Original Article

The Von Willebrand Factor Antigen Plasma Concentration: a Monitoring Marker in the Treatment of Aortic and Mitral Valve Diseases

(von Willebrand factor / aortic valve stenosis / vWF multimers / valvular disease / coagulation / mitral valve regurgitation)

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Abstract. Von Willebrand disease is a commonly inherited bleeding disorder caused by defects of von Willebrand factor (vWF). In the most common valve diseases, aortic valve stenosis (AVS) and mitral valve regurgitation (MVR), a bleeding tendency has been described in a number of patients. This has been associated to a high turbulence of blood flow through the compromised valve, promoting degradation of vWF with loss of high-molecular-weight multimers of vWF (HMWM), leading to an acquired von Willebrand syndrome (AvWS). We analysed three groups of patients, one affected by AVS, treated with transcatheter aortic valve implantation (TAVI), the second group of patients affected by MVR, treated with Mitraclip® mitral valve repair. The third group was represented by patients also affected by AVS, but not eligible for TAVI and treated with standard surgery. A fourth group of patients that underwent percutaneous coronary intervention (PCI) with stenting was used as a control. Our results demonstrated that the level of vWF measured as antigen concentration (vWF:Ag) increases in all cohorts of patients after treatment, while in control PCI patients, no modification of vWF:Ag has been registered. Western blot analysis showed only a quantitative loss of vWF in the pre-treatment time, but without significant HMWM modification. The monitoring of the vWF:Ag concentration, but not the quality of HMWM, can indicate the status of blood flow in the treated patients, thus introducing the possibility of using the vWF antigen detection in monitoring the status of replaced or repaired valves.

Introduction

Von Willebrand disease is a commonly inherited bleeding disorder caused by either a qualitative or quantitative defect of von Willebrand Factor (vWF), an adhesive plasma protein important for effective primary haemostasis (Lison et al., 2012). vWF has a multimeric structure with binding sites for platelets, collagen, Factor VIII and heparin (Solomon et al., 2011). An unexpected bleeding tendency during surgical intervention of aortic valve replacement (Fisher et al., 2001; Tsai, 2003) in patients affected by aortic valve stenosis (AVS) has been described (Zheng et al., 2001; Vincentelli, et al., 2003). However, some researchers reported a significant discrepancy between bleeding symptoms and the prevalence of vWF abnormalities in patients with aortic valve defects (Mendolicchio and Ruggeri, 2005). This phenomenon could be associated with the loss of

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Abbreviations: ADAMTS-13 – thrombospondin type 1 motif, member 13, AVS – aortic valve stent, AvWS – acquired von Willebrand syndrome, ER – endoplasmic reticulum, HMWM – high-molecular-weight multimers, MVR – mitral valve regurgitation, PCI – percutaneous coronary intervention, PVT – Policlinico Tor Vergata, TAVI – transcatheter aortic valve implantation, vWD – von Willebrand disease, vWF – von Willebrand factor, vWF:ag – von Willebrand factor antigen concentration, vWF:RCo – vWF ristocetin cofactor.

high-molecular-weight multimers of vWF (HMWM) (Van Belle et al., 2015). This condition, referred to as acquired von Willebrand syndrome (AvWS), could be responsible for the increased bleeding predisposition and related symptoms.

Physiologically, the pro-vWF protein is produced by endothelial cells and megakaryocytes in the endoplasmic reticulum (ER), then modified in the Golgi compartment, where the multimers are assembled, and secreted in the bloodstream. The vWF multimers are cleared from the plasma predominantly by targeting the liver. Clearance is achieved by different mechanisms, mainly correlated with the glycosylation state of the protein (Denis et al., 2008), and independently by the multimer size (Lenting et al., 2004). The lack of HMWM can occur through multiple pathophysiological mechanisms. The shear stress induced by blood flow disturbances (Ruggeri, 2002) changes the shape of vWF molecules from a coiled to an elongated filament. The unfolded vWF molecule could expose a specific site (located in the A2 domain of the protein) to disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13 (ADAMTS-13), resulting in a proteolysis process with reduction of HMWM. This condition could lead to the rare AvWS bleeding syndrome. About 270 cases of AvWS have been reported worldwide described by the ISTH Registry of AVWS (Federici et al., 2000), and in the literature (Veyradier et al., 2000; Mohri, 2003; Tiede et al., 2008; Tiede, 2012). Forty % of AvWS cases in cardiovascular pathology correlate with the aortic valve stenosis (Velik-Salchner et al., 2008), followed by mitral valve regurgitation (MVR) (Mehta et al., 2019). The latter is the second leading cause of valvular heart disease in the United States affecting 50 % of patients after myocardial infarction (Maganti et al., 2010). To treat these pathological conditions, two possibilities are currently available: a classical surgical approach (Salomon et al., 1978) involving open-heart surgery, or a transcatheter replacement/repair approach. Recently, two techniques to treat AVS have been established, the transcatheter aortic valve implantation (TAVI) (Cribier et al., 2002), and the transcatheter mitral valve repair technique, using Mitraclip® (Pregowski and Witkowski, 2013). The standard laboratory analysis of vWF is mainly based on the plasma vWF antigen determination (vWF:Ag) assay, the vWF ristocetin cofactor (vWF:RCo) assay, and the Factor VIII activity assay, which are used worldwide in standard clinic. In the text, we use vWF:Ag referring to the vWF plasma concentration.

In any case, laboratory assays do not give information about the alteration of the vWF multimer size, but only about the quantitative presence of the factor (antigen) and its activity as cofactor. The detection of the multimeric organization of vWF protein can be done by using denaturing SDS agarose gel (2.5 mm thick), performed in a vertical electrophoresis chamber with a run length of 25 cm at least, followed by Western blot analysis (Ledford-Kraemer, 2010; Ott et al., 2010).

In this study, we analysed the concentration of vWF antigen and the size of the related multimers in different cohorts of AVS patients with the indication for TAVI or standard surgery approach, and patients suffering from mitral regurgitation treated with Mitraclip®. The analysis has been performed before and after the treatment in order to verify the modification of vWF antigen characteristics.

Material and Methods

Study subjects

The study was carried out by the Department of Cardiac Surgery and the Unit of Interventional Cardiology at the Policlinico Tor Vergata (PTV) Hospital. The Ethics Committee approval from the PTV Hospital was obtained, and all enrolled patients signed the informed consent. The experimental study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Blood samples for the coagulation tests were collected by the Laboratory of Clinic Biochemistry at the PTV Hospital.

In the first phase of the study, a first group of patients with severe aortic valve stenosis and a second group with mitral regurgitation were enrolled by using standard inclusion criteria. Further, patients were selected according to the degree of the aortic stenosis, or mitral valve regurgitation, both classified as severe according to ESC/EACTS heart valve disease guidelines. Bleeding history was recorded in the six months before surgery, following the protocol proposed by Koscielny (2009). As exclusion criteria, the presence of coagulation disorders, active-phase endocarditis and treatment with anticoagulants (without interruption) at least 10 days before surgery were considered. On the basis of all the abovementioned criteria, homogeneous groups of patients were established.

Blood sampling

Blood samples were collected from each patient in tubes containing 0.109 M sodium citrate (3.2%) as anticoagulant in the 9 : 1 ratio (BDvacutainer®, Becton, Dickinson and Co., Franklin Lakes, NJ). Venepuncture was performed at three different time points; before surgery (baseline, T0), 24 h (T1) and 48 h (T2) after surgery for both TAVI and Mitraclip® procedures. The samples were centrifuged at 3,000 *g* for 15 min and then stored at −80 °C until use.

Von Willebrand factor antigen assay, ristocetin cofactor activity and Factor VIII assay

vWF:Ag was determined using a fully automated turbidimetric immunoassay (HemosIL von Willebrand Factor Antigen, Instrumentation Laboratory-Werfen Co., Italy) in the ACL TOP platform (Instrumentation Laboratory-Werfen Co.). Reference range: 0.66–1.76 U/ml.

vWF:RCo was determined using a fully automated turbidimetric immunoassay (HemosIL von Willebrand Factor Ristocetin Cofactor Activity kit) in the ACL TOP platform (Instrumentation Laboratory-Werfen Co.). Reference range: 0.60 – 2.39 U/ml.

Factor VIII was determined using the HemosIL Factor VIII Deficient Plasma kit (Instrumentation Laboratory-Werfen Co.), based on activated partial thromboplastin time (aPTT assay) in the ACL TOP platform (Instrumentation Laboratory-Werfen Co.). Reference range: 0.50–1.50 U/ml. FVIII-deficient plasma was used to substitute for potential deficiency of other clotting factors; in fact, as known, activated partial thromboplastin time (aPTT) depends exclusively on the FVIII activity and results elongated if FVIII activity is decreased.

Statistical analysis

A SPSS software package version 20 (IBM, New York, NY) was used for statistical analysis. Two non-parametric tests (Friedman test and Wilcoxon test) were performed. The significance level (P value) for these tests was $P < 0.05$.

Western blot analysis of von Willebrand factor multimers

Western blot analysis was performed using a vertical gel apparatus (Hoefer SE600, Hoefer Inc., Holliston, MA). A 2-cm base of acrylamide (4%) was poured below the resolving agarose gel (1%) to avoid its collapse. The stacking was 1.2% agarose gel, and 6.00 U/ml of vWF (based on vWF:Ag measurement) was dissolved in 25 µl of BPB (bromophenol blue in standard urea loading buffer). Electrophoresis was performed with an overnight run (5 mA, 4 °C). The gel was then transferred onto a membrane (Amersham Hybond, Ge Healtcare, Little Chalfont, UK) for 3 h, at 60 V, 4 °C, using 5 l of transfer buffer. The transferred membrane was blocked with PBS TWEEN 0.01% + 5% of milk for 2 h. The anti-vWF antibody (Takara, Shiga, Japan) was dissolved in 10 ml (1 µg/ml final concentration) of reconstituted milk and incubated overnight at 4 °C. After washing, the membrane was incubated with 20 ml of fresh milk and 1 : 10,000 of goat anti-mouse HRP secondary antibody (Molecular Probes, Eugene, OR) (Coppola et al., 2012). After washing, the membrane was covered with 5 ml of ECL (Perkin Elmer, Waltham) and used for X-ray film exposure.

Results

After application of the inclusion/exclusion criteria (see Methods), three groups of patients were established. Group 1 included 14 patients affected by AVS and treated with TAVI (Sambu and Curzen, 2010); group 2 comprised 12 patients with MVR treated with Mitraclip® (Stewart and Jenkins, 2016); group 3 comprised 15 AVS patients not eligible for percutaneous procedure that underwent surgical aortic valve implantation. The control group (group 4) consisted of patients subjected to percutaneous coronary intervention (PCI) with stenting. The TAVI group was composed by patients with an average age of 83 years with 50 % of gender distribution and the Mitraclip group with an average age of 71 years with 60 % of male distribution. The other two groups had a mean of 75 years and a gender distribution of 50 %.

Concentration of vWF

Measurement of the vWF:Ag concentration in the pre-surgery time (T0), compared to the post-surgery time points (T1, T2), showed interesting results. In TAVI patients, we obtained a median value of 1.60 U/ml at T0 (min 0.63 U/ml, max 2.79 U/ml). At time T1, the median value was 2.41 U/ml (min 1.35 U/ml, max 3.81 U/ml). Values measured at time T2 were similar to those of T1, with a median value of 2.64 U/ml (min 1.40 U/ml, max 3.59 U/ml) (Fig. 1A). These data, highlighted by the median bars of the graph in Fig. 1A, show that there is a significant difference between values obtained at time T0 compared to those obtained after surgery (T1, T2), thus indicating an increase or "recovery" of vWF:Ag. In Mitraclip® patients, similar results were obtained; the median value at T0 was 1.40 U/ml (min 0.51 U/ml, max 2.28 U/ml), at time T1, the median value was 2.08 U/ml (min 1.56 U/ml, max 3.36 U/ml), and at time T2, the values remained similar to those measured at T1 (Fig. 2A), indicating that a saturating concentration had been reached.

A different representation of these data, expressed as a ratio between T1 and T0, shows the presence of a recovery of the vWF:Ag concentration in all patients, but with a different ratio. The slope of the lines from time T0 to T1 indicates the increase of vWF:Ag, demonstrating the efficiency of recovery (Figs. 1C, 2C). In the TAVI group, three different groups of recovery can be identified, one between 80–120 %, the second between 30–60 %, and a small group in which the ratio is between 10–20 % (Fig. 1C), while the recovery in Mitraclip® patients displays a more random distribution. In the group of patients that underwent standard surgery, the mean concentration of vWF:Ag at time T0 was 1.49 U/ml (min 1.17 U/ml, max 2.65 U/ml). At time T1, the median concentration was 1.79 U/ml (min 1.07 U/ml, max 3.01 U/ml), and at T2, the median value was 2.43 U/ml (min 1.62 U/ml, max 3.00 U/ml) (Fig. 3A). These results indicate an improvement of vWF:Ag also in this group of patients. In the control group without valve pathology but subjected to a cardiovascular stenting procedure (Fig. 3C), no significant changes in the vWF:Ag concentration values were obtained.

vWF ristocetin cofactor assay

vWF:Rco was measured for all study participants. In TAVI patients, the concentration of vWF:Rco at time T0 was within the normal reference range (0.60–2.39 U/ml), with a median value of 1.15 U/ml (min 0.48 U/ml, max

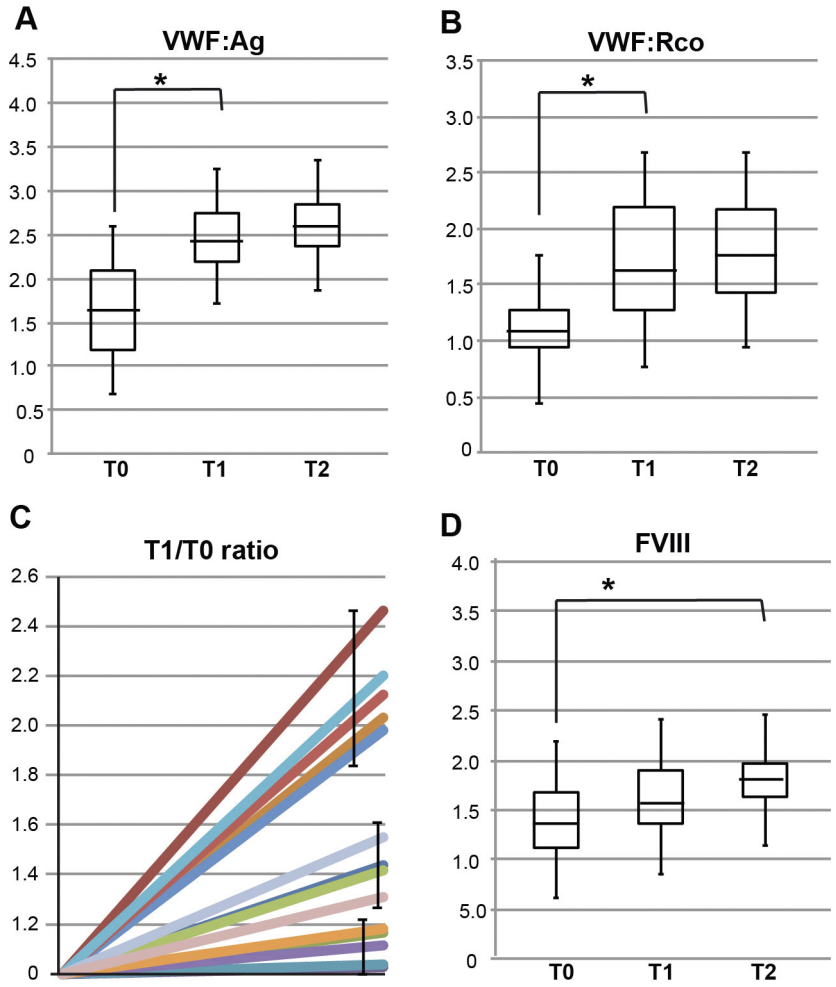

Fig. 1. Analysis of coagulation parameters in TAVI patients. The distribution of the values is represented as box plots. The median values are showed by the horizontal bar inside the box. The vertical bars at the bottom and top of the box represent the range of the values. Measurement was performed in pre-treatment stage (time T0), 12 h after surgery (time T1) and 24 h after surgery (time T2). (**A**) vWF:Rco median values show the increase of vWF after the TAVI treatment. (**B**) Concentration of vWF:Ag showing the increase of the factor after the TAVI treatment. (**C**) vWF:Ag ratio. The concentration of vWF measured at time T0 and T1 was used to calculate the relative increase of the factor in each patient. The slope of the line and the endpoint show how the vWF increases. (**D**) Determination of FVIII activity in TAVI patients; median values are representative of the three time points. The significance level (P value) was $P < 0.05$ (asterisks in figure).

1.34 U/ml); at time T1, a median value of 1.54 U/ml (min 0.98 U/ml, max 2.93 U/ml) was obtained; finally, at time T2, a median value of 1.68 U/ml was observed (min 1.04 U/ml, max 2.94 U/ml) (Fig. 1B). In Mitraclip® patients, the concentration of vWF:Rco at time T0 was within the normal reference range, with a median value of 1.03 U/ml (min 0.28 U/ml, max 1.2 U/ml); at time T1, a median value of 1.25 U/ml (min 0.43 U/ml, max 1.50 U/ml) was obtained; at time T2, a median value of 1.29 U/ml was obtained (min 1.06 U/ml, max 1.76 U/ml) (Fig. 2B). The analysis of vWF:Rco in the control group of patients (PCI) did not show significant variations (Fig. 3D).

Analysis of vWF:RCo/vWF:Ag showed variable results in all group of patients, but inside the normal ranges.

Determination of FVIII activity

Samples of the 14 TAVI patients showed, at time T0, a median value of 1.37 U/ml (min 0.43 U/ml, max 3.73 U/ml), and at time T1, a median value of 1.60 U/ml (min 0.36 U/ml, max 2.02 U/ml). Finally, at time T2, a median value of 1.73 U/ml was obtained (min 1.31 U/ml, max 3.45 U/ml), as shown in Fig. 1D. Mitraclip® patients showed a median value of 1.10 U/ml at time T0, a median value of 1.47 U/ml at time T1 (min 1.15 U/ml, max 2.14 U/ml), and at time T2, a median value of 1.53 U/ml (min 1.19 U/ml, max 2.68 U/ml) (Fig. 2D).

vWF multimeric Western blot analysis

The vWF multimers are based on a 250 KDa monomer. The multimerization leads to the formation of pro-

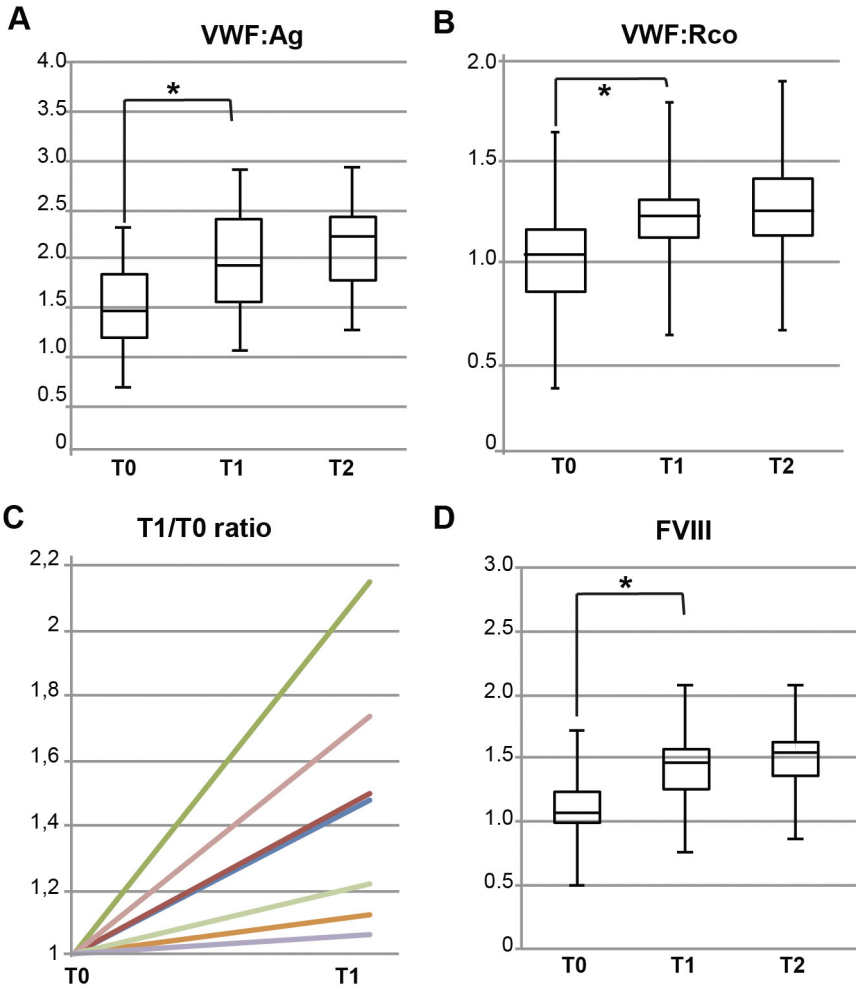

Fig. 2. Analysis of coagulation parameters in Mitraclip®. Values are represented, as specified in Fig. 1, as box plots. Measurement was performed in pre-treatment stage (time T0), 12 h after surgery (time T1) and 24 h after surgery (time T2). (**A**) vWF:Rco median values show the increase of vWF after the Mitraclip® treatment. (**B**) Concentration of vWF:Ag showing the increase of the factor after the Mitraclip® treatment. (**C**) vWF:Ag ratio. The concentration of vWF measured at time T0 and T1 was used to calculate the relative increase of the factor in each patient. The slope of the line and the endpoint show how the vWF increases. (**D**) Determination of FVIII activity. Median values in the three time points evaluated. The significance level (P value) was $P < 0.05$ (asterisks in figure).

teins with an extremely large weight, up to 20,000 kDa. These large proteins cannot be visualized using standard denaturing acrylamide gel electrophoresis. Indeed, it is also difficult to run samples in agarose SDS gel in a normal agarose electrophoresis apparatus, since in this case it is not possible to finely control the thickness of the gel. Technically, the only way is to use a large vertical acrylamide box adapted for the use of agarose (see Material and Methods for details). This approach gave us the possibility to resolve high-molecular-weight vWF multimers when visualized in Western blot analysis.

Western blot analysis was conducted using 6 µl of plasma obtained from patients at times T0 and T2 and from a normal control. The results showed an evident difference in the total quantity of vWF, measured as vWF:Ag, and consequently, of the higher multimers (Fig. 4A). Indeed, higher mass bands are represented in reason of the total quantity of the antigen, so their presence is affected when the total antigen is diminished. To better evaluate the size of vWF multimers, a second experiment was performed; in this case, the total quantity of the protein was normalized according to the values of the vWF:Ag level for all samples. The results showed that the numbers of high-molecular-weight bands after normalization seemed not to be affected (Fig. 4B). SDS agarose gel, performed using a prolonged (20%) electrophoresis, was set up for both groups of patients (TAVI, Mitraclip®; Fig. 4C–D). After the transfer and protein revelation, we used densitometry to analyse the quantity and molecular mass of the vWF multimers. The densitometric profile showed that all higher bands were present at similar levels in the two samples (Fig. 4D).

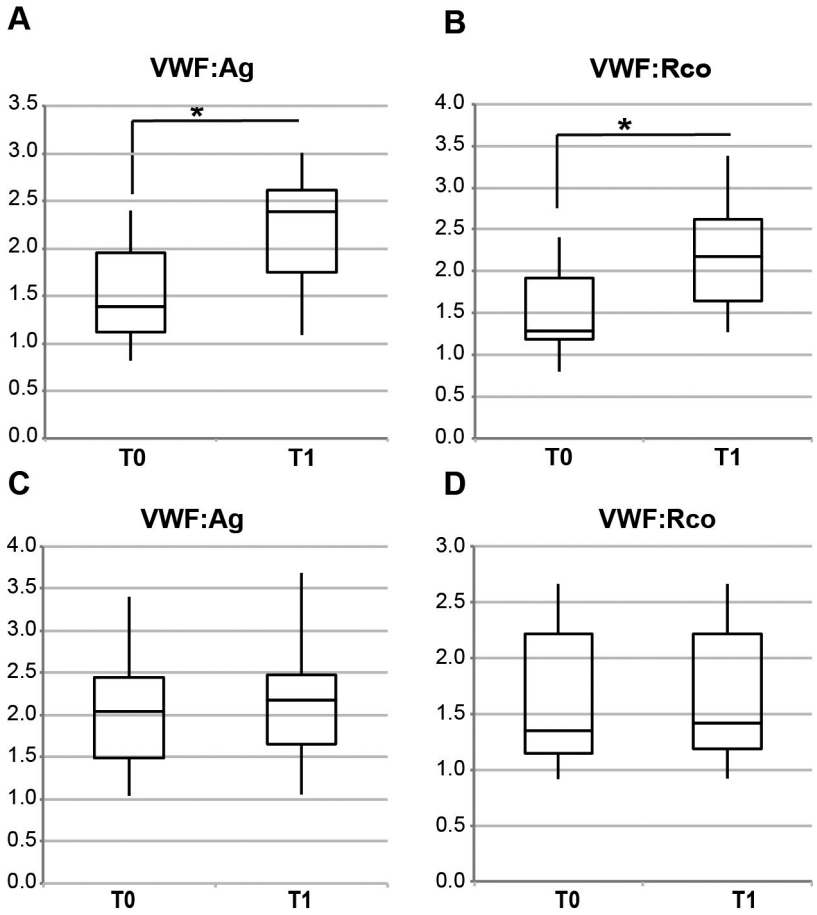

Fig. 3. Analysis of coagulation parameters in patients treated with standard surgery and PCI technique. The values are expressed using the same system as in Figs. 1 and 2. Box plots represent values in the pre-surgery stage (time T0), and 24 h after surgery (time T1). (**A**) Concentration of vWF:Ag, showing the increase of the factor after standard surgery. (**B**) Concentration of vWF:Ag, showing the increase of the factor after standard surgery. (**C**) Values of vWF:Ag in the control group (PCI) showing a non-significant change. (**D**) vWF:Rco median values of the control group, again showing a non-significant modification of the values. (Results are in default instrument units, median values.) The significance level (P value) was $P < 0.05$ (asterisks in figure).

Also, in higher exposure films, the numbers of bands were similar among samples before and after the treatment (Fig. 4E).

Discussion

In the general population, von Willebrand disease (vWD) has a prevalence between 0.01 % and 1.3 %, while the AvWS prevalence is between 0.04 % and 0.13 %; therefore, the latter is very rare and difficult to notice. According to the ISTH registry, almost 21 % of AvWS cases are estimated in pathologies that require a form of heart surgery; other causes are lymphoproliferative (48 %), myeloproliferative (15 %), and other neoplastic (5 %) and autoimmune disorders (2 %) (Federici et al., 2000; Tiede et al., 2011). In valve pathologies, the high pressure gradient, generated across the stenotic or regurgitating valves, causes high shear forces, which can induce degradation of the vWF protein, also by activation of platelets, leading to adsorption of the vWF multimers onto their surface (O'Brien and Etherington, 1992; Federici, 2006; Franchini and Lippi, 2007), or other unknown mechanisms. Moreover, it has already been described that the reversal of aortic stenosis leads to amelioration of bleeding gastrointestinal angiodysplasia (Anderson et al., 1996).

Indeed, our results demonstrate that in selected patients, with aortic valve stenosis or mitral regurgitation, the values of vWF antigen are noticeably lower before the valve repair/substitution when compared to those measured after (24 or 48 h) TAVI and Mitraclip® procedures. Moreover, also in the group of patients that underwent surgical valve substitution, the vWF antigen concentration improves after surgery, demonstrating that the factor concentration improvement is not dependent on the technique used, but it may be due to the decrease of the blood flow turbulence. Consistently, the control group submitted to percutaneous coronary intervention

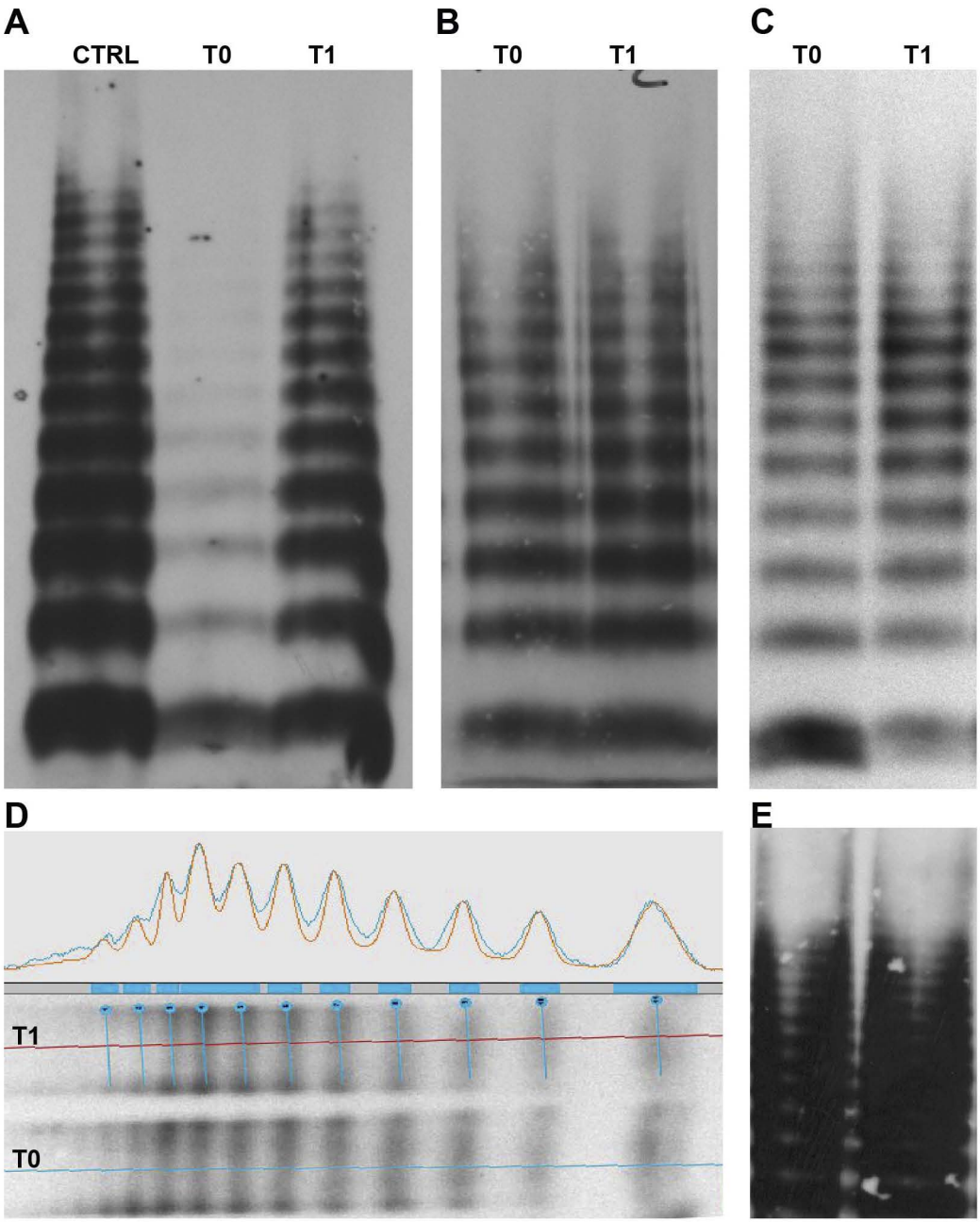

Fig. 4. Western blot analysis**.** (**A**) Agarose gel performed using 4 µl control plasma in loading buffer (lane 1) and plasma from a patient treated with TAVI (lanes 2, 3). The lower band is a 250 kDa mass band. T0 is the untreated patient, T1 24 h after valve intervention. The control plasma is from an unrelated unaffected patient. (**B**) Gel electrophoresis of samples from the TAVI patient normalized for the content of vWF:Ag. (**C**) Long run of a representative TAVI patient for the detection of high-molecular-weight bands. (**D**) Long run of a representative Mitraclip® patient, also showing densitometry analysis, demonstrating the presence of high-molecular-weight bands also in the T1 condition. (**E**) Long exposure time to detect high-molecular-weight bands missed in the standard exposure that shows no change between T0 and T1.

(PCI) with stenting did not show a significant change of vWF:Ag values, indicating that when the blood flow is not compromised, the value of the antigen is not affected.

The vWF protein is probably subject to active degradation in the stage before the valve repair/substitution (Federici, 2006; Franchini and Lippi, 2007), and this phenomenon could be responsible for the lower concentration of the antigen at time T0, still not sufficient to induce AvWS with clinical relevance. The degradation of the protein does not involve a selective loss of high-molecular-weight multimers, but a strong general decrease in the total protein. In experiments in which the stenosis was induced artificially, a loss of HMWM was observed over a short time of 5–30 min (Van Belle et al., 2015). In our patients, the degree of valvular failure very likely evolved into a general lack of the factor, also

determining the diminishment of the HMWM available for normal coagulation. Considering the median values of vWF:Ag in the three patient groups at time T0 (1.60 U/ml, 1.40 U/ml, 1.49 U/ml), there is an evident decrease in the vWF antigen in respect to values measured in the normal controls (median value of 2.07 U/ml). Since the diminishment of the vWF antigen seems to be correlated with the level of valvular impairment, if prolonged in time and not treated, the worsening of the valvular function could lead to a haemorrhagic phenotype during the surgery. Tiede and colleagues (Tiede et al., 2011) in their clinical practice focused the attention on testing patients with AvWS-associated disorders prior to major surgery, because a reduced function/antigen ratio could indicate structural or functional disorders, even if the absolute activity is within normal limits. The transcatheter replacement/repair approach is well tolerated by aged and critical patients, and it also gives notable results in reactivation of the vWF antigen plasma concentration, avoiding the development of AvWS.

Indeed, the most eligible diagnostic procedure to evaluate the state of cardiac artery, aortic valve and blood fluxes is computed sonography. In most cases, when the problem is evidenced by sonography, it is already in an advanced state. Our study shows that the value of vWF:Ag is reduced in the presence of untreated severe valvulopathies, and higher levels of vWF:Ag are restored after valve substitution or repair. In valve compromised patients, continuous monitoring of the stability of vWF:Ag value, reached after valve repair/substitution, could indicate the state of blood flux related to the valvular function. If a new decrease of the antigen is observed, this could indicate deterioration of the valve.

A clinical trial should be first established, monitoring the treated patients for both vWF:Ag levels and echocardiogram imaging, or standard clinical assessment. The data obtained could be used to check out a relationship between blood and clinical parameters. If confirmed, this could give the possibility to follow patients over time and plan an early intervention.

Even if the group of patients studied was not extremely large, there are numerous patients that can be collected according to the selection criteria in our hospital, which represents one of the major referring points for central Italian population.

In conclusion, this work can be considered a starting point suggesting a new potential diagnostic use for vWF:Ag for detection of the valvular functioning state in AVS and mitral regurgitation patients after the valve replacement or repair.

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M. A. Perrone[#] and F. G. Viola[*] equally contributed to the work.

Conflicts of interest

All authors disclose no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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