Platelet activation by collagen is increased in retinal vein occlusion

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Summary

Retinal vein occlusion (RVO) is the most common retinal vascular disorder second to diabetic retinopathy. The main risk factors in patients with RVO are hypertension, diabetes, hyperlipidemia, increased blood viscosity and glaucoma. The pathogenesis of RVO has not yet been clarified. In these events platelets could play a very important role. In the present study the platelet response to collagen was deeply investigated. Experiments were carried out on a selected group of RVO patients, which were compared to a group of healthy subjects matched for age, sex, clinical and metabolic characteristics. In resting and activated platelets of both groups of subjects p72syk phosphorylation, phospholipase Ca2+ phosphorylation, protein kinase Ca activation, intracellular calcium levels and nitric oxide formation were measured. Results show that platelets of patients were more responsive to collagen or ADP than healthy subjects and that the response was significantly different (p<0.0005) at low concentrations of these agonists. In platelets of patients stimulated with collagen increased phosphorylation of p72syk and phospholipase Ca2 was found. Also protein kinase Ca was more activated in patients. In addition intracellular calcium rise induced by collagen was significantly higher in patients than in healthy subjects. RVO patients showed a lower basal level of nitric oxide both in resting and stimulated platelets compared to healthy subjects. Altogether these results suggest that the platelet hyperaggregability described in patients might be an important factor in the development of RVO contributing to the thrombogenic effects.

Keywords

Collagen, human platelets, retinal vein occlusion

Introduction

Retinal vein occlusion (RVO) is the second most common retinal vascular occlusive disease after diabetic retinopathy affecting not only elderly, but also young patients (1). The exact pathogenesis of RVO is unclear. However, the three classical factors involved in thrombogenesis, stasis, vessel damage and hypercoagulability have been described in RVO patients. Increased risk of RVO in patients with hypertension, diabetes, hypercholesterolemia, hyperhomocysteinemia, history of cardiovascular disease and antiphospholipid syndrome disorders has been shown (2–9). In all these conditions platelet activation occurs and plays an important role. Following injury to blood vessels and in some pathological conditions, platelets adhere to the exposed subendothelial connective tissue, collagen in particular, undergo activation, through a tyrosine kinase-dependent mechanism leading to the release of several biologically active substances, and aggregate. Collagen is a vessel wall protein that directly activates platelets. Platelet activation by exposed collagen after vessel injury is believed to be an early step in cardiopathogenic disease (10). Collagen-platelet interaction involves several platelet-surface glycoproteins including the integrin α,β3, and the non-integrin receptor glycoprotein VI. Gibbins et al. (11) proposed a model for collagen-induced signalling in human platelets, in which receptor clustering induced by collagen leads to tyrosine phosphorylation of the Fc receptor γ-chain, possible by as src family kinase, allowing binding of p72syk, which becomes tyrosine phosphorylated and activated. The signal is transduced to other tyrosine kinases and adaptor proteins leading to tyrosine phosphorylation and activation of phospholipase C (PLC) γ2, as shown by experiments carried out in mouse platelets deficient in Fc receptor γ-chain or p72syk (12). Likely the tyrosine protein kinase p72syk and PLCγ2 can be considered two important enzymes of the signalling pathway primed by collagen. The aim of the present study was to evaluate the platelet response to collagen and the steps involved in the signalling transduction. The p72syk and PLCγ2 phosphorylation and activation, the calcium rise and nitric oxide formation in collagen-challenged platelets of a selected group of patients affected with RVO were measured.
Patients and methods

Patients

The study population consisted of 38 patients with a first RVO, including central retinal vein occlusion (n=18) and branch retinal vein occlusion (n=20), recruited from the Thrombosis Center of the Department of Internal Medicine of the University of Genoa. The diagnosis of RVO was made within three days of the onset of symptoms in the Department of Neurosciences, Ophthalmology and Genetics of the University of Genoa. The patients underwent a complete ophthalmological evaluation. This included best-corrected visual acuity, an anterior segment examination, intraocular pressure recorded with a Goldmann applanation tonometer, relative afferent papillary defect, detailed fundus evaluation by direct and indirect ophthalmoscopy and fluorescein angiography. Each patient received a detailed systemic examination with particular attention to cardiovascular and neurologic abnormalities and underwent laboratory evaluation (clinical and metabolic parameters, coagulation profile and homocysteine levels) at one month to three months after a diagnosis of RVO. The control population comprised 40 subjects age-matched without history of thromboembolic diseases, glaucoma and malignancy. Information on history of hypertension, diabetes, peripheral or coronary artery disease, history of other vascular occlusive events, such as cerebrovascular disease and history of high levels of cholesterol and triglycerides were obtained for patients and healthy subjects. Body mass index was measured for all study participants. Subjects were classified as non-smokers if they had stopped smoking at least one year before enrolment. Hypertension was defined as a blood pressure >140/90 mmHg or if the patient was on antihypertensive therapy. Hypercholesterolemia was defined by a fasting plasma cholesterol level greater than 200 mg/dl or the intake of lipid-lowering drugs. Subjects were classified as diabetics when being treated for insulin- or non insulin-dependent diabetes mellitus. Exclusion criteria, applied equally to patients and healthy subjects included primarily the presence of antiplatelet therapy, glaucoma or other local factors predisposing to thrombosis, renal dysfunction (serum creatinine >2 mg/dl), malignancy, intake of vitamin B12/B6, folate and drugs known to influence plasma homocysteine concentrations, such as fribates, methotrexate, trimetoprin, estrogen, carbamazepine, phenytoin, and tricyclic antidepressants. Patients and healthy subjects gave their informed consent for this study. All subjects lived in Liguria and Southern Piemonte (North West Italy) and none of them were on antithrombotic therapy at the time of the study.

Blood collection and preparative procedure

Washed platelets were prepared from platelet-rich plasma (PRP) as detailed elsewhere (13). PRP, obtained by centrifugation of the whole blood at 100 g for 25 minutes (min) and added to 1 µl/ml apyrase and 1 µM prostaglandin E2, was centrifuged at 1,000 g for 15 min. Pellet was washed once with pH 4.8 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose) and centrifuged at 1,000 g for 15 min, then resuspended in pH 7.4 Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM glucose, 10 mM HEPES). The final pH of the washed platelet suspension was 7.4. The platelet count number ranged between 290–310/ml either in patients and healthy subjects. All chemicals, if not otherwise indicated, were from Sigma-Aldrich Co. (St. Louis, MO, USA).

Measurement of platelet aggregation

Platelet aggregation, performed in a Menarini Aggreccoder PA-3210 aggregometer, was monitored according to Born's method (14) and quantified by the light transmission reached within 3 min. PRP was preincubated with saline for 2 min at 37°C before collagen (from equine tendon, Mascia Brunelli S.p.A., Milan, Italy) or ADP addition.

Total protein-tyrosine phosphorylation measurement

Washed platelets (1.0 x 10^9 platelets/ml), prewarmed with saline or 100 µM genistein at 37°C for 15 min, were stimulated for 15 min at 37°C with varying collagen concentrations under unstirring conditions. Incubation was stopped by addition of 2×Laemmli-SDS reducing sample buffer. Samples heated for 5 min at 100°C, were separated by 5–10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Running was performed in the presence of phosphotyrosine molecular weight standard markers. Blots were blocked for 30 min at 37°C in 5% fat-free dry milk dissolved in TBST (Tris buffer saline, 10 mM Tris, 150 mM NaCl pH 7.6, containing 0.1% Tween 20) and then incubated overnight at 4°C with antiphosphotyrosine 4G10 clone at 1/1,000 dilution (Upstate, Charlottesville, VA, USA). Membranes were extensively washed and incubated for 1 hour (h) at room temperature with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA, USA). After further washings, blots were developed using the Amersham ECL system (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK) and revealed by the Bio-Rad Chemi-Doc. The optical density of bands was quantified with the related software package.

Immunoprecipitation procedures

Washed platelets (1.0×10^9 platelets/ml), prewarmed for 15 min at 37°C with saline, were incubated for 15 min at 37°C with collagen as indicated. Incubation was stopped by adding equal volume of lysis mixture (0.5% SDS, 1% Triton X-100, 0.75% sodium deoxycholate, 10 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride, 50 mM NaF, 200 µM Na3VO4, 100 µM leupeptin, 100 µg/ml aprotinin, 10 µM staurosphorine). Lysates, after a brief centrifugation, were treated with 1.0 µg of anti p72syk (Upstate) or anti PLCγ2 antibodies for 2 h at 4°C. The immunocomplexes were precipitated with 100 µl of protein A-Sepharose CL-4B (Amersham Pharmacia). After 1 h on ice, immunoprecipitates were washed with 1 ml of IP-wash 1 (10 mM pH 7.4 Tris/HCl, 150 mM NaCl, 0.5% Triton X-100), followed by IP-wash 2 (10 mM pH 7.4 Tris/HCl, 750 mM NaCl, 0.5% Triton X-100) and finally again with IP-wash 1. Immunoprecipitates were extracted with 100 µl of 2×Laemmli-SDS reducing sample buffer, heated at 60°C for 20 min and resolved on 5–10% SDS-PAGE. Gels were transferred to nitrocellulose membranes and blots were treated as detailed above. Blots were then stripped by incubation with 62.5 mM pH 6.7 Tris/HCl, 2% SDS, 100 mM β-mercaptoethanol for 30 min at 50°C, reprobed with antibodies against anti-
p72syk or anti PLCγ2 and protein bands revealed as above described.

Detection of p47pleckstrin in [32P] labelled platelets
Washed platelets (2.5 x 10^8 platelets/ml), resuspended in pH 7.4 Hepes buffer containing 1 mM EGTA and 5% platelet-poor plasma (PPP), were incubated at 37°C with 1 mCi/ml [32P] phosphoric acid (Perkin-Elmer Life Science, Shelton, CT, USA), under gentle shaking. After 60 min platelets, washed once, were resuspended to 2.0 x 10^8 platelets/ml in the same buffer. Samples were prewarmed with saline for 15 min at 37°C and then incubated for 15 min at 37°C with collagen as indicated. Incubation was stopped by adding equal volume of lysis mixture. The subsequent immunoprecipitation was performed as above detailed with antibody raised against pleckstrin 25 clone (Santa Cruz). The heated immunoprecipitates were resolved on 5–10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and the [32P] phosphorylated pleckstrin was revealed by the Packard Cyclone Storage Phosphor system and quantified with the related software package. This was followed by Western blotting with anti-pleckstrin 25 clone at 1/1,000 dilution and protein bands revealed as described.

Measurement of intracellular calcium levels
Intracellular Ca²⁺ concentration was measured as previously described with light modifications (15). Washed platelets (3.0 x 10^8 platelets/ml), resuspended in pH 7.4 Hepes buffer, were incubated with 1 µM FURA 2/AM (Calbiochem-Merck Biosciences GmbH, Schwalbach/Ts, Germany), for 60 min at 37°C. Prostaglandin E₁ (2 µM final concentration) and EGTA (1 mM final concentration) were added before centrifuging loaded platelets for 15 min at 1,100 g. The pellet, resuspended at 2.0 x 10^8 platelets/ml in pH 7.4 Hepes buffer containing 1 mM CaCl₂, was preincubated at 37°C for 5 min with saline, then collagen was added. FURA 2-loaded platelet fluorescence was followed at 37°C in unstirred conditions for 15 min in a Perkin –Elmer Fluorescence Spectrometer model LS50B with excitations at 340 nm and 380 nm and emission at 510 nm. The fluorescence of fully-saturated FURA 2 (Fₘₐₓ) was obtained by lysing the cells with 50 µM digitonin in the presence of 2 mM Ca²⁺, while Fₘᵦ was determined by exposing lysed platelets to 20 mM EGTA. The fluorescence was fully quenched with 5 mM Mn²⁺ to give the autofluorescence value. A software combined with the fluorescence spectrometer converted data into cytosolic Ca²⁺ concentration. The Kᵩ value for FURA 2 and Ca²⁺ was 135 nM.

Measurement of nitrite + nitrate (NOx) production
Washed platelets (1.0 x 10^8 platelets/ml), resuspended in pH 7.4 Hepes buffer containing 2 mM CaCl₂, and prewarmed at 37°C for 10 min with saline, were incubated with 40 µM L-arginine and varying collagen concentrations for 15 min at 37°C under mild shaking without stirring. Incubation was stopped by sonicating samples on ice. To measure the total NOx content of samples, suitable aliquots of the supernatant were added to equal volumes of pH 9.7 assay buffer (15 g/l glycine-NaOH) containing cadmium beds (Fluka Chemie AG, Buchs, Switzerland) and incubated overnight at room temperature under horizontal shaking. Cadmium, which catalyzes the chemical reduction of nitrate to nitrite (16), was activated immediately before each experiment by subsequent washings with 0.2 N H₂SO₄, bidistilled water and pH 9.7 assay buffer. NOx formation was measured at 540 nm by the Griess colorimetric detection (17) of nitrite, that is as stable derivative of NO. A sodium nitrite calibration curve was used.

Statistical analysis
Data are the mean ± SD and statistical analysis was performed using the unpaired Student’s t-test, considering significant the difference at 5% level (p < 0.05).

Results
Clinical and metabolic characteristic of subjects
A group of 38 RVO patients was compared with a group of 40 age-matched healthy subjects. Clinical and metabolic parame-
ters of RVO patients and healthy subjects, described in Table 1, show that the two groups differed with respect to the number of habitual smokers, and subjects with diabetes, hypertension or hyperlipidemia. In addition, the plasma homocysteine level was significantly higher in RVO patients (p <0.0005), while serum folate level was significantly (p< 0.0005) lower.

**Effect of collagen or ADP on platelet function**
Platelet function was evaluated “in vitro” in the selected group of RVO patients compared to age-matched healthy subjects. Results demonstrated that platelets of RVO patients are more responsive to collagen or ADP than healthy subjects. As shown in Figure 1, the response to collagen (A) or ADP (B) is dose-dependent in both groups and it is higher in patients than in controls at all tested concentrations. However, the difference appears to be significant (p <0.0005) at low concentrations of collagen (0.5–1.0 µg/ml) or ADP (0.5–1.0 µM).

**Total protein-tyrosine phosphorylation**
One of the early events derived from platelet treatment with agonists is a great increase in the protein-tyrosine phosphorylation. The stimulation of platelets with collagen leads to a concentration-dependent increase in total protein tyrosine phosphorylation (Fig. 2). In the upper panels the results of one experiment, representative of six similar phosphotyrosine immunoblots, are shown, and the electrophoretic pattern of platelets of one patient is compared with that of one healthy subject. In Figure 2 data (mean ± SD of six separate determinations for each group of subjects) show that the total tyrosine phosphorylation is significantly increased in patients (bottom panel). The proteins with molecular masses of p53–62, p72 and p145 KDa seem to be particularly involved (upper panels). As expected, the protein tyrosine kinase inhibitor genistein greatly decreases protein tyrosine phosphorylation both in patients and in controls. Thus we examined closely the response to collagen, deeply investigating the p72syk/PLCγ2 pathway.

**Phosphorylation of p72syk**
p72syk is a tyrosine protein kinase rapidly activated following stimulation of platelets with agonists. In particular p72syk has been implicated in collagen-induced platelet signalling (12, 18). In platelets challenged with collagen p72syk becomes phosphorylated at tyrosine residues and activated. In our experimental conditions, in which platelets are stimulated under unstirring conditions of R VO patients and healthy subjects, described in Table 1, show that the two groups differed with respect to the number of habitual smokers, and subjects with diabetes, hypertension or hyperlipidemia. In addition, the plasma homocysteine level was significantly higher in RVO patients (p <0.0005), while serum folate level was significantly (p< 0.0005) lower.

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conditions, p72syk phosphorylation gradually increases up to 10 µg/ml collagen both in healthy subjects and in RVO patients (Fig. 3, bottom). However, the p72syk phosphorylation in platelets of the patient, who is representative of seven other patients (Fig. 3, upper panels), appears to be higher than that of the control subject. The difference in the p72syk phosphorylation between the two subjects is marked in the range 0.5–5.0 µg/ml collagen.
Figure 5: Activation of PKC. [32P]-Labelled washed platelets (2.0×10^8 platelets/ml), preincubated for 15 min at 37°C with saline, were stimulated for 15 min at 37°C with collagen as indicated. Pleckstrin was immunoprecipitated with the specific antibody and phosphorylated p47pleckstrin revealed in a Cyclone Phosphor Imager System. Blots are representative of four independent experiments performed on four subjects for each group. In the upper panels the densitometric scanning ± SD of the p47 pleckstrin phosphorylation of four experiments is shown. § p < 0.025; # p <0.0025; * p <0.0005 vs. none; † p <0.0005 vs. healthy subjects.

Figure 6: Effect of collagen on intracellular [Ca^{2+}] elevation. FURA 2-loaded washed platelets (2.0×10^8 platelets/ml), resuspended in pH 7.4 Ca^{2+}-free Heps buffer and preincubated for 5 min at 37°C, were stimulated with collagen as indicated. Data are the mean ± SD of seven experiments carried out in duplicate performed on seven subjects for each group. * P<0.0005 vs. healthy subjects.
Phosphorylation of PLCγ2
p72syk mediates tyrosine phosphorylation of PLCγ2. This mechanism represents a general strategy for tyrosine phosphorylation of PLCγ2 in stimulated platelets (12, 18, 19). Thus we wanted to evaluate the extent of phosphorylation of this phospholipase. Data of Figure 4 show that the PLCγ2 phosphorylation is greatly marked in platelets of the RVO patient. The difference between the patient and the control subject is significant at all tested concentrations of collagen (bottom panels). Similar results have been obtained in five other experiments carried out on different patients and healthy subjects (upper panels).

Activation of PKC
The PLCγ2 activation leads to the generation of two important second messengers, inositol 1, 4, 5 trisphosphate, which mediates the translocation and the activation of PKC. PKC promotes the phosphorylation of several proteins, among them p47pleckstrin. Thus, the p47pleckstrin phosphorylation can be considered indicative of PKC and PLCγ2 activation. Figure 5 shows that collagen dose-dependently induces PKC activation both in patients and in healthy subjects. However, the effect is more evident in the patient, as low collagen concentrations (0.5–1.0 µg/ml), which are ineffective in the healthy subject, induce the p47pleckstrin phosphorylation in the patient. In addition, the phosphorylated pleckstrin is clearly evident in resting platelets of the patient, while this band does not appear in the healthy subject, suggesting that platelets of the patient are much more responsive to collagen than healthy subject ones.

Intracellular calcium rise
Figure 6 shows changes of the intracellular calcium level in platelets resuspended in calcium-free medium and treated with collagen in unstirring conditions. Collagen produces a gradual rise of the intracellular calcium concentration. The effect is dose- and time-dependent. It is noteworthy that the calcium rise evoked by collagen is significantly (p < 0.0005) higher in patients than in healthy subjects at all tested concentrations of the agonist. For instance in platelets stimulated with 10 µg/ml collagen (15 min at 37°C) the intracellular calcium concentration reaches the level of 148 ± 13 nM in healthy subjects and 220 ± 18 nM in RVO patients, respectively (p <0.0005). Moreover, in the presence of 1 mM extracellular calcium, the basal calcium and the calcium level evoked by collagen is significantly (p <0.0005) higher than in the absence. In addition, the calcium rise is higher in RVO patients than in healthy subjects (Fig.7), suggesting that not only calcium release from intracellular stores but also calcium influx is increased in patients.

NOX formation
Nitric oxide produced by platelets acts as a negative feedback mechanism to inhibit platelet adhesion and aggregation (20). Previously it was shown that collagen inhibits NOX production in whole platelets (21). Thus we wanted to quantify the concentration of this radical in RVO patients during platelet stimulation by this agonist. Data of Figure 8A show that the NO basal level is significantly (p <0.0005) lower in patients than in healthy subjects. Since collagen dose-dependently decreases NOX formation (21), in activated platelets a further significant decrease in NO basal level was measured both in RVO patients and in controls (Fig. 8B). However, NO formation was always lower in patients: in platelets from patients treated with 10 µg/ml collagen NO level is 121 ± 23 pmol/2.0 x 10⁸ platelets/ml, that corresponds to the half of the basal level measured in healthy subjects.

Discussion
Collagen is the most thrombogenic component of the subendothelial layer following vascular injury. Collagen supports platelet adhesion to the subendothelium and induces aggregation, secretion and procoagulant activity. The interaction between collagen and platelets is mainly mediated by two receptors, the integrin α₂β₁ and glycoproteinVI. Previous data (21) have suggested that glycoprotein VI seems to play a central role in the signalling pathway leading to the formation of com-

![Figure 7: Effect of collagen on intracellular [Ca²⁺] elevation in the presence of extracellular calcium.](image-url)
pounds, such as calcium and NO which are very important in the regulation of platelet function. The glycoprotein VI activation by collagen stimulates a cascade of events including the activation of p72syk and PLCγ2 (12). PLCγ2 leads to the formation of the two second messengers diacylglycerol, which activates PKC, and inositol 1, 4, 5 triphosphate, which induces calcium release from the dense tubular system. In addition collagen stimulates platelet calcium influx (22). Data reported in Figures 6 and 7 show that collagen induces intracellular calcium rise in platelets stimulated under mild conditions. The collagen effect is dose- and time-dependent. Moreover, the response to the agonist of platelets of RVO patients is significantly higher than that of healthy subjects at all tested concentrations of collagen (0.5–10 µg/ml), both in the absence and in the presence of extracellular calcium. Previously it was found that intracellular calcium rise induced by collagen regulates the L-arginine/NO pathway, modulating NO synthesis through the inhibition of L-arginine uptake (21). It is in this way that increased calcium levels are associated with a reduced NO formation (Fig. 7). Nevertheless, the unpaired NO bioavailability could also depend on increased reactive oxygen species formation, as occurs in platelets activated by agonists (23). Unfortunately, at the present we have no data on reactive oxygen species level in resting and activated platelets of RVO patients. The activation of platelets by collagen results in a significant increase of tyrosine phosphorylation on several cellular proteins, among which p72syk and PLCγ2 (Fig. 2). The phosphorylation and activation of p72syk is an early signal transduction event that could be mediated by various tyrosine kinases, including p60src (24). The role of p72syk activation in platelets stimulated by collagen has been well established as p72syk catalyzes the tyrosine phosphorylation of downstream substrates, including PLCγ2 (12). Since p72syk and PLCγ2 are two important enzymes of the signalling cascade primed by collagen, their activation in RVO patients was tested. The obtained results show that the phosphorylation and activation of p72syk and PLCγ2 by collagen are increased in platelets of patients. The difference between patients and healthy subjects is marked at low agonist concentrations. Altogether data from our study show that in RVO patients collagen stimulates platelet activation and aggregation by enhancing the tyrosine phosphorylation of specific signalling enzymes such as p72syk and PLCγ2, by rising intracellular calcium levels and by reducing NO formation.

RVO is an important cause of permanent visual loss. It is considered a disease of the elderly but in 90% of cases occurs in patients younger than 50 years of age. It is known that a large part of these patients have systemic vascular diseases such as arterial hypertension, diabetes mellitus (5), hyperhomocysteinemia (6–8), coronary artery disease and peripheral vascular disease (25, 26). The role of coagulation factors in the development of RVO remains unclear. The major problem of these studies is the inadequate number of patients to define an association and the presence of opposite data in the literature. Most of these studies are retrospective. Moreover, an association between antithrombin S, protein C and RVO was never demonstrated (27). Several studies on platelet aggregation in RVO patients have produced conflicting results. Walsh et al. (28) have shown that neither ADP, collagen, nor epinephrine stimulation of platelet aggregation has risen. On the contrary, Watson et al. (29) and others (30–32) demonstrated increased platelet activation in RVO. In addition a significant increase in the formation of small platelet aggregates in RVO patients compared with the control subjects was demonstrated by Yamamoto et al. (33). In agreement with the majority of previous studies (29–32), we have shown that platelets of RVO patients are more responsive to collagen or ADP than those in healthy subjects (Fig. 1). Likely, hyperhomocysteinemia together with other factors such as diabetes, hypertension and/or hypercholesterolemia could play a role. In recent years a great number of studies has defined elevated plasma homocysteine as an important and independent risk factor similar to smoking or hyperlipidemia for atherosclerotic vascular diseases and venous thrombosis (34–36). Hyperhomocysteinemia can be caused by a wide range of disorders, the most important of which are genetic defects of the enzymes involved in homocysteine metabolism and deficiencies of the vitamin co-factors of these enzymes. The defi-
Table 1: Clinical and metabolic characteristics of retinal vein occlusion patients and healthy subjects.

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<th>Healthy subjects</th>
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<td>Number (n)</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Male/female</td>
<td>14/26</td>
<td>14/24</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63.3 ± 7.2</td>
<td>67.3 ± 11.2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.8 ± 3.7</td>
<td>24.4 ± 3.6†</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>0</td>
<td>4*</td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>0</td>
<td>4*</td>
</tr>
<tr>
<td>Dyslipidemia (n)</td>
<td>0</td>
<td>12*</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>3</td>
<td>23*</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.2*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>191 ± 10</td>
<td>235 ± 32*</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>54 ± 14</td>
<td>61 ± 15*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>101 ± 79</td>
<td>124 ± 68*</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>339 ± 52</td>
<td>347 ± 49*</td>
</tr>
<tr>
<td>Prothrombin time</td>
<td>98 ± 2</td>
<td>110 ± 9*</td>
</tr>
<tr>
<td>Partial thromboplastin time</td>
<td>27 ± 3</td>
<td>34 ± 5*</td>
</tr>
<tr>
<td>Factor VII activity</td>
<td>125.9 ± 23.6</td>
<td>131.5 ± 34.2*</td>
</tr>
<tr>
<td>Homocysteine (µM)</td>
<td>11.8 ± 2.3</td>
<td>16.2 ± 5.3*</td>
</tr>
<tr>
<td>B12 (pg/ml)</td>
<td>498 ± 274</td>
<td>426 ± 151*</td>
</tr>
<tr>
<td>Serum folate (ng/ml)</td>
<td>7.9 ± 3.3</td>
<td>3.9 ± 1.1*</td>
</tr>
<tr>
<td>BRVO (Branch retinal vein occlusion)</td>
<td>0</td>
<td>20*</td>
</tr>
<tr>
<td>CRVO (central retinal vein occlusion)</td>
<td>0</td>
<td>18*</td>
</tr>
</tbody>
</table>

*P < 0.0005 vs. healthy subjects.

cies of the 5, 10 methylene tetrahydrofolate reductase (MTHFR677TT) due to the C677T mutation and that of the cystathionine β synthase are determinants of the homocysteine levels. However, only the mutation MTHFR677TT seems to be associated with increased risk of RVO (37). In addition, nutritional deficiencies can be the cause of elevated plasma homocysteine concentrations: plasma folate, vitamins B6 and B12 relate inversely to the plasma homocysteine levels. This inverse relation was found in the patient group of this study, as in these patients low plasma folate concentrations correspond to high homocysteine levels (Table 1). Likely the deficiency of folate will result in the reduction of methylenetetrahydrofolate co-factor. Failure of remethylation will therefore promote homocysteine accumulation. Thus, high homocysteine levels can produce oxidative damage to vascular endothelial cells “in vitro” (38) and “in vivo” (39). Homocysteine itself may induce platelet aggregation (40) and potentiates the aggregating effect of agonists (41).

It is known that many haemostatic parameters are modified in non-insulin-dependent diabetes mellitus. In particular, spontaneous aggregation (42) and platelet hyperactivity in response to different agonists (43, 44) have been shown. It has been suggested that in hypertensive patients platelet hyperaggregation can be a risk factor (45) and antiplatelet therapy may be considered useful (46). Oxidative stress, which is enhanced in hypertension, could contribute to platelet activation (47). Moreover, various reports (48, 49) have shown that hypercholesterolemic PRP showed increased aggregation without an increase in the platelet number. Likely hyperaggregability is caused by high levels of LDL-cholesterol, described to promote aggregation (50), arachidonic acid mobilization from phospholipids and increased thromboxane A2 production (51).

The efficacy of antiplatelet treatment in patients with RVO is controversial. Hayreh et al. (52) concluded in their investigation on the haematologic abnormalities associated with various types of RVO that the treatment of these patients with platelet aggregating agents may adversely influence the visual outcome, without any evidence of protective or beneficial effect. On the contrary, other authors (31, 33) suggested that the platelet aggregation inhibitors ticlopidine or beraprost can be considered effective in the treatment of ocular vein occlusion.

In conclusion, platelet hyperaggregability can be an important factor in the pathogenesis of impending RVO. Likely antiplatelet therapy may be used in the management of this haemodynamic alteration.

References
Leoncini et al. Platelet hyperaggregability in retinal vein occlusion