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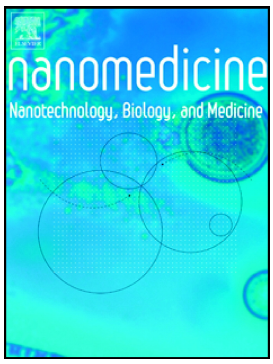

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Sensing adhesion forces between erythrocytes and γ' fibrinogen, modulating fibrin clot architecture and function

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Abbreviations: γ', gamma prime fibrinogen; AFM, atomic force microscopy; γA, the most common fibrinogen variant; RBCs, red blood cells; CVD, cardiovascular diseases; FXIII, factor XIII; SEM, scanning electron microscopy, LSCM, laser scanning confocal microscopy.

Abstract

Plasma fibrinogen includes an alternatively spliced γ-chain variant (γ'), which mainly exists as a heterodimer (γAγ') and has been associated with thrombosis. We tested γAγ' fibrinogen-red blood cells (RBCs) interaction using atomic force microscopy-based force spectroscopy, magnetic tweezers, fibrin clot permeability, scanning electron microscopy and laser scanning confocal microscopy. Data reveal higher work necessary for RBC-RBC detachment in the presence of γAγ' rather than γAγA fibrinogen. γAγ' fibrinogen-RBCs interaction is followed by changes in fibrin network structure, which forms an heterogeneous clot structure with areas of denser and highly branched fibrin fibers. The presence of RBCs also increased the stiffness of γAγ' fibrin clots which are less permeable and more resistant to lysis than γAγA clots.

The modifications on clots promoted by RBCs-γAγ' fibrinogen interaction could alter the risk of thrombotic disorders.

Keywords: cell adhesion, atomic force microscopy, magnetic tweezers, fibrin clot, γ' fibrinogen.

Background

Fibrinogen is a 340 kDa homodimeric plasma glycoprotein, composed of three pairs of polypeptide chains, denoted Aα, Bβ and γ, linked together by 29 disulphide bonds[1]. A common variant of the fibrinogen is produced by alternative splicing of mRNA of the γ-chain, called γ' fibrinogen[2]. The γ' isoform has a substitution of the final 4 carboxyl-terminal amino acid residues (AGDV) of the prevalent γA-chain with a different 20-amino acid residue sequence (VRPEHPAETEYDSLYPEDDL), which includes a high proportion of negatively charged residues and two sulfated tyrosines. This variant accounts for 8-15% of all plasma fibrinogen, largely in the form of the heterodimer γAγ', while the homodimer γ'γ' accounts for less than 1% of total plasma fibrinogen[3]. γ' fibrinogen has distinct biochemical and biophysical properties that distinguish it from the more common γA isoform[4, 5, 6]. Studies have highlighted that abnormal plasma levels of γAγ' fibrinogen are associated with thrombotic diseases, such as coronary artery disease[7], myocardial infarction[8] and stroke[9]. Elevated levels of γ' fibrinogen in patients with cardiovascular disease (CVD) were independent of the total plasma fibrinogen concentration[7]. This could indicate that the proportion of γ' variant in plasma may be a risk factor for cardiovascular pathologies. However, reduced concentrations have been associated with microangiopathy syndrome[10] and deep vein thrombosis[11]. Studies by Appiah *et al*. did not support the hypothesis that γ' fibrinogen influences CVD events through its prothrombotic properties. Rather, γ' fibrinogen concentrations seem to reflect general inflammation that accompanies and may contribute to atherosclerotic CVD, instead of γ' fibrinogen being a causal risk factor[12]. In addition to this, Walton *et al*. found that elevated levels of γAγA fibrinogen promoted arterial thrombosis *in vivo*, whereas γAγ' fibrinogen did not[13]. Nevertheless, as γ' chain modulates clot formation *in vitro*, further studies are required to elucidate its functional properties and its possible relationship with bleeding disorders or thrombosis.

The formation of a thrombus and subsequent impairment of blood flow to vital organs are the immediate cause of CVD events[14]. The architecture of the clot network affects clot stability, particularly its viscoelastic or mechanical properties and fibrinolytic characteristics[15, 16]. These

aspects can determine if the clot is broken down by fibrinolysis or if it will lead to occlusion or embolization[17]. Other studies have indicated the clinical relevance of fibrin clot structure. It has been shown that tightly packed and rigid clots with thinner fibers and low porosity are less susceptible to the action of thrombolytic agents[18, 19], and a prolonged clot lysis time has been associated with CVD events[20]. Several studies have shown that clots produced from γAγ' fibrinogen have a different structure and behave differently from γAγA clots[4, 5, 6]. Fibrin clots formed in the presence of the fibrinogen γ' variant are more resistant to fibrinolysis, due to reduced binding of plasminogen and tissue plasminogen activator, leading to reduced plasmin generation[21]. Fibrinogen γ' variant is associated with a heterogeneous, non-uniform clot structure, with more branch points than clots formed by γA chains[6, 22, 23].In addition, γ' fibrinogen binds to thrombin exosite II[24]. The binding of thrombin to the γ' chain has been reported to modulate thrombin activity, particularly towards substrates that require exosite II interaction such as coagulation factors FV and FVIII (but not those dependent on exosite I interactions, e.g. fibrinogen and factor FXIII), which may influence clot structure[6]. However, some of the effects of γ' fibrinogen on clot structure have been shown to be independent of thrombin, as clots produced with snake venom enzyme ancrod, which cleaves fibrinopeptide A from fibrinogen but not fibrinopeptide B, show similar structural abnormalities as those produced with thrombin[22]. On the other hand, it has been shown that the high affinity binding of thrombin to γAγ'-fibrin protects thrombin from inhibition by antithrombin, raising the possibility that γAγ'-fibrin serves as a scavenger and reservoir for thrombin in the clot[25].

Fibrinogen is thought to contribute to CVD, not only through altering the fibrin network structure, but also through several other mechanisms, such as promoting red blood cell (RBC) aggregation[26]. Although several studies on isolated fibrin clots have been performed, *in vitro* experiments using fibrin blood clots containing RBCs are scarce in the literature. RBCs play a role in blood coagulation by increasing blood viscosity, which is associated with propensity for clot formation[27]. RBCs have been shown to interact specifically with fibrinogen, with *ca.*20,000 binding sites *per* RBC and a dissociation constant, K_d, of approximately 1.3 μM[28]. We have identified a specific receptor for fibrinogen on the surface of RBCs[29, 30, 31].

Despite their significance in thrombosis and hemostasis, relatively few studies have been conducted to describe the effects of RBCs on blood clot structure, particularly with different fibrinogen variants. One complication of studying RBC-rich thrombi is that incorporation of RBCs into a thrombus seems uncontrolled and variable. This scarcity may also be, in part, due to the fact that the presence of RBCs in a clot often interferes with the techniques available to monitor clot structure[32].

The present study aimed to investigate, through atomic force microscopy-based force spectroscopy, the adhesion of RBCs in the presence of γAγA or γAγ' fibrinogen variants. Moreover, we aimed to understand the effect of RBC-γAγ' fibrinogen interactions on fibrin clot structure, properties and function through scanning electron microscopy, laser scanning confocal microscopy, magnetic tweezers and permeability studies.

Methods

Purification of γAγA and γAγ' fibrinogen

The γAγA and γAγ' fibrinogen variants were purified from human plasminogen-depleted fibrinogen using anion-exchange chromatography (Millipore, Billerica, MA) as previously described[22, 23]. Detailed description of the method can be found in Supplementary Material.

Human RBCs isolation

Blood from healthy donors was obtained, with written informed consent, at the Instituto Português do Sangue e da Transplantação (IPST, Lisbon) and at the University of Leeds. This study was approved by the joint Ethics Committee of Faculdade de Medicina da Universidade de Lisboa and Centro Hospitalar Lisboa Norte, and by the University of Leeds Medical School Ethical Committee. RBCs were isolated through standard procedures[29, 30, 31].

Atomic force microscopy (AFM) – Cell-Cell adhesion

AFM studies were performed on a NanoWizard II atomic force microscope with a CellHesion 200 module (JPK Instruments, Berlin, Germany), mounted on an Axiovert200 inverted optical microscope (Zeiss, Jena, Germany). RBCs were attached to tipless cantilevers through the functionalization with concanavalin A (for details see Supplementary Material). RBCs on the substrate were adhered to poly-L-lysine coated glass slides. RBC-RBC adhesion was measured in the presence of different fibrinogen variant concentrations, from 0 to 1.0 mg/ml. Detailed description of the method can be found in Supplementary Material.

Magnetic tweezers

To examine the viscoelastic properties of the fibrin clots, magnetic tweezers were used. The magnetic microrheometer operates by applying a force of 40 pN to a 4.5 µm superparamagnetic bead (Dynabeads M-450 Epoxy, Invitrogen, Paisley, UK) using a magnetic field gradient generated by four electromagnets in 2D directions. The device was used coupled with an

Olympus IX71 inverted microscope (Olympus, UK) incorporating an ultralong working objective (40× magnification) and a CCD camera. Particle tracking, electromagnet control and image analysis were performed in real time using custom written Labview 7.1 software (National Instruments, Newbury, UK). Fibrin clots were formed with and without RBCs. The viscoelastic properties were measured using the procedure of Evans *et al*.[33]. The parameters G', G'' and Tan δ were calculated at a frequency of 0.1 Hz. The displacement of 8 random magnetic beads was measured *per* sample, and each sample was studied at least in triplicate. Please find detailed description in Supplementary Material.

Fibrin clot permeability

To study the clot permeability, samples were prepared in triplicate, according to the standardized protocol published by the Fibrinogen and FXIII Subcommittee of the International Society on Thrombosis and Hemostasis (ISTH)[34]. Purified fibrinogen samples (final concentration of 1 mg/ml) were incubated with 1 U/ml human thrombin (Calbiochem, Nottingham, UK) and 2.5 mM $CaCl_2$. Samples were placed in mechanically scratched open clotting tips for 120 min, at room temperature, in a humidity chamber. A detailed description of the method can be seen in Supplementary Material.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to further investigate the structure of clots formed with γAγA and γAγ′ fibrinogen in the presence or in the absence of RBCs.

Clots were generated by adding 100 µl of γAγA and γAγ' fibrinogen variants (final concentration of 1 mg/ml) to 10 µl of activation mixture (human thrombin 1 U/ml and 10 mM $CaCl_2$, final concentrations, in TBS (50 mM Tris, 100 mM NaCl, pH 7.5)). All details of the procedure are described in Supplementary Material.

Laser scanning confocal microscopy

Fibrin clots were prepared according the procedure described in Supplementary Material. Three random areas of the clot were visualized under higher magnification. 20 μm optical z-stacks were obtained and projected into 2D images. In order to obtain 3D images of the fibrin clots, 40 μm z-stacks were also performed. Confocal microscopy was also used to assess, in real time, the rate of fibrinolysis following the procedure also described in Supplementary Material.

Statistical Analysis

Descriptive statistics are given as mean ± standard error of mean (SEM). Unpaired Student's *t*-test was used for statistical analysis of comparisons between two groups. Non-parametric analysis was also performed in cell-cell adhesion data, applying the Mann Whitney U test. P-values below 0.05 were considered to indicate statistical significance. Statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA, USA).

Results

RBC-RBC adhesion is altered in the presence of fibrinogen variants

Atomic force microscopy experiments were performed to investigate RBC cell-cell adhesion and the influence of γAγ' and γAγA fibrinogen variants in the cells adhesion. We evaluated the interaction between RBCs, in the presence of different concentrations of γAγA and γAγ' fibrinogen variants, up to1.0 mg/ml, by force-distance retract curves (Figure 1).

AFM data showed that, in the presence of low concentrations of γAγ' fibrinogen, the work (thermodynamic parameter corresponding to the area under the curve on a force × distance plot, Figure 1B) required to detach one RBC from another was higher than in the presence of the same concentration of γAγA fibrinogen (0.87 ± 0.07 fJ *vs.* 0.55 ± 0.04 fJ, p=0.0275, for 0.1 mg/ml fibrinogen, and 1.03 ± 0.06 fJ *vs.* 0.65 ± 0.07 fJ, p<0.0001, for 0.4 mg/ml fibrinogen) (Figure 1C). At higher concentrations of fibrinogen variants, statistically significant differences between both variants were not observed (for 0.4 mg/ml fibrinogen, 0.71 ± 0.05 fJ for γAγ' *vs.* 1.10 ± 0.13 fJ for γAγA, p=0.144; for 0.7 mg/ml fibrinogen, 0.57 ± 0.07 fJ for γAγ' *vs.* 0.86 ± 0.04 fJ for γAγA, p=0.248). γAγA fibrinogen concentrations showed a clear dose-response for interactions with RBCs, whereas γAγ' had a dual effect at low and high concentrations (Figure 1C). Values of the maximum detachment force (schematically explained in Figure 1B) were also calculated. As shown on Figure 1D, statistically significant differences between the values of maximum RBC-RBC detachment force in the presence of γAγ' or γAγA fibrinogen were found at all fibrinogen concentrations: for 0.1 mg/ml fibrinogen, 141.8 ± 17.2 pN for γAγA *vs.* 173.6 ± 0.85 pN for γAγ', p = 0.0279; for 0.4 mg/ml fibrinogen, 246.3 ± 5.64 pN for γAγA *vs.* 203.7 ± 0.70 pN for γAγ', p < 0.0001; for 0.7 mg/ml fibrinogen, 141.2 ± 5.35 pN for γAγA *vs.* 170.3 ± 0.95 pN for γAγ', p < 0.0001; and, for 1 mg/ml fibrinogen, 182.9 ± 9.80 pN for γAγA *vs.* 205.5 ± 1.90 pN for γAγ', p = 0.0054). With the exception of the data obtained for 0.4 mg/ml fibrinogen, the values of maximum detachment force obtained for γAγA fibrinogen were always lower than those of γAγ' fibrinogen.

After the initial maximum detachment force, two types of jumps could be observed on the force curves (Figure 1B): jumps preceded by a ramp of force or jumps preceded by a force plateau. We considered a membrane tether as a step in force detected after a force plateau of 0.25 µm in distance. Jump events were assigned to the unbinding of membrane ligand-receptor interactions without a preceding membrane deformation, while membrane tether events corresponded to situations where a membrane tether was extruded before the unbinding of the ligand-receptor complex (Figure 1B)[35]. A combination of each of the parameters extracted from the force-distance curves (maximum detachment force, number of jumps events/curve × mean force of jumps and number of membrane tethers events × mean force of tethers) could be considered as the value of the work needed to detach one cell from another. Similarly to the values of work (Figure 1C), the same profile is observed for the values of force of jumps and membrane tethers events with increasing fibrinogen concentrations when comparing both fibrinogen variants As depicted in Figure 1E,F, at lower fibrinogen concentrations, jumps and membrane tethers displayed higher forces for γAγ' fibrinogen, when compared to γAγA variant, both for 0.1 mg/ml fibrinogen (jumps, 68.52 ± 2.42 pN *vs.* 40.40 ± 0.80 pN, $p < 0.0001$; membrane tethers, 79.80 ± 7.06 pN *vs.* 47.56 ± 3.37 pN, $p < 0.0001$) and 0.4 mg/ml fibrinogen (jumps, 80.82 ± 3.45 pN *vs.* 74.94 ± 3.31 pN, $p = 0.0061$; membrane tethers, 91.91 ± 6.32 pN *vs.* 68.08 ± 9.69 pN, $p = 0.0069$). Figure 1E,F also shows that, at higher fibrinogen concentrations, similarly to the values of work, higher forces of jumps and membrane tethers were measured for γAγA fibrinogen, when compared to the γAγ' variant. This becomes evident at 0.7 mg/ml of fibrinogen (jumps, 70.94 ± 4.36 pN *vs.* 24.01 ± 0.77 pN, $p < 0.0001$; membrane tethers, 117.75 ± 14.64 pN *vs.* 36.47 ± 5.13 pN, $p < 0.0001$) and at 1.0 mg/ml of fibrinogen (jumps, 201.51 ± 8.54 pN *vs.* 93.41 ± 2.85 pN, $p < 0.0001$; membrane tethers, 313.95 ± 58.05 pN *vs.* 135.51 ± 7.98 pN, $p > 0.05$).

RBCs influence γAγ' fibrin clot mechanical properties

The viscoelastic properties of γAγA and γAγ' fibrin clots were examined using a magnetic microrheometer and the influence of RBCs was studied. In agreement with previous findings[22],

we observed that γAγA clots were stiffer than γAγ' clots (storage modulus, G', 1.076 ± 0.065 Pa *vs.* 0.512 ± 0.051 Pa, respectively; $p < 0.0001$; Figure 2). The presence of RBCs in the γAγA fibrin clots did not statistically change their stiffness. On the other hand, a significant increase in the stiffness of γAγ' clots was observed in clots with RBCs, when compared to those without RBCs (1.220 ± 0.104 Pa *vs.* 0.512 ± 0.051 Pa, respectively; $p < 0.0001$). There were no significant differences on storage modulus G' between γAγA and γAγ' clots in the presence of RBCs; however, there was a trend for the γAγ' clots to be stiffer than γAγA clots (Figure 2).

In addition, we observed a statistically significant change on loss tangent (Tan δ) comparing γAγA clots with RBCs and without RBCs (0.460 ± 0.066 *vs.* 1.013 ± 0.126, respectively; $p = 0.0008$), but only a trend for higher Tan δ in γAγ' with RBCs than for this variant in the absence of RBCs (data not shown).

RBCs reduce γAγ' fibrin clot permeability

To characterize the fibrin network of γAγA and γAγ' fibrin with and without RBCs, permeability experiments were performed. The results for the Darcy constant (K_s), which represent the pore size of the fibrin clot, are shown in Figure 3.

Importantly, in the presence of RBCs, normalized K_s was higher for γAγA fibrin clots with RBCs ($(2.44 \pm 0.3.1) \times 10^{-9}$ cm^2) than for γAγ' fibrin clot with RBCs ($(1.40 \pm 0.10) \times 10^{-9}$ cm^2) ($p = 0.0001$). Thus, when comparing clots of both fibrinogen variants with RBCs, the γAγ' clots were less porous.

RBCs influence γAγA and γAγ' fibrin clot structure

Scanning electron microscopy data showed differences between the structures of the fibers formed by γAγA or γAγ' fibrinogen (different magnification micrographs from each sample are shown in Figure S1 in the supplementary information).

We next investigated the structure of plasma clots formed *in vitro* in the presence of RBCs to determine the effect that these cells might have on clot structure *in vivo*. RBCs tended to be heterogeneously spread throughout the sample, particularly in clots produced with γAγ' fibrin

(Figure 4). Upon the addition of 20% RBCs, the structure of the γAγ' clot became more heterogeneous, with densely packed fiber regions as well as large holes where more RBCs were located (Figure 4 A, C, E). γAγ' fibrin fibers were thinner and the network of the clot showed more interconnections with RBCs compared to γAγA fibers. On the micrographs of Figure 4 (panels B, D and F), it can be noticed that γAγ' fibrin clots contained more free fiber ends and there were more fiber-to-RBC points of connections, when compared to γAγA fibrin clots, resulting in "clustered" cell arrangements.

In order to measure the diameter of the fibers, twenty fibers from 25000× magnification images were assessed on 2 different clots from each condition and in 3 different zones of the clot. Average fiber diameters were smaller in γAγ' fibrin compared to the γAγA variant (18.29 ± 1.32 nm *vs.* 41.10 ± 1.58 nm, $p < 0.0001$; Figure 5). The presence of RBCs in the fibrin clot caused a decrease of the γAγA fibers diameters compared to the clot without RBCs (41.10 ± 1.58 nm in the absence of RBCs *vs.* 35.39 ± 2.46 nm in their presence, $p = 0.044$) (Figure 5). The presence of RBCs on clots with γAγ' fibers did not statistically change the diameter of the fibers compared to the clots without cells. However, clots with RBCs and prepared with γAγ' fibrinogen led to thinner fibers than those produced with γAγA fibrinogen and RBCs (35.39 ± 2.46 nm for γAγA *vs.* 15.23 ± 0.98 nm for γAγ', $p < 0.0001$; Figure 5).

Increased fibrinolytic resistance of γAγ' fibrin clots in the presence of RBCs

We used laser scanning confocal microscopy to study clot structure and resistance to fibrinolysis in the presence and absence of RBCs for each fibrin variant.

Figure 6 shows representative examples of the confocal micrographs obtained for fiber clots made with γAγA and γAγ' purified fibrinogen variants. In clots formed with γAγA fibrinogen, the area occupied by the fibers seems to be larger (Figure 6A) than for the clots with γAγ' fibrinogen (Figure 6B). However, these differences were not statistically significant. In agreement with SEM imaging and with previous studies[22], there was a clear difference between the structures of γAγA and γAγ' fibrin. The γAγA fibrin clots were composed of longer and straighter fibers than γAγ' fibrin clots. The later showed fibers that were shorter and clustered,

producing larger pores. However, we did not find differences regarding the fiber density comparing γAγA with γAγ' fibrin clots (Figure S2).

Relevant was to study the micrographs of fibrin clots from γAγA and γAγ' fibrinogen variants in the presence of 2% of RBCs, as shown on Figure 6 (C,D). In the presence of γAγA fibrinogen, the network of the fibers seemed straighter and denser. In clots with γAγ' fibers, we observed that the fibers were more clustered and the RBCs were more visible. We also studied fibrin clots in the presence of 5% and 10% of RBCs by LSCM; however, the high number of RBCs did not allow clearly differentiating fibrin fibers from RBCs. Thus, technical limitations restricted us to increase the amount of RBCs closer to the physiological range.

Finally, we studied fibrinolysis of clots made with both fibrinogen variants (Figure 7). In line with previous studies[21], a significant increase in lysis time was observed for γAγ' compared with γAγA fibrin clots (15.82 ± 1.48 min *vs.* 10.54 ± 0.44 min, respectively; $p = 0.0267$). During the experiment, we observed a non-uniform lysis of the γAγ' fibrin clot, taking more time to achieve complete lysis than the γAγA clot (videos in Supplementary information).

We measured the lysis time for γAγA and γAγ' clots in the presence of 5% (data not shown) and 10% of RBCs. Under these conditions, there were no significant differences in fibrinolysis times in the presence of RBCs, when compared to the clots without RBCs. Statistical significance was observed in fibrinolysis times for γAγA *vs.* γAγ' fibrin clots in the presence of RBCs (12.03 ± 0.49 min *vs.* 18.30 ± 1.47 min; $p = 0.0476$).

Discussion

In this study, we evaluated the interaction of purified γAγ' and γAγA fibrinogen with RBCs, as well as their influence on the structure and function of clots formed with both variants. They show a discernible effect of the presence of RBCs on fibrin clot structure and function. To pursue this study further, we purified the main component of plasma fibrinogen, γAγA, and its splice-variant, γAγ'. Cell-cell adhesion studies were performed to evaluate the relative roles of both fibrinogen variants on the interaction between RBCs. We also evaluated the changes of biophysical properties of the clots, by studying their permeability and viscoelastic properties. By laser confocal microscopy and scanning electron microscopy, we directly observed the effects of RBCs on the architecture of clots formed from γAγ' and γAγA fibrinogens.

AFM data showed that in the presence of low concentrations of γAγ' fibrinogen (≤ 0.4 mg/ml), the force of the binding between two RBCs was higher than with γAγA fibrinogen. Statistically significant differences between both fibrinogen variants at all fibrinogen concentrations were obtained for the values of force for the maximum cell-cell detachment, jumps and membrane tether events. Although these three parameters contribute to the values of the work necessary to completely detach two RBCs, no statistically significant variations were obtained for this parameter at fibrinogen concentrations ≥ 0.7 mg/mL.

Thus, at lower fibrinogen concentrations, γAγ' fibrinogen binds more promptly to RBCs, inducing higher RBC-RBC adhesion than γAγA fibrinogen. RBCs adhesion has a clear γAγA fibrinogen dose response curve, whereas γAγ' did not. This could be due to differences in fibrinogen variant molecular conformations and net protein electrostatic charge, which could lead to different binding affinities at different fibrinogen concentrations. It is known that RBCs present sialic acid residues on their surface, contributing to their negative surface charge, which induce repulsive electrostatic forces that minimize RBC aggregation under blood flow conditions[36]. The binding of the more negatively charged γ' fibrinogen to RBCs may increase the overall negative cell surface charge. This may overcome the strong RBC-RBC interactions observed at lower γAγ' fibrinogen concentration, by lowering cell-cell adhesion forces, due to

increased electrostatic repulsion. On the other hand, γAγA fibrinogen lacks the negatively charged sequence of γ' chain, leading to an increase of RBC-RBC adhesion upon increasing its concentration.

Previous studies have shown that RBCs influence the viscoelastic properties of the clot[32, 37]. Our evaluation of the viscoelastic properties of the clots revealed that γAγA fibrin clots are stiffer than γAγ' fibrin clots in the absence of RBCs, in agreement with previous studies on the effect of γAγ' on clot stiffness[22]. Our data shows that γAγ' clots are inherently weaker. This is likely due to differences in fibrin fiber structure. We recently demonstrated that fibrinogen γ' influences intrafibrillar structure of fibrin and significantly reduces the average number of protofibrils packed within the fibrin fiber, thereby influencing clot stiffness[38]. In γAγA fibrin clots, more protofibrils are packed within fibrin fibers, which increases the rigidity and the viscosity of the clot, when compared to γAγ' fibrin clots. In this study, the presence of RBCs did not influence the stiffness of the γAγA fibrin clots. In γAγ' fibrin clots, the presence of RBCs resulted in a significant increase in stiffness. This indicates that the combination of γAγ' fibrinogen variant and RBCs may affect clot structure and mechanical properties. The stiffening of the γAγ' fibrin clots in the presence of RBCs may be related to rearrangements of protofibril packing or significant modifications in clot structure. The imaging by SEM of the clot structure in the presence of RBCs, compared to its absence, showed a looser structure in clots produced with γAγ' fibrinogen, which may be more prone to creep, as well as more fiber interconnections, which turn the clot more rigid. Our data indicates that RBCs are not a mere bystander in blood clot formation. Indeed, other studies have shown that RBCs influence fibrin network structure and pore size[39, 40]. A decrease in the permeability of fibrin clots after the addition of RBCs was shown to be proportional to the total RBC surface area and their total concentration[40]. Here, we show that the addition of RBCs promotes the decrease of permeability. Importantly, we show that the γAγ' clots with RBCs were less porous than γAγA clots with RBCs. This could potentially lead to pro-thrombotic clots, due to a higher resistance to fibrinolysis[41]. In agreement with this, we also found an increase in the lysis time of γAγ' clots with RBCs, when compared to γAγA clots with RBCs.

According with previous studies[22, 23], SEM and confocal microcopy showed that the clots formed by γAγA and γAγ' fibers have a different structure. The incorporation of RBCs has an important impact on fibrin fiber size and in its network structure. Regions of higher and lower RBC density were clearly visible in the clots made with γ' fibrinogen. In the presence of RBCs, the γ' fibrin clots were more heterogeneous, seeming to form a looser structure when compared with the clots of γA fibrinogen. These findings are supported by our AFM data. At high γAγ' fibrinogen concentrations, the work necessary to detach two RBCs was the same that the one achieved in the presence of γAγA fibrinogen. In agreement with this, the clot might become looser, with a higher ability to deform and embolise, eventually blocking a downstream blood capillary.

The γAγ' fibrin clots contain a large number of free fiber ends. Fiber ends are normally found at low levels in scanning electron micrographs of fibrin clots, and have been suggested to arise from early truncation of protofibril formation or lateral aggregation[42, 43]. It was previously proposed that the negative charge of γAγ' fibrinogen on the D-D interface may disrupt oligomerization and, therefore, interrupt the continuous growth of the protofibril, leading to highly branched fibers[44, 45].

This study has shown that the incorporation of RBCs into the clot affects fibrin fiber formation. RBCs induced thinning of fibers in fibrin clots formed with γAγA, but not with γAγ' fibrinogen. However, in the presence of RBCs, γAγA fibers remained significantly thicker than γAγ' fibers. Here, we could conclude that thinner fibrin fibers (such as γAγ' fibrin fibers) form highly branched networks. We propose that growing fibers surround RBCs, interacting with them through an RBC surface receptor. We have already reported the existence of a receptor for fibrinogen on RBCs[29, 30, 31]:RBCs specifically bind fibrin(ogen) via an integrin receptor with a β_3 subunit[29], possibly$\alpha_v\beta_3$[31].

Previous studies indicated that fibrin clots with increased fiber branching and resistance to fibrinolysis are a risk factor for thrombosis[15]. Fibrinolysis data showed that the lysis time of a γAγ' fibrin clot is longer than for a γAγA clot, as already reported[4, 5]. The presence of RBCs seems to increase the lysis time. However these studies were conducted with a 10% hematocrit,

due to technical limitations, considerably below the normal hematocrit of ~40% RBCs. Aleman *et al*. and Tutwiler *et al*. suggested that RBCs cause direct and complex effects on clot structure and stability[46, 47]. Furthermore, RBCs can support thrombin generation and decrease fibrinolysis, suggesting that RBCs may promote fibrin deposition during venous thrombosis[46]. Other authors report the role of RBCs on stabilizing clots based on suppressed tissue plasminogen activator(tPA)-induced fibrinolysis in RBC-modified fibrin structures. They also observed, using eptifibatide inhibition, that RBCs modulate fibrinolysis through a specific fibrinogen receptor[41]. Collet *et al.* demonstrated that fibrin clots with a tight fibrin conformation made of thinner fibers were lysed at a slower rate than those with a loose fibrin conformation made of thicker fibers, although the overall fibrin content remained constant[48]. The γAγ' clot is more heterogeneous than the γAγA clot. The densely packed areas with fibers and RBCs may impair the diffusion of fibrinolytic agents. However, at the same time, there are areas with large holes, where their diffusion may be increased. It was suggested that the slower lysis of γ' clots is due to delayed fibrinopeptide B release, leading to a delay of plasminogen binding and activation[21].

Some of these findings may provide a mechanism underpinning the epidemiological data suggesting that a high ratio between γAγ' and γAγA fibrinogen variants may be a risk factor for thrombotic disease[7, 8, 9]. Further studies on the prothrombotic nature of RBCs and on fibrinogen variants are necessary to fully understand the contribution of the γ' chain and RBCs to different etiologies of thrombotic diseases.

In conclusion, the incorporation of RBCs into a fibrin clot significantly affects the clot structure and its mechanical properties. RBCs interact differently with clots made with γ-chain fibrinogen splice variants, which may explain some of the cardiovascular clinical associations that have been reported with these fibrinogen variants.

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Figure legends

Figure 1 | AFM cell-cell adhesion data. A) Schematic representation of the adhesion between two RBCs (one chemically attached to the cantilever and the other on the poly-L-lysine-treated substrate) in the presence of fibrinogen variants. B) Representative force-distance curve with the relevant analyzed parameters, namely, work of cell-cell detachment (grey area), maximum detachment force, jumps and membrane tethers (insets magnify examples of a jump and a membrane tether event on the force-distance curve). C) Work necessary to overcome RBC-RBC adhesion in the presence of different concentrations of γAγA or γAγ' fibrinogen (*, p = 0.0275; ***, p < 0.0001). D) Maximum detachment force values to detach two RBCs (*, p = 0.0279; **, p= 0.0054; ***, p < 0.0001. E) Jumps and F) membrane tethers force data (**, $p \leq 0.0069$; ***, p < 0.0001) in the presence of each fibrinogen variant or in their absence. Values are presented as mean ± standard error of mean (SEM).

Figure 2 | Viscoelastic properties of the purified γAγA and γAγ' fibrinogen clots. Storage modulus G' (elastic modulus) data are presented in the presence of 0.5 % RBCs and in the absence of RBCs (***, p < 0.0001 for γAγA *vs.* γAγ' and for γAγ' *vs.* γAγ' + RBC). Fibrinogen concentration was 0.5 mg/ml. Values are presented as mean ± SEM.

Figure 3 | Normalized permeability data (normalized in percentage against Ks of each variant without RBCs) in the presence of RBCs for fibrin clots formed with purified γAγA or γAγ' fibrinogen. Darcy constant, K_s, represents the average size of the pores within the clot (*, p= 0.0319). Values are presented as mean ± SEM.

Figure 4 | Scanning electron micrographs of fibrin fiber clots with 20% of RBCs. Fibers were formed from 1.0 mg/ml fibrinogen variants γAγA (A, C and E) and γAγ' (B, D and F). White arrows in A indicate the more homogeneous structure in the γAγA clot network, when compared with the areas of small and large holes, pinpointed by red arrows in B,, associating a more heterogeneous structure to the γAγ' clots. The free fiber ends and the fiber-to-RBC points of connections are represented as small circles, being more frequent for γAγ' (red circles in F) than for γAγA (white circles in E). Magnifications: A and B, 2500×; C and D, 5000×; E and F, 25000×.

Figure 5 | Diameters of purified γAγA and γAγ' fibrin fibers in the absence or in the presence of 20% of RBCs. Fiber diameters were measured from 20 random fibers of each image using a 25000× magnification (*, $p = 0.0439$; ***, $p < 0.0001$).

Figure 6 | Laser scanning confocal microscopy images of fiber clots formed with purified γAγA (A and C) and γAγ' (B and D) fibrinogen, at 1.0 mg/ml fibrinogen concentration. Alexa 488-labelled fibrin fibers are shown in green. Confocal micrographs of fiber clots with 2% of RBCs formed with 1.0 mg/ml of purified γAγA (C) and γAγ' fibrinogen (D). Alexa 488-labelled fibrin fibers are shown in green. RBCs were labeled with Vibrant DiD cell-labeling solution (red). Z-stack images at 40 µm and 63× magnification are shown in panel A and B and Z-stacks images at 20 µm, also with 63× magnification are shown in panels C and D.

Figure 7 | Fibrinolysis time rates were assessed by laser scanning confocal microscopy from clots formed with purified γAγA and γAγ' fibrinogen, in the absence and presence of RBCs (10%). The serial time scans were taken with 40× magnification. Values are shown as mean ± SEM (*, $p \leq 0.0476$).

Figures

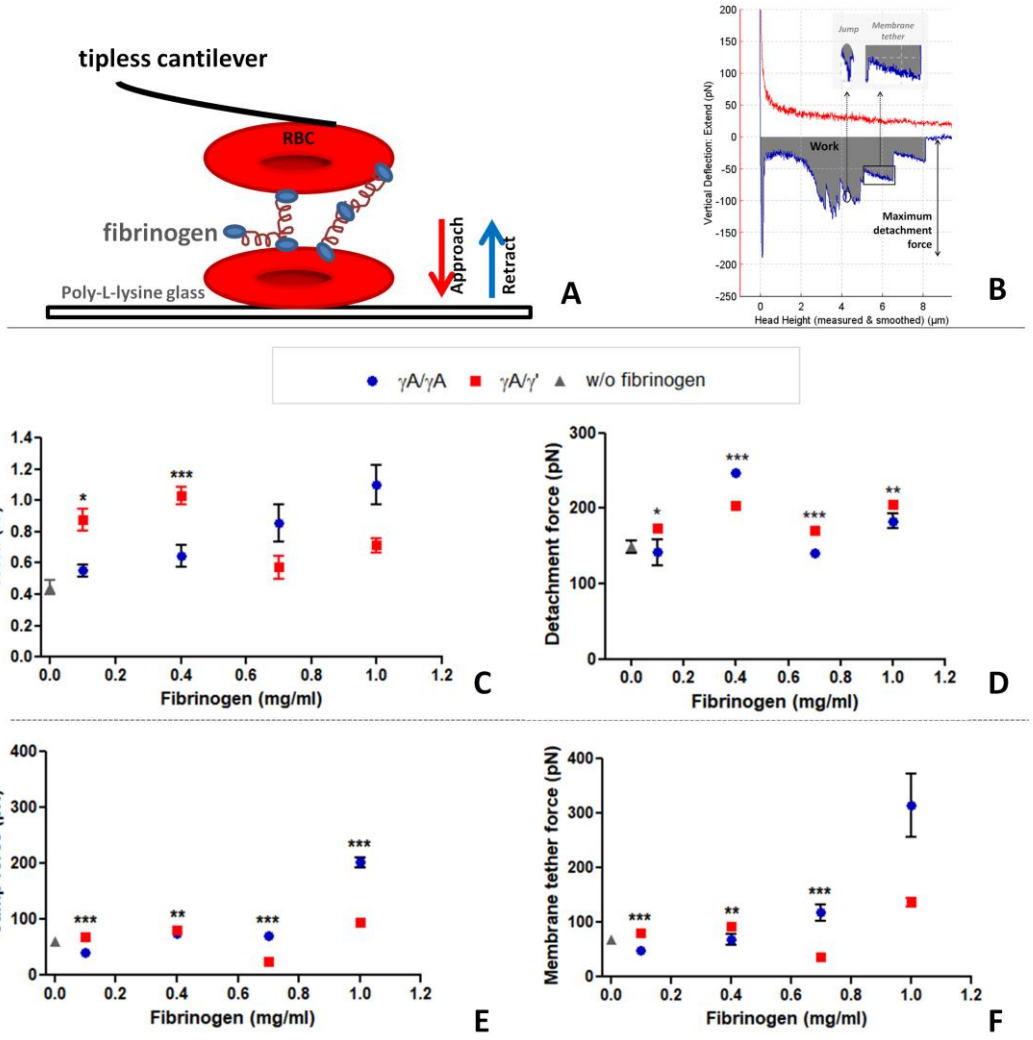


Figure 1

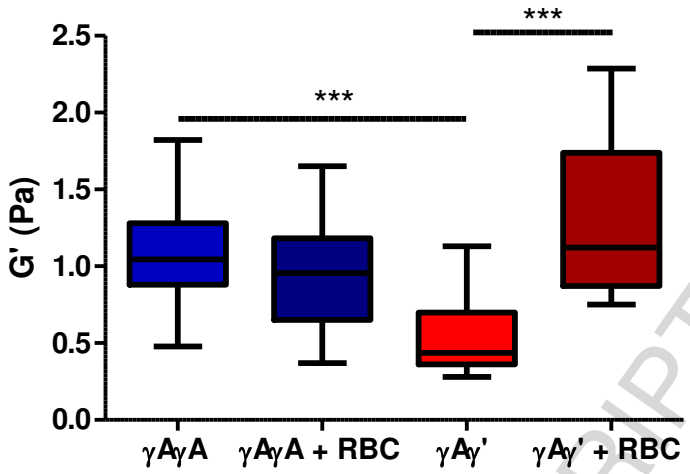


Figure 2

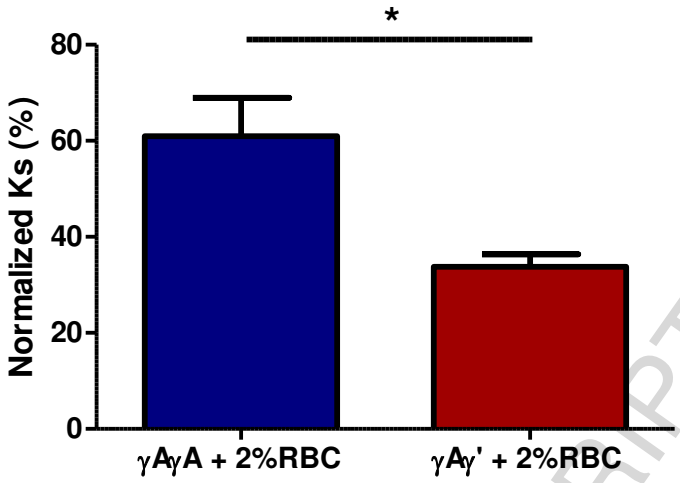
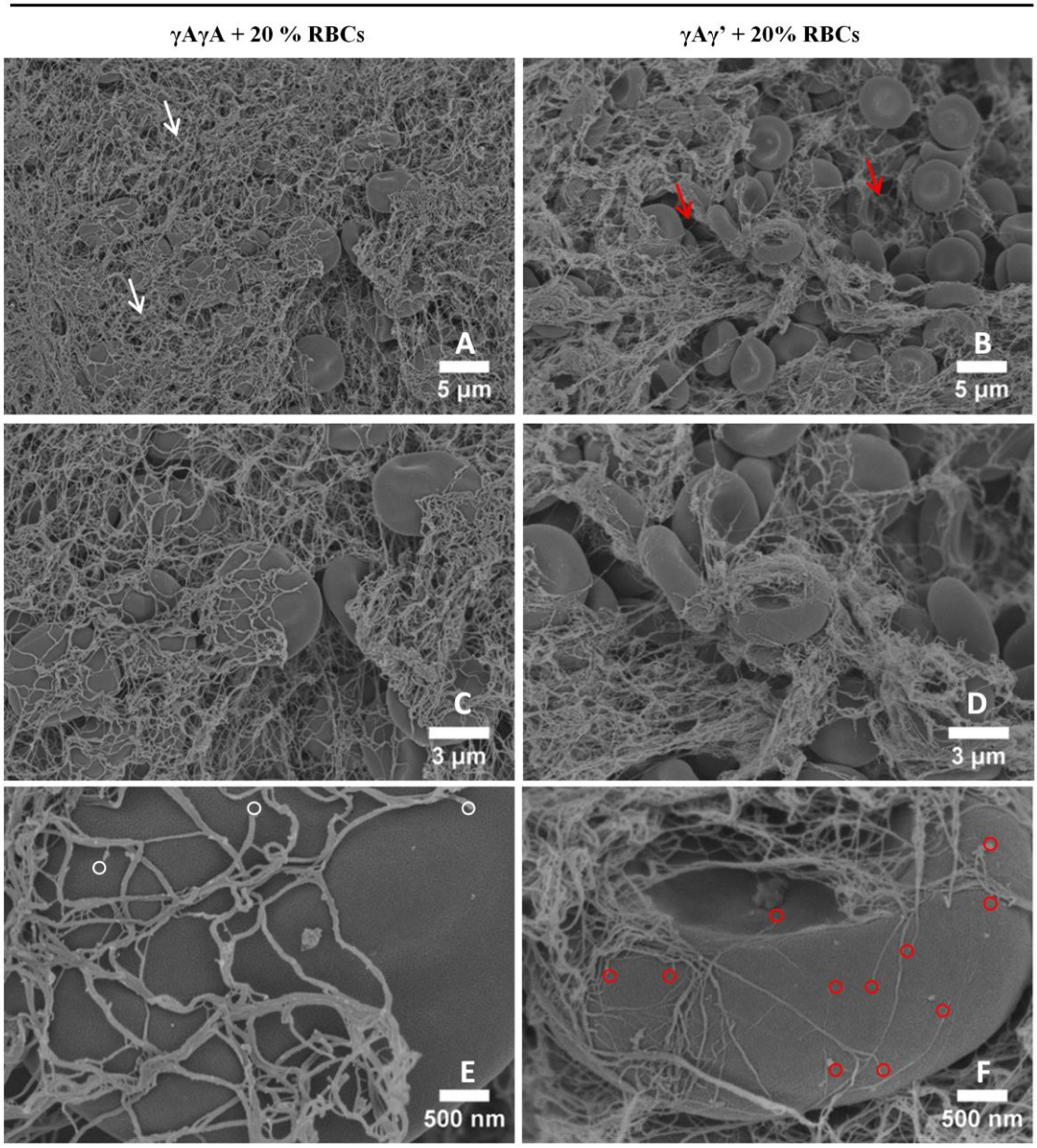


Figure 3



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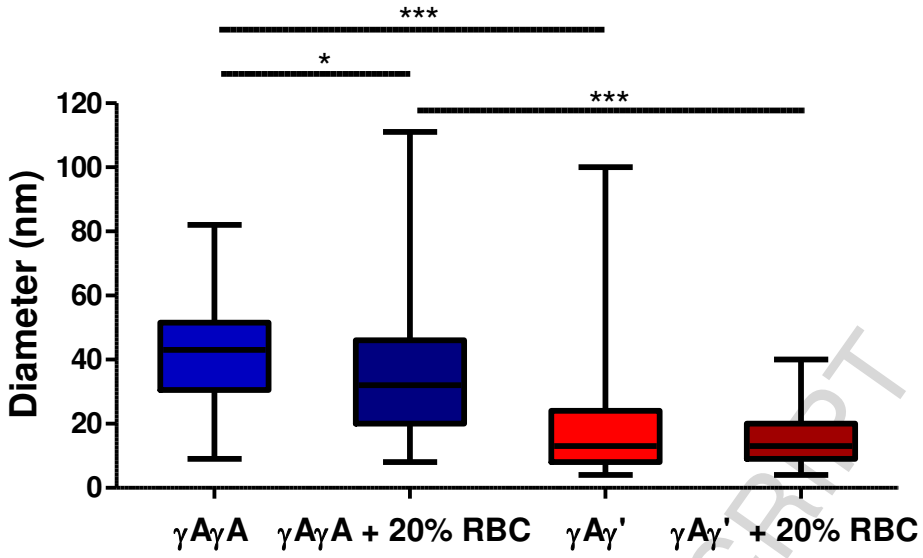

Figure 5

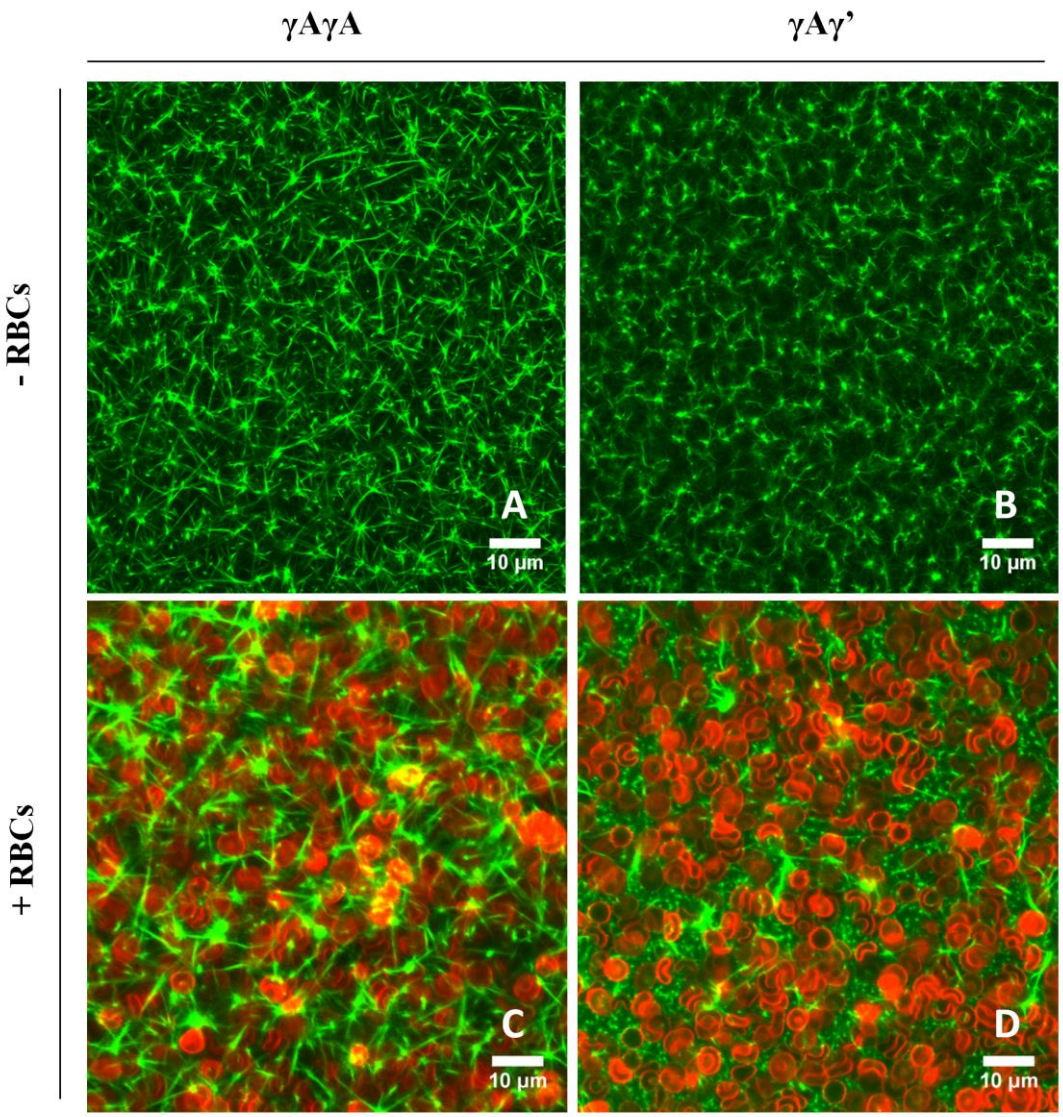

Figure 6

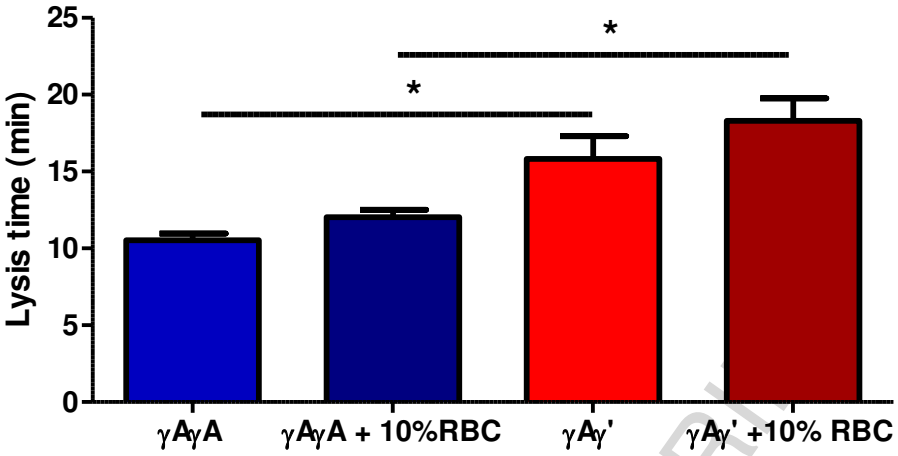

Figure 7

Graphical abstract description

Comparison of the interaction between red blood cells (RBCs) and γAγA or γAγ' fibrinogen. Schematic representation of the adhesion forces between two RBCs in the presence of γAγ' fibrinogen, which affects clot properties and function. Changes on fibrin network structure, decreased fiber diameter, increased fibrinolysis and decreased permeability by γAγ' fibrinogen-RBC interactions were encountered. The nanotechnology-based approach was essential to understand that the RBCs interact differently with clots made with γ' chain fibrinogen splice variant instead of the common variant, γA. This may explain some of the cardiovascular clinical associations that have been reported for γ' fibrinogen.