Novel regulators of megakaryopoiesis: The road less traveled by
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Pathogen reduction treatment using riboflavin and ultraviolet light impairs platelet reactivity towards specific agonists in vitro

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Abstract

**BACKGROUND:** Recent studies showed that Mirasol pathogen reduction treatment leads to increased P-selectin expression and increased oxygen and glucose consumption in resting platelets. This study investigates the effect of PRT on platelet activation.

**STUDY DESIGN AND METHODS:** Untreated or Mirasol-treated platelets were analysed at different time points during storage. Microaggregation upon stimulation with PMA, convulxin and ristocetin was measured. Alpha granule contents and release upon thrombin stimulation were assessed by flow cytometry and Western blotting. Platelet spreading was determined on collagen-coated glass slides.

**RESULTS:** Mirasol PRT led to spontaneous aggregation (hyper-reactivity), as measured by flow cytometry in the absence of agonist throughout storage time. PMA-induced aggregation was significantly higher in Mirasol PRT platelets compared to controls. Aggregation in response to convulxin and ristocetin was significantly lower and directly influenced by storage time after Mirasol PRT, compared to untreated stored platelet concentrates. Despite the reported hyper-reactivity of resting platelets, platelet activation with thrombin at day 8 after Mirasol PRT resulted in less P-selectin positive platelets. Furthermore, platelet factor 4 (PF4) secretion was reduced upon thrombin stimulation at day 8 after PRT compared to controls. Significantly decreased spreading of Mirasol PRT platelets over collagen-coated slides was observed directly after PRT and persisted throughout storage.

**CONCLUSION:** Mirasol PRT leads to hyper-reactive platelets, probably caused by continuous basal degranulation through storage time. This results in a reduction in the degranulation capacity upon acute stimulation, which influences platelet spreading, but not overtly microaggregation. The clinical relevance needs to be investigated.
Introduction

Pathogen-reduction treatment (PRT) is of great importance for platelet concentrates, because they can only be used for up to 7 days after preparation, making extensive microbial and protozoan screening a challenge. Furthermore, platelet concentrates have to be stored at room temperature, which increases the chance of inoculum survival. Improved donor screening and pathogen testing of transfusion products has greatly reduced the risk of transfusion-mediated infections\(^1\;^2\). However, a residual risk of pathogen transmission through transfusion products remains\(^3\;^6\). To further increase safety, new technologies aimed at the reduction of pathogens have been developed. Several PRT systems have been developed on the basis of either ultraviolet (UV) light alone, or in combination with cross-linking agents. Mirasol PRT uses a combination of riboflavin (Vitamin B\(_2\)) with subsequent UV light illumination, which induces damage to nucleated cells in three different ways. First, UV light causes thymine dimerization leading to DNA damage. Second, overall cell damage is generated by reactive oxygen species, which are formed between riboflavin after UV-illumination and oxygen in the platelet transfusion product. Finally, riboflavin intercalates into nucleic acid strands and oxidizes guanine bases upon illumination resulting in more damage to the cells\(^7\;^9\).

Mirasol PRT efficacy has been shown by spiking platelet concentrates with viruses or bacteria followed by PRT resulting in successful pathogen reduction\(^10\;^11\). Several studies have subsequently assessed the quality of PRT platelets (PRT-PLTs) in resting state, stored for up to seven days after PRT. Increased expression of P-selectin (CD62P), higher lactate concentrations and increased glucose and oxygen consumption, as well as lower ATP, were found over storage time after PRT\(^12\;^16\). Proteomic analysis also revealed a PRT-specific increased phosphorylation of p38MAPK\(^17\), a protein involved in granule secretion and clot retraction\(^18\). A randomized controlled clinical trial evaluating the performance and safety of Mirasol PRT-PLTs showed that the corrected count increment (CCI) after transfusion of PRT-PLTs was significantly lower when compared to reference PLTs\(^19\). No significant bleeding risk was detected, although this study was too small to reach
substantial power. Larger studies in the Netherlands (PREPARES study) and Italy (IPTAS study) are currently being performed to study the clinical efficiency of Mirasol PRT.

Platelet activation and aggregation is regulated by specific agonists binding to receptor molecules on the platelet surface such as β1 and β3 integrins, GPVI and vWF receptor. Upon vascular damage, the exposed subendothelium is rich in von Willebrand factor (vWF) and collagen. Adhesion to vWF via the vWF receptor on the platelet surface slows down platelets at the site of damage, followed by firm adhesion through platelet integrin α2β1 and GPVI-mediated binding to collagen. Collagen binding induces cytoskeletal remodelling, leading to platelet spreading and activation. Upon platelet activation, alpha and dense granules fuse with the platelet membrane and release their contents such as platelet factor 4 (PF4) and ADP, attracting and activating more platelets. Following alpha granule fusion, molecules present on the granule membrane are exposed at the platelet surface, amongst others CD62P, subunits of the vWF receptor, CD31 (PECAM) and αIIbβ3 integrin subunits (fibrinogen receptor)\textsuperscript{20-25}. Thrombin, generated through the coagulation system, is a potent platelet activator when binding to its receptors PAR1 and PAR4, inducing an increased release of alpha granule contents, calcium and ADP\textsuperscript{26;27}. All these pathways ultimately synergize to activate platelet integrin αIIbβ3, which binds to fibrinogen and is essential for thrombus formation.

As it is known that Mirasol PRT affects the quality of resting platelets, it is crucial to determine the effects of PRT on platelet activation in response to agonists. Here, we used extensive in vitro testing to determine the functionality of Mirasol PRT-PLTs.

Materials and methods

**Preparation of platelet concentrate transfusion products and Mirasol PRT**

Whole blood (target volume, 500 mL) was collected in CPD anticoagulant in a bottom and top system (C3941, Fresenius, Emmer-Compascuum, The Netherlands). After overnight hold, the units were centrifuged and a buffy coat
(BC) of 50 mL and a hematocrit of 42% was produced. Five BCs and a unit of plasma were pooled in a pooling system (C5000, Fresenius) and after centrifugation the platelet-rich plasma was passed through a leukoreduction filter to a storage bag to make the final platelet concentrate. This represents one standard platelet concentrate or unit as routinely produced at Sanquin Blood Bank. We pooled six platelet concentrates in a 2-L container (R4R2041, Fenwal, Mont Saint Guibert, Belgium) and split them in 6 equal units to minimize donor variation. Three of these units were left untreated while the other three were Mirasol-treated (TerumoBCT, Zaventem, Belgium) by adding 35 mL of a solution of 500 mmol/L riboflavin and illuminating with ultraviolet-B light with a total energy of 6.2 J/cm². The units were stored on a flat bed shaker at room temperature away from direct light sources. At day 2, 5 and 8 of storage, one independent set of Mirasol-treated and control concentrates was used for in vitro experiments as described below. This experimental set-up was performed a total of three times (n=3).

**Flow cytometry phenotyping of resting platelets and flow cytometry based platelet aggregation assay (FCA)**

1x10⁶ platelets were incubated in 100 µl PBS + 1% human serum albumin (HSA) with a cocktail of antibodies directed against known platelet receptors, using a 1:200 dilution for 10 minutes at room temperature. Samples were subsequently diluted 1:10 with 900 µl PBS + 1% HSA and measured on the flow cytometer specified below. Antibodies used were: CD61-FITC, CD41-PerCP Cy5.5, CD42b-APC, CD62P-APC (all from BD Biosciences, San Jose, CA, USA) and CD31-Pacific Blue (Biolegend, San Diego, CA, USA).

FCA was conducted as previously described²⁸. In brief, platelets were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) or with PKH26 for 15 minutes at room temperature. Labelled platelets were washed and incubated with or without agonists in the presence of plasma. Agonists used to induce aggregation were PMA, convulxin and ristocetin, as described and specified previously²⁸. Incubation was conducted at 37°C with shaking. Subsequently, platelets were fixed at different time points in 0.5% paraformaldehyde (PFA) and samples were measured by flow cytometry.
(LSRII + HTS, BD Biosciences). Data was analyzed with FlowJo software version 9.2 (Tree Star Inc., Ashland, OR, USA).

**Platelet degranulation**

Platelets were centrifuged (450xg, 20 minutes) in warm Piperazine-N,N′-bis(2-ethanesulfonic acid (PIPES) saline glucose buffer containing 300µM prostaglandin E1 (Calbiotech, Spring Valley, CA, USA) and washed once in the same buffer. After centrifugation, platelets were resuspended in warm PIPES saline glucose buffer. 0.02U/ml thrombin was added to the same amount of either control or Mirasol PRT-PLT suspensions to induce alpha granule release. Releasate was collected over a time period of four hours. Samples were taken at day 2, 5 and 8 of storage for flow cytometry analysis and Western blotting as specified below.

Flow cytometry samples were prepared by incubation with monoclonal CD62P antibody (Sanquin Pelikines, Sanquin, Amsterdam, The Netherlands) followed by fixation with 1% paraformaldehyde (PFA). Samples were measured on a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and the percentage of CD62P positive platelets over time was used as readout.

Protein extracts for Western blotting analysis were obtained by centrifugation of platelet suspensions at 450xg before and after incubation with thrombin for up to 4 hours. Platelets were resuspended in lysis buffer containing 10% glycerol and 1% NP-40. To measure PF4 release, supernatants were collected after centrifugation and protein was precipitated using four volumes of ice-cold acetone (Bufa, IJsselstein, The Netherlands), followed by overnight precipitation at -20°C. Samples were then centrifuged for 30 minutes at 20,000xg and pellets resuspended in lysis buffer containing glycerol and NP-40. Platelet lysates and releasate samples were subsequently separated on 4-20% gradient gels (BioRad, Veenendaal, The Netherlands), transferred onto nitrocellulose membranes (IBlot, Invitrogen, Bleiswijk, The Netherlands) and probed for PF4(Abcam, Cambridge, UK), and actin (Sigma Aldrich, Zwijndrecht, The Netherlands) followed by goat-anti mouse HRP secondary antibody (Dako, Glostrup, Denmark). Membranes were developed by Enhanced Chemiluminescence (Pierce, Rockford, IL, USA). Quantitation was
performed after measuring band intensity from three independent experiments by using Fiji software\textsuperscript{29}, setting respective controls to 100 per time-point, day and experiment analysed.

**Collagen-spreading**
Glass slides were coated with collagen I solution (Stem Cell Technologies, Grenoble, France) over-night at 4\textdegree{}C. The next day, control or Mirasol PRT-PLTs were added to the glass slides and fixed with 1\% paraformaldehyde (Applied Biosystems, Darmstadt, Germany), either directly after seeding or after 30 minutes incubation time. Platelets were stained for actin (Phalloidin-633, Molecular probes, Invitrogen, Bleiswijk, The Netherlands) and imaged using a confocal microscope (LSM 510, Carl Zeiss, Sliedrecht, The Netherlands). Surface area coverage was measured from three independent experiments using Fiji software\textsuperscript{29} on three representative optical fields per condition.

**Statistical analysis**
Results were analyzed using GraphPad Prism version 5 software (San Diego, CA, USA). Paired t-test was used and differences were considered significant with a p value <0.05.

**Results**

**PRT leads to platelet hyper-reactivity**
Mirasol PRT and control platelets were sampled at day 2, 5 and 8 of storage, in three independent experiments. Previous reports showed increased alpha granule fusion with the platelet membrane after PRT in resting platelets\textsuperscript{13}. In accordance with these studies, we found that the percentage of CD62P positive platelets was overall elevated on Mirasol PRT-PLTs when compared to controls throughout storage time (Fig.1). These results suggest that Mirasol PRT induces mild basal degranulation in resting-state platelets. To confirm this, we explored the expression of known platelet receptors which are also contained in platelet granules, and that upon granule fusion relocate to the
platelet surface. IIbβ3 Integrin (CD41 and CD61) increased throughout storage time in Mirasol PRT-PLTs, although this increase was significant only for CD61 at day 5 of storage (Fig.1). There was also a significant increase in CD42b, one of the subunits of the vWF receptor, at day 5 of storage (Fig.1). Expression of CD31, however, was not significantly altered (Fig.1), probably due to its lower abundance compared to the other receptors analysed. These results imply relocation of receptor molecules to the platelet membrane due to the basal degranulation found in Mirasol PRT-PLTs.

Next, aggregation of PRT-PLTs was assessed using a novel flow-cytometry based platelet aggregation assay. Compared to controls, Mirasol PRT-PLTs showed spontaneous aggregation without addition of agonists throughout storage (Fig.2, Unstimulated). In the presence of agonists, PRT-PLTs differed in response compared to control platelets. Induction of aggregation with PMA, which binds to and activates IIbβ3 integrin, led to hyper-reactivity in Mirasol PRT-PLTs at day 2 of storage and normalised upon subsequent storage (Fig. 2). The aggregation capacity of Mirasol PRT-PLTs upon activation with convulxin, which binds to and activates GPVI, was lower compared to controls (Fig. 2). The same effect was observed with ristocetin, which mediates vWF binding to GPIb-IX-V (Fig. 2). In summary, we found that Mirasol PRT leads to hyper-reactivity resulting in spontaneous aggregation and alpha granule fusion with the platelet membrane in resting platelets. This hyper-reactivity was observed on the first days after PRT upon integrin-dependent platelet activation, whereas vWF and GPVI dependent aggregation were reduced upon storage.

**PRT induces exhaustion of the alpha granule content over storage time**

Next, platelet activation through the thrombin receptors PAR1 and PAR 4 was investigated. The reactivity of PRT-PLTs to thrombin was assessed by measuring the increase in CD62P positive platelets over a time course of 4 hours. Upon addition of thrombin, the increase in CD62P positive platelets at day 2 and 5 of storage was equal for Mirasol PRT-PLT and controls (Fig.3A). At day 8, however, significantly less CD62P positive platelets were detected upon thrombin addition compared to controls (Fig.3A). In parallel, the ability of platelets to secrete alpha granule contents was determined by the release of the alpha granule protein PF4. To ensure that differences in released PF4
were due to the treatment of the platelets and not to fluctuations in the amount of platelets used, we also probed the platelet pellet for the cytoskeletal protein actin and calculated the release of PF4 relative to actin (Fig. 3B). At day 2 and 5 of storage, Mirasol PRT-PLTs secrete equal amounts of PF4 upon stimulation with thrombin compared to controls (Fig.3B & C). At day 8, however, a significant decrease in PF4 secretion was detected in PRT-PLTs compared to controls (Fig.3B & C). This effect was also seen, although to a lesser extent, when platelet concentrates were treated with UV light only in the absence of riboflavin (data not shown). Furthermore, riboflavin addition without UV illumination did not affect the platelet concentrates as measured by the increase in CD62P positive platelets or PF4 release upon thrombin stimulation.

Fig. 1. Surface marker expression in resting platelets. Surface marker expression in resting platelets was measured with flow cytometry and is depicted as percentage of CD62P positive platelets or mean fluorescence intensity (MFI) as measured in three independent experiments. Untreated controls of each experiment were set to 100% and represented by the grey filling, and the variable of Mirasol treated samples is shown as percentage of controls. * p<0.05
In order to assess whether thrombin-mediated alpha granule fusion with the membrane was impaired in Mirasol PRT-PLTs or whether alpha granule contents of PRT-PLTs are reduced during storage time, we evaluated the amount of PF4 retained in the platelet lysates prior to thrombin addition. Equal amounts of PF4 compared to actin were measured in control and Mirasol

Fig. 2. Flow cytometry-based platelet aggregation test. Aggregation in resting state without addition of agonists measuring the percentage of aggregates by flow cytometry over an incubation time of 6 minutes is depicted in the upper panel (Unstimulated). Aggregation of platelets from untreated controls (■) and Mirasol PRT (□) units upon addition of agonists PMA, convulxin and ristocetin over an incubation time of 5 minutes is depicted below. Aggregation was measured by flow cytometry as percentage of double colored events. Mean and SEM of three independent experiments are shown. * p<0.05
PRT-PLTs on day 2 (Fig.3D). At day 5 and 8 of storage, a tendency towards less PF4 retention in Mirasol PRT-PLTs was detected compared to controls, although significance was not reached (Fig.3D).

**Spreading on collagen is impaired in Mirasol PRT-PLTs**

Adhesion and spreading on subendothelial collagen is a crucial first step in wound closure, which prompted us to test whether PRT-PLTs are still capable of this interaction. Control and PRT-PLTs were incubated on collagen I-coated glass-slides followed by actin-staining, and the platelet area coverage was assessed using confocal microscopy. After 30 minutes of incubation, a 65% decrease in area coverage was detected in PRT-PLTs compared to controls at day 2 after PRT (Fig. 4A&B). Decreased spreading was persistent over the whole storage period of 8 days (Fig. 4A&B), indicating that PRT has a direct effect on the capacity of cytoskeletal remodelling in platelets following adhesion to collagen.

Taken together, these results show that Mirasol PRT leads to hyper-reactivity of platelets, probably caused by continuous basal degranulation through storage time culminating in a reduced degranulation capacity upon acute stimulation, which influences platelet spreading, but not overtly microaggregation.

**Discussion**

A crucial step in initiating wound closure is the adhesion and spreading of platelets to collagen exposed in the subendothelium at sites of damage\textsuperscript{26,27}. The major collagen receptor on platelets is GPVI\textsuperscript{30}. Our experimental setup used collagen I-coated glass surfaces to specifically study spreading induced by collagen under static conditions. We observed that Mirasol PRT negatively affects the capacity of platelets to spread over collagen as early as day 2 of storage, persisting over the storage time of 8 days. Furthermore, stimulation of GPVI by convulxin\textsuperscript{31} led to significantly decreased microaggregation after 8 days of storage. This strongly suggests that PRT has a specific deleterious effect on GPVI signalling. These results may also indicate that reduced
Fig. 3. Alpha granule fusion with the platelet membrane and granule content release.

(A) Control (●) or Mirasol PRT (□) platelets were activated with thrombin for 4 hours. CD62P expression was measured with flow cytometry and the percentage of CD62P positive platelets are displayed. (B) PF4 secretion relative to actin in control (●) or Mirasol PRT-PLTs (□) activated with thrombin for 4 hours at day 2, 5 and 8. PF4 secretion was measured relative to actin for control and Mirasol PRT-PLT. PF4 secretion from Mirasol PRT-PLTs is shown relative to control. Control is set to 100% and represented by grey filling. (C) Representative images of immunodetection of PF4 release upon activation with thrombin in control or Mirasol PRT platelets 2, 5 and 8 days after PRT and actin as control for equal loading. (D) Immunodetection with PF4 in control or Mirasol PRT platelet lysates at day 2, 5 and 8. * p<0.05, ** p<0.01, n.s.: not significant
spreading on collagen could be due to defective GPVI-dependent signalling and/or cytoskeletal rearrangement. Adherence of platelets is further facilitated by subendothelial vWF binding to the vWF receptor on the platelet surface. The expression of the vWF receptor subunit CD42b (GPIb) was only significantly increased by Mirasol PRT at day 5 of storage. On the other hand, vWF-receptor function, as tested by aggregation in response to ristocetin, was lower compared to controls. Mirasol PRT-PLT not only display defects in collagen-mediated adhesion, but also reduced vWF receptor function, which is important for initial adherence of platelets to a damaged vessel.

Another functional characteristic of platelets is to aggregate with each other in order to form thrombi and facilitate wound closure. This type of aggregation is induced and mediated by fibrinogen, a component of the coagulation cascade binding to CD41 and CD61 (integrin αIIbβ3) on the platelet surface. We also detected increased CD61 expression in resting platelets during storage. Aggregation in the presence of PMA, which induces integrin αIIbβ3 activation, resulted in significantly higher aggregate formation at day 2 of storage and

![Fig. 4. Platelet spreading on collagen-coated glass slides.](image)

(A & B) Control (■) or Mirasol PRT (□) platelets were seeded onto glass-slides coated with collagen I. Samples were taken 2, 5 and 8 days after PRT. Directly after seeding and after 30 minutes incubation, platelets were fixed and stained for actin. Imaging was performed on a confocal microscope using 100-fold magnification. Scale bars represent 10 M. Area surface coverage was calculated from three representative images in three independent experiments. * p<0.05, ** p<0.01
aggregation comparable to controls over the rest of the storage time. This can be explained by the detected hyper-reactivity of PRT-PLTs and suggest that integrin function is not deleteriously affected by Mirasol PRT. In accordance with our findings, a previous study investigated Mirasol PRT-PLTs under flow over rabbit aorta endothelium. It was found that at a light dose of 6.2 J per cm², which was also used in our study, adhesion, spreading and thrombus formation of Mirasol PRT-PLTs was similar to controls¹³ indicating that impaired GPVI and vWF receptor signalling may be compensated for by integrin-mediated adhesion. In addition, a study employing impedance aggregometry described no defect in thrombus formation of Mirasol PRT-PLTs³². These studies underline that platelet adherence to sites of wounding and thrombus formation are multi-factorial processes, which are influenced by a multitude of positive and negative signals in vivo. Therefore, it is possible that Mirasol PRT-PLTs retain enough functional capacity when transfused. The specific functional facets of Mirasol PRT-PLTs as a transfusion product however should still be closely monitored in all clinical studies that are currently conducted.

Platelet PAR1 and PAR4 receptors bind and are activated by the cleaving actions of thrombin, resulting in activation and subsequent fusion of alpha granules with the platelet membrane, which can be measured by an increase in the alpha granule membrane protein CD62P³³. Subsequently, alpha granule contents, such as PF4, are released. At day 8 of storage, less CD62P positive platelets were detected in response to thrombin in Mirasol PRT-PLTs compared to controls. In parallel, PF4 release upon thrombin stimulation was also significantly lower at day 8 of storage compared to controls. Vice versa, resting platelets displayed an increased population positive for CD62P, most prominently at day 5 and day 8. Taken together, these results strongly suggest that resting PRT-PLTs are hyper-reactive and therefore degranulate continuously during storage time. This mild but gradual secretion ultimately leads to severely reduced granule content in PRT-PLTs resulting in decreased granule release in response to agonists.

Transfusion product safety has greatly improved over time, but residual risks of pathogen transmission persist. The emergence of pathogen reduction treatments (PRT) offers an effective way to disable replication of pathogens in
platelet concentrates, thereby ensuring safe transfusions. Previous research showed that PRT efficiently inhibits pathogen replication\textsuperscript{10,11} but also impairs the quality of the stored platelets after PRT in vitro\textsuperscript{13,14,34}.

Other aspects where PRT was clearly beneficial include the improvement of safety and decreased side effects of platelet transfusions caused by residual amounts of leukocytes, which can cause antibody generation against the human leukocyte antigen (HLA)\textsuperscript{35}. These antibodies may cause graft rejection after transplantation\textsuperscript{36} or platelet refractoriness, shown as poor increment in platelet counts after transfusion. While leukoreduction greatly decreased the risk for alloimmunization and transfusion-associated graft-versus-host disease, a residual risk remains\textsuperscript{37}. Mirasol PRT-PLTs has been shown to completely prevent alloimmunization after platelet transfusions and therefore reduced the amount of rejected transplants in a rat heart transplantation study\textsuperscript{36}.

Furthermore, PRT is of great importance in a globalized society with increasing transcontinental travel. Mirasol efficiently inhibits pathogen replication not only of bacteria and viruses, but also of parasites such as \textit{Trypanosoma cruzi}\textsuperscript{3} and \textit{Plasmodium falciparum} as well as \textit{Plasmodium yoelii}\textsuperscript{4}. While PRT can prevent transfusion-transmitted infections with these parasites in endemic countries, it has also become of great importance in non-endemic countries, such as the United Kingdom, where de novo infections with malaria were reported\textsuperscript{5}. Taken these considerations into account, improving the safety of transfusion units using PRT is clearly beneficial.

Our study shows that Mirasol PRT leads to hyper-reactive platelets, which spontaneously aggregate and degranulate over storage time. Preliminary results from a patient study indicated lower increment of Mirasol PRT-PLTs compared to controls\textsuperscript{38}. It would be interesting to study if storage time after PRT correlates with these lower increments and evaluate the clinical consequences after transfusion of Mirasol PRT-PLTs on the prevention or treatment of bleeding. We are therefore currently conducting a large patient study with bleeding as the primary endpoint [PREPARReS] to investigate the clinical relevance of these findings.
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Authorship contributions: SZ performed experiments, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript; IMdC performed experiments, analyzed and interpreted the data and revised the manuscript; PFvdM participated in discussions and revised the manuscript; DdK provided materials and equipment and revised the manuscript; BBD performed experiments; LG designed the research, performed experiments, analyzed and interpreted data and revised the manuscript; DCT designed the research, analyzed and interpreted data and revised the manuscript. Terumo BCT provided disposables en illuminators at no cost. This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project INCOAG (grant 01C-201), and supported by the Dutch Heart Foundation.
Reference List


