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Plasminogen activator-coated nanobubbles targeting cell-bound β2-glycoprotein I as a novel thrombus-specific thrombolytic strategy

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Running heads rtPA-coated NPs targeting β2GPI lyse APS and aHUS thrombi

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Contributions
PM, RC, PLM, MC and FT designed the study; MA prepared and characterized the nanobubbles; PD, VDL, KM, SC and K S-J performed the in vivo experiments and analyzed the data; FS and CTA performed the in vitro fibrinolytic and thrombolytic assays and analyzed the data; VST surgically removed the thrombi from patients; FT and PM wrote the draft of the manuscript; RC, MC, PLM, KM and K S-J revised the manuscript

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Data-sharing statement
All the data obtained in this study have been included in the article and the supplementary appendix and are available upon request to the corresponding author.

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Abstract

Beta2-glycoprotein I (β2-GPI) is a serum protein widely recognized as the main target of antibodies present in patients with anti-phospholipid syndrome (APS). β2-GPI binds to activated endothelial cells, platelets and leukocytes, key players in thrombus formation. We developed a new targeted thrombolytic agent consisting of nanobubbles (NBs) coated with recombinant tissue plasminogen activator (rtPA) and recombinant antibody specific for cell-bound β2-GPI. The therapeutic efficacy of targeted nanobubbles was evaluated in vitro, using platelet-rich blood clots, and in vivo in three different animal models: 1) thrombosis developed in a rat model of APS; 2) ferric chloride-induced mesenteric thrombosis in rats, and 3) thrombotic microangiopathy in a mouse model of atypical hemolytic uremic syndrome (C3-gain-of-function mice). Targeted nanobubbles bound preferentially to platelets and leukocytes within thrombi and to endothelial cells through β2-GPI expressed on activated cells. In vitro, rtPA-targeted NBs (rtPA-tNBs) induced greater lysis of platelet-rich blood clots than untargeted NBs. In a rat model of APS, administration of rtPA-tNBs caused rapid dissolution of thrombi and, unlike soluble rtPA that induced transient thrombolysis, prevented new thrombus formation. In a rat model of ferric chloride triggered thrombosis, rtPA-tNBs, but not untargeted NBs and free rtPA, induced rapid and persistent recanalization of occluded vessels. Finally, treatment of C3-gain-of-function mice with rtPA-tNBs, that target β2-GPI deposited in kidney glomeruli, decreased fibrin deposition, and improved urinalysis data with a greater efficiency than untargeted NBs. Our findings suggest that targeting cell-bound β2-GPI may represent an efficient and thrombus-specific thrombolytic strategy in both APS-related and APS-unrelated thrombotic conditions.

Keywords
Thrombus-specific thrombolytic strategy; targeted nanoparticles; nanobubbles, Beta2-glycoprotein I; recombinant tissue plasminogen activator; animal models
**Introduction**

Anti-phospholipid syndrome (APS) is an autoimmune disease characterized by antibody-mediated vascular thrombosis and adverse pregnancy outcomes including fetal loss, pre-eclampsia, pre-term delivery, and intrauterine growth restriction\(^1\).\(^2\). Vascular thrombosis is a serious and often recurrent medical condition that affects relatively young individuals with important social and clinical implications\(^3\).\(^4\). Vessel occlusion by blood clots is the most common clinical manifestation observed in a 10-year prospective study of a thousand APS patients followed in various University Hospitals in Europe\(^5\). Although thrombi may potentially form in all arteries and veins of the vascular tree, clinical observation of APS patients has revealed 40% localization in certain districts of the circulatory system, that are responsible for stroke, myocardial infarction, deep vein thrombosis, pulmonary embolism, and other less frequent vascular manifestations\(^5\).

Evidence collected from clinical studies and animal models of APS has documented the critical role played by antibodies to beta2-glycoprotein I (β\(^2\)-GPI) in thrombus formation. Medium to high titer antibodies are detected in APS patients with increased risk of thrombosis and are listed among the classification criteria for the diagnosis of the disease\(^6\). β\(^2\)-GPI is a five-domain serum protein now recognized to be the main target of anti-phospholipid antibodies. Four domains (DI-IV) are composed of short consensus repeats of approximately 60 amino acids shared with other members of the complement (C) control protein family, while the fifth domain contains a phospholipid-binding site that interacts with the membrane of various cell types involved in thrombus formation including endothelial cells, platelets, and leukocytes\(^7\).\(^8\). The binding of β\(^2\)-GPI to endothelial cells requires cell priming with LPS, as documented by the analysis of the *in vivo* protein biodistribution in mice\(^9\), and may also be induced by proinflammatory and physical stimuli such as surgery\(^10\). An epitope exposed in the open form of β\(^2\)-GPI on the N-terminal DI is the preferential target of the pathogenic antibodies that induce thrombus formation\(^11\). The critical role played by the C system in this process is supported by the finding that vascular thrombosis, caused by passive administration of patients’ antibodies or a monoclonal antibody to DI in an animal model of APS, is not observed in C-deficient animals or in animals receiving a non-C fixing antibody\(^11\).\(^12\). Despite the beneficial effect of long-term anticoagulation with vitamin K antagonists, recurrent thrombosis has been reported in 30-40% of high-risk patients with triple APL-positivity\(^5\) and remains a problem to be solved. Resolution of thrombi formed in carotid and cerebral arteries, and less frequently in coronary arteries, that cause serious clinical consequences including death, still represents an unmet clinical need. Thrombolytic agents administered to dissolve thrombi and surgical intervention aimed...
at removing blood clots in medium-large arteries in a limited number of patients unresponsive to pharmacologic treatment are the therapies currently used to control thrombosis in APS patients. However, despite the beneficial effect observed in patients treated with recombinant tissue plasminogen activator (rtPA), this therapy has significant limitations in safety and efficacy. Bleeding is a serious side effect frequently observed in patients with ischemic stroke receiving rtPA and can be reduced, but not abolished, using a lower dose of the drug. Moreover, the rapid clearance of rtPA from the circulation, the relative resistance of large vessels to recanalization and the modest response observed in approximately 40% of patients with small vessel occlusions represent additional limitations of the thrombolytic therapy.

The aim of this work was to devise a strategy to selectively deliver rtPA at sites of vessel occluding thrombi in an attempt to reduce the systemic side effects of the thrombolytic therapy and to make the treatment more effective. The therapeutic approach was based on the administration of polymer-shelled nanobubbles conjugated with rtPA and a recombinant antibody specific for β2-GPI bound to activated endothelial cells lining the occluded vessel, and to activated platelets and leukocytes that accumulate within the thrombus. Moreover, considering that β2-GPI binds to these cells independently of antiphospholipid antibodies, we investigated whether this type of engineered nanobubbles had also a beneficial effect in thrombosis models unrelated to APS.
Methods

Nanobubbles preparation and characterization
Nanobubbles (NBs) with a perfluoropentane core and a chitosan shell were prepared by tuning a method previously reported and used at the final concentration of $4\times 10^{11}$ NBs/ml saline. Further details are available in the Online Supplementary Methods.

Patients’ sera
Serum samples were obtained from 5 APS patients with medium-high titers antibodies to DI domain of $\beta_2$-GPI after obtaining informed consent and were previously shown to induce clot formation in rats. The ethical committee of Istituto Auxologico Italiano approved the study.

Human thrombi
Three patients aged 60-72 years with clinical atherosclerotic disease, undergoing thrombectomy for thrombotic occlusion of descending thoracic aorta, popliteal or femoral arteries gave written informed consent to use surgically removed thrombi for research purposes. In vitro clots were prepared from freshly collected citrated human blood by the addition of thromboplastin and CaCl$_2$. Two different types of clots were generated: a) blood clots prepared under static conditions, referred to as platelet-poor clots, and b) blood clots prepared under flowing conditions (Chandler loop), that have been shown to resemble arterial thrombi, and referred to as platelet-rich clots. Patient’s thrombi and in vitro generated clots (prepared from 3 different donors) were fixed for 24 hours in 10% buffered formalin, snap-frozen and embedded in OCT medium (Diagnostic Division; Miles Inc).

Immunofluorescence analysis
Patients’ thrombi, in vitro blood clots and kidneys from C3 gain-of-function (GOF) mice were examined by immunofluorescence as detailed in the Online Supplementary Methods.

In vitro fibrinolytic and thrombolytic assays
The fibrinolytic and thrombolytic activities of rtPA-coated NBs were estimated as previously reported. Further details are available in the Online Supplementary Methods.
Animal models
Experimental thrombosis models were established in male Wistar rats (270-290g) kept under standard conditions in the Animal House of the University of Trieste, Italy, and in C3 GOF mice at Newcastle University, UK. The in-vivo procedures were performed in compliance with the guidelines of European (86/609/EEC) and Italian (Legislative Decree 116/92) laws and were approved by the Italian Ministry of University and Research (Prot. N° 910/2018PR, rat models) and by the ethics committee of the Comparative Biology Centre of Newcastle University (United Kingdom’s Home Office granted license PD86B3678, mice model). The study was conducted in accordance with the Declaration of Helsinki.

APS model
We used a previously published model of APS 12. Further details are available in the Online Supplementary Methods

Ferric chloride induced thrombosis
The experiments were carried out according to Li et al 22, in anesthetized rats following an incision made through the abdominal wall to exteriorize the ileal mesentery 23. Further details are available in the Online Supplementary Methods

C3 GOF mouse model of atypical hemolytic uremic syndrome.
The C3 GOF mouse model of atypical hemolytic uremic syndrome (aHUS) has been previously published 24. Further details are available in the Online Supplementary Methods

Nanobubbles distribution in rat
In vivo biodistribution studies were performed in 2 anesthetized rats per group that received an intraperitoneal injection of LPS followed by either tNBs conjugated with 3 nmol of cyanine 5.5 or saline and euthanized 2 hours later. The organs were analyzed ex vivo by IVIS Lumina III (PerkinElmer, Milan, Italy) 25,26.

Statistical analysis
Data are presented as mean ± SD. Difference between groups was assessed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test for pairwise comparisons. Survival estimates were calculated according to Kaplan-Meier and compared with the log-rank test. A two-
sided p value of 0.05 was considered significant. Statistical analyses were done with GraphPad Prism 9 (San Diego, CA).

**Results**

**Expression of β2-GPI on thrombi**

In the initial experiments, we sought to determine whether β2-GPI may represent a potentially valuable target for antibody-coated thrombolytic nanobubbles. For this purpose, we searched for the presence of β2-GPI in the thrombi surgically removed from three patients with arterial thrombotic occlusions (Figure 1A) or in vitro formed blood clots with different composition (Figure 1B). Staining of patient’s thrombus sections with antibodies to β2-GPI and to either fibrin or CD9 (to detect platelets and leukocytes) revealed co-localization of β2-GPI with platelets and leukocytes but not with fibrin. Deposits of β2-GPI on nucleated cells were also observed by nuclear staining with DAPI (Figure 1A). The preferential deposition of β2-GPI on platelets and nucleated cells was confirmed by the more intense staining of *in vitro* clots formed under flow conditions (Chandler thrombi), which resemble platelet-rich arterial thrombi, as compared to platelet-poor clots generated under static conditions (Figure 1B).

**Preparation and targeting properties of NB formulations**

A recombinant scFv-Fc miniantibody (MBB2) containing the hinge-CH2-CH3 domains of human IgG1 engineered from scFv isolated from phage display library was selected to functionalize the NBs as ligand for bound β2-GPI 11. To avoid C activation by the MBB2/β2-GPI complex, a CH2deleted variant of MBB2 (MBB2ΔCH2) was conjugated to NBs via a covalent bond. TEM analysis of the NBs showed no difference in the morphology of targeted and untargeted NBs (Figure 2A). Likewise, the two types of NBs had a similar size with an average diameter of 363.5 ± 10.6 nm for the untargeted NBs and 359 ± 12.5 nm for the targeted NBs and both the polydispersity index, a measure of particle size distribution, and the zeta potential, an indicator of particle charge, were within the same range (Figure 2B). Analysis of NBs interaction with thrombi revealed a significant binding of MBB2ΔCH2-coated NBs that was inhibited by 10-fold excess soluble MBB2ΔCH2, but not by an unrelated recombinant antibody 27, 28, preincubated with the clot section (Figure 2C), supporting the targeting specificity for β2-GPI. Search for binding of tNBs was extended to thrombi induced *in vivo* by patients’ sera containing antibodies to β2-GPI. Rhodamine 6G was administered to anesthetized rat to stain platelets and leukocytes prior to infusion of antibodies to β2-GPI. Targeted or control NBs loaded
with coumarin 6 were infused immediately after formation of thrombi in mesenteric microvessels. Analysis of NBs distribution in rats revealed selective co-localization of tNBs and platelets/leukocytes in blood clots while untargeted NBs were practically undetectable (Figure 2D and Online supplementary Videos S1-2).

**Preparation and evaluation of in vitro thrombolytic activity of rtPA-coated NBs**

To selectively deliver the thrombolytic agent at site of thrombi and avoid its release into the circulation, rtPA was covalently conjugated to NBs shell exploiting two binding methods. The fibrinolytic activity of the two types of rtPA-coated NBs was investigated by a turbidimetric clot lysis assay in which the NBs were added to plasma prior to clot formation. As shown in Online supplementary Figure S1, type B rtPA-NBs (carbodiimide-mediated amide bond) displayed a concentration-dependent fibrinolytic activity, which was comparable to that of soluble rtPA, whereas type A rtPA-NBs (amino-reductive reaction) were inactive at all tested concentrations. Based on these results, type B rtPA-NBs coated with MBB2ΔCH2 (rtPA-tNBs) or untargeted (rtPA-NBs) were used for all the in vitro and in vivo experiments.

The physico-chemical properties of rtPA-tNBs, including the size, the polydispersity index and the zeta potential, were essentially similar to those observed with rtPA-NBs (Figure 3A). The encapsulation efficiency, expressed as percentage amount of rtPA loaded on NBs / total amount of rtPA, was over 90% and the loading capacity, expressed as percentage amount of rtPA loaded / total weight of NBs, was about 3.5%. These data did not change when rtPA was loaded on tNBs. The amount of rtPA bound to NBs stored at 4°C was quantified and functionally evaluated at different time points and found to be stable for up to 6 months.

The in vitro thrombolytic activity of targeted and untargeted rtPA-NBs was investigated in a model consisting of platelet-rich blood clots bathed in autologous plasma. Upon addition of the fibrinolytic agent to the plasma surrounding the clot, the degree of lysis by targeted NBs was greater than that of untargeted NBs and was comparable to that of soluble rtPA (Figure 3B). The thrombolytic activity of untargeted rtPA-NBs can be most likely attributed to the static conditions of the in vitro clot lysis and to the continued presence of the thrombolytic agent in the plasma surrounding the clot.

**Effect of targeted NBs on thrombi in the rat model of APS**

To investigate the thrombolytic effect of rtPA-tNBs in the rat model of APS, NBs were infused intravenously after thrombus formation, approximately 30 min after the injection of antibodies to β2-GPI, and the presence and size of rhodamine 6G-stained thrombi in mesenteric vessels were
monitored over time. Administration of rtPA-tNBs caused thrombolysis within 1 min (Video S3) as opposed to the less rapid dissolution of thrombi caused by soluble rtPA (Video S4), whereas untargeted rtPA-NBs were ineffective (Video S5). The dissolution of the thrombi, obtained with rtPA-tNBs and soluble rtPA, was confirmed by quantitative analysis of rhodamine 6G intensity staining (Figure 4A). A point to emphasize is that the dose of rtPA bound to NBs was 10 times lower than that of the soluble rtPA. Moreover, as shown in Figure 4A and Videos S3-4, soluble rtPA exhibited a transient thrombolytic effect that lasted less than 3 minutes and was followed by the formation of new thrombi. Conversely, rtPA-tNBs induced fast thrombolysis and prevented the formation of new thrombi during the 90-minutes observation period (Figure 4A). Untargeted rtPA-NBs failed to lyse the thrombi at all time points (Figure 4A and Online supplementary Video S5).

We further analyzed the efficacy of rtPA-tNBs on blood vessels occlusion by evaluating the number of vessels with markedly reduced or absent blood flow. The results presented in Figure 4B show that treatment with rtPA-tNBs resulted in vascular recanalization and restoration of blood flow in over 80% of occluded vessels whereas both rtPA and rtPA-NBs were ineffective.

The endothelium of the mesenteric vessels examined at the end of the experiment was still covered by tNBs indicating a stable interaction of β2-GPI with endothelial cells (Figure 5). This finding might explain the prolonged profibrinolytic effect observed after administration of rtPA-tNBs. No sign of vascular leakage, assessed by extravascular diffusion of free Rhodamine 6G, or blood extravasation was seen in the ileal mesentery of rats treated with rtPA-tNBs throughout the experimental procedure despite the micro-traumas caused by the tissue manipulation during the mesentery exteriorization from the abdominal cavity to petri dishes for the intravital microscopy analysis.

It is important to underline that only a small percentage of the infused tNBs localized in the thrombi while a large proportion was cleared by the liver and, to a lesser extent, by the lung (Online supplementary Figure S2).

**Effect of targeted NBs on ferric chloride thrombosis**

Having found that β2-GPI was expressed on thrombi independently of the presence of antibodies to this protein, we sought to determine whether rtPA-tNBs may also be effective in lysing blood clots induced by ferric chloride (FeCl3) applied to the rat mesentery. As in the case of APS, the NBs were infused soon after thrombus formation, which occurred within 10 minutes after removal of the chemical compound applied to the mesentery. Unlike rtPA and untargeted NBs, rtPA-tNBs localized at the thrombus site (Figure 6A) and induced rapid and persistent thrombolysis (Online supplementary Video S6-8). The greater efficacy of rtPA-tNBs was also confirmed by the
substantial decrease in the percentage of occluded vessels that was not seen using either soluble rtPA or untargeted rtPA-NBs (Figure 6B).

**Effect of targeted NBs on the C3 GOF murine model of aHUS.**

Immunofluorescence analysis confirmed that both β2-GPI and fibrin were co-localized within the glomeruli and renal vessels (Online supplementary Figure S3), thus proving the rationale to test the *in vivo* therapeutic effect of rtPA-tNBs.

Mice exhibiting active disease (evidenced through an active urinary sediment) were randomized into three groups receiving saline, rtPA-NBs or rtPA-tNBs. As predicted, a modest reduction in fibrin deposition with rtPA-NB treatment was observed due to the thrombolytic effects of rtPA. However, rtPA-tNBs, which enabled targeted therapy due to the addition of antibodies to β2-GPI, significantly attenuated fibrin deposition within the glomeruli when compared to untreated animals and rtPA-NB treated animals (Figure 7A-B).

Survival analysis showed that C3 GOF mice treated with rtPA-tNBs had reduced mortality in comparison to saline treated or rtPA-NB treated animals (Online supplementary Figure S4A); the difference, however, did not reach statistical significance because of the small number of animals. Urinalysis data are shown in Online supplementary Figure S4 (B-D). Saline group exhibit persisting hematuria, which reached clinical end point in 5 out of 6 mice (Online supplementary Figure S4D). In contrast, 3 out of the 5 animals receiving rtPA-tNBs showed improvement in hematuria after the second dose of the thrombolytic agent.

**Discussion**

Nanoparticles are being developed as a novel therapeutic tool to deliver a sufficient amount of thrombolytic drugs to vessel occluding thrombi and to reduce and possibly avoid serious side effects associated with the administration of soluble drugs. This therapeutic approach can be made more effective by coating nanoparticles with ligands that bind with reasonably good affinity to target molecules expressed on blood clots. The data presented here provide evidence that rtPA-coated nanobubbles targeting cell-bound β2-GPI clear occluded vessels and re-establish blood flow in APS and non-APS thrombosis models.

Chitosan-shelled NBs employed in this study have been largely used thanks to their stability, biocompatibility, low immunogenicity and biodegradability and have been adopted in different biomedical and pharmaceutical fields. The NB good biocompatibility is related to their
components, such as chitosan and phospholipids, that are biodegradable, safe and admitted by the Regulatory Agencies FDA and EMA. Indeed, chitosan-shelled NBs have previously been shown to be non-cytotoxic on different cell lines and no signs of acute toxicity was observed after intravenous or intradermal administration in animal models. The approximately 400 nm size of the NBs has the advantage of preventing or markedly reducing their renal excretion and their distribution is favored by the physical property of soft particles. An additional advantage of the polymer-shelled NBs is to be easily modified by covalently binding antibodies, aptamers, peptides and small molecules that allow selective localization. Equally important is the characteristic of the NBs to be filled with safe and biologically inert gases or vaporizable compounds such as perfluorocarbons, sulfur hexafluoride, air and carbon dioxide, enabling them to be good ultrasound reflectors and to be used as an ultrasound imaging probe to localize thrombi and monitor their dissolution.

Platelets and fibrin, being the major components of vascular thrombi, have been investigated as potential targets for the local delivery of nanoparticles coated with thrombolytic agents. Monoclonal antibodies and peptides reacting with fibrin have proven successful for the selective delivery of nanoparticles to thrombi for theranostic purposes. Satisfactory results have also been obtained targeting the platelets with a monoclonal antibody to the GPIIb/IIIa receptor expressed on both quiescent and activated platelets. Unfortunately, treatment with this antibody is often associated with bleeding, which can be markedly reduced by targeting the platelets with a single chain fragment variable that binds the activated form of GPIIb/IIIa. Thrombus dissolution with minimal hemorrhagic risk has also been induced by microbubbles loaded with thrombolytic agents and targeted to blood clots by the arginine-glycine-aspartic acid-serine peptide. However, this therapeutic approach has the limitation of having been tested on thrombi induced by either chemical or physical vascular injury, but not in models relevant to human diseases. We addressed this issue by selecting β2-GPI as a target for the delivery of rtPA-coated nanobubbles to APS and non-APS thrombi. The advantage of β2-GPI over other targets is to interact with different cells involved in thrombus formation including platelets, endothelial cells and leukocytes. These cells express several receptors for β2-GPI such as ApoER2, Toll-like receptors 2 and 4, annexin A2, glycoprotein Iba, and LRP8, though their in vivo relevance in the procoagulant effect of anti-β2-GPI antibodies in APS patients remains to be established. The endothelium is an important target of β2-GPI and represents the initial site of clot formation triggered by the interaction of antibodies with cell-bound β2-GPI. It is important to emphasize that binding of β2-GPI to endothelial cells requires cell activation by LPS and possibly proinflammatory cytokines, and is independent of
antibodies. This is consistent with the finding reported here that β2-GPI is localized on both renal vascular endothelium and glomeruli of a mouse model of aHUS in the absence of specific antibodies. Activation seems to be also required for the binding of β2-GPI to platelets, as suggested by the detection of this molecule on platelets incubated with thrombin receptor activating peptide, but not on resting platelets. This observation is supported by our data showing that β2-GPI is expressed in platelet-rich thrombi such as human arterial thrombi or in vitro formed platelet-rich blood clots (Chandler thrombi). However, platelets do not seem to be the only target of β2-GPI in thrombi as positive staining was seen in thrombus-associated nucleated cells, which include monocytes and neutrophils, known to be involved in thrombogenesis by expressing and/or releasing tissue factor and neutrophil extracellular traps.

The rtPA-coated tNBs induced faster dissolution (within 1 min) of thrombi in the mesenteric microcirculation in the rat model of APS than soluble rtPA, whereas untargeted rtPA-NBs were ineffective. These results clearly indicate that targeted NBs are able to deliver rtPA to blood clots localized in large arteries as well as in microvessels of various organs observed in the classic APS, and in the more severe catastrophic antiphospholipid syndrome. The in vivo effect of rtPA was transient because re-thrombosis occurred within few minutes and persisted for the whole observation period. On the contrary, administration of targeted NBs prevented the formation of new thrombi despite the presence of circulating thrombus-inducing antibodies to β2-GPI. This protection against re-thrombosis is most likely due to the binding of rtPA-bearing tNBs to β2-GPI deposited on the endothelium at sites of vascular thrombi, thereby creating a profibrinolytic shield. Moreover, the fast and persistent re-canalization of occluded vessels by targeted NBs was achieved with a dose of rtPA that was 10-fold lower than that of soluble rtPA, which is expected to significantly reduce the bleeding risk as suggested by our failure to detect bleeding in the ileal mesentery during the in vivo experiments. This greater efficiency can be explained by the reduced dispersion and the selective delivery of the thrombolytic agent bound to NBs at sites of thrombus formation. One possible indication of this therapeutic approach in the clinical setting might be APS patients undergoing vascular surgery, who are known to be at high risk of re-thrombosis as a result of β2GPI deposition on activated endothelium followed by the binding of pathogenic antibodies and C activation.

Another important finding of this work is the ability of targeted NBs to lyse thrombi in non-APS models such as ferric chloride-induced thrombosis and the more clinically relevant atypical hemolytic uremic syndrome in C3 GOF mice that manifests with hematuria, proteinuria, high creatinine level, hemolysis, fibrin deposition in the glomeruli and occasional intravascular
thromboses. As anticipated by our previous observations that chemical and physical stimuli can promote β2-GPI deposition on endothelial cells \(^{10, 12}\), it was not surprising to discover that the targeted NBs were able to dissolve blood clots formed in vessels where the local application of ferric chloride leads to activation of endothelial cells and binding of β2-GPI. Our data also show that thrombotic microangiopathy (TMA) observed in the C3 GOF mouse model of aHUS is a predisposing condition for the deposition of β2-GPI on endothelial cells of glomeruli. The role of bound β2-GPI is to focalize the delivery of rtPA-coated NBs at sites of fibrin clots where they induce fibrin dissolution. The successful reduction of fibrin deposition within the microvasculature of the glomeruli in C3 GOF mice suggests this therapy could be a useful adjunct in the treatment of thrombotic microangiopathies. For C-mediated TMAs, a C-inhibiting therapy will still be required to extinguish the disease process through restoring C regulation. However, restoration of C regulation takes time and thus a fast-acting prophylactic treatment with targeted fibrinolytic NBs could reduce the fibrin burden within the glomeruli, thereby attenuating renal ischemic injury and thus end organ damage. This targeted therapy could be extended to patients with secondary TMAs to reduce ischemic injury in the interim, whilst the precipitating factor of the TMA is identified and subsequently removed. Collectively, any reduction in end organ damage will translate to improved clinical outcomes.

In conclusion, rtPA-coated polymer-shelled NBs targeted to β2-GPI expressed on activated endothelial cells, platelets and leukocytes have been shown to be effective in dissolving thrombi and prevent rethrombosis in rat models of APS and ferric chloride thrombosis, as well as in removing fibrin deposits in the kidneys of mice that develop aHUS. Targeting cell-bound β2-GPI may represent an efficient and safe strategy to selectively deliver a fibrinolytic agent at sites of thrombotic vessel occlusion as well as at sites at risk of developing thrombosis such as injured vascular districts, where activated endothelial cells, along with activated platelets and leukocytes adhering to their surface, might promote a strong thrombogenic environment.
References


Figure legends

Figure 1. Detection of β2-GPI on thrombi by immunofluorescence analysis. Clot sections were double stained with rabbit antibody to β2-GPI and either antibody to fibrin or to CD9, to investigate the localization of β2-GPI on fibrin, platelets and leucocytes. DAPI was used to stain cell nuclei. The thrombi were obtained from two different sources: A) three patients undergoing surgical thrombectomy; B) *in vitro* blood clots generated under static (platelet-poor) or flow (platelet-rich) conditions (see Methods for additional details). Representative images of thrombus section from one patient showing absence of co-staining of β2-GPI and fibrin. Arrows highlight the co-localization of β2-GPI with CD9-positive structures and arrowheads show the co-localization of β2-GPI with DAPI-positive nucleated cells.

Figure 2. Physico-chemical characteristics and binding of NBs to thrombi. A) TEM images showing similar morphology of untargeted (NBs) and targeted NBs (tNBs); B) Average size, size distribution (polydispersity index) and particle charge (zeta potential); MBB2ΔCH2 denotes the recombinant CH2-deleted scFv-Fc miniantibody against the DI domain of β2-GPI. C) In vitro binding of tNBs to patient’s thrombus sections and inhibition by soluble MBB2ΔCH2. Tissue sections were pre-incubated either with MBB2ΔCH2 or an unrelated recombinant antibody (unrelated MB) (100 µg/ml) for 15 minutes prior to exposure to tNBs containing 10 µg/ml MBB2ΔCH2 for further 60 minutes; D) In vivo co-localization of platelets and leukocytes (stained in red with rhodamine 6G) and NBs loaded with coumarin 6 (green) on thrombi induced in rats by administration of antibodies to β2-GPI.

Figure 3. Physico-chemical characteristics and functional activity of NBs coated with rtPA. A) Size and characteristics of rtPA-tNBs and rtPA-NBs (see legend to Figure 2 for further details). B) Thrombolytic activity of rtPA-bound NBs and soluble rtPA on blood clot formed under flow conditions (Chandler loop). NBs (500 ng/ml bound rtPA) and soluble rtPA (500 ng/ml) were added
to the plasma surrounding the clot and the percent lysis was determined at the indicated intervals as detailed in Methods. The results are presented as mean ± SD of three different experiments.

\*p<0.05 using one-way ANOVA followed by Student-Newman-Keuls test.

**Figure 4.** Effect of targeted and untargeted NB coated with rtPA (0.1 µg/g body weight) and of soluble rtPA (1 µg/g body weight) on thrombus dissolution and vascular occlusion in the rat APS model. Thrombosis was induced by administration of anti-β2-GPI antibodies and treatment with thrombolytic agents was started after thrombus formation as detailed in Methods. A) Changes of fluorescence intensity of thrombi shown in videos S3-5 during the first 15 minutes after thrombolytic treatment. Note the rapid and persistent decrease of fluorescence intensity in rats receiving targeted NBs (rtPA-tNBs), whereas soluble rtPA produced only a transient thrombolysis and untargeted NBs (rtPA-NBs) were ineffective. B) Effect of NBs and soluble rtPA on vascular occlusion during a 90-minutes follow-up, as assessed by blood flow measurement. Consistent with the data of panel A, only rtPA-tNBs caused a marked and significant reduction of occluded vessels at all time points. The results in panel B are presented as mean ± SD of experiments conducted in three rats. \*p<0.05, **p<0.005 using one-way ANOVA followed by Student-Newman-Keuls test.

**Figure 5.** Localization of targeted NBs on endothelium and vascular thrombi during thrombolytic treatment in a rat model of APS. Thrombus formation and NBs deposits were followed by intravital microscopy and the images were collected 90 minutes after injection of NBs. Residual intravascular thrombi are visualized in red by in vivo staining with rhodamine 6G and NBs loaded with coumarin 6 in green. Arrows show the co-localization of rtPA-tNBs and residual vascular thrombi and arrowheads highlight the localization of rtPA-tNBs on activated endothelium. Note the absence of untargeted NBs and the presence of occluded vessels in rtPA-NB treated animal.

**Figure 6.** Localization of targeted and untargeted NBs (without rtPA) on vascular thrombi induced by ferric chloride and effect of rtPA-NBs, rtPA-tNBs and soluble rtPA on vascular thrombotic occlusion. A) Intravascular thrombi are visualized in red by in vivo staining with rhodamine 6G and
in green by coumarin 6-loaded tNBs. The images were collected 30 minutes after injection of NBs.

Note the absence of thrombus green staining in rats that received untargeted NBs.

B) Time-course of vascular occlusion after treatment with rtPA-coated targeted or untargeted NBs (0.2 µg/g body weight) or soluble rtPA (2 µg/g body weight), as assessed by intravascular microscopy analysis. A significant reduction in the number of occluded vessels was seen in rats treated with targeted NBs (rtPA-tNBs) but not in those treated with untargeted NBs or soluble rtPA. The results are presented as mean ± SD of experiments conducted in three rats. *p<0.05, ** p<0.005 using one-way ANOVA followed by Student-Newman-Keuls test.

Figure 7. rtPA-tNBs dissolve clots in the C3 GOF mouse model of aHUS. A. Representative image of glomerular fibrin deposition in saline-treated aHUS mice (n=3), aHUS mice treated with rtPANBs, (0.5µg/g body weight; n=6) or aHUS mice treated with rtPA-tNBs (0.5µg/g body weight; n=5). B. Densitometry analysis of glomerular fibrin deposition, 87 glomeruli scored in saline treated C3 GOF, 358 glomeruli scored in rtPA-NB, 459 scored in rtPA-tNB. *p<0.05, ** p< 0.005, *** p <0.0001 using one-way ANOVA followed by Student-Newman-Keuls test.
Figure 2.

A

NBs

B

<table>
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<tr>
<th></th>
<th>Average diameter ± SD (nm)</th>
<th>Polydispersity index ± SD</th>
<th>Zeta potential ± SD (mV)</th>
<th>MBB2dCH2 (mg/ml)</th>
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<tr>
<td>tNBs</td>
<td>359 ± 12.5</td>
<td>0.122 ± 0.003</td>
<td>+29.67 ± 2.05</td>
<td>0.1</td>
</tr>
<tr>
<td>NBs</td>
<td>364 ± 10.6</td>
<td>0.116 ± 0.004</td>
<td>+31.44 ± 2.11</td>
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</tbody>
</table>

C

- tNBs + unrelated MB
- tNBs + MBB2dCH2

D

- Platelets/Leukocytes
- Nanobubbles

- tNBs
- NBs
**Figure 3.**

### A

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<tr>
<th>Sample</th>
<th>Average diameter ± SD (nm)</th>
<th>PDI ± SD</th>
<th>Zeta potential ± SD (mV)</th>
<th>NB concentration (10^{11}/ml)</th>
<th>MBB2dCH2 (mg/ml)</th>
<th>rtPA (mg/ml)</th>
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<tbody>
<tr>
<td>rtPA-tNBs</td>
<td>356.7 ± 9.5</td>
<td>0.123 ± 0.003</td>
<td>+ 28.65 ± 1.22</td>
<td>4</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>rtPA-NBs</td>
<td>360.2 ± 12.3</td>
<td>0.125 ± 0.002</td>
<td>+ 29.03 ± 0.87</td>
<td>4,1</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

### B

**Lysis (%)**

- rtPA
- rtPA-NBs
- rtPA-tNBs

- **90 minutes**: rtPA > rtPA-NBs > rtPA-tNBs
- **180 minutes**: rtPA > rtPA-tNBs > rtPA-NBs

*Significant differences indicated by asterisks.*
Figure 5.

Thrombi

Nanobubbles

tNBs

NBs
Figure 6.

A

Thrombi

Nanobubbles

tNBs

NBs

B

rtPA-tNBs

rtPA

rtPA-NBs

% of occluded vessels

Time (minutes)

0 15 30 45 60 90

0 15 30 45 60 90

0 15 30 45 60 90

* * * * * * * * * * * * * * * *
SUPPLEMENTARY APPENDIX

Plasminogen activator-coated nanobubbles targeting cell-bound β2-glicoprotein I as a novel thrombus-specific thrombolytic strategy

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Supplementary Methods

Nanobubbles preparation and characterization

Targeted NBs were prepared by chemical conjugation of MBB2DCH2, a CH2-deleted variant of scFv-Hinge-CH2-CH3 recombinant antibody directed against the DI domain of β2-GPI. The recombinant antibody was oxidized by periodate oxidation and conjugated by reductive amination to chitosan amino groups. NBs were then loaded with rtPA from Boehringer Ingelheim (Milan, Italy; 1mg/ml) to the final concentration of 100 μg/ml NB suspension through covalent conjugation of the thrombolytic agent either by amino-reductive reaction (Type A) or by carbodiimide-mediated amide bond formation (Type B). The amount of rtPA loaded on NBs was measured by the Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL USA). One ml of the BCA reagent was added to 100 μl of the sample and reacted for 30 min at 37 °C. After cooling to room temperature, the chromogenic product was measured by an UV-Vis spectrophotometer at 562 nm. The rtPA concentration was calculated with reference to a calibration curve constructed with rtPA standard solutions in the concentration range between 0 - 200 μg/ml. Fluorescent NBs formulations were prepared by labeling the NB core with coumarin 6. The encapsulation efficiency and loading capacity of the rtPA-loaded NBs were calculated using the following equations: Encapsulation efficiency = [amount of rtPA loaded/total amount of rtPA] x 100 and Loading capacity = [amount of rtPA loaded/total weight of NBs] x 100. NB formulations were characterized determining their physico-chemical properties. The size, polydispersity index and surface charge were determined by dynamic light scattering technique (DLS) using a 90 Plus instrument (Brookhaven, NY, USA). The analyses on diluted NB samples (1:30 v/v in filtered water) were performed at a scattering angle of 90°C and a temperature of 25°C. Nanobubble morphology was investigated by transmission electron microscopy (TEM) using a High Resolution JEOL 300 kV microscope. The diluted NBs were stained with osmium tetroxide aqueous solution (0.1% v/v) before TEM analysis. After dropping the sample on the copper grid, it was blotted onto a filter paper and air dried before analysis. The physical stability of NB formulations stored at 4° C was investigated up to 6 months, evaluating the physico-chemical parameters and the rtPA content and activity of NBs over time.

Immunofluorescence analysis

Seven μm sections of patients’ thrombi or in vitro blood clots were stained with the following antibodies (5μg/ml): FITC-labeled mouse anti-human CD9 (ImmunoTools, Friesoythe, Germany); rabbit anti-human β2-GPI (Sigma, Milan, Italy) revealed by CY3-labeled sheep anti-rabbit IgG
The slides were mounted with the Mowiol-based antifading medium (Sigma) and the images were acquired with the fluorescence microscope Leica DM2000 equipped with Leica DFC420 camera.

For studies in C3 gain-of-function (GOF) mice, 5µm cryosections from mouse kidneys were mounted on a Shandon ColorFrost Plus microscope slide (Thermo Scientific), and stored at -80°C. To detect fibrin, thawed tissue sections were fixed in acetone, blocked for 30 minutes in rabbit serum and then incubated with sheep anti-human fibrinogen (Bio-Rad, Milan, Italy) for 1 hour, followed by rabbit anti-sheep IgG Alexa 555 (Abcam, Cambridge, UK). After incubation with the secondary antibodies, the slides underwent repeated washing with PBS, then stained with DAPI, covered with glass coverslip and fluorescence images were taken at x20 on Leica DM2000 LED using a Leica DFC 7000 T camera. Densitometry analysis of Glomerular Fibrin deposition was performed using Fiji (Image J, NIH).

In vitro fibrinolytic and thrombolytic assays

The experiments to investigate the functionality of rtPA-coated NBs were carried out in microplate wells. NBs loaded with rtPA (5 µl) or soluble rtPA (5 µl) were added to human plasma (50 µl) and clot formation was induced by 10 µl thromboplastin (Recombiplastin, Wefen, Milan, 1:1000 final dilution) and 50 µl CaCl$_2$ (20 mM). The rate of lysis (1/lysis time) was calculated by the changes in optical density measured at 405 nm.

The thrombolytic activity of targeted and untargeted rtPA-NBs was evaluated in models consisting of FITC-labeled platelet-rich human blood clots (Chandler thrombi, ~100 µl) submerged in autologous plasma (0.49 ml). In these experiments NBs or rtPA (10 µl) were added to the bathing plasma, thereby mimicking the thrombolytic process. The percent of lysis was determined at fixed intervals by measuring the release of FITC-labeled fibrin degradation products.

APS model

The APS model was established in rats that were intraperitoneally injected with LPS (2.5 mg/kg) and then anesthetized with Zoletil (Virbac, 25 mg/Kg) and Xylazine (Rompun, 7.5mg/kg). Afterwards, they received a slow infusion of Rhodamine 6G (Sigma) into the jugular vein, to stain leukocytes and platelets, followed by infusion of 1 ml of pooled sera from 5 APS patients containing antibodies to β2-GPI into the carotid artery to cause thrombus formation, which occurred in a time range of 15-25 min. Targeted and untargeted NBs (30 µg of bound rtPA in 300 µl saline; ~0.1 µg/g body weight) or free rtPA (300 µg of rtPA in 300 µl saline; ~1 µg/g body weight) were then infused
into the carotid artery and the formation of thrombi in the mesenteric vessels was monitored by intravital microscopy. The percentage of occluded vessels was evaluated by measuring the blood flow.

**Ferric chloride induced thrombosis**

Thrombosis was induced in anesthetized rats following an incision made through the abdominal wall to exteriorize the ileal mesentery. After careful analysis to ascertain the integrity of mesenteric vasculature, bands of filter paper soaked with 0.6% ferric chloride (FeCl₃) were placed on the ileal mesentery for 3 minutes. Eight to 10 minutes after removal of the filter paper, generated thrombi were visualized by intravital staining with Rhodamine 6G. Targeted and untargeted NBs (60 μg of bound rtPA in 300 μl saline; ~0.2 μg/g body weight) or free rtPA (600 μg of rtPA in 300 μl of saline; ~2 μg/g body weight) were injected soon after thrombus formation. The percentage of occluded vessels was evaluated by measuring the blood flow.

**C3 GOF mouse model of atypical hemolytic uremic syndrome**

The model of atypical hemolytic uremic syndrome (aHUS) was established in mice. In brief, a point mutation in the C activation protein C3 (C3.p. D1115N), associated with aHUS in man, was transferred to C57Bl/6J mice. The mice develop a spontaneous thrombotic microangiopathy, with microvascular fibrin deposition in the glomeruli. The phenotype can be rescued through C5 inhibition or C5 genetic knockout, thus akin to aHUS in man. Mice that developed spontaneous disease (identified by haematuria >80g/L for 48 hours) were entered into the study. Mice were randomized to receive rtPA-tNBs or rtPA-NBs (0.5μg/g body weight) or saline. The animals were dosed via the intraperitoneal route on days 0, 2 and 5 (total of 3 doses) and then culled 24 hours after the last injection. Mouse kidneys were harvested, placed in an embedding cassette, immersed in OCT and frozen on dry ice. The tissue was then stored at -80 until immunofluorescence analysis as detailed above.
Supplementary Results

Figure S1: Fibrinolytic activity of two different types of rt-PA-coated NPs.
Increasing concentrations of either rtPA-NB (A) or rtPA-NB (B) were added to plasma, which was then clotted by thromboplastin and CaCl₂. Soluble rt-PA was tested in parallel for comparison. Clot lysis was monitored by the changes in optical density and expressed as lysis rate (i.e., 1/lysis time in minutes). Optical density was measured at 405 nm. The results are presented as mean of 3 independent experiments.
Figure S2: In vivo biodistribution of tNBs.

Cyanine 5.5-labeled tNBs were injected in LPS-stimulated rats and the near infrared fluorophore cyanine 5.5 was detected by optical imaging. The figure shows localization of the nanostructures in heart, kidneys, lungs, liver, spleen and brain as compared with organs from LPS-stimulated rats receiving either NBs or PBS and documents the typical elimination by macrophages in liver and lungs.
Figure S3: β2-GPI in kidneys from C3 GOF mice.
Cryosection from mouse kidney were incubated with sheep anti-human fibrinogen followed by rabbit Anti-Sheep IgG Alexa 555 to detect fibrin deposits (red) and with rabbit anti human β2-GPI revealed by sheep anti-rabbit IgG CY3 to detect β2-GPI (green). Note localization of fibrin and β2-GPI in the glomeruli and renal vessels.
Figure S4: rtPA-tNBs dissolve clots in the C3 GOF mouse model of aHUS

A) Survival data of C3 GOF mice given saline only (n=6; Black), treated with rtPA-NB (n=7; green) or treated with rtPA-tNB (n=5; Blue). (B) Densitometry analysis using Adobe Photoshop CS3 from digital photograph of Combistix (Siemens) after 1 minute exposure to collected urine. The inverse ratio in ‘eye drop’ red channel (3 spatial reads) was interpolated from the reference scale (digital photograph) using 4PL. Hematuria (left panel) and proteinuria (right) of C3 GOF mice
treated with rtPA-tNB. Orange lines and ! denotes loss of mouse, treatment is indicated by arrows and substance indicated to the left. ^ and purple line denotes intervention by vet (mouse removed from study at this point). (C). hematuria (left panel) and proteinuria (right) of C3 GOF mice treated with rtPA-NB. (D). hematuria (left panel) and proteinuria (right) of C3 GOF mice given saline control. Note: When urine volume was limited, hematuria analysis was favored over proteinuria. No signal on combistix or no urine was entered as zero/blank.
Movie S1. Targeted NB accumulation in thrombi developed in a rat model of APS.

Targeted NBs labeled with coumarin 6 were injected into rat soon after the formation of thrombi induced by the administration of antibodies to β2-GPI and their in vivo distribution was followed by intravital microscopy. The NBs start localizing on thrombi already 30 seconds after injection and accumulate progressively being clearly visible after 15 min.

Movie S2. Untargeted NB accumulation in thrombi developed in a rat model of APS.

The experiment was conducted as reported in the legend to movie S1. Note that the untargeted NBs were barely detectable on intravascular thrombi.

Movie S3. Effect of rtPA-tNB on thrombus dissolution and vascular occlusion in APS model.

Targeted NBs bearing rtPA were injected into rat soon after the formation of thrombi induced by the administration of antibodies to β2-GPI and their ability to dissolve the formed thrombi was monitored with intravital microscopy. Note the rapid dissolution of the thrombi that occurred within 1 min after the injection of rtPA-tNB and persisted up to the 90-min observation.

Movie S4. Effect of rtPA on thrombus dissolution and vascular occlusion in APS model.

The experiment was conducted as reported in the legend to movie S3. Note that soluble rtPA caused a less rapid dissolution of thrombi than rtPA-tNB that was nearly complete 15 min after rtPA injection, but failed to prevent formation of new thrombi clearly seen at 90 min post-injection.

Movie S5. Effect of rtPA-NB on thrombus dissolution and vascular occlusion in APS model.

The experiment was conducted as reported in the legend to movie S3. Untargeted NBs coated with rtPA failed to dissolve the thrombi that persisted throughout the experiment.

Movie S6. Effect of rtPA-tNB on vascular thrombi induced by ferric chloride.

Targeted NBs bearing rtPA were injected into rat soon after the formation of thrombi induced by the local application of ferric chloride and their thrombolytic effect was evaluated using intravital microscopy. Dissolution of thrombi started to be seen 1 min after NB administration and was definitely more marked at 15 min persisting until 90 min.

Movie S7. Effect of rtPA on vascular thrombi induced by ferric chloride.
The experiment was conducted as reported in the legend to movie S6. The thrombolysis induced by soluble rtPA started to be seen 15 min after the injection of the fibrinolytic agent and was no longer observed at 90 min.

**Movie S8. Effect of rtPA-NB on vascular thrombi induced by ferric chloride.**

The experiment was conducted as reported in the legend to movie S6. Note that the markedly reduced blood flow caused by thrombi remained unchanged following the administration of the untargeted NBs coated with rtPA.