patients were prospectively enrolled using a centralized telephone registration system. A list of participating centres is provided in Supplementary material online, Appendix. Stent thrombosis was defined according to Academic Research Consortium (ARC) criteria for definite stent thrombosis. Data were collected according to a standardized protocol and was entered in a central electronic database (Open Clinka, Leuven Coordinating Centre, Leuven, Belgium). All patients provided written informed consent. Funding was provided by the European Commission under the Seventh Frame Work Programme (Grant agreement number 260309 PRESTIGE). As an additional control group, we also included analysis of thrombus aspirates from patients with spontaneous myocardial infarction undergoing thrombus aspiration in non-stented vessels segments. Recruitment of these patients occurred at a single centre (Deutsches Herzzentrum München, Munich, Germany) and did not comprise part of the PRESTIGE project.

Histopathological sampling and analysis

After crossing the lesion with a standard guide-wire, a thrombectomy catheter was advanced to the target lesion. Thrombus aspiration was performed using a manual suction device according to standard practice. The catheter type used was at the discretion of the cardiologist and was not recorded in the database. Thrombus was collected according to a standardized protocol, fixed in formalin 4%, and transported to the core laboratory for thrombus analysis (Deutsches Herzzentrum München, Munich, Germany). Here thrombus specimens were embedded in paraffin after 48 h of formalin fixation. Thrombus aspirates were processed and analysed wherever practicably possible. Some thrombi were received as small fragments and could not be processed for histological analysis. The smallest thrombus processed successfully had a cross-sectional area of 0.01 mm² at the time of image acquisition. Serial cross sections of all thrombi were cut with 5 μm thickness. Paraffin-embedded tissue sections were deparaffinized by immersion in xylene and rehydrated in decreasing concentrations of ethanol. Sections were stained with H&E, Luna or Carstairs. Reagents were provided by EMS (Hatfield, USA). Slides were mounted with colourless mounting medium (Pertex).

For immunohistochemistry, antigen retrieval was performed using heat and citrate buffer. Specimens were washed in PBS and blocked with 5 μg/mL anti-mouse CD16/32 (eBioscience) and 1% BSA (PAA Laboratories) in PBS for 30 min. Primary antibodies were incubated for 1 h at room temperature, and sections were then washed in PBS containing 0.1% Tween. Secondary antibodies were incubated for 1 h at room temperature. Samples were mounted using an anti-fade mounting medium (DAKO) and sealed with a coverslip. Fibrin/fibrinogen was stained using a rabbit polyclonal antibody (DAKO #A0080). Neutrophil extracellular traps were identified by their expression of neutrophil elastase (NE) using a rabbit polyclonal neutrophil elastase antibody (Abcam #Ab68672). Platelets were identified using a rabbit polyclonal CD41 antibody (Acris #APS4811PU-N). Rabbit IgG was used for control stains (Supplementary material online, Figure S1). Goat anti-rabbit Alexa Fluor 594 (Invitrogen) was used as secondary antibody. DNA was stained with Hoechst 33342 Solution (Invitrogen). Cells and neutrophil extracellular traps (NETs) were quantified in four visual fields using a 40× objective (176 × 131 μm). Data were normalized to square meter thrombus area. For the assessment of cell counts, coefficient of variation for inter- and intra-observer variability was 0.78 and 0.77%, respectively. The following preconditions had to be fulfilled for quantification of NET formation: (i) attendance of filamentary structured extracellular DNA, (ii) respective DNA had to originate from cells that stained positive for a neutrophil marker, and (iii) filamentary structures had to be decorated with a marker for neutrophil granule proteins such as NE. Eosinophils were counted on whole sections stained with Luna and the results were presented as cells/mm² thrombus area. The percentage of fibrinogen, erythrocyte, and platelet area in relation to the whole thrombus area was investigated in overview images of the thrombi taken with the above-mentioned camera and analysed using Cap-Image 7.1 software (Dr Zeintl, Heidelberg, Germany) and Adobe Photoshop. Images were acquired using either a Zeiss Imager M2 Axio epifluorescence microscope and processed using AxioVision AxioVision SE64 Rel. 4.9 software, or a Leica DMRB epifluorescence microscope equipped with a Zeiss AxioCam and processed using AxioVision 4.6 software (Zeiss).

Histological features and comparisons of interest

Patients were categorized according to timing of ST—as early (<30 days) and late (>30 days) post stent implantation (on the basis that mechanistic factors tend to be different between early and late ST)—and type of stent at the index procedure (DES or bare metal stent). Further categorization of timing of ST was performed in accordance with ARC criteria. In a detailed analysis, we then compared the number of leukocytes, neutrophils, NETs, and eosinophils between (i) early and late ST, (ii) DES and bare metal stent, and (iii) DES types.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics) version 23.0 (IBM, Ehningen, Germany) and Prism 5 (GraphPad Software, La Jolla, USA). Data were tested for normal distribution using the Kolmogorov–Smirnov test. For comparison of continuous data between two groups, we used Student’s t-test or Mann–Whitney U-test, for three and more groups, we used analysis of variance ANOVA or Kruskal–Wallis test, according to the distribution of the data. For comparison of categorical data between two groups we used χ² or Fisher’s exact test as appropriate. For comparisons of multiple groups, post hoc testing was performed applying the methods of Bonferroni (Dunn’s test). Significance level was set at a two-sided α of 0.05.

Results

Five hundred and forty-one patients presenting with ST to participating centres between December 2010 and February 2014 were included in the PRESTIGE registry. Thrombus samples were collected from 294 patients. A total of 41 patients were excluded because thrombus specimens were too small for analysis. Overall, thrombus from 253 patients was available for histological analysis. Baseline patient characteristics are shown in Table 1. Procedural characteristics at the time of the ST are shown in Table 2. Baseline laboratory values are shown in Table 3.

As an additional control group, we also included analysis of thrombus aspirates from 104 patients with spontaneous myocardial infarction undergoing thrombus aspiration in non-stented vessel segments. The baseline characteristics of these patients are shown in Supplementary material online, Table S1.
Histological characterization of coronary stent thrombosis

Data are shown as median [Q1, Q3] or n (%). Percentages were calculated on the basis of patients with available information. MI, myocardial infarction; DES, drug-eluting stent.

Table 1  Baseline clinical and demographic characteristics of patients with analysable thrombus aspirates according to presentation as early and late stent thrombosis

|                          | Early (<30 days), n = 79 | Late (>30 days), n = 174 | P-value |
|--------------------------|--------------------------|---------------------------|---------|
| Age                      | 66 [57.74]               | 62 [54.72]                | 0.94    |
| Sex                      |                          |                           |         |
| Male                     | 54 (68.4)                | 143 (82.2)                | 0.014   |
| Coronary artery disease, n (%) |                       |                           |         |
| 1-vessel                 | 36 (48.0)                | 97 (59.1)                 | 0.24    |
| 2-vessel                 | 25 (33.3)                | 46 (28.0)                 |         |
| 3-vessel                 | 14 (18.7)                | 21 (12.9)                 |         |
| Multi-vessel disease     | 39 (52.0)                | 67 (40.9)                 | 0.11    |
| History of coronary bypass | 5 (6.4)                  | 16 (9.2)                  | 0.46    |
| Ejection fraction <30%   | 2 (2.7)                  | 4 (2.4)                   | >0.99   |
| Risk factors             |                          |                           |         |
| Diabetes                 | 30 (38.5)                | 35 (20.2)                 | 0.002   |
| Hypertension             | 42 (55.3)                | 69 (41.3)                 | 0.043   |
| Ex-/smoker               | 51 (65.4)                | 123 (72.3)                | 0.22    |
| Hypercholesterolaemia    | 66 (83.5)                | 157 (90.2)                | 0.13    |
| Clinical presentation    |                          |                           |         |
| Unstable angina pectoris | 3 (3.8)                  | 6 (3.5)                   | 0.629   |
| Non-ST-elevation MI      | 9 (11.5)                 | 28 (16.2)                 |         |
| ST-elevation MI          | 66 (84.6)                | 139 (80.3)                |         |
| Antiplatelet therapy     |                          |                           |         |
| Aspirin                  | 69 (87.3)                | 138 (80.7)                | 0.20    |
| ADP-receptor antagonist  | 65 (82.3)                | 43 (25.0)                 | >0.001  |
| Clopidogrel              | 43 (66.2)                | 26 (60.5)                 |         |
| Prasugrel                | 7 (10.8)                 | 11 (25.6)                 |         |
| Ticagrelor               | 15 (23.1)                | 6 (14)                    |         |
| Dual antiplatelet therapy| 60 (75.9)                | 36 (20.9)                 | <0.001  |
| Coexisting conditions    |                          |                           |         |
| Renal failure (GFR < 30 mL/min) | 6 (7.8)                | 10 (5.8)                  | 0.580   |
| Dialysis                 | 1 (1.3)                  | 2 (1.1)                   | >0.99   |
| Stroke                   | 6 (7.7)                  | 9 (5.2)                   | 0.57    |
| Autoimmune disease       | 1 (1.4)                  | 5 (2.9)                   | 0.67    |
| Active malignancy        | 3 (3.9)                  | 5 (3.0)                   | 0.71    |
| Stent type               |                          |                           |         |
| Bare metal stent         | 22 (27.8)                | 57 (32.8)                 | <0.001  |
| First-generation DES     | 4 (5.1)                  | 49 (28.2)                 |         |
| Second-generation DES    | 45 (57.0)                | 47 (27.0)                 |         |
| Unknown DES type         | 8 (10.1)                 | 13 (7.5)                  |         |
| Presentation at index intervention |            |                           | 0.026   |
| Stable angina pectoris   | 18 (24.0)                | 49 (28.2)                 |         |
| Unstable angina pectoris | 6 (8.0)                  | 21 (13.0)                 |         |
| Non-ST-elevation MI      | 23 (30.7)                | 23 (14.3)                 |         |
| ST-elevation MI          | 28 (37.3)                | 68 (42.2)                 |         |
| EF <30% at index PCI     | 2 (2.7)                  | 3 (1.8)                   | 0.65    |

Data are shown as n (%). Percentages were calculated on the basis of patients with available information.

Table 2  Procedural characteristics of patients with analysable thrombus aspirates according to presentation as early and late stent thrombosis

|                          | Early (<30 days), n = 79 | Late (>30 days), n = 174 | P-value |
|--------------------------|--------------------------|---------------------------|---------|
| Target vessel            |                          |                           |         |
| Left anterior descending | 46 (63.0)                | 70 (41.9)                 | 0.02    |
| Left circumflex          | 7 (9.6)                  | 18 (10.8)                 |         |
| Right coronary           | 19 (26.0)                | 74 (44.3)                 |         |
| Bypass graft             | 1 (1.4)                  | 5 (3.0)                   |         |
| TIMI flow pre-intervention |                        |                           |         |
| 0                        | 67 (89.3)                | 140 (82.4)                | 0.20    |
| I                        | 0 (0.0)                  | 7 (4.1)                   |         |
| II                       | 4 (5.3)                  | 16 (9.4)                  |         |
| III                      | 4 (5.3)                  | 7 (4.1)                   |         |
| Procedure                |                          |                           |         |
| Thrombus aspiration performed |                  |                           |         |
| Balloon angoplasty       | 70 (92.1)                | 146 (85.4)                | 0.14    |
| Additional stent implanted |                                    |                           |         |
| Use of glycoprotein receptor antagonists | 11 (16.4) | 41 (28.1)                | 0.07    |

Data are shown as n (%). Percentages were calculated on the basis of patients with available information.

TIMI, thrombolysis in myocardial infarction.

Frequency of stent types and timing of stent thrombosis

In terms of timing of ST, 79 (31.2%) thrombus specimens were from patients presenting with early ST and 174 (68.8%) from late ST. In terms of stent type, 166 (65.6%) thrombus samples were from DES and 79 (31.2%) from bare metal stents; 8 (3.2%) were of unknown stent type (Figure 1A). The proportion of early and late ST was broadly similar in samples from DES and bare metal stents (Figure 1B and C). The breakdown of stents according to the specific type of DES is shown in Figure 1A; overall, everolimus-eluting stents were most commonly represented. Early stent thrombosis cases in sirolimus- and paclitaxel-eluting stents were poorly represented; early and late ST were more equally represented in newer-generation DES (everolimus, zotarolimus eluting) (Figure 1D).

Histological analysis of thrombus specimens

Thrombus specimens from patients with ST were subjected to systematic histological analysis; representative samples are displayed in Figure 2. Thrombus specimens displayed a compact, heterogeneous morphology. Specimens were rich in platelets (grey/blue to navy staining in Carstairs and red staining in CD41-specific immunohistochemistry) (Figure 2A and B). Mean platelet coverage was 57% of...
thrombus area. Fibrin/fibrinogen (FGN) represented a major component (red staining in Carstairs and in FGN-specific immunohistochemistry) ([Figure 2A] and [C]). Clusters of erythrocytes were present; however, the distribution was heterogeneous ([Figure 2A]). Comparison of the erythrocyte, platelet, and fibrin/fibrinogen components of thrombus area is shown in [Figure 2D].

Table 3  Laboratory parameters of patients with analysable thrombus aspirates according to presentation as early and late stent thrombosis

|                          | Early (<30 days), n = 79 | Late (>30 days), n = 174 |
|--------------------------|--------------------------|--------------------------|
| CRP (mg/L)               | 5.0 [2.1, 15.5]          | 2.2 [0.8, 10.0]          | 0.006
| Leukocytes (10⁹/L)       | 10.9 [8.6, 14.4]         | 10.8 [8.2, 14.3]         | 0.71
| Platelets (10⁹/L)        | 270.5 [224.5, 328.3]     | 223.5 [178.0, 268.0]     | <0.001
| CKmax (U/L)              | 527.0 [178.8, 1629.0]    | 628.5 [211.5, 1664.8]    | 0.71
| CK-MBmax (U/L)           | 78 [30.0, 200.0]         | 95 [43.0, 191.0]         | 0.62
| Troponin T (ng/mL)       | 3.2 [0.7, 31.4]          | 4.74 [1.0, 24.2]         | 0.32

Data are shown as median [Q1, Q3].
Leukocytes and neutrophil extracellular traps in thrombus specimens

Overall in histological and immunohistochemical analyses of ST specimens, inflammatory cells were prominently represented. Cells were present in significant numbers and were mostly found in clusters or layers (Figure 3, Supplementary material online, Table S2). The majority of leukocytes were neutrophils as identified by staining for NE (Figure 3A). Regarding the timing of ST, there was no difference in the numbers of leukocytes (early: 2260 ± 1550 per mm² vs. late: 2486 ± 1578 per mm²; P = 0.44) and neutrophils (early: 1364 ± 923 per mm² vs. late: 1428 ± 1023 per mm²; P = 0.81) (Figure 3B). Overall, the number of leukocytes and neutrophils was not significantly different according to presentation with acute, subacute, late or very late ST (Supplementary material online, Figure S2); there was no correlation between leukocyte counts and time interval from index stenting (Supplementary material online, Figure S3). Neither were there significant differences according to antiplatelet therapy at the time of presentation (Supplementary material online, Figure S4). Regarding stent type, there was no significant difference in the numbers of leukocytes (DES: 2538 ± 1798 per mm² vs. bare metal stents: 2218 ± 1567 per mm²; P = 0.33) and neutrophils (DES: 1429 ± 1041 per mm² vs. bare metal stents: 1393 ± 931 per mm²; P = 0.97) (Figure 3C, Supplementary material online, Table S2). However, patients with spontaneous myocardial infarction (MI) had smaller numbers of leukocytes and neutrophils in comparison with ST patients (Figure 3B and C). In patients with very late ST, there were numerically more leukocytes with DES vs. bare metal stents but this was not statistically significant.
Drug-eluting stents type had no significant influence on the overall number of inflammatory cells observed (Supplementary material online, Figure S6 and Table S3).

Neutrophil extracellular traps were also frequently observed in the thrombus specimens (Figure 4). Overall, 59 (23.3%) thrombus specimens were found to have extracellular DNA originating from neutrophils. There were no significant differences in the numbers of NETs according to timing of ST (early: 27 ± 21 per mm² vs. late: 26 ± 16 per mm²; P = 0.75) (Figure 4B) or stent type (DES: 25 ± 18 per mm² vs. bare metal stents: 29 ± 19 per mm²; P = 0.15) (Figure 4C), or compared with patients with spontaneous myocardial infarction (54 ± 53 per mm²; P = 0.13) (Figure 4C).

Eosinophil accumulation in thrombus specimens

Using Luna staining, we identified eosinophils in thrombus specimens (Figure 5A). There was no significant difference in the number of eosinophils according to the timing of ST (early 107 ± 181 per mm²; late 61 ± 73 per mm²; P = 0.63) (Figure 5B; Supplementary material online, Figure S3C and Table S2). Neither was there an overall difference in the number of eosinophils according to the stent type (DES: 82 ± 136 per mm² vs. bare metal stents: 67 ± 89 per mm²; P = 0.69) (Figure 5C; Supplementary material online, Figure S3C and Tables S2 and S3). Patients with spontaneous Ml had similar numbers of eosinophils in comparison with ST patients (Figure 5C). In patients with very late ST, there were numerically more eosinophils in DES vs. bare metal stents but this was not statistically significant (Supplementary material online, Figure S5); however, in terms of DES stent type, we found numerically more eosinophils in aspirates from sirolimus-eluting (83 ± 70 per mm²) and everolimus-eluting stents (80 ± 109 per mm²) compared with paclitaxel- (41 ± 54 per mm²) and zotarolimus-eluting stents (48 ± 44 per mm²) (P = 0.14, Figure 5D; Supplementary material online, Figure S3D and Table S3).

There was no difference between durable polymer DES compared with bioabsorbable polymer DES (Figure 5E, P = 0.58).

Discussion

With analysis of samples from 253 patients, the present report represents the largest analysis of thrombus specimens from patients presenting with ST following percutaneous coronary intervention. The main findings are as follows: (i) thrombus aspirates were heterogeneous in composition containing platelet-rich thrombus, fibrin/fibrinogen fragments, erythrocytes, and inflammatory cells; (ii) the composition of thrombus from patients with early and late ST as well as with thrombosis in bare metal and drug-eluting stents was broadly similar; (iii) leukocytes were present in thrombus samples in substantial numbers, with neutrophil subpopulations accounting for the majority of cells, highlighting the important role of inflammatory cell recruitment in ST; (iv) for the first time, we could demonstrate that, as is the case in native vessel thrombosis, NETs are important components of stent formation.
thrombus aspirates with these structures present in approximately 1-in-4 samples; and (v) eosinophils are typically present in both bare metal and drug-eluting stent thrombus specimens, with higher numbers in patients with very late ST thrombosis in sirolimus- and everolimus-eluting stents.

The PRESTIGE consortium was established to investigate and reduce the incidence of ST after coronary intervention across Europe. The central work of the clinical work package was based around a prospective multicentre registry of patients presenting with ST to participating centres across Europe. In the setting of this registry, thrombus specimens retrieved from catheter thrombectomy were systematically collected and analysed in a central core laboratory. Through a Europe-wide multicentre collaboration involving the cooperation of a large network of participating clinical centres it was possible to collect 253 analysable ST thrombus aspirates—by far the largest number of aspirates from patients suffering a ST reported in the literature.

A number of issues should be considered in more detail when interpreting the data. First, the heterogeneous nature of the thrombus specimens is consistent with prior reports involving smaller numbers of patients. The central components were platelet-rich thrombus, fibrin/fibrinogen fragments, trapped erythrocytes, and inflammatory cells of leukocyte lineage. In terms of thrombus area, the greatest contribution was from platelets and fibrin/fibrinogen. This is not surprising as it is well known that activated platelets directly promote blood coagulation leading to subsequent fibrin formation, which plays a central role in thrombus growth and stabilization in human atherothrombosis.  

![Figure 4](https://example.com/figure4.png)

**Figure 4** Detection of neutrophil extracellular traps in stent thrombus specimens. (A) Immunofluorescence images of neutrophil extracellular traps stained for neutrophil elastase and DNA (Hoechst). Extracellular DNA originates from neutrophil elastase positive neutrophils. Arrowheads, nuclei; arrows, neutrophil extracellular trap fibres. Bars, 5 μm; (B) number of neutrophil extracellular traps in early \((n = 23)\) vs. late \((n = 37)\) stent thrombosis \((P = 0.75)\); (C) quantification of neutrophil extracellular traps in thrombi derived from drug-eluting stents \((n = 36)\), bare metal stents \((n = 23)\), and spontaneous myocardial infarction \((\text{spont. myocardial infarction}) \,(n = 25)\) \((P = 0.13)\); data are shown as mean ± SD, each symbol in (B) and (C) represents one individual patient.
Secondly, it is increasingly recognized that immune cells participate in thrombosis, including native human coronary arteries.\(^18,19\) Leukocytes can induce local thrombosis as an intravascular defence strategy to pathogen infection.\(^6\) Innate immune cells activate procoagulant pathways to compartmentalize, retain and kill pathogens and trigger coagulation in models of vessel thrombosis.\(^20\) However, whether immunothrombosis-related pathways are also involved in human ST has been a poorly defined issue. In our analysis leukocytes, and specifically neutrophils, represented a hallmark of thrombus aspirates from both bare metal stents and DES. Moreover, analysing leukocyte counts according to timing of ST and type of underlying stent showed no significant difference in cell counts between the different groups. This suggests that leukocyte recruitment is likely a component of the final common pathway in ST irrespective of the initial anatomopathological trigger. In addition, in our analysis the numbers of leukocytes and neutrophils was significantly higher in patients with ST compared with spontaneous MI. This suggests that the immune response may play a relatively more important role in ST when compared with spontaneous MI.

Interestingly, we could demonstrate for the first time that NETs are important components of ST aspirates. Recent research has shown that neutrophils are able to build NETs through a specific type of cell death termed NETosis. These procoagulant extracellular DNA matrices represent a catalytic platform able to bind and activate platelets and other procoagulant effectors like FXII, thereby contributing to both venous and arterial thrombosis.\(^14,20–23\) Mangold et al. correlated high NET burden in aspirated thrombus from patients with acute myocardial infarction with larger infarct size and lesser ST-segment resolution underlining the potential clinical relevance of these structures.\(^24\) Overall, NETs were observed in approximately 1-in-4 thrombus samples in our study. The finding that the majority of aspirates did not contain NET-positive thrombi might be explained by concomitant early administration of heparin in patients with ST undergoing angioplasty in the setting of myocardial infarction. Heparin anticoagulant is known for its direct interaction with NETs, causing their degradation both in vitro\(^25\) and in vivo.\(^14\) Interpatient differences in DNase activity within the circulatory system might also account for the variability of this finding. DNase is able to cleave extracellular DNA and Mangold et al. showed that a higher DNase activity correlated negatively with coronary thrombus NET burden.\(^24\) Taken together, these observations suggest that pharmacological targeting of immunothrombosis may represent a realistic target for novel therapies. Inhibition of triggers of immunothrombosis, such as extracellular nucleic acids activating...
the contact phase, may not only result in efficient anticoagulation in the setting of ST but might also yield less therapy-associated bleeding. Future studies should evaluate whether inhibition of immunothrombosis pathways is effective and safe in clinical practice.

Thirdly, observations in relation to the eosinophil subpopulation of leukocytes were interesting for a number of reasons. Eosinophils were observed at some level across the spectrum of ST cases examined demonstrating a role of these cells in ST in general. Moreover, there were no significant differences in the number of eosinophils observed in early vs. late ST or in bare metal vs. drug-eluting stents. However, interestingly, examination of eosinophil counts according to DES subtypes in patients with very late ST showed numerically higher counts in ST occurring within sirolimus- and everolimus-eluting stents. Indeed, hypersensitivity reactions (e.g. type IVb delayed hypersensitivity reactions) with vasculitis of the vessel wall and lymphocytic and eosinophilic infiltration has previously been observed in autopsy specimens and preclinical studies after DES implantation leading to late ST.\textsuperscript{9,26,27} Moreover, a prior thrombus aspirate study by Cook et al. showed higher levels of eosinophils in patients with ST in sirolimus-eluting stents. A possible explanation is that both sirolimus- and everolimus-eluting stents utilize polymer coatings with methacrylate components, and this might represent a stimulus for hypersensitivity reactions.\textsuperscript{7,9,10}

It is important to interpret the results of our analysis in the context in which the samples were obtained. For example, overall more samples were available from patients with late ST when compared with early ST. Although it is well documented in clinical studies that the majority of ST events occur during the initial 30 days after implantation,\textsuperscript{1,2} this distribution depends to some extent on the duration of follow-up available, with late ST increasingly better represented with increasing overall duration of follow-up. In addition, it might be hypothesized that patients with acute ST and clear mechanical risk factors were less likely to be represented in the registry. In addition, important differences were observed in relation to stent type. In this respect, the high representation of thrombus from everolimus-eluting stents most likely reflects the high usage of these stents in clinical practice. Similarly, the relative over-representation of late ST samples in patients treated with sirolimus- or paclitaxel-eluting stents reflects the low usage of these devices during the enrolment period of the study when newer-generation DES were the dominant devices in clinical use.

Our study has some additional important limitations. First, only patients with successful thrombus aspiration were eligible for inclusion. This impacts on the generalizability of the data. Second, analysis of thrombus is restricted to retrievable pieces of thrombus and theoretically differences might exist between thrombus fragments that are removed vs. those that are retained or displaced. Third, findings in relation to thrombus components remain observational in nature and caution must be used in interpreting comparative findings between different timings and stent types. Fourth, analysis is limited to the description of aspirated cell types, which are interpreted in isolation from information regarding the underlying pathology of the vessel wall; this might have been derived from intravascular imaging data. Fifth, although flow cytometry analysis of thrombus could have provided additional useful data, due to logistical considerations related to multicentre recruitment such an analysis was not planned as part of the current study. Finally, our analysis did not report thrombus age at the time of aspiration. This is because methods for adjudication of thrombus age are not standardized and robustness of these observations is unclear.

In conclusion, we present a comprehensive analysis of the largest series of thrombus samples from patients presenting with ST in the literature to date. The main finding was that thrombus samples were heterogeneous in composition with platelet-rich thrombus, fibrin/fibrinogen fragments, and erythrocytes accounting for the largest volume of the thrombus samples. Moreover, leukocyte recruitment was a hallmark of human ST and NETs, central effectors of immunothrombosis, could be detected in human ST for the first time, supporting their relevance in the pathophysiology of this condition. Eosinophils are also recruited in ST, indicating that allergic reactions could contribute to ST, with differential eosinophilic counts according to DES type, suggesting that hypersensitivity reactions might be more important with certain types of polymer-based stents. Notwithstanding the multifactorial nature of the process of ST, the findings of the present study suggest that immune cells could represent an important target for the prevention of ST in future experimental research and clinical trials.

Supplementary material
Supplementary material is available at European Heart Journal online.

Funding
The research leading to these results has received funding from the European Union Seventh Framework Programme FP7/2007-2013 under grant agreement n° HEALTH-F2-2010-260309 (PRESTIGE), Funding to pay the Open Access publication charges for this article was provided by the European Commission under the Seventh Frame Work Programme (Grant agreement number 260309 PRESTIGE).

Conflict of interest: R.A.B. reports receiving lectures fees from B. Braun Melsungen AG, Biotronik, and Boston Scientific. A.G. reports advisory board membership, lecture fees, travel bursary, research support from Abbott Vascular, Boston Scientific, The Medicines Company, Eli Lilly, Daiichi-Sankyo, and Medtronic. D.T. reports personal fees from AstraZeneca, Bayer, BMS, Boehringer-Ingelheim, Daiichi-Sankyo, Eli Lilly, MSD, Otsuka, and Pfizer. L.F.J reports grants from Sanofi and Bristol-Myers-Squibb. P.G.S. reports personal fees from AstraZeneca, Bayer, Boehringer-Ingelheim, Bristol-Myers-Squibb, Daiichi-Sankyo, GlaxoSmithKline, Lilly, Merck-Sharpe-Dohme, Novartis, Otsuka, Pfizer, Roche, Medtronic, Vivus, Janssen, Orexigen and Regado, grants and personal fees from Sanofi and Servier, personal fees and non-financial support from The Medicines Company. C.V. reports personal fees from Lilly and Novartis. A.K. reports submission of patent applications in relation to drug-eluting stent technology.

References
1. Tada T, Byrne RA, Simunovic I, King LA, Cassese S, Joner M, Fusaro M, Schneider S, Schulz S, Ibrahim T, Ott I, Massberg S, Laugwitz KL, Kastrati A. Risk of stent thrombosis among bare-metal stents, first-generation drug-eluting stents, and second-generation drug-eluting stents: results from a registry of 18,334 patients. JACC Cardiovasc Interv 2013;6:1267–1274.
2. Raber L, Magro M, Stefanini GG, Kalesan B, van Domburg RT, Onuma Y, Wenaesper P, Daemen J, Meier B, Jun P, Serruys PW, Windecker S. Very late coronary stent thrombosis of a newer-generation everolimus-eluting stent compared with early-generation drug-eluting stents: a prospective cohort study. Circulation 2012;125:1109–1121.
3. Schulz S, Schuster T, Mehlhi J, Byrne RA, Ellert J, Massberg S, Goedel J, Bruskina O, Ulm K, Schomig A, Kastrati A. Stent thrombosis after drug-eluting stent
implantation: incidence, timing, and relation to discontinuation of clopidogrel therapy over a 4-year period. Eur Heart J 2009;30:2714–2721.

4. Windecker S, Kohl P, Afonso J, Collet JP, Cremer J, Falk V, Filipatos G, Hamm C, Head SJ, Juni P, Kappetein AP, Kasprzak A, Knuuti J, Landmesser U, Laufer G, Neumann FJ, Richter DJ, Schauerte P, Sousa Uva M, Stefanini GG, Taggart DP, Torracca L, Valgimigli M, Wijns W, Witkowska A, Authors/Task Force m. 2014 ESC/EACTS Guidelines on myocardial revascularization: The Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS).Developed with the special contribution of the European Association of Percutaneous Cardiovascular Interventions (EAPCI). Eur Heart J 2014;35:2541–2619.

5. Cook S, Ladich E, Nakazawa G, Eshtehardi P, Neidhart M, Vogel R, Togni M, Wenaweser P, Billinger M, Seiler C, Gay S, Meier B, Pichler WJ, Juni P, Virmani R, Windecker S. Correlation of intravascular ultrasound findings with histopathological analysis of thrombus aspirates in patients with very late drug-eluting stent thrombosis. Circulation 2009;120:391–399.

6. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. Nat Rev Immunol 2011;13:34–45.

7. Otsuka F, Yahagi K, Ladich E, Kutys R, Alexander R, Fowler D, Virmani R, Joner M. Hypersensitivity reaction in the US Food and Drug Administration-approved second-generation drug-eluting stents: histopathological assessment with ex vivo optical coherence tomography. Circulation 2015;131:322–324.

8. Otsuka F, Byrne RA, Yahagi K, Mani H, Ladich E, Fowlow D, DR, Kutys R, Xhepa E, Kastrati A, Virmani R, Joner M. Neoatherosclerosis: overview of histopathologic findings and implications for intravascular imaging assessment. Eur Heart J 2015; doi:10.1093/eurheartj/ehv205.

9. Finn AV, Joner M, Nakazawa G, Koldogie F, Newell J, John MC, Gold HK, Virmani R. Pathological correlates of late drug-eluting stent thrombosis: strut coverage as a marker of endothelialization. Circulation 2007;115:2435–2441.

10. Joner M, Finn AV, Farb A, Mont EK, Kolodgie FD, Ladich E, Kutys R, Skaaria K, Gold HK, Virmani R. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. J Am Coll Cardiol 2006;48:193–202.

11. PRESTIGE Consortium, Adriaenssens T, Byrne R. Prevention of late stent thrombosis secondary to a sirolimus-eluting stent: should we be cautious? Circulation 2004;109:701–705.

12. Wilson GJ, Nakazawa G, Schwartz RS, Huijbregts B, Rofr H, Herbst TJ, Baim DS, Virmani R. Comparison of inflammatory response after implantation of sirolimus- and paclitaxel-eluting stents in porcine coronary arteries. Circulation 2009;120:141–149, 1, 2.

Appendix

PRESTIGE consortium members

Partners

Deutsches Herz-Zentrum München (DHM), Azienda Ospedaliera Papa Giovanni XXIII (BER), Samodzielny Publiczny Zakład Opieki Zdrowotnej Szpital Uniwersytecki W Krakowie (KRAK), St. Antonius Ziekenhuis Nieuwegein (NIE), University of Leicester (ULEIC), Universitäts-Herzzentrum Freiburg-Bad Krozingen GmbH (UHZ), Institut national de la santé et de la recherche médicale (INSM), Rigas Tехniska Университет, Kizyme S.A. (KIZ), Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt GmbH (HMGU), Katholieke Universiteit Leuven (K.U.LEUVEN), Servicio Madrileño de Salud: Hospital Universitario Clínico San Carlos (SC) and Hospital Universitario La Princesa (HULP), BIOTRONIK SE & Co. KG (BIO), neoplas GmbH (NEO).

Investigators

Belgium: Tom Adriaenssens (K.U.LEUVEN), Ian Buyschaetzel (ZNA Middelheim), Michel Blaauw (initially KIZ, now Synolacos Pharma), Dries De Cock (K.U.LEUVEN), Jo Dens (Oost-Limburg Hospital, Genk), Emanuele Barbato (Cardiovascular Center, OLV Gent), Walter Desmet (K.U.Leuven), Sandrine Gautier (initially KIZ, now Synolacos Pharma), Paul Vermeersch (ZNA Middelheim), Peter Sinnaeve (K.U.LEUVEN), Czech Republic: Ota Hlíniak (St Anne University Hospital, Brno), France: Helene Abergel (INSERM), Laurent Feldman (INSERM), Martine Jandrot-Perrus (INSERM), Didier Letourneur (INSERM), Pierre Mangin (INSERM); Véronique Olivier (INSERM), Caroline Roques (INSERM); Germany: Robert A. Byrne (DHM), Sue Charradtrate (initially DHM, now Klinikum der Universität München), Matthias Gratz (BIO); Michael Joner (DHM), Adnan Kastrati (DHM), Elisabeth Kennerknecht (DHM), Ildiko Konrad (DHM), Tobias Koppa (DHM), Steffen Massberg (initially DHM, now Klinikum der Universitats München), Franz-Josef Neumann (UHZ), Vasili Ntziachristos (HMGU), Sheryl Opinaldo (initially DHM, now Klinikum der Universität München), Robert Sinnaeve (K.U.LEUVEN), Julia Riegger (initially DHM, now Klinikum der Universität München).
Massive vegetation in device-related endocarditis

Julian O.M. Ormerod1*, Amar Keiralla1, Raman Uberoi2, and Timothy R. Betts1

1Oxford Heart Centre, Oxford University Hospitals, Oxford, UK; and 2Department of Interventional Radiology, Oxford University Hospitals, Oxford, UK

* Corresponding author. Tel: +44 1865 741166, Fax: +44 1865 23029, Email: julian.ormerod@ouh.nhs.uk

A 71-year-old man was transferred to the extraction centre with Staphylococcus aureus endocarditis and a 3.5 × 1.7 cm vegetation adherent to the ventricular lead of his 7-year-old secondary-prevention dual chamber ICD (see Panel A and Supplementary material online, Videos S1–S3). He had been initially managed medically but the vegetation increased in size. He was refused surgical extraction due to frailty and multiple co-morbidities (severe LV dysfunction, cachexia, previous hypoxic brain injury, and chronic pulmonary disease). Although the potential haemodynamic and infective consequences of embolization of the vegetation were a contraindication to percutaneous extraction, his deteriorating condition justified this novel approach. Initially, unsuccessful attempts were made to capture the vegetation with several types of snare. The leads were then extracted using a locking stylet and laser sheath and the vegetation, having briefly adhered to the tricuspid valve, disappeared from transoesophageal echo view. Pulmonary angiography located it in the origin of the left lower pulmonary artery (Panel B). It was captured with a tri-snare (Panel C) and pulled back into the IVC. The right iliac vein diameter was too small to pull it further with the snare. A 14 mm Cobra balloon was tracked beyond the vegetation and inflated. This was used alongside the snare to pull the compressed mass into the femoral vein without further embolization, where it was removed in three pieces by open venotomy (Panel D). The patient was extubated the same day, made a full recovery and eventually discharged home.

Supplementary material is available at European Heart Journal online.