Thrombosis Research 173 (2019) 42–47

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Link between von Willebrand factor multimers, relapses and coronary microcirculation in patients with thrombotic thrombocytopenic purpura in remission

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ARTICLE INFO

Keywords:
Thrombotic thrombocytopenic purpura (TTP)
von Willebrand factor
ADAMTS13

ABSTRACT

Introduction: ADAMTS13 deficiency results in unusually large von Willebrand factor (ULVWF) multimers in the circulation and a higher risk of microthrombi due to high shear stress. In patients treated for acquired thrombotic thrombocytopenic purpura (TTP), a persistently severe ADAMTS13 deficiency (< 10%) in remission is associated with more relapses. A reduced plasma ADAMTS13 activity and increased VWF levels are associated with a higher risk of myocardial infarction. Assessing coronary flow reserve (CFR) enables a better cardiovascular risk stratification: a lower CFR correlates inversely with cardiovascular risk. The aim of the study was to establish whether patients with TTP in remission have an impaired coronary microcirculation, in terms of a lower CFR, and whether there is any correlation between ADAMTS13 activity, the presence of ULVWF multimers, and the occurrence of relapses.

Methods: The clinical information and hemostatic parameters of 24 patients with TTP in remission managed at our center were analyzed. The CFR was assessed in a subgroup of the TTP patients and compared with a control group consisting of 50 healthy volunteers.

Results: The CFR was statistically lower in patients in remission of TTP than in controls, but there were no differences between TTP patients with normal and lower CFR. The occurrence of relapses correlated with the presence of ULVWF multimers and with a residual ADAMTS13 activity.

Conclusions: When compared with healthy controls, TTP patients in remission have an impaired coronary microcirculation and the occurrence of relapses in the former reveal the presence of ULVWF multimers.

1. Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare life-threatening disease [1]. The disorder is characterized by thrombocytopenia, microangiopathic hemolytic anemia, microvascular thrombosis, and consequent organ dysfunction [2]. The disease is due to a lack of ADAMTS13 (A Disintegrin and Metalloprotease with ThromboSpondin type 1motif), which is needed to cleave von Willebrand factor (VWF) [3–7]. A deficiency in this protease, or an immune inhibition of its activity [8], lead to the adhesion and aggregation of platelets to ultralarge von Willebrand factor (ULVWF) multimers, which results in thrombosis (especially in the microcirculation) due to high shear stress.

ULVWF multimers are not usually detectable in the plasma of patients with acute TTP because they have been incorporated in the VWF-rich thrombotic material [9], but they appear *de novo* in periods of remission [9,10]. Budde et al. found that the triplet structure of the VWF multimer pattern can vary, depending on the residual proteolytic activity of ADAMTS13 or other proteases [11,12]. In patients with TTP, a severe ADAMTS13 deficiency is by itself enough to expose patients to relapse, and the risk of relapse is still increased if associated with anti-ADAMTS13 antibodies [13].

There is clinical evidence of a cardiac involvement in acquired TTP, involving arrhythmias, sudden death, or heart failure, for instance [14–16]. Hughes et al. retrospectively reviewed 41 TTP patients,

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https://doi.org/10.1016/j.thromres.2018.11.005
Received 20 June 2018; Received in revised form 18 October 2018; Accepted 5 November 2018
Available online 12 November 2018

showing that higher levels of troponin T and anti-ADAMTS13 IgG were associated with microvascular thrombi, high mortality and acute morbidity [17].

The arterial involvement of the VWF-ADAMTS13 complex has been confirmed in various animal models. To give an example, a deficient plasma ADAMTS13 activity and increased plasma VWF levels were associated with a higher risk of myocardial infarction in mice [18]. To date, however, no one has investigated the persistence of a coronary microvascular dysfunction in patients with TTP in remission [19,20]. Monitoring ADAMTS13 activity and VWF multimer patterns in TTP patients in remission could be important for the purpose of identifying any subclinical cardiac dysfunction, even after the acute disease has regressed.

Assessing coronary flow reserve (CFR) has been recognized as a valid tool for detecting the onset of coronary microvascular dysfunction [21]. Measuring the CFR on transthoracic Doppler echocardiography (TDE) enables a functional assessment of the microvascular circulation [22]. As already reported for other diseases [23–25], the CFR could be clinically relevant to the management and risk assessment of the delicate asymptomatic remission phase of TTP.

We studied 24 TTP patients in remission to see if CFR could be used as a marker of coronary microvascular dysfunction, even in the absence of acute coronary disease. Patients were assessed in terms of ADAMTS13 activity, anti-ADAMTS13 antibodies (Ab), VWF multimer pattern, and clinical behavior.

2. Materials and methods

2.1. Study design and patients

A prospective cohort study was conducted on 24 consecutive patients referred to the First Internal Medicine Department at Padua University and City Hospital between April 2016 and April 2017 with acquired TTP in remission [26,27]. We considered with previous diagnosis of acquired TTP presenting, in the acute phase, microangiopathic hemolytic anemia with moderate or severe thrombocytopenia, associated with organ dysfunction, confirmed by a severe ADAMTS13 deficiency (< 10%), associated with the presence of anti ADAMTS13 autoantibodies. The remission was characterized by a clinical response after cessation of plasma-exchange (PEX), maintained for > 30 days and a normalization of laboratory parameters. Patients were compared with a control group consisting of 50 healthy volunteers recruited from personnel at the department matched for age and sex. All patients and controls were asymptomatic with no cardiovascular disease, judging from their clinical history, physical examination, and electrocardiograms (ECG). All participants had a normal ECG at rest and during adenosine-induced hyperemia. Exclusion criteria for all subjects included any of the following conditions: cerebral vascular disease, carotid artery bruit, peripheral bruit or abnormal pulse, history of angina or myocardial infarction, alcohol intake > 10 oz. per week [28]. Patients and control subjects came from the same geographic area (northeast Italy). Clinical data on patients' cardiac condition during acute TTP, the number of previous TTP relapses, and previous treatments were collected retrospectively by reviewing their medical records. Information on any cardiac involvement during acute TTP was unfortunately only available for some patients, however, and was consequently disregarded for the purposes of the present paper.

Laboratory parameters were routinely collected prospectively during the CFR assessment in the follow-up, including troponin I levels.

All patients gave their written informed consent to the study, and all related procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000. The two study populations were compared in terms of their clinical characteristics, laboratory findings, and CFR assessment.

2.2. ADAMTS13 assays

ADAMTS13 activity was ascertained using a chemically synthesized fluorescence-quenching substrate FRETSVWF73 (Peptide International, Lexington, KY) [29], as described elsewhere [30] (in-house normal levels 65–130%, tested on 50 healthy volunteers; severe deficiency was defined as < 10%). Patients have been categorized in three groups: Normal (ADAMTS13 activity > 65%); Undetectable (ADAMTS13 activity < 5% or < 20% in presence of positive inhibitory anti-ADAMTS13 antibodies) [31]; Reduced (ADAMTS13 activity between 20 and 65%).

Anti-ADAMTS13 IgG Ab titers were measured in all patients using the ADAMTS13-inh ELISA kit from Technoclone (Wien, Austria), as previously described [30]. Inhibitor titer of antibody was above 17 U/ml valued on the same sample group.

2.3. VWF multimer analysis

VWF multimer analysis was performed on 1.2% SDS-agarose (LGT agarose Type VII; Sigma, Munich, Germany) gels to detect ULVWF multimers according to Budde et al. [32]. After electrophoresis, VWF multimers were transferred to nitrocellulose filters by electroblotting, incubation and washing steps in low-fat milk with a long half time. The filters were incubated overnight in a 1:2500 dilution of polyclonal rabbit anti-human VWF antibody (DakoCytomation, Glostrup, Denmark), then in the second antibody, diluted 1:2500 (Goat anti-rabbit IgG HRP conjugate; BioRad Laboratories, Hercules, CA, USA), placed in the video-detection system (BioRad, Chemidoc Touch Imaging System), and overlaid with a solution of luminol and iodophenol (both from Sigma-Aldrich Chemie, Steinheim, Germany) in Tris buffer. Patients' VWF multimers were compared with reference pooled plasma (pooled from 70 healthy controls) run on the same gel. Quantitative, densitometric gel analysis of all samples was performed using software provided with the video-detection system (AlphaView Q stand Alone; Alpha Innotec, San Jose, CA, USA). For densitometric analysis, small, intermediate, and large multimers were defined as oligomers 1–5, 6–10, and > 10, respectively [32–34]. The multimer patterns were examined by the first two authors, blinded to the patients' disease status, and the population was dichotomized by the presence or absence of ULVWF multimers.

2.4. CFR assessment by transthoracic Doppler echocardiography

Images were obtained in the distal part of the left anterior descending coronary artery (LAD) using a 7 MHz transducer. The coronary blood flow rate was obtained using color Doppler flow-mapping guidance, and a sample volume was located within the color signal in the LAD artery by the pulse-wave Doppler. After obtaining baseline flow velocity recordings, adenosine was administered by intravenous infusion (140 mg/kg/min) for 3 min to obtain hyperemic Doppler flow profiles. Adenosine is thought to be an important regulatory substance, matching coronary blood flow to alterations in myocardial O_2 supply and/or demand. Adenosine is a ubiquitous purine base that has many physiological actions in the body, including arterial vasodilation in all vascular beds, with the exception of the kidneys. Myocardial ischemia causes an immediate breakdown of adenosine triphosphate and generates adenosine, thereby producing coronary vasodilation and restoring flow. On this premise, exogenously administered adenosine produces vasodilation by interacting with the adenosine receptors in the cell wall. Exogenously administered adenosine has a very short half-life (< 10 s) and produces maximal or near-maximal coronary vasodilation in a dose-dependent fashion. Adenosine relaxes the coronary arteries of various species through A_2 receptors. This coronary vasodilatory action of adenosine has both extracellular Ca-dependent and -independent components. Adenosine, an endothelium-independent vasodilator, induces maximal coronary microvascular dilation and is

used clinically to assess CFR. The underlying mechanism for production of CFR impairment by adenosine echocardiography is a greater coronary flow increase in the normal arteries and a lesser increase in the stenotic arteries. In the absence of significant coronary stenosis, CFR impairment is indicative of microvascular dysfunction. There is considerable evidence that coronary flow reserve, endothelium-independent vasodilation, is diminished in chronic heart failure and in several diseases associated to coronary microvascular involvement [35]. To account for differences in cardiac work, resting diastolic coronary blood flow velocities were normalized to the corresponding rate-pressure product by dividing the resting blood flow velocity by the rate-pressure product (an indicator of cardiac work), multiplied by a linear factor of 10,000, in each individual patient. The corrected CFR was calculated as the ratio between the hyperemic and normalized resting diastolic flow velocities. A CFR ≤2.5 was considered abnormal, and the population was dichotomized according to this cut-off. CFR ≤2.5 has been suggested as a cut point for microvascular dysfunction, being the lower limit of normal flow reserve in arteries free of obstructive coronary artery disease. The lower limit has been demonstrated with positron emission tomography (the gold standard for CFR evaluation) as well as with other techniques [36–39]. All patients abstained from caffeine-containing drinks for at least 24 h before being tested [21,22].

2.5. Statistical analysis

All statistical analyses were performed on the parameters recorded at the time of a patient's follow-up. Nominal variables were compared with the X^2 or Fisher's exact tests, as appropriate. Continuous variables were compared with the *t*-test or the Mann-Whitney *U* test, where appropriate. Bivariate correlations were assessed using Pearson's coefficient (r). The analysis of correlation between ADAMTS13 activity levels and time from the last relapse and from last treatment with rituximab was performed according to linear regression model. The statistical analyses were done with the SPSS (Statsoft Inc., Tulsa, OK, USA) software v.23. p-Values < 0.05 were considered statistically significant.

3. Results

The patients' clinical and laboratory findings are summarized in Table 1.

Among 24 patients (M/F 5/19, age 47.8 ± 10.5), twenty-three patients had acute idiopathic TTP, and one had TTP secondary to ticlopidine therapy; 11 patients had experienced previous relapses, with a median of 72 (3–144) months. All patients had previously been treated with steroids and plasma exchange, 7 (29%) patients received rituximab therapy and the median from the last course of rituximab was 48 (24–120) months. One patient was treated with rituximab during the acute phase of first occurrence, six during first relapse and, among these, one patient received rituximab, for the second time, as a pre-emptive therapy, during clinical remission. Sixteen (67%) patients were still being treated with antiplatelet therapy (aspirin 100 mg). Platelet counts and troponin I levels were in the normal range in all patients.

Median ADAMTS13 activity was 79% (range 7–130); it was below 20% in 5 (21%) patients, with a median of 12% (range 7–18). The median Ab titer was 6 U/ml (range 3–117); 4 patients (17%) had Ab titers above 17 U/ml, with a median of 104 U/ml (range 49–117) and ADAMTS13 activity < 20%. The median VWF:Ag level was 182 (range 50–237), and ULVWF multimers were detectable in 11 patients (46%) (Fig. 1).

Table 1
Characteristics of the study sample. Normal ADAMTS13 activity group: ADAMTS13 > 65%; reduced ADAMTS13 activity group: ADAMTS13 activity between 20 and 65%. Undetectable ADAMTS13 activity group: ADAMTS13 activity < 5% or < 20% in presence of inhibitory anti-ADAMTS13 antibodies. Cardiovascular characteristics are available for 23 patients.

TTP patients (n 24)	
Age, years, mean ± SD	48 ± 10.5
M/F	5/19
Relapses per patients, mean ± SD	1.5 ± 2.6
Time (months) from last relapse per patients, median (range)	72 (3–144)
Antiplatelet therapy, n (%)	16 (67)
Rituximab, n (%)	7 (29)
Time (months) from last Rituximab course per patients, median (range)	48 (24–120)
Cardiovascular characteristics	
BMI, median (range)	28 (19–39)
Current smokers, n (%)	9 (39)
Hypertension, n (%)	8 (35)
Hyperlipidemia, n (%)	6 (26)
Diabetes mellitus, n(%)	2 (9)

3.1. Coronary flow reserve

CFR assessment based on TDE was performed in 17 patients because seven patients refused consent to the CFR test. The mean of CFR was 2.8 ± 0.7; six patients (35%) had a CFR below the cut-off of 2.5 and their mean adenosine-stimulated peak diastolic velocity was 60 ± 17 cm/s. When the hemodynamic parameters of TTP patients in remission and controls were compared, there was a statistically significant difference in adenosine-stimulated peak diastolic velocity ($p < 0.0001$), mean CFR, and number of individuals with CFR ≤2.5: all 3 values were statistically lower ($p < 0.0001$) in patients than in controls (Table 2). Table 3 shows the clinical and laboratory findings in TTP patients by CFR (≤ or > 2.5): no differences emerged between these two groups of patients. In particular, there were no differences between patients with ADAMTS13 ≥ 65% or < 20%.

3.2. Hemostatic and clinical parameters

Table 4 shows the comparison between the different categories of patients, as explained in the Materials and methods.

One or more relapses were most frequently associated with patients whose enzyme activity was reduced ($p < 0.001$), and with the presence of ULVWF multimers ($p = 0.03$). Patients with a history of relapses were also more likely to have been treated with rituximab ($p = 0.023$).

ADAMTS13 activity levels correlated inversely with anti-ADAMTS13 Ab ($p = 0.001$, $r^2 = 0.3955$), and number of relapses per patient ($p = 0.003$, $r^2 = 0.3337$), while they correlated directly with age ($p = 0.03$, $r^2 = 0.215$). ADAMTS13 Ab also correlated directly with the number of relapses per patient ($p = 0.001$, $r^2 = 0.396$). ADAMTS13 activity levels did not correlate with both time from last relapse ($p = 0.326$, $r^2 = 0.024$) and from last rituximab course ($p = 0.369$, $r^2 = 0.024$).

4. Discussion

In patients with a history of TTP, particular attention has to be paid to clinical signs of a risk of relapse, especially in patients with persistently reduced ADAMTS13 activity, with or without evidence of anti-ADAMTS13 Ab [13]. As concerns cardiac damage, Hughes et al. [17] reported that a rise in troponin T levels in TTP patients is a sign of myocardial necrosis associated with microvascular thrombi. Mortality and acute morbidity were found associated with higher troponin T levels on admission and an increase in IgG Ab to ADAMTS13. Benhamou et al. [15] suggested that high troponin I levels at presentation are an independent factor associated with a three-fold increase in the risk of death or refractoriness. Previous case reports have described sudden death as being the likely result of arrhythmias associated with small coronary vessel platelet thrombi and myocardial ischemia [40–43].

It is not known, however, whether there is persistent, chronic damage to the cardiac microcirculation, or whether this may be associated

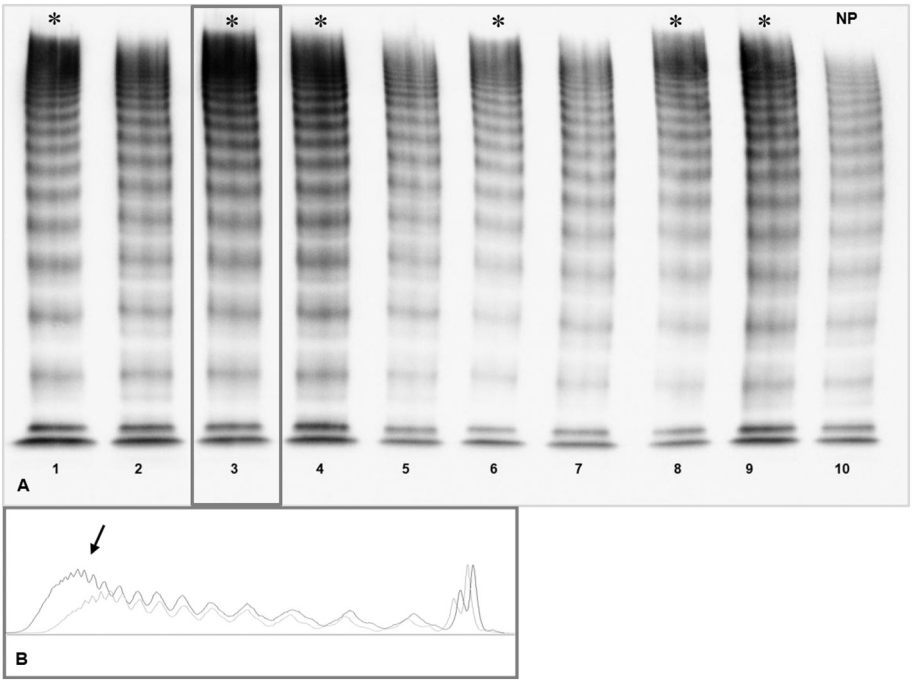

Fig. 1. A) Low-resolution gels show presence of ultralarge multimers (*) in some TTP patients in remission, compared to normal plasma (NP). B) Densitometric tracing of sample 3. Note the ULVWF multimers identifiable with an arrow from a series of high peaks on the left side of the densitogram.

with deficient ADAMTS13 activity levels, high titers of anti-ADAMTS13 IgG, the presence of ULVWF multimers, or the number of relapses.

Over the years, the prognostic role of CFR has become increasingly clear. CFR is a predictive index of major cardiovascular events, including death. This has also been demonstrated in many systemic, inflammatory and endocrine and dysmetabolic diseases, some of which (obesity, diabetes) notoriously exacerbate the cardiovascular risk due not to atherosclerotic disease in the epicardial vessels, but to a microvascular dysfunction. This could be the "link" between systemic inflammatory disease and a higher incidence of cardiovascular events [23,24]. Vianello et al. [25] also showed that the CFR of patients with myeloproliferative neoplasms (essential thrombocythemia and polycythemia vera) was lower than in controls.

Microvascular coronary dysfunction is associated with an annual 2.5% incidence rate of major cardiovascular events, including death, non-fatal myocardial infarction, non-fatal stroke, and congestive heart failure. In the absence of obstructive coronary artery disease or known cardiomyopathy, the traditional risk factors for acute coronary disease are linked by the presence of systemic inflammation, oxidative stress, and immune system activation, which promote the onset of atherosclerosis (endothelium-dependent microvascular dysfunction), and a lower CFR (endothelium-independent microvascular dysfunction), both associated with adverse clinical outcomes.

Table 3
Comparison between patients classified by their normal *vs.* reduced CFR. At least 1 cardiovascular risk factor = current smokers, hypertension, hyperlipidemia, diabetes mellitus; ns = not significant.

	CFR > 2.5	CFR ≤ 2.5	p
N, (%)	11 (65)	6 (35)	–
Mean ± SD	3.1 ± 0.6	2.05 ± 0.3	–
M/F	2/9	2/4	ns
Age, mean ± SD	48.1 ± 13.6	50.2 ± 6.5	ns
At least 1 cardiovascular risk factor	9 (81)	4 (67)	ns
Normal ADAMTS13 activity group	5 (45)	4 (66)	ns
Reduced ADAMTS13 activity group	3 (27)	0	ns
Undetectable ADAMTS13 activity group	3 (27)	2 (33)	ns
Antiplatelet therapy	8 (73)	4 (67)	ns
Previous rituximab	5 (45)	1 (17)	ns
Relapses ≥ 1	7 (64)	2 (33)	ns
ULVWF multimers	7 (64)	2 (33)	ns
Basal peak diastolic velocity, cm/s, mean ± SD	20.2 ± 4.1	16.9 ± 4.1	ns
Adenosine-stimulated peak diastolic velocity, cm/s, mean ± SD	63.8 ± 15	54.2 ± 20.1	ns

None of our TTP patients in remission had evidence of cardiac damage, such as clinical signs, raised troponin levels, electrocardiographic alterations. They did have a slightly increased adenosine-stimulated

Table 2
Comparison between TTP patients and controls. Values are reported as mean ± SD or median (Q1, Q3), and n with frequency (%).

	Controls (n = 50)	Patients (n = 17)	p
Basal heart rate, beats/min	75 (67–82)	75 (63–88)	0.8
Adenosine-stimulated heart rate, beats/min	96 (86–110)	87 (70–91)	0.2
Basal systolic blood pressure, mm Hg	120 (110–127)	120 (102–127)	0.9
Adenosine-stimulated systolic blood pressure, mm Hg	100 (95–115)	105 (97–112)	0.8
Basal diastolic blood pressure, mmHg	80 (67–83)	70 (62–80)	0.3
Adenosine-stimulated diastolic blood pressure, mm Hg	60 (60–70)	70 (60–75)	0.4
Basal peak diastolic velocity, cm/s	22 (18–28)	21 (15–22)	0.7
Adenosine-stimulated peak diastolic velocity, cm/s	84 (64–97)	63 (45–75)	< 0.0001
Mean CFR	3.8 ± 0.7	2.8 ± 0.6	< 0.0001
CFR ≤2.5, n (%)	0 (0)	6 (35)	< 0.0001

Table 4
Comparison between patients classified by their hemostatic and clinical parameters. Patients with reduced ADAMTS 13 activity have not been considered in this analysis; ns = not significant. Two patients presented a relative decrease of multimers and they have not been included in the analysis according presence/absence of ULVWF.

	Normal ADAMTS13 activity group	Undetectable ADAMTS13 activity group	p
N, (%)	15 (62.5)	5 (21)	–
ADAMTS13 activity, %, median (range)	108 (70–130)	12 (7–18)	–
Age, years, median (range)	51 (32–70)	48 (32–51)	ns
M/F	3/12	0/5	ns
Antiplatelet therapy	10 (67)	4 (80)	ns
Previous rituximab	3 (20)	2 (40)	ns
Relapses ≥ 1	3 (20)	5 (100)	0.004
ULVWF multimers	5 (33)	5 (100)	0.038
CFR ≤ 2.5	4 (27)	2 (40)	ns

	Absence of ULVWF multimers	Presence of ULVWF multimers	p
N, (%)	11 (50%)	11 (50%)	–
Age, years, median (range)	46 (32–67)	49 (32–70)	ns
M/F	3/8	1/10	ns
Antiplatelet therapy	6 (54.5)	9 (82)	ns
Previous rituximab	3 (27)	3 (27)	ns
Relapses ≥ 1	2 (18)	8 (73)	0.03
Undetectable ADAMTS13activity group	0	5(45)	0.038
CFR ≤ 2.5	4 (36)	2 (18)	ns

	Relapses < 1	Relapses ≥ 1	p
N, (%)	13 (54%)	11 (46%)	–
Age, years, median (range)	46 (32–70)	49 (32–59)	ns
M/F	3/10	2/9	ns
Antiplatelet therapy	7 (54)	9 (82)	ns
Previous rituximab	1 (8)	6 (55)	0.023
ULVWF multimers	3 (2)	8 (73)	0.03
Undetectable ADAMTS13 activity group	0	5 (45)	0.04
CFR ≤2.5	4 (31)	2 (18)	ns

peak diastolic velocity, although no significant differences were apparent in their baseline rates. In addition, the CFR was more reduced in patients than in controls, indicating an impaired structural microcirculation, or vasodilation of microcirculation, as physiologically expected to occur in conditions of greater cardiac work. Assessing the CFR with adenosine does not enable a distinction to be drawn between endothelium-dependent and endothelium-independent changes, while the finding of impaired structural microcirculation can be considered the first detectable anomaly in the process leading to microvasculopathy.

Although the patients' hemodynamic findings were not significantly pathological, they pointed to an abnormal situation by comparison with the controls, that was more evident when the patients' mean age and the anti-platelet therapy chronically used by the majority of them were taken into account. International guidelines [26] recommend aspirin only in the acute phase of TTP, given the discrepancy between the pathogenesis of microcirculatory thrombus formation and the drug's action.

Reduced CFR is a prognostically negative cardiac index even in non-cardiac patients with or without risk factors. A reduced CFR, being an index of coronary microvascular dysfunction, is reasonably a negative fact in itself. For the same reason, we think that aspirin can be useful, even if we do not recommend its use in all TTP patients in follow-up, according to our results.

For the CFR to have a scientifically-demonstrable role as a marker of coronary microvascular dysfunction in TTP patients in remission, we would need to study larger samples of patients. It is certain that patients who experience more relapses have cardiac microcirculation injury during acute episodes, as demonstrated by their ECG changes and higher troponin I levels [15]. It is therefore conceivable that these patients may suffer from chronic damage, probably associated with lower ADAMTS13 levels, the presence of ULVWF multimers, and a lower CFR. Morici et al. [14] claimed that local factors might influence cardiac involvement in patients with TTP, as well as their typical systemic ADAMTS13 deficiency. This would mean that it may be mandatory to monitor these patients carefully for cardiovascular events even during remission, especially if they have risk factors for coronary disease.

During remission, VWF multimer analysis is not used for prognostic purposes, although it provides a “snapshot” of both VWF activity and ADAMTS13 proteolytic activity. In 1982, Moake et al. [9] found ULVWF multimers in the plasma of TTP patients in remission, whereas the situation in the acute phase is characterized by a relative loss of high-molecular-weight multimers due to multimers being involved in microthrombi. Classifying our cases by their ULVWF multimer patterns, we provide the first demonstration of a statistically significant correlation with previous relapses. This picture is consistent with the finding reported by Lotta et al. that TTP patients' ULVWF multimer ratio was lower in the acute phase, and higher than normal in remission [10]. VWF multimer analysis is not routinely done for TTP patients in remission because it has to be done at specialized centers. A new C.E. certified method to run multimers gel on an equipment present in many biochemical laboratory is now used and, if adequate for the identification of the ULVWF multimers, could make this assay available in many laboratories [44].

Bearing in mind the variability and fluctuation of ADAMTS13 and its inhibitor levels in TTP patients in remission, however, it may be appropriate to use this method as an additional tool to help pinpoint the best time to start relapse prevention treatment.

Rituximab has shown its benefits in patients with acute TTP who experience a suboptimal response to the standard treatment, or even as a front-line option. It aids the episode resolution, reduces PEX requirement, and decreases the 1-year relapse rate by diminishing the production of anti-ADAMTS13 antibodies and restoring.

ADAMTS13 activity [31]. According to recent studies, the use of preemptive rituximab, initiated after observation of a progressive decrease in ADAMTS13 levels, is still debated. Hie et al. [45] showed that preemptive infusions of rituximab in remission significantly decrease TTP relapse rate. On the contrary, Lym et al. [46] recommended against the use of rituximab for this indication because of the marginal benefit for relapse-free survival and due to the not known natural history of ADAMTS13 activity following recovery from acquired TTP. In this study there isn't correlation between ADAMTS13 activity levels and both time from last course of rituximab, that was administrated at least 2 years before, and from last relapse. Considering our findings, clinicians could be encouraged to consider preemptive rituximab in TTP patients in remission with persistent ADAMTS13 deficiency to avoid relapses and to protect coronary microcirculation, especially if other cardiovascular risk factors exist. This study is limited by the small number of patients with an abnormal CFR, which limits its statistical power and prevents us from establishing a precise hematological parameter potentially directly correlating with the CFR. That said, the low incidence of TTP in our local population needs to be considered.

In conclusion, to the best of our knowledge, this study is the first to demonstrate that, when compared with healthy controls, TTP patients in remission have an impaired coronary microcirculation and reveal the presence of ULVWF multimers.

Acknowledgements

I. Di Pasquale designed the research, analyzed and interpreted the data and wrote the manuscript; U. Budde interpreted the data and

critically reviewed the manuscript; U. Budde, I. Di Pasquale, R. Dittmer, S. Schneppenheim performed multimeric analysis of VWF and interpreted the data; AM Lombardi performed ADAMTS13 assays; G. Famoso performed CFR assessment; F. Tona interpreted the data and critically reviewed the manuscript; A. Bertomoro interpreted the data and wrote the manuscript; F. Tona and I. Bertozzi performed statistical analysis; F. Fabris designed the study, interpreted the data and critically reviewed the manuscript. All authors read and critically reviewed the original manuscript and its revised versions.

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