Clinical aspects of blood activation in open-heart surgery
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Chapter 2

Adhesion of thrombotic components to the surface of a clinically used oxygenator is not affected by Trillium coating

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ABSTRACT

The Trillium® coating is designed to minimize adsorption of protein and the attachment of cells and other particles. The present study was undertaken to investigate the effect of surface coating on the adhesion of thrombotic components (activated platelets, white blood cells and fibrin) to the surface of a clinically used oxygenator.

Twenty patients undergoing elective coronary artery bypass grafting (CABG) were randomized to one of the two oxygenator groups: non coated (NC, n=10) or Trillium® coated (TC, n=10). Platelet and white blood cell counts and factor XIIa concentrations were determined prior to the induction of anesthesia and at the end of cardiopulmonary bypass (CPB). Binding of activated platelets, white blood cells and fibrin to the artificial surfaces was quantified by means of antibody binding and histological validation was achieved by scanning electron microscopy.

Patient demographic and CPB data were similar for the two groups. No significant differences between the groups were found for any of the tested thrombotic components. However, observations from our scanning electron microscopy suggested release of formed particles from the Trillium® coated surface.

Primary adhesion of activated platelets, white blood cells and fibrin to the artificial surface of the venous blood inlet from an oxygenator is not affected by the Trillium® surface coating under conditions of full systemic heparinization.
INTRODUCTION

Cardiopulmonary bypass (CPB) is accompanied by an interaction between blood and artificial surfaces. The exposure of blood to foreign surfaces, as well as non-physiological flow conditions during CPB is considered detrimental since they will activate the cellular and humoral inflammatory systems, thus, resulting in inflammatory reactions and organ dysfunction.\textsuperscript{1,2} Coating of the inner surfaces of the bypass circuit may help to mitigate these side-effects.\textsuperscript{4,5}

The Trillium\textsuperscript{®} (Medtronic, Minneapolis, MN) surface coating has been developed to minimize adsorption of proteins and adherence of cells and other particles. This surface coating is a new procedure involving two polymers. The first polymer is a primer based upon polyethyleneimine that is modified hydrophobically in order to enhance strong binding to artificial materials. The second polymer being anionic, thus, strongly adhering to the cationic primer, consists of 3 blood-compatible functional groups: sulphonate groups, polyethylene oxide (PEO) chains and heparin molecules from a porcine source. Sulphonate groups yield a characteristic anticoagulant effect to polymers by mimicking the functional groups responsible for heparin’s anticoagulant action.\textsuperscript{6} Moreover, sulphonate groups are negatively charged and they are believed to contribute to thromboresistance.\textsuperscript{7} PEO chains are highly hydrophilic and, thus, minimize the interaction with the aqueous environment. The presence of hydrated PEO chains minimizes protein adsorption.\textsuperscript{8} Heparin is modified to co-polymerize with sulphonate groups and PEO monomers. Theoretically, the advantage of this coating should not only be related to the presence of heparin, but even more so to the endothelium-like properties of the negatively charged surface.

In a bovine model of CPB without blood anticoagulation by heparin, Trillium\textsuperscript{®} coating was shown to completely prevent thrombus formation on connectors, even under conditions of stagnant blood flow.\textsuperscript{9} In another bovine model, using low systemic heparin concentrations, fibrin deposits or clots were either absent or significantly reduced in different elements (connectors, reservoirs, oxygenator) of the Trillium\textsuperscript{®} coated circuits when compared to corresponding parts of the control group.\textsuperscript{10}

The present study was undertaken to investigate the binding of activated platelets, white blood cells, and fibrin on Trillium\textsuperscript{®} coated and non coated surfaces in patients...
subjected to elective CPB with full systemic heparinization. In addition to surface thrombotic components, white blood cells and platelets were analysed to evaluate the loss of circulating cells by adhesion or aggregation. Concentrations of factor XIIa were studied to evaluate the activation of the intrinsic coagulation pathway.

PATIENTS, MATERIALS AND METHODS

The present study was carried out as a substudy of a randomized trial to investigate the biocompatibility of the Trillium® and the Carmeda® coating in patients undergoing elective coronary artery bypass grafting (CABG) assisted by CPB. The main results have been published elsewhere.¹¹,¹²

The original randomized trial

Seventy-one patients were included and randomly allocated to one of the three groups: non coated oxygenator (n=25), Trillium® coated oxygenator (n=25) and Carmeda® coated oxygenator (n=21).

Inclusion criteria were the following: age 21-75 years, elective coronary artery bypass surgery, left ventricular ejection fraction (LVEF) > 30%, body surface area (BSA) > 1.66 m² and preoperative hemoglobin levels > 12 g/dL. Exclusion criteria were the following: combined valve surgery or aneurysmectomy, redo operations, insulin-dependent diabetes mellitus, creatinine plasma level > 300 μmol/L, preoperative intra-aortic balloon pumping, preoperative use of non-steroid anti-inflammatory drugs, preoperative use of warfarin or ‘heparin resistance’, preoperative immunosuppressive therapy > 24 hrs, allergic reactions and chronic obstructive pulmonary disease.

All extracorporeal bypass circuits consisted of a hollow-fiber oxygenator (Affinity®, Medtronic, Minneapolis, MN). The oxygenator and heat exchanger were non coated, ie, without surface modification (n=25), Trillium® coated (n=25) or Carmeda® coated (n=21). The additional components of the extracorporeal circuit (all non coated) were identical for all patients. They included a soft-shell venous reservoir, two additional reservoirs, an arterial line filter (Affinity®, 38 micron, non coated), tubing system (Medtronic) and an arterial blood roller pump (Terumo/Sarns, Ann Arbor, MI). One of the additional reservoirs was used to collect shed blood, which was processed by a cell saver
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before being returned into the systemic circulation after termination of the bypass procedure. The other reservoir was used to collect aortic root (systemic) blood, which was returned into the systemic circulation via the soft-shell venous reservoir during the bypass procedure. The extracorporeal system was primed with 500 mL Ringer's lactate solution, 1 L Haemaccel (Behring, Malburg, Germany), 100 mL mannitol 20% (w/v), 50 mL of sodium bicarbonate 8.4% (w/v) and 200 mL aprotinin (2 x 10^6 KIU Trasylol®; Bayer, Leverkusen, Germany). Magnesium sulphate 4 mmol/10 kg (ie, 24 < x < 32 mmol) and 10000 IU bovine heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) were added to the priming solution. The total priming volume was 1.85 L. All patients received 300 IU/kg heparin (Leo Pharmaceutical Products) before cannulation of the aorta. CPB was initiated when the activated clotting time (Hemochron 401, ITC, Edison, NJ) amounted to 480 sec or longer. During CPB and after aprotinin, the activated clotting time was maintained at values longer than 750 sec by means of the administration of additional heparin when required.13 Moderate hypothermia (30-34 °C) was applied to all patients. The non-pulsatile flow rate was maintained between 2.0 and 2.4 L/min/m^2 squared during the cooling and rewarming phases. Myocardial protection was achieved using cold (4-8 °C) crystalloid cardioplegia solution (St. Thomas). Shed blood in the surgical field was processed by a cell saver (HaemoLite 2 plus, Haemonetics Corp., Braintree, MA). Processed blood was returned into the systemic circulation of the patients immediately after CPB. After weaning from CPB and decannulation, heparin was neutralized with protamine sulphate at a 1:1 ratio.

Arterial blood samples were obtained before induction of anesthesia and at termination of CPB. To facilitate comparison between plasma samples, platelet and white blood cell counts and concentrations of factor XIIa end CPB were corrected for hemodilution by hemoglobin concentration.

Blood samples for platelet counts, white blood cell counts and hemoglobin were collected in 5 mL glass vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson, San Jose, CA), and analysed on a Celldyn 4000 (Abbot, Mijdrecht, The Netherlands).

Blood samples (4.5 mL) for the determination of concentrations of factor XIIa were collected in 5 mL glass vacutainer tubes (Becton Dickinson) containing indomethacin.
hirudin citrate medium. Cell-free plasma aliquots (1 mL) were prepared by centrifugation (11 min at 1100 g and 4 °C) and stored at -80 °C until use. During activation of the intrinsic coagulation pathway (Hageman Factor, FXII) β-factor XIIa is cleaved from α-factor XIIa and released in the blood. β-factor XIIa can be determined by substrate Z-Lys-Phe-Arg-pNA.2HCL, which is converted to a yellow coloured product. Factor XIIa activity is expressed as percentage of human plasma activated by celite, known to be a very potent activator of the clotting system and, as such, used in the activated clotting time test tubes. This maximally activated plasma (100%) is stepwise diluted against FXII-deficient plasma (0%) (Sigma Diagnostics, St Louis, MI, USA). To avoid substrate conversion by kallikrein, the kallikrein inhibitor aprotinin (200KIU/ml) (Bayer, Leverkusen, Germany) was added to the reaction mixture.

The current study

The current substudy consists of 20 patients of the original study of whom parts of the venous blood inlet of the oxygenator were available for analysis. Ten patients had been randomized to the non coated oxygenator and ten to the Trillium® coated oxygenator (Table 1).

After CPB, the circuit was emptied and within 45 min a segment of the polycarbonate venous blood inlet was detached from the oxygenator, rinsed gently with 500 mL NaCl 0.9% solution (Baxter, Utrecht, The Netherlands) and stored at -80 °C until analysis. For scanning electron microscopy, segments were fixated with 2% glutaraldehyde in 0.1 M cacodylate buffered saline solution, pH 7.4. There was no period where stagnant blood was left sitting in contact with the sample material. The mentioned segments were chosen because they were easy to remove for analysis and for standardization without touching the contact surface.

Surface thrombotic components (PAC-1, CD11b, fibrin)

Thrombotic components adhering to the venous blood inlet from the oxygenator were quantified using a method previously described by Spijker et al.14 and Amoroso et al.15, by means of antibody binding to a platelet receptor (PAC-1), to a white blood cell receptor (CD11b), and to fibrin. PAC-1 recognizes an epitope on the glycoprotein IIb/IIIa complex.
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of activated platelets at or near the platelet fibrinogen receptor. These three antigens contain the major thrombotic components. Prior to antibody binding, the venous blood inlet samples from the oxygenators were rinsed carefully and then exposed to fluorescent-labelled antibodies against PAC-1, CD11b or fibrin.

**Table 1. Demographic and cardiopulmonary bypass data.**

<table>
<thead>
<tr>
<th>Coating of the oxygenator</th>
<th>Non coated (n=10)</th>
<th>Trillium® coated (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.2 (8.7)</td>
<td>60.1 (10.8)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>88.3 (9.9)</td>
<td>87.1 (11.6)</td>
</tr>
<tr>
<td>Body surface area (m²)</td>
<td>2.05 (0.13)</td>
<td>2.06 (0.16)</td>
</tr>
<tr>
<td>Platelets (10⁹/L)</td>
<td>282.1 (82.8)</td>
<td>231.8 (56.5)</td>
</tr>
<tr>
<td>White blood cell count (10⁹/L)</td>
<td>7.6 (1.8)</td>
<td>6.6 (0.98)</td>
</tr>
<tr>
<td>Hemoglobin (mmol/L)</td>
<td>9.2 (0.83)</td>
<td>9.0 (0.54)</td>
</tr>
<tr>
<td>Cardiopulmonary bypass (min)</td>
<td>88 (19.1)</td>
<td>93.5 (19.2)</td>
</tr>
<tr>
<td>Aortic crossclamping (min)</td>
<td>56 (13.4)</td>
<td>59.9 (18.0)</td>
</tr>
<tr>
<td>Shear stress (N/m²) at blood viscosity of 3x10⁻¹ Ns/m²</td>
<td>2.51 (0.16)ᵃ</td>
<td>2.52 (0.19)ᵃ</td>
</tr>
<tr>
<td>Shear stress (N/m²) at blood viscosity of 4x10⁻¹ Ns/m²</td>
<td>3.34 (0.21)ᵃ</td>
<td>3.36 (0.26)ᵃ</td>
</tr>
</tbody>
</table>

ᵃCalculated values were comparable to the shear stress in large arteries under physiological conditions. No significant differences were found between the groups for any of the variables. Data are presented as numbers or as means with standard deviations in parentheses.
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The antibodies used were anti-human platelet GPIb/IIa (PAC-1, Becton Dickinson, San Jose, CA), anti-human CD11b (Dako, Glostrup, Denmark) and anti-human fibrin (American Diagnostica Inc, Stamford, CT). These antibodies were labelled by reaction with Samarium-N1 ITC chelate (anti-GPIb/IIa and anti-CD11b) or Europium-N1 ITC chelate (anti-fibrin) from EG&G Wallac (Perkin Elmer, Groningen, The Netherlands), according to the supplier’s instructions.

Each piece of the venous blood inlet from the oxygenator was washed with Assay Buffer (50 mM Tris, 150 mM NaCl, 1 mg/mL BSA and 20 μM diethylenetriamine pentaacetic acid (DTPA), pH 7.8) and then incubated with assay buffer containing 0.02 μg/mL of antibody. The samples were incubated overnight at 2-8 °C and then washed four times with wash buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.8). The Sm and Eu labels were eluted from the test materials by incubation for 15 min at room temperature with a so-called Enhancement Solution (EG&G Wallac) and the amounts of label were determined by measuring Sm and Eu-dependent time-resolved fluorescence with a Victor² multilabel counter (EG&G Wallac).

Fluorescence values were converted into amounts of antibody using calibration curves of the label and pre-determined values for the amount of label per weight unit of antibody. The dimensions of the segments of the venous blood inlet from the oxygenators were measured and the surface area was calculated. These data were used to calculate the number of ng of IgG bound/cm² surface area. Histological validation was achieved by scanning electron microscopy (Jeol 6301 F, Jeol Ltd, Tokyo, Japan).

Reference values for surface thrombotic components

Reference values were obtained from a series of similar experiments with the reference materials polydimethylsiloxane (PDMS, Eriks, Alkmaar, The Netherlands) as positive control and polyethylene (LDPE, ET311350, Goodfellow, Cambridge, UK) as negative control in stagnant blood. In these experiments, we measured antibody binding to a platelet receptor (PAC-1), to a white blood cell receptor (CD11b), and to fibrin. The PDMS experiments for antibody binding to PAC-1, CD11b, and fibrin were performed in 10, 10, and five healthy volunteers; the LDPE experiments in 10, 10, and three healthy volunteers. The participants had not received any medication in the two weeks before the
experiments. A whole blood sample was taken and heparin (3 IU/mL) was added as an anticoagulant. Blood was diluted 1:1 with carbonate-buffered Ringer’s lactate solution. According to Belanger and Marois, PDMS and LDPE appear to be suitable reference materials for the evaluation of the *in vitro* and *in vivo* biocompatibility of biomaterials.

**Other measurements**

Platelet counts, white blood cell counts, hemoglobin and concentrations of factor XIIa had been determined in the context of the original randomized trial and were used in the current study for analysis.

During CPB, the shear stress conditions ($\tau$) were calculated at the venous blood inlet of the oxygenator, without conditions of turbulence, according to the equation: $32 \times \frac{Q \eta}{\pi} \times D^3$, where $Q$ = flow, $\eta$ = blood viscosity and $D$ = inlet diameter.

**Statistical evaluation**

Data was analysed using SPSS, release 11.0 (SPSS Inc., Chicago, IL). Demographic and CPB data are reported as means with standard deviations. Outcome data (platelet counts, white blood cell counts, concentrations of factor XIIa and surface thrombotic components) are presented as medians with interquartile ranges. Platelet and white blood cell counts and concentrations of factor XIIa at the end of CPB were corrected for hemodilution (hemoglobin). For blood cell counts and concentrations of factor XIIa, statistical analyses were performed concerning the change ($\Delta$) of that variable relative to the baseline value ($t=0$) per individual patient. No formal sample size was performed. The actual sample size was determined by the availability of material suitable for analysis. Comparisons between (treatment) groups were made by applying the Independent-Samples t-test. Statistical significance was achieved at $p<0.05$ (two-sided). The results of the control assays (reference values) are expressed as ranges.
RESULTS

Clinical results, blood cell counts

The two oxygenator groups (NC; n=10 and TC; n=10) were comparable with regard to: gender, age, body surface area, platelet counts, white blood cell counts, hemoglobin, concentrations of factor XIIa, and surgical data (CPB times, aortic crossclamping times and shear stress during CPB; Table 1). By the end of CPB, platelet and white blood cell counts and concentrations of factor XIIa were similar for the two groups (Figures 1-3).

Figure 1. Platelet counts in patients undergoing cardiopulmonary bypass (CPB). Platelet counts before bypass after induction of anaesthesia (baseline value: 'pre') and at the end of bypass after release of the aortic crossclamp (end CPB). End CPB blood samples were corrected for hemodilution. The measurements were made in 10 patients treated with a non coated oxygenator (NC: white bars) and in 10 patients treated with a Trillium® coated oxygenator (TC: striped bars). Median values and interquartile ranges are presented. No significant differences were present between the two oxygenator groups.

Surface thrombotic components

Binding of activated platelets (GpIIbIIIa), white blood cells (CD11b) and fibrin to the venous blood inlets of non-coated (NC) and coated (TC) oxygenators was identical, no significant differences between the two oxygenator groups were found (Figure 4).

Scanning electron microscopy of the non coated surface showed more sharply defined cellular structures, while the coated surface revealed more blurry cellular structures (Figure 5).
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**Figure 2.** White blood cell counts in patients undergoing cardiopulmonary bypass (CPB). White blood cell counts before bypass after induction of anaesthesia (baseline value: ‘pre’) and at the end of bypass after release of the aortic crossclamp (end CPB). End CPB blood samples were corrected for hemodilution. The measurements were made in 10 patients treated with a non-coated oxygenator (NC: white bars) and in 10 patients treated with a Trillium® coated oxygenator (TC: striped bars). Median values and interquartile ranges are presented. No significant differences were present between the two oxygenator groups.

**Figure 3.** Concentrations of factor XIIa in patients undergoing cardiopulmonary bypass (CPB). Concentrations of factor XIIa before bypass after induction of anaesthesia (baseline value: ‘pre’) and at the end of bypass after release of the aortic crossclamp (end CPB). End CPB blood samples were corrected for hemodilution. The measurements were made in 10 patients treated with a non-coated oxygenator (NC: white bars) and in 10 patients treated with a Trillium® coated oxygenator (TC: striped bars). Median values and interquartile ranges are presented. No significant differences were present between the two oxygenator groups.
Figure 4. Adhesion of thrombotic components to the surface of an oxygenator. Adhesion of platelets (GpIIbIIIa), white blood cells (CD11b) and fibrin to the venous blood inlet of a clinically used oxygenator. Measurements were made in 10 non coated (NC: white bars) and in 10 Trillium® coated oxygenators (TC: striped bars). Median values and interquartile ranges are presented. No significant differences were present between the two oxygenator groups.

The binding of activated platelets to the non coated (NC group) and coated (TC group) surfaces achieved values between the reference values of PDMS (range: 0.42 to 0.91) and those of LDPE (range 0.03 to 0.88). Binding of white blood cells to the tested surfaces was below that of PDMS (range: 0.98 to 1.67) and in the same order of magnitude as that of LDPE (range: 0.29 to 0.70). The fibrin binding corresponded with the lower range of PDMS reference values (range: 0.16 to 1.35) and was in the same range as that of the LDPE values (range: 0.29 to 0.40).
Figure 5. Scanning electron microscopy of the surface of a venous blood inlet of a clinically used oxygenator. Panels A and B: non coated surface showing more sharply defined cellular structures (A, original magnification x60; B, original magnification x200). Panels C and D: coated surface showing more blurry cellular structures (C, original magnification x60, D, original magnification x200). Panel A: sizes of the white spots range between 5 and 30 μm, indicating platelet aggregates and leucocytes. The bigger spots, ranging between 20 and 30 μm, are fingerprints, containing no three-dimensional structures. Panel B: details of leucocytes and aggregates, without visible pseudopodes. Panel C: Sizes of the white spots range between 5 and 50 μm. All the spots seem to be fingerprints. No three-dimensional structures were observed. Panel D: visible particles are not of cellular nature.
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DISCUSSION

In the present study, we observed that binding of activated platelets, white blood cells and fibrin to the artificial surface of the oxygenator’s venous blood inlet is not affected by the Trillium® surface coating. The attachment of thrombotic components proved very modest, and consistently less than that of the positive reference material PDMS. In both groups, the adhesion of white blood cells was most pronounced when compared with that of platelets and fibrin.

Also, we did not observe significant differences in platelet and white blood cell counts between the patients subjected to non coated oxygenators and those exposed to Trillium® coated oxygenators, respectively. This observation is supported by that of Ereth et al.18 who also did not find significant differences in platelet and white blood cell counts during CPB in a similar clinical trial on Trillium® coated versus non coated oxygenators. By contrast, Palanzo et al.19 found a significantly decreased platelet count in the patient group treated with non coated oxygenators.

Mueller et al.20 used an ex vivo, partial CPB bovine model without systemic heparinization. In his study, Trillium® coated connectors showed statistically significantly better antithrombotic properties when exposed to flowing and stagnant blood, compared to non-coated connectors. Using an animal model (calves) at low systemic heparinization, Tevaeearai et al.19 found significantly more clots in the connectors, the reservoir, the heat exchanger and the oxygenator of a non coated CPB circuit than in a Trillium® coated system.

The Trillium® surface has been designed in order to minimize adsorption of proteins and the attachment of cells. In the present clinical study, we were unable to detect reduced fibrin and cell deposition by analysis of antibody binding. A possible explanation can be offered by full systemic heparinization, which reduces deposition of thrombotic components. Another possible explanation may be offered by the improvements of the modern CPB system by using more biocompatible materials containing polycarbonate, polyvinyl chloride, polyethylene and polypropylene.20-22 Possibly, the benefit of coating on heat exchanger - stainless steel - is more pronounced than on polycarbonate. On most parts of the circuits, however, additional benefits of the Trillium® coating may be minimal.
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and difficult to demonstrate under clinical circumstances and in limited numbers of patients.

Other variables and properties of the CPB circuit may also affect the efficacy of coating procedures. It has, for instance, been shown that suction can cause considerable activation of the coagulation pathway. In the present study, suction (wound) blood was processed by a cell saver before being returned into the systemic circulation after the bypass procedure had been terminated. This treatment rules out the possibility that blood activation related to clotting, platelet degranulation, complement activation and hemolysis during the bypass procedure is caused by reinfusion of the processed wound blood. Cell adhesion and intrinsic coagulation are more or less independent from other sources of activation. Nevertheless, we tried to minimize physiological blood activation by using a soft-shell reservoir, by reducing air contact and by separating the suction blood.

The systemic blood contained identical concentrations of factor XIIa in the coated and non-coated groups, indicating a similar activation of the intrinsic coagulation pathway.

Initiation of CPB causes a certain 'first pass effect', blood coming into contact with the bare artificial surface. Initially, this effect is most intensive, although it will diminish after some time. For instance, strong shear stresses could have been responsible for detachment of thrombotic material after initial binding. The scanning electron microscopic images indicated detachment of thrombotic material from the Trillium® coated surface, as reflected by the blurry aspect of the cellular structures on the coated surface. Accordingly, after initial binding, those blurry prints could have been left behind by the thrombotic material.

In this study, we analysed the inner surface of the venous blood inlet of a clinically used oxygenator. In our opinion, that piece of material was easy to isolate from the oxygenator and allowed reproduction in a standardized way without the danger of looking at 'preferred' or 'shunted' pathways through the oxygenator. Our calculations (Table 1) indicated that the shear stress was similar to or higher than the shear stress in the arterial system.

Accordingly, in spite of the Trillium® coating’s antithrombotic activity, a certain equilibrium will have been obtained after about 90 min (similar to most CPB times in the
present study). The present study strongly suggests that a similar equilibrium is achieved in the two treatment groups.

**CONCLUSION**

On the basis of our data, we conclude that, after 90 min of CPB, initial adhesion of thrombotic components to the surface of an oxygenator is not affected by the Trillium<sup>®</sup> surface coating under conditions of full systemic heparinization. However, persistent attachment of cells seems to be reduced by Trillium<sup>®</sup> coating, which may prevent thrombotic accumulation.

**ACKNOWLEDGEMENTS**

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