A heparin-bonded vascular graft generates no systemic effect on markers of hemostasis activation or detectable heparin-induced thrombocytopenia–associated antibodies in humans

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Objectives: **Almost a third of patients who undergo peripheral bypass procedures do not have suitable veins, making the use of prosthetic materials necessary. Prosthetic materials can cause platelet adhesion and activation of the coagulation cascade on the graft. One potential strategy to reduce this thrombogenicity is to covalently bind heparin to the endoluminal surface of grafts. This human in vivo study examined systemic effects of the endoluminal heparin and addressed whether graft implantation results in (1) a measurable reduction of systemic markers of hemostasis activation compared with control grafts and (2) antibody formation against heparin, potentially responsible for heparin-induced thrombocytopenia (HIT).**

Methods: **The study included 20 patients undergoing femoropopliteal bypass grafting, of whom 10 received a standard Gore-Tex Thin Walled Stretch Vascular Graft (W. L. Gore & Associates, Flagstaff, Ariz) and 10 received a heparin-bonded expanded polytetrafluoroethylene (ePTFE) graft (Gore-Tex Propaten Vascular Graft). Blood samples were drawn before and directly after the operation and at days 1, 3, 5, and week 6 after surgery. Established markers of in vivo activation of platelets and blood coagulation (prothrombin fragment 1+2, fibrinopeptide A, soluble glycoprotein V, thrombin-antithrombin complexes, and D-dimers) were measured using standard commercially available techniques. Antiplatelet factor 4/heparin antibody titers were measured using a commercially available enzyme-linked immunosorbent assay, and platelet counts were determined.**

Results: **No statistical differences were observed in any of the markers of in vivo activation of platelets and blood coagulation between patients receiving Propaten or control ePTFE. Moreover, no antibodies against heparin could be demonstrated up to 6 weeks after implantation.**

Conclusions: **No measurable effect of heparin immobilization on systemic markers of hemostasis was found using a heparin-bonded ePTFE graft in vivo. Also, no antibodies against heparin could be detected up to 6 weeks after implantation. (J Vasc Surg 2008;47:324-9.)**

Almost a third of patients who need peripheral arterial reconstructive operations do not have suitable autologous veins available for grafting.[1] For that reason, prosthetic grafts, such as those made from polytetrafluoroethylene (PTFE), are frequently used in arterial bypass procedures. Autologous veins, however, have better patency than prosthetic grafts. A recent review comparing venous and PTFE above knee femoropopliteal bypasses reported 5-year primary patency rates of 74% and 39%, respectively.[2]

Apart from this clinical evidence, laboratory models also have shown that PTFE grafts are substantially more thrombogenic compared with autologous veins. Prosthetic materials can cause platelet adhesion and activation of the coagulation cascade on the graft.[3] One potential strategy to reduce this thrombogenicity is to covalently bind heparin to the endoluminal surface of grafts.

Heparin is a polysaccharide anticoagulant with potent inhibitory effects on coagulation and a long history of clinical use in the prevention and treatment of thrombosis.[4] Long-term systemic use of heparin is hampered by the immunogenicity of the heparin/platelet factor 4 (PF4) complex. Heparin-induced thrombocytopenia without (HIT) or with thrombosis (HITT) is a serious complication of heparin administration, with an estimated incidence of approximately 3%, although the percentage of patients experiencing serious symptoms is much lower. Even short-term administration of heparin may be associated with HIT

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Competition of interest: Dr H. J. M. Verhagen received a consulting fee from W. L. Gore & Associates.

This study was part of an unrestricted educational grant from W. L. Gore & Associates.

The preliminary results of this study were part of the PhD thesis of Dr Jan Heyligers, publicly defended at the University of Utrecht, September 29, 2006. The study was presented at the Spring 2007 Peripheral Vascular Surgery Society Meeting, Baltimore, Md, Jun 6-9, 2007.

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0741-5214/$34.00

doi:10.1016/j.jvs.2007.10.005

or generation of antibodies against the PF4 complex without clinically overt symptoms. These antibodies lead to platelet activation resulting in thrombocytopenia as a consequence of immune-mediated clearance and consumption. Furthermore, the coagulation cascade may also be activated, resulting in thrombosis, which occurs in approximately one-third of patients with HIT.[5] These thromboses are clinically relevant in about 50% of the patients, and these patients have a poor prognosis: 20% will lose a limb, and 30% eventually die.[6]

Heparin-bonded (or heparinized) grafts have shown favorable results with respect to graft patency in animal models and humans compared with untreated vascular grafts.[7-9] In fact, a recent analysis showed that the heparinized graft used in this study gives patency rates comparable with rates for autologous veins.[10] The beneficial effects of heparinized grafts appear particularly caused by a substantial reduction in acute graft thrombosis within weeks after implantation. Clinical evidence thus suggests that heparin-bonded grafts are superior to untreated grafts. Studies on the mechanism by which heparin immobilized on the grafts prevents graft thrombosis are scarce.

A new expanded PTFE (ePTFE) graft with long-term bonding of heparin accomplished by covalent linkage of the anticoagulant is commercially available in several European countries and has been approved by the United States Food and Drug Administration for use in the United States since 2006. We recently developed a human ex vivo model comparing this heparin-bonded graft with untreated ePTFE and concluded that heparin bonding substantially reduces the thrombogenicity of ePTFE grafts.[11] Whether this translates in a systemic anticoagulant effect is unknown and was the focus of our study.

The fact that the heparin is covalently bonded to these grafts raises an important question with respect to antigenicity. It is unknown whether surface-bonded heparin is able to elicit an immunogenic response similar to that of heparin in solution. Thus, this study examined systemic effects of the local presence of heparin and specifically addressed whether graft implantation results in (1) a measurable reduction of systemic markers of hemostasis activation compared with control grafts and (2) antibody formation against heparin, potentially responsible for HIT. The thrombotic markers used in the proposed study are generally accepted as predictors of thrombotic complications in vivo.[12]

MATERIAL AND METHODS

Study population. The study population included 20 patients, all of whom signed a patient informed consent form to participate in the study. Each study subject received an infrainguinal bypass with a Gore-Tex e-PTFE Vascular Graft (W. L. Gore & Associates, Flagstaff, Ariz). Ten subjects received a Gore-Tex Propaten Vascular Graft, and 10 subjects received a Gore-Tex Thin Walled Stretch Vascular Graft. Patients were assigned to two groups in alternating order, and the first treatment was selected by computer-assisted randomization. They were sequentially included. Patients were excluded if aged <18 years old, if no informed consent could be given, or if they had a known history of heparin allergy. All patients were included in the University Medical Center Utrecht. Three vascular surgeons performed the operations.

All patients were on standard intraoperative and postoperative regimens as applied in our institution: 5000 IU of unfractionated heparin was administered just before clamping, and afterwards, low-molecular-weight heparin (LMWH) was given once daily at a fixed time during the day (17:00 hours) during the entire hospital stay. The two study groups were therefore comparable in terms of postoperative medication.

Vascular grafts. All vascular grafts used in the study were 6-mm Gore-Tex e-PTFE Vascular Grafts from the standard hospital inventory. Patient assignment to a specific graft-type treatment group, Gore-Tex Propaten Vascular Graft or Gore-Tex Thin Walled Stretch Vascular Graft, followed a systematic, alternating-type methodology initiated at the beginning of the study to establish the starting treatment group assignment and the order of subsequent treatment group assignments. Surgeons were aware of the material used; however, because outcome was determined only by laboratory assays on blood samples, which were blinded to the laboratory team, a lack of bias was assured.

Blood sampling. Peripheral blood samples were collected preoperatively, directly postoperatively, before administration of 2500 or 5000 IU once daily postoperative LMWH (Fragmin, Pfizer Health AB/Eisai, Woodcliff Lake, NJ) for thromboprophylaxis, and at subsequent postoperative intervals of 1, 3, and 5 days, and at 6 weeks. Patients who weighed >75 kg received 5000 IU of LMWH, those <75 kg received 2500 IU. Because both groups were treated according to this standard regimen, the influence of the LMWH on outcome was, theoretically, neutralized.

All postoperative blood samples were collected immediately before administration of daily LMWH during the hospital stay. Blood was obtained from the antecubital vein according to standardized venipuncture procedures and collected in tubes containing sodium citrate. Although this study was not performed blinded for the surgical team, the samples were blinded before the laboratory analyses were done.

Systemic marker assays. Prothrombin fragment 1+2 (F_{1+2}), which is removed from prothrombin when this proenzyme is activated to the enzyme thrombin, was measured with an F_{1+2}-specific enzyme-linked immunosorbent assay (ELISA; Enzygnost F1+2 Micro, Dade Behring, Marburg, Germany). Fibrinopeptide A (FPA), which is removed from fibrinogen upon conversion to fibrin, was measured with an FPA ELISA assay (Zymutest FPA, Hyphen Biomed, Andresy, France). Thrombin-antithrombin (TAT) complexes, which reflect the amount of thrombin generated, were measured with a TAT-specific ELISA (Diagnostica Stago, Asnieres-Sur-Seine, France). Soluble glycoprotein V (sGPV), which is removed from the platelet surface following platelet activation, was measured with an

sGPV ELISA Kit (Diagnostica Stago). Levels of D-dimer were measured with an Asserachrom D-DI ELISA kit (Diagnostica Stago). Antibodies against the PF4 complex were measured by a sensitive ELISA assay (GTI Diagnostics, Waukesha, Wisc), which is able to detect low-titer antibodies, and is nearly as sensitive as the serotonin-release assay, which is considered the golden standard of HIT diagnostics. Platelet counts were determined according to standard hospital methods.

Follow-up. Patients underwent physical examination by a medical doctor directly after operation, before discharge, and at the outpatient clinic after 6 weeks to check the status of the graft implanted.

Statistical analyses. Data for systemic markers in the peripheral blood samples from patients with Gore-Tex Propaten and Gore-Tex Thin Walled Stretch Vascular Grafts were compared at each time point with a standard *t* test. Progression of platelet counts in time was assessed by repeated one-way analysis of variance (ANOVA) with the Tukey post-test. $P < .05$ was considered statistically significant.

RESULTS

The study initially included 21 patients (8 women), and their average age was 72 years (range, 62-78 years), Patients receiving ePTFE or Propaten grafts were comparable in terms of age and sex distribution. All patients had uncomplicated procedures, and all bypasses were implanted infrainguinally. All patients were discharged ≤1 week after operation.

One patient was excluded and analyzed separately, and 20 patients were analyzed as a group. A regular ePTFE graft was implanted in the excluded patient, but a systemic heparin infusion was needed during 4 days postoperatively owing to the anticipated risk of progressive leg ischemia after the procedure. All markers were measured in this patient because a decline in platelet count or an antibody against heparin might be detected. This patient also had an uneventful recovery.

At the long-term follow-up (6 weeks), all grafts were patent, as was concluded from the physical exam at the outpatient clinic. If patency of the graft was in doubt, duplex ultrasound scanning was to be performed, but this was not necessary in any of the patients.

No substantial or statistically significant differences were observed in any of the markers of in vivo activation of platelets and blood coagulation between patients receiving Propaten or control ePTFE grafts (Fig 1). Platelet counts stayed relatively constant after surgery. In the Propaten group, a 20% decrease in platelet count was observed at day 1 after surgery compared with baseline values, but this difference was not statistically significant, and platelet counts were already normalized again at day 3. At day 5, platelet counts were 23% higher compared with baseline values in the Propaten group ($P < .05$, Fig 2).

In contrast, the patient who was not included in the study population because of the administration of intravenous heparin, yet analyzed, showed a progressive decrease in platelet count after surgery, with a percentage nadir value of 55% compared with the platelet count before surgery (Fig 3). Moreover, antibodies against heparin were detected at day 3 and 5, without clinically overt manifestations of HIT. At week 6, the amount of platelets almost returned to the preoperative count and antibodies against heparin could still be detected. On the other hand, no antibodies against heparin could be demonstrated up to 6 weeks after implantation in the primary study population.

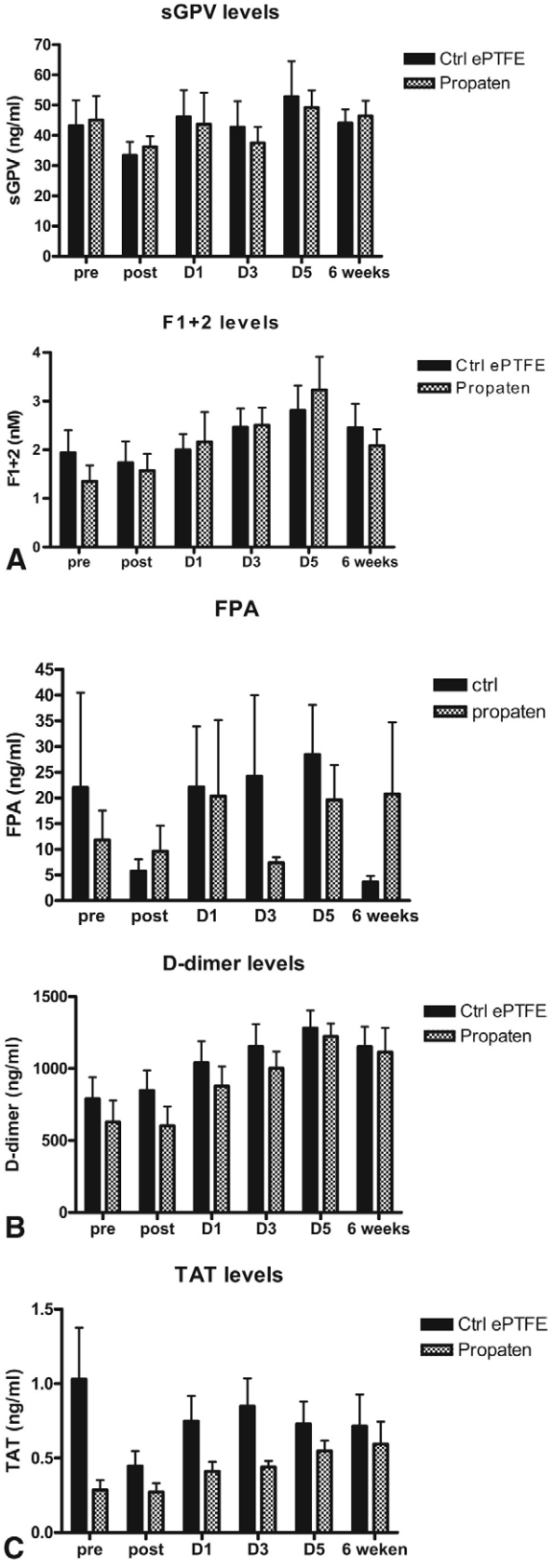
DISCUSSION

This study evaluated in a human in vivo setup whether implantation of an infrainguinal heparinized graft results in (1) a measurable reduction of systemic markers of hemostasis activation compared with control grafts and (2) antibody formation against heparin, potentially responsible for HIT.

Recent data show that the short-term 1-year primary patency of below knee Propaten bypass grafts is about 80%, which is comparable with vein grafting.[10] The reason for this improved patency compared with standard ePTFE grafts (66% primary patency) is unknown. From a prospective multicenter trial describing a 5-year follow-up study comparing heparinized Dacron (DuPont, Wilmington, Del) grafts with PTFE grafts, we know that the beneficial effects of the heparin graft were particularly evident in the early weeks after implantation and sustained thereafter.[9] This suggests that the thrombogenicity reduction due to the heparinization resulting in a decreased occurrence of acute graft thrombosis is at least partly responsible for the improved patency in favor of the heparin-bonded Dacron graft. Another explanation for the improved patency might be the reduction of the development of intimal hyperplasia, because heparin is known to reduce the migration of smooth muscle cells that are responsible for the formation of intimal hyperplasia.[13-17]

Our research group, however, recently concluded from a human ex vivo model comparing heparinized with untreated ePTFE that heparin bonding substantially reduces the thrombogenicity of ePTFE grafts,[11] but it was still unknown whether this translates into a systemic anticoagulant effect. It is also unknown whether antibodies can be formed against the locally bonded heparin. This might be of great clinical importance, not only because of the possible development of HIT(T) but also because of antibody formation against heparin that is clinically nonrelevant but which might imply a life-time prohibition of heparin use.

We could not demonstrate an effect of heparin immobilization on systemic activation of hemostasis using markers of platelet activation (sGPV), thrombin generation (F_{1+2}), thrombin inactivation (TAT), and fibrin generation (FPA). In contrast, our recent ex vivo study did show profound inhibitory effects of heparin immobilization on platelet deposition and fibrin formation.[9] The reason that the local antithrombotic effects of the immobilized heparin does not translate to a detectable reduction of markers of platelet activation or fibrin generation measured in the systemic circulation may be explained by a dilutional effect.



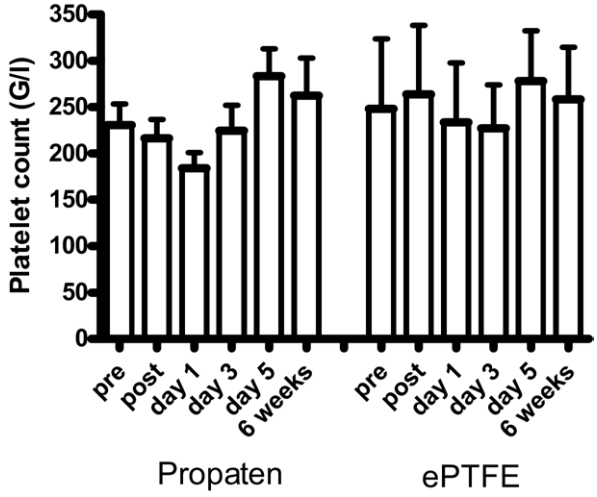


Fig 2. Mean platelet count of patients receiving either a Propaten (W.L. Gore & Associates, Flagstaff, Ariz) or expanded polytetrafluoroethylene *(ePTFE)* graft at various times before and after surgery. Error bars indicate standard error of mean.

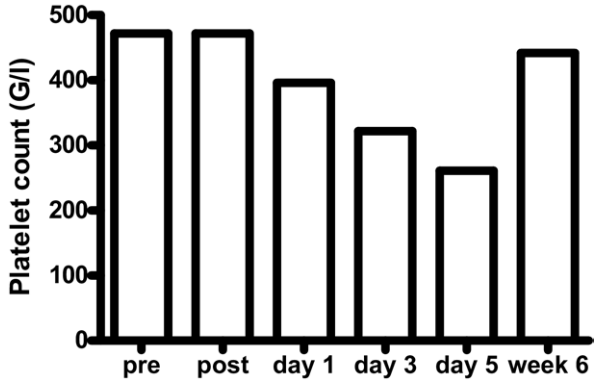


Fig 3. Absolute platelet count of a patient who received an expanded polytetrafluoroethylene *(ePTFE)* graft but was excluded from the analysis because systemic heparin was needed for 4 days postoperatively. A 45% decline in platelet count was observed at day 5, which recovered up to normal levels at week 6; moreover, no heparin/platelet factor 4 complex antibodies developed in this patient.

The implanted heparinized grafts only constitute a minor fraction of the entire vasculature, and this may explain why the local inhibition of platelet activation and fibrin generation by the heparin on the graft does not result in a

Fig 1. Mean levels of markers of coagulation or platelet activation in patients receiving plain expanded polytetrafluoroethylene *(ePTFE)* or Propaten grafts (W. L. Gore & Associates, Flagstaff, Ariz) at various times after surgery compared with preoperative levels. Error bars indicate standard error of mean. s*GPV,* soluble glycoprotein V; *F1+2,* prothrombin fragment 1+2; *FPA,* fibrinopeptide A; *TAT,* thrombin-antithrombin complexes.

detectable systemic antithrombotic effect. A theoretic approach to investigate an in vivo antithrombotic effect of heparinized grafts would involve sampling of blood right after the prosthesis, which is in practice not feasible.

Alternatively, the present study may be underpowered to detect differences. The study was designed as an observational study, and in the relatively small number of subjects studied, no differences or trends towards differences in the measured markers were observed. If a much larger study were to show differences, they would likely be small and not have substantial clinical relevance.

A potential confounder in our study is the administration of LMWH heparin as thromboprophylaxis. Administration of LMWH could potentially affect the hemostasis markers as measured in our study. The goal of our study, however, was to investigate a potential effect of heparin-bonded grafts in patients undergoing standard medical care, because it is with this regimen that substantial differences in patency between heparin-bonded and bare grafts have been observed. Thus, although postoperative LMWH is a potential confounder, it is part of standard medical care in patients who receive ePTFE or Propaten grafts. We attempted to minimize LMWH interference with the measurement of hemostasis markers by sampling just before the daily LMWH injection, when circulating LMWH would be at the lowest possible level. We believe this approach results in a fair comparison. In addition, it would be ethically unacceptable to withhold LMWH in these patients because of the risk of graft-related and deep vein thrombosis.

We did not detect any antibodies against heparin/PF4 in the patients who received a Propaten graft, even after 6 weeks of implantation. We did observe a 20% decrease in platelet count in the Propaten group 1 day after surgery, but this decrease was not statistically significant and resolved again at day 3; thus, we do not believe that this temporal drop in platelet count reflects the activity of very low titer (undetectable by our assay) heparin/PF4 antibodies. That we did not detect antibodies against heparin/PF4 even at 6 weeks after surgery might imply that the immobilized heparin does not elicit an immune response such as frequently observed with systemic (intravenous) heparin. The apparent nonimmunogenic nature of the Propaten grafts contrasts with grafts to which heparin is coated noncovalently. With these grafts, the heparin was observed to be immunogenic as a result of leakage of heparin from the graft.[18,19] This leakage does not occur with Propaten grafts to which heparin is bonded covalently.[7,8]

Although systemic heparin infusion is only possible for a few days because of the high risk of antibody development against heparin/PF4 potentially leading to HIT(T), implantation of Propaten grafts does not result in formation of these antibodies even after 6 weeks. The immobilized heparin is still active after 6 weeks as shown by animal experiments.[7] To our knowledge, this is the first report of surveillance of antibody development after prolonged (6 weeks) in vivo exposure to immobilized heparin.

A single patient did receive intravenous heparin after implantation of an ePTFE graft and had a substantial drop (45%) in platelet count associated with the development of low-titer antiheparin/PF4 antibodies in the absence of overt HIT, indicating that clinically silent HIT(T) can be detected with our assay methods.

CONCLUSION

The results of this study indicate that heparin-coated vascular grafts do not result in a measurable systemic thromboresistant effect and also do not result in development of HIT-inducing antibodies even after 6 weeks of implantation. The reason for the lack of immunogenicity of immobilized heparin is unclear, but is beneficial because this does not prohibit future use of intravenous heparin.

AUTHOR CONTRIBUTIONS

Conception and design: JH, TL, HV, PG, FM
Analysis and interpretation: JH, TL, CW
Data collection: JH, TL, CW
Writing the article: JH, TL
Critical revision of the article: JH, TL, HV, CW, PG, FM
Final approval of the article: JH, TL, HV, PG, CW, FM
Statistical analysis: JH, TL, CW
Obtained funding: PG, FM
Overall responsibility: JH, FM

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Submitted Jun 21, 2007; accepted Oct 8, 2007.

DISCUSSION

Dr Karthik Kasirajan (Atlanta, Ga): I thank the authors for getting me the manuscript in a timely fashion. And I certainly think that this is a very timely paper given recent FDA approval for the Propaten graft. In their paper, the authors attempt to study if the surface heparin would elicit the same immunogenic response as systemic administration of heparin. Heparin-bonded ePTFE grafts are compared in humans to standard thin wall stretch ePTFE grafts implanted in a similar location. The authors have evaluated the systemic markers of hemostasis and antibody formation against heparin in 20 patients, which is about 10 patients in each arm.

A 20% drop in platelet counts seen in the Propaten group did not achieve statistical significance This drop was noted in the early postoperative repair. The authors in conclusion attempt to explain the lack of systemic effects of heparin as possibly secondary to dilution or secondary to the covalent bonding of heparin to the Propaten grafts. That brings me to a few questions.

What is the overall incidence of HIT I and HIT II seen on a general population of patients receiving heparin? I assumed this is quite low. We use heparin almost on a daily basis and rarely see a patient with overt manifestations of HIT. The reason I asked this question is, given the low incidence of HIT and the fact that you have only 10 patients in each arm, is it possible that you have a type 2 error or a false-negative result given the small sample size? That's my first question.

It is also my understanding that the HIT syndrome is an immune-mediated response and not a dose-dependent phenomenon. Patients with a prior HIT syndrome cannot receive even small dose of heparin after the initial diagnosis has been established. Hence, I'm not sure I agree with the argument that covalent bonding or dilution prevents the manifestation of HIT in patients receiving the Propaten graft. Hence, I feel that this study has not sufficiently powered to demonstrate a HIT response. Given the authors' conclusion, and the title of their paper, do the authors feel confident, based on the study, to implant a Propaten graft in the patient with a known HIT syndrome? Thank you very much.

Dr Felix Schlosser: You asked what the incidences of HIT I and HIT II were after intravenous heparin and if there could be possibly a type 2 error in our study. Well, I have to agree that the number of patients in our study is low and therefore there could be a type 2 error. However, to our knowledge, this is the first study that has surveillance of such a long time in these patients for antibody formation. There is no better evidence available at this moment. The number of patients is low; however, our evidence is the first evidence that has followed these patients and these growth markers for quite a long time, and we are at this moment collecting more data. We are collecting the 1-year data of those patients, and we hope to show more improved evidence when we have collected all these data.

The other part of your question, the patients that received intravenous heparin: I don't know the exact number of how many of these patients develop heparin-induced thrombocytopenia, but a large proportion of these patients, around 20% to 30%, will develop it after a prolonged time of heparin dosages. They will develop antibodies against the heparin/platelet factor 4 complex; however, not all of these patients develop heparin-induced thrombocytopenia. About 1% to 2% of patients that received heparin during a prolonged time developed a heparin-induced thrombocytopenia, and 50% of those patients suffered from clinically relevant thrombosis. If the number of patients that develop antibodies against the heparin is about 20% and the number of patients that really suffer from heparin-induced thrombocytopenia is 1% to 2%, it's only a small percentage of the total amount of patients that develop antibodies against the heparin/platelet factor 4 complex. We have in our study 10 patients who did not develop those antibodies or other blood products that were side effects of the heparin that was covalently bonded to the prosthesis. There are few patients that develop those antibodies, and if there was a patient that developed antibodies, there is also a small chance that these antibodies will lead to heparin-induced thrombocytopenia.

It is also important to realize that we do not state or conclude that covalent bonding or dilution of heparin prevents the development of HIT. What we conclude from our study is that in these patients no antibodies against heparin could be detected, even after exposure of 6 weeks. This is the first human study, as far as we know, that looked for detection of antibodies against heparin in this timeframe.

We do not feel confident to implant a heparin-bonded graft in patients with known HIT on the basis of these data, simply because this study does not address this question and therefore can not be recommended.

Dr Allen Hamdan (Boston, Mass): I have just a couple of questions. I assume you used ELISA test, and why not the serotonin release assay as part of it? And can you give a little bit more information on the patient that was excluded? I didn't quite understand what happened.

Dr Schlosser: Thank you for the question. You asked why we're using ELISA. The sensitivity and the specificity ELISA is quite good. Sensitivity is 99% to detect antibodies against the heparin/platelet factor 4 complex, and specificity is 100%. The excluded patient had a risk to develop progressive leg ischemia, and therefore the surgeon started heparin infusion during day 1 until day 4. Because she got heparin intravenously, she was excluded from the analysis, because otherwise, the results would change our outcome drastically.

Dr. Hamdan: But that patient did develop HIT? One of the conclusions would be maybe systemic heparinization with a heparin-bonded graft increases risk.

Dr. Schlosser: That patient did not develop HIT, but developed antibodies against heparin/platelet factor 4 complex. I can add that this patient did not have a Propaten graft but a thin stretchable graft. It is clear that the cause of the antibodies in these patients were from the intravenous heparin.