

## VON WILLEBRAND FACTOR CLEAVING PROTEASE ACTIVITY IN THE PHYSIOPATHOLOGY OF MICROANGIOPATHIC DISORDERS

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**Abstract** The von Willebrand factor cleaving protease (VWFPC) modulates the von Willebrand factor (VWF) multimeric size in normal plasma. VWFPC activity levels are decreased in different physiological and pathologic situations. Different techniques have been developed to unfold the purified VWF (perfusion at high shear rate, dialysis against urea in nitrocellulose filters), to detect the VWFPC activity on it (multimeric analysis of VWF, collagen binding to VWF assay) and to use the patient plasma both as the source of the enzyme and substrate. In this paper we compared the above mentioned methods with new ones: normal plasma dialyzed on membranes instead of purified VWF, dialysis of the samples against urea in tubing instead of nitrocellulose filters, and sonicated plasma to remove the endogenous VWF. The perfusion assay and detection by multimeric analysis showed a limit of detection (25%) of VWFPC activity. Dialysis against urea in both supports and detection by multimeric analysis, showed a better limit of detection (3%), but the recovery of the samples was not as efficient in nitrocellulose filters as it was in tubing. The detection by collagen binding to VWF has more advantages because it allows to analyze more samples than the multimeric analysis does in the same assay. The dialysis of plasma by membranes to obtain the source of exogenous VWF requires no complex equipment. The method, which uses patient plasma as the source of the enzyme and substrate, was inapplicable in our experience because the values could not be interpolated in the reference curve.

**Key words:** von Willebrand factor, cleaving protease, thrombotic thrombocytopenic purpura

**Resumen** *Detección de la actividad de la proteasa que cliva al factor von Willebrand en la patofisiología de las microangiopatías.* La proteasa que cliva al factor von Willebrand (VWFPC) controla el tamaño multimérico del factor von Willebrand (VWF) en plasma normal. Sus niveles se encuentran disminuidos en diferentes situaciones fisiológicas y patológicas. Diferentes técnicas se desarrollaron para desplegar al VWF purificado (perfusión a alta fuerza de cizallamiento, diálisis contra urea en filtros de nitrocelulosa), para detectar la actividad de la VWFPC (análisis multimérico del VWF, enlace del VWF al colágeno) y para usar el plasma del paciente como fuente de enzima (VWFPC) y de sustrato (VWF). Este trabajo compara los métodos descritos con métodos nuevos: plasma dializado en membranas en lugar de VWF purificado, diálisis de la muestra contra urea en tubos, en lugar de filtros de nitrocelulosa y plasma sonificado para remover el VWF endógeno. El ensayo de perfusión y la detección por análisis multimérico mostró un límite de detección de la actividad de la VWFPC del 25%. La diálisis contra urea en ambos soportes y la detección por análisis multimérico arrojó un límite mejor (3%), pero la recuperación de las muestras no fue tan eficiente en filtros de nitrocelulosa como en tubos. La detección por enlace del VWF al colágeno posee mayores ventajas al analizar más muestras en el mismo ensayo. La diálisis de plasma en membranas, para obtener VWF exógeno, no requiere equipos complejos. El método que usa el plasma del paciente como fuente de enzima y sustrato fue inaplicable en nuestras manos ya que los valores no pudieron ser interpolados en la curva de referencia.

**Palabras clave:** factor von Willebrand, proteasa, púrpura trombocitopénica trombótica

VWF is a glycoprotein circulating in plasma as a series of multimers ranging from 500 to 20 000 kD<sup>1</sup>. VWF precursor is synthesized as a very large protein in en-

dothelial cells and megakaryocytes<sup>2,3</sup>. An important mechanism for depolymerization of the large multimers is the limited proteolysis by a VWFPC present in plasma. The apparent molecular weight of the enzyme is approximately 300 kD as estimated by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis<sup>1</sup>. The enzyme is found to specifically cleave the Y (842)-M (843) peptide bond in the A2 domain<sup>4</sup>. The shear stress may be a modulator of VWF size in the circulation because it increases the VWF susceptibility to proteolysis<sup>5</sup>. Proteolytic activity has a pH optimum at 8 to 9. The pro-

Received: 27-VI-2002

Accepted: 3-III-2003

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tease is activated by preincubation with divalent ions in the following order:  $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$ . It is inhibited by peptidyl diazomethyl ketones as Z-Phe-Phe-CHN<sub>2</sub> and chelating agents as EDTA, EGTA<sup>1,5</sup>.

The active VWFCP is detected in human plasma, serum, cryoprecipitate supernatant, defibrinated plasma, and commercial plasma preparations including solvent-detergent-treated and methylene-blue-treated plasma<sup>1,6</sup>. When the protease is deficient in the plasma, ultralarge forms of VWF are released from damaged vascular endothelium<sup>7,8</sup> and may cause thrombus formation because they aggregate platelets under high shear stress conditions<sup>9</sup>.

Two primary mechanisms for deficiency of VWFCP activity were identified, constitutive deficiency and presence of an acquired immunoglobulin inhibitor<sup>6,10</sup>. Recent studies showed complete constitutional deficiency of VWFCP activity in patients with familial thrombotic thrombocytopenic purpura (TTP)<sup>11,12</sup> and the presence of autoantibodies inhibiting VWFCP in patients with nonfamilial TTP<sup>6,10,13</sup>. At the beginning, some authors as Furlan<sup>6,11</sup> and Tsai<sup>10</sup> proposed that VWFCP levels could discriminate between TTP and hemolytic uremic syndrome (HUS). They demonstrated the deficiency of VWFCP in the plasma of patients with acute, sporadic, chronic relapsing, and familial TTP and they found that it was measurable in normal amounts in acute sporadic HUS. Nevertheless, in recent studies low levels of VWFCP activity were also found in patients with HUS<sup>14</sup>. Demonstration of its activity requires to subject substrate VWF to mechanical<sup>15,5</sup> or chemical denaturation<sup>1,16</sup> to unfold VWF and to allow better access to the cleavage site. *In vitro*, the protease cleaves VWF under non-physiological conditions, such as low ionic strength in the presence of 1 to 1.5 M urea<sup>1,6</sup> or guanidine HCl<sup>16</sup>.

Till now, the methods developed to test the VWFCP effect on the VWF do not join all the necessary conditions: high sensibility, infallibility, and availability of the necessary substructure, quick and low cost. In the present report, we used the published techniques to unfold the purified VWF (perfusion at high shear rate, dialysis against urea in nitrocellulose filters), to detect the VWFCP activity on it (multimeric analysis of VWF, collagen binding to VWF assay) and to use the patient plasma both as the source of the enzyme and substrate. In addition we introduced the following modifications: filtered normal plasma instead of purified VWF, dialysis against urea in tubing of the samples instead of nitrocellulose filters and plasma sonicated to remove the endogenous VWF.

## Materials and Methods

### Materials

Type I agarose, type III collagen (from calf skin), 4-Chloro-1-naphthol, Tween 20, dialysis tubing D-9277 with diameter size of

6mm (retain most proteins of mol. wt. 12 000 D or greater) were from SIGMA. Polyclonal antibodies (rabbit anti-human VWF, FITC rabbit anti-human VWF, biotinylated goat anti-rabbit IgG), and streptavidin-biotin-horse radish peroxidase complex (ABCComplex/HRP) were from DAKO. Polystyrene beads with diameter size of 2.837m were from Polysciences, Inc. (Warrington, PA). Sephacryl S-1000 and Protein-G Sepharose 4B were from Pharmacia. Medicell membranes with diameter size of 20mm (retain most proteins of mol. wt. 1 000 000 D or greater) were from Spectrum Medical. Nitrocellulose filters with pore size of 0.025 and with size diameter of 25mm were from MILLIPORE.

All other reagents were of analytical grade.

### Plasma preparation

Venous blood was collected from healthy volunteers donors, into 1:10 volume trisodium citrate to achieve a final concentration of 129mM. Platelet-poor plasma was obtained by centrifugation at 2 500g for 60 minutes at 4 °C, after which the plasma was transferred to another tube, it was centrifuged at 10 000 for 30 minutes and stored at -80 °C. Samples should contained less than 1000 platelets/mL. Pooled normal plasma (PNP) (n=15) was used as reference for VWF:CB.

### Preparation of exogenous VWF

*Purified VWF.* VWF was isolated from fresh frozen plasma of normal volunteers following the procedures described by Thorell and Blombäck<sup>17</sup>. The eluted fractions containing VWF were pooled and concentrated using 20 % PEG 20 000 and dialyzed against 0.15M NaCl, pH 7.2 overnight.

*Dialyzed plasma by membranes.* Protease-depleted plasma by Medicell membranes, was used to replace the purified VWF. About 1mL of citrated plasma was depleted of VWFCP by dialysis against 0.15M NaCl, pH 7.2 for 19 hours. This method was developed at IHEMA (not published yet).

### Assay of VWFCP activity

The assay of VWFCP activity was performed in diluted plasma samples after activation by barium chloride and incubation with VWF substrate as previously described<sup>1</sup> with some modifications. Curves with normal plasmas (n=10) were constructed undiluted and diluted from 1:2 to 1:32 with 0.15M NaCl, 10 mM Tris-HCl solution, pH 7.4. When the variations between the normals were estimated, a PNP was used to estimate the VWFCP %. BaCl<sub>2</sub> (final concentration 0.01M) was added to each diluted plasma sample and then it was incubated at 37 °C for 30 minutes.

In a set of experiments, curves with normal plasmas (n=3) were constructed diluted from 1:2 to 1:32 with 0.15M NaCl, 10 mM Tris-HCl solution, pH 7.4 and Pefabloc SC 1mM.

### Degradation of VWF from plasma

The degradation of VWF was performed by sonication using the method described by Casillas et al<sup>18</sup>.

### Preparation of samples to be tested with exogenous VWF

Aliquots (148mL) of diluted plasma samples, after activation, by barium chloride were incubated with 36mL of exogenous VWF at a final concentration of 1U/mL to evaluate VWFCP activity.

### Preparation of samples without exogenous VWF

500mL of the test plasma were placed in dialysis casing and so they were used as a source of the enzyme and substrate,

negating the need for purified VWF or other preparation of normal plasma VWF<sup>19</sup>. The degree of VWF cleavage by the protease is then quantitated by exploiting the property of larger multimers to bind preferentially to human collagen type III<sup>20,21</sup>.

#### *Conformational change of VWF*

##### Perfusion at high shear rate

The perfusion assay was developed using the method previously described<sup>15</sup>.

##### Urea treatment

##### i) Dialysis on hydrophilic filter

100 mL of the test samples were dialyzed on the surface of a hydrophilic filter for 19 hours at 37 °C against 1.5M urea and 5mM TRIS (pH 8). The reaction was stopped by the addition of EDTA (final concentration 20mM, pH 7.4)<sup>6</sup>.

##### ii) Dialysis in tubing

Aliquots (184mL) of the test samples were dialyzed in tubing, which were immersed in 1.5 M urea 5mM Tris-HCl, pH 8, for 19 hours at 37 °C. Then, the samples were dialyzed against 1.5M NaCl, (pH 7.4). The reaction was stopped by the addition of EDTA (final concentration 20mM, pH 7.4).

#### *Laboratory test to detect the effect of VWF<sub>FCP</sub> on VWF*

##### Multimeric analysis of VWF

A multimeric analysis of normal VWF, subject to proteolytic degradation, was performed on SDS-1% agarose gel electrophoresis and it was visualized by immunoenzymatic stain, like it was previously described<sup>22</sup>. VWF multimers were measured by densitometrical scanning of the stained gels with a Sharp Scanner (*JX 330*, Hamburg, Germany) using the software *ImageMaster* (*Pharmacia*, Newcastle, England). The used software gave the RF value automatically. The control in each running was the purified VWF (100% of present multimers) that was considered as RF=0.

##### Binding of VWF to collagen (VWF:CB)

Collagen has been shown to bind VWF, with the preferred binding with high molecular weight forms of VWF<sup>23,20</sup>. This assay is based on modifications of the VWF:CB assay previously described<sup>24</sup>. 7mL of buffer Horm (isotonic glucose solution, pH 2.7-2.9) were added to 1mL of a collagen solution (100mg/mL in isotonic glucose solution, pH 2.7-2.9), the mix was incubated 5 minutes at 37 °C. Finally 1000mL of polystyrene beads were washed and resuspended in 5mL of this last solution. The beads were coated by re-suspending them in 10 % BSA diluted in an ELISA buffer and they were incubated for 30 minutes.

A calibration curve was set up always using PNP (1:20 to 1:640). Aliquots of 20mL of beads were added to the treated samples and to PNP, and they were incubated gently rocking at room temperature for 1 hour in Eppendorf tubes. After the beads were washed (2 times), they were incubated with anti-VWF-FITC diluted 1:100 in ELISA buffer containing 0.1% BSA, in the darkness for 30 minutes at 37 °C. Finally the beads were centrifuged, washed with ELISA buffer with 0.1% BSA, re-suspended in 500mL of Isoflow and transferred to polyethylene tubes. All samples were measured by flow cytometry (*FACSCAN*, *Becton Dickinson*) and analyzed with the *Cell Quest* software. The results were expressed as the median of fluorescence.

The 1-U/mL VWF:CB represents the loss of large VWF multimers, which is directly proportional to VWF<sub>FCP</sub> activity, so 1-U/mL VWF:CB = 0 corresponds to 100% of multimers.

##### Assay for VWF:Ag

This assay is based in the method previously described<sup>24</sup>. The solutions and materials used were identical to the VWF:CB assay previously described.

##### *Statistical Analysis*

The values were expressed in terms of media  $\pm$ SD, and the significant differences were examined by the paired *t* test. P values below 0.001 were considered to be statistically significant. Inter-assay variation, expressed as between-run coefficient of variation, was determined by using normal and patients samples, they were frozen in aliquots and then subsequently thawed and tested repeatedly on different days. Intra-assay variation, expressed as within-run coefficient of variation, was determined using those samples, which were tested repeatedly on the same day.

## Results

#### *Detection of VWF<sub>FCP</sub> activity after perfusion at high shear rate by multimeric analysis of VWF*

As previously described, in each curve the control RF=0 was the purified VWF. In all the assays, undiluted normal plasma was considered as 100% VWF<sub>FCP</sub>. A quantification curve (n=10) was made with normal plasma (undiluted to 1:16) + purified VWF (Figure 1, curve a). The curve exhibits a good correlation (coefficient=0.96). No statistical differences (P=0.278) were found between the media  $\pm$  SD of purified VWF and purified VWF + normal plasma (diluted in 1:8), suggesting that the limit of detection (P<0.001) was 25% of protease activity according to purified VWF + normal plasma (diluted 1:4). Figure 1, curve b, represents the quantification curve of sonicated samples and it presented a good correlation (coefficient=0.96). No statistical differences were found, for all the dilutions of quantification curve, between RF of sonicated plasma and non-sonicated plasma. No statistical differences were found, between RF of normal plasma dilutions with and without Pefabloc (data not shown).

No statistical differences of VWF:Ag were found between sonicated and non sonicated samples (data not shown).

#### *Detection of VWF<sub>FCP</sub> activity after dialysis against urea in nitrocellulose filters by multimeric analysis of VWF*

A quantification curve (n=10) was made with normal plasma (undiluted to 1:32) + purified VWF (Figure 1, curve c). The correlation coefficient of the curve was 0.99. The obtained RF values by dialysis against urea in nitrocellu-

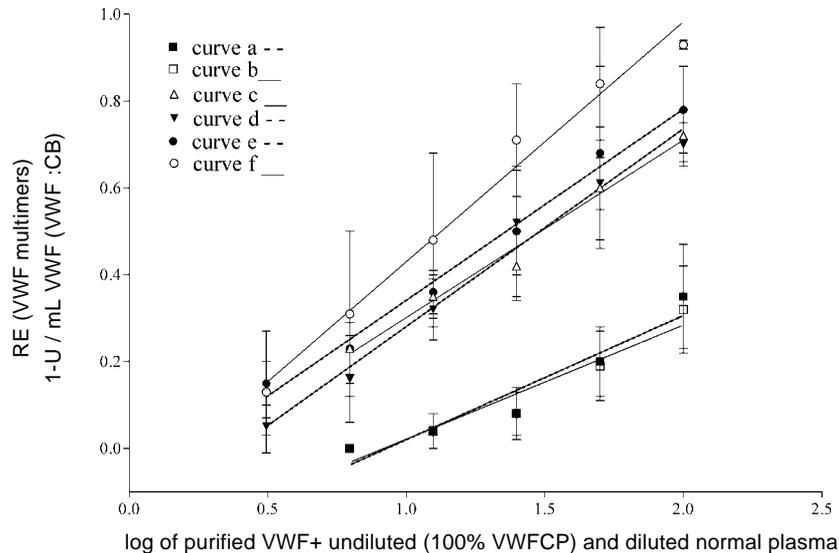


Fig.1.– VWF cleaving-protease. Different methods for its quantification and detection. Correlation between log % VWF:CB (source VWF + normal plasma dilutions) and RF or 1-U/mL VWF (VWF:CB), each point reflects the mean of ten values. Curves: a perfusion of purified VWF and plasma detected by multimeric analysis, b perfusion of purified VWF and sonicated plasma detected by multimeric analysis, c dialysis against urea in nitrocellulose membranes of purified VWF and plasma detected by multimeric analysis, d dialysis against urea in tubing of purified VWF and plasma detected by multimeric analysis, e dialysis against urea in tubing of dialyzed plasma by membranes and plasma detected by multimeric analysis, f dialysis against urea in tubing of purified VWF and plasma detected by VWF:CB.

lose filters method were significantly different ( $P < 0.001$ ) from all the curve dilutions regarding purified VWF. The limit of detection was 3% of proteasic activity according to purified VWF + normal plasma (diluted 1:32). No statistical differences of VWF:Ag were found between treated and untreated samples (data not shown).

*Comparison of the RF values obtained by perfusion at high shear rate and dialysis against urea in nitrocellulose filters. Detection by multimeric analysis of VWF*

The comparison of the RF values of plasma samples ( $n=10$ ), which were exposed to both methods to get the conformational change of VWF, showed that dialysis against urea in nitrocellulose filters method presented statistically significant differences ( $P < 0.001$ ) regarding perfusion at high shear rate.

No statistical differences of VWF:Ag were found between treated and untreated samples (data not shown).

*Detection of VWF:CB activity after dialysis against urea in tubing by multimeric analysis of VWF*

A quantification curve ( $n=10$ ) was made with normal plasma (undiluted to 1:32) + purified VWF (Figure 1, curve

d). It presented a correlation coefficient of 0.99. The obtained RF values by dialysis against urea in tubing method were significantly different ( $P < 0.05$ ) regarding purified VWF. The limit of detection was 3% of proteasic activity according to purified VWF + normal plasma (diluted 1:32). No statistical differences of VWF:Ag were found between treated and untreated samples (data not shown).

The comparison of RF values derived from the dialysis against urea in tubing method with two different exogenous VWF sources; purified (Figure 1, curve d) and dialyzed plasma by membranes (Figure 1, curve e), showed that no statistical differences were found between the dilutions of both quantification curve (purified VWF or dialyzed plasma by membranes) except at 1:32 plasma dilution ( $P < 0.001$ ) which corresponds to the limit of detection (3 % of proteasic activity). The Figure 1, curve e, shows a correlation coefficient of 0.99.

No statistical differences of VWF:Ag were found between samples with purified and dialyzed plasma by membranes (data not shown).

*Detection of VWF:CB activity after dialysis against urea in tubing by VWF:CB*

Results suggested that all the 1-U /mL VWF (VWF:CB) values obtained from the quantification curve dilutions,

Table 1.– Statistical comparisons between slopes of different VWFCP activity assays.

| Curves | Methods  | Slope $\pm$ standard error |
|--------|--|----------------------------|
| a      | Perfusion of purified VWF and plasma detected by multimeric analysis.  | 0.29 $\pm$ 0.05*           |
| b      | Perfusion of purified VWF and sonicated plasma detected by multimeric analysis                               | 0.26 $\pm$ 0.04*           |
| c      | Dialysis against urea in nitrocellulose membranes of purified VWF and plasma detected by multimeric analysis | 0.41 $\pm$ 0.03*           |
| d      | Dialysis against urea in tubing of purified VWF and plasma detected by multimeric analysis                   | 0.46 $\pm$ 0.03*           |
| e      | Dialysis against urea in tubing of dialyzed plasma by membranes and plasma detected by multimeric analysis   | 0.44 $\pm$ 0.02*           |
| f      | Dialysis against urea in tubing of purified VWF and plasma detected by VWF:CB.                               | 0.55 $\pm$ 0.03            |

\*Paired t-test, relative to f curve used as reference, n=10.

were significantly different ( $P < 0.001$ ) from the purified VWF (Figure 1, curve f). The limit of detection (3% of protease activity) corresponds to purified VWF + plasma 1:32. No statistical differences of VWF:Ag were found between treated and untreated samples (data not shown).

When patient's plasma was submitted to dialysis against urea in tubing method, within-run (n=15) coefficient of variation was 13.6%, and between-run (n=15) coefficient of variation was 18 %. For normal plasma within-run (n=17) coefficient of variation was 5.4 %, and between-run (n=17) coefficient of variation was 3.5%.

*Comparison of quantification curves obtained by different methods to get the conformational change of VWF and to detect the VWFCP effect on VWF*

The slope of the quantification curve that was obtained by dialysis against urea in tubing and detection by VWF:CB was significantly different ( $P < 0.001$ ) from the slopes of quantification curves given by the others methods above described (Table 1).

*Dialysis against urea in tubing applied to plasma samples without exogenous VWF. Detection by VWF:CB*

Undiluted and diluted (1:2) normal plasma samples were submitted to the conformational change of VWF by dialysis against urea in tubing. A PNP reference curve was used to interpolate these samples and extrapolate the 1-U/mL (VWF:CB) results. The fluorescence medians of the samples were under the 1:640 PNP dilution (data not shown).

No statistical differences of VWF:Ag were found between treated and untreated samples (data not shown).

## Discussion

A VWFCP activity has been identified in normal plasma, which degrades VWF at position 842-843 of the subunit<sup>4</sup>. In vivo this protease appears to play a critical role by regulating the size of VWF multimers for optimal efficiency in platelet adhesion to the subendothelium under high shear conditions<sup>25</sup>. Deficiency of VWFCP (ADAMTS13) is the molecular mechanism responsible for TTP<sup>26</sup>. Recently, Mannucci<sup>27</sup> measured the VWFCP in a population of healthy controls from newborns to elderly. The protease levels were low in newborns but became normal within 6 months. In healthy people the VWFCP was decreased since 65 years old, and in pregnancy it was lower in the last 2 trimesters than in the first. In cirrhosis, uremia, acute inflammation and postoperative period the VWFCP activity was also found decreased. Liver and renal diseases and inflammatory states are not unusual in thrombotic microangiopathies, but low protease values in these states do not mean TTP. In addition, the protease may be low in patients with HUS and renal or liver failure, therefore this observation seems to be opposed to the idea that normal protease levels accompany HUS<sup>6, 10, 11</sup>. Remuzzi et al<sup>28</sup> found that deficient ADAMTS13 activity does not distinguish TTP from HUS, at least in the recurrent and familial forms.

As VWFCP activity levels are decreased in different physiological and pathologic situations, we think that is very important to find the most effective method to test the VWFCP activity. Therefore, these protease levels could be related with clinics features in the diagnostic of patients.

The purpose of the present study was to find a sensitive method that does not require high technology to detect VWFCP activity.

Kempfer and Tsai<sup>5,15</sup> measured the protease in plasma samples by perfusion at high shear rate. Since their observations, we attempted to use this method for assay the VWFCP quantification in normal plasma dilutions in which purified VWF was added. Nevertheless, we found that the limit of detection was low (25%).

Plasma sonication allows to obtain plasma samples free of VWF activity without modification of protease activity. We removed the endogenous VWF<sup>18</sup> because at plasma dilutions lower than 1:20, endogenous VWF interferes in the VWFCP measurement, especially if it is decreased or if the cleavage site is disturbed. The obtained results suggested that the VWFCP activity was not affected by sonication. Remuzzi et al<sup>28</sup> confirms their results by evaluating the cleavage of recombinant VWF A1-A2-A3 domains, which overcomes possible artifacts of the VWF:CB due to the presence of endogenous undegraded VWF in test samples.

In our experience, the method described by Furlan<sup>1</sup> showed a lower limit of detection of VWFCP activity (3%) than perfusion at high shear rate (25%). Nevertheless, this method practically fails due to evaporation or hydration of the samples during dialysis against urea buffer. Obert<sup>25</sup> placed the nitrocellulose filters in Petri boxes, but we employed this alternative and did not improve the recovery of the samples. A similar limit of detection of VWFCP activity was obtained replacing nitrocellulose filters by dialysis tubing and it did not fail.

Since the preparation of purified VWF needs a complex substructure, we removed the plasma VWFCP by dialysis in membranes to obtain the source of exogenous VWF.

For the VWFCP activity detection we used the VWF:CB assay. It has a limit of detection similar to the obtained limit by multimeric analysis. In addition, this method allows to include much more samples than the multimeric VWF assay does and it is faster too (7 hours instead of 48 hours). We have observed that between-run and within-run precision data of the method were suitable.

Rick<sup>19</sup> developed a VWF:CB that allows a quantitative test that can be performed in less than a day. In our experience, this technique was inapplicable because the values of the treated plasma were under the limit of detection of the reference curve of VWF:CB.

In conclusion, to quantify the VWFCP activity, we advise using dialysis against urea in tubing to unfold VWF and VWF:CB to detect the remanent VWF. VWF:CB technique by flow cytometry is not available in all laboratories and it can be replaced by an ELISA method in micro wells<sup>29</sup>. To the best of our knowledge, our data provide a demonstration that dialyzed plasma in membranes could be used instead of purified VWF.

**Acknowledgements:** This work was supported by grants from the Alberto J. Roemmers Foundation, René Barón Foundation and CNICT, National Research Council, Argentina.

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*We have to shed the utopian imagery of the past 50 years. Health for all is unattainable. A disease-free world is an illusion. Death in good health is a contradiction. Health, life, and living with disease have to be purchased. Our technological development has overtaken our ethics.*

Tenemos que desprendernos de la imagen utópica de los pasados 50 años. La salud para todos es inalcanzable. Un mundo libre de enfermedades es una ilusión. La muerte en buena salud es una contradicción. La salud, la vida y vivir con una enfermedad se deben comprar. Nuestro desarrollo tecnológico ha sobrepasado nuestra ética.

Imre Loeffler

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(Número del 28 de julio, dedicado a la salud de los excluidos; acceso libre: [www.bmj.com](http://www.bmj.com))