# von Willebrand factor-cleaving protease (ADAMTS13) activity in normal non-pregnant women, pregnant and post-delivery women

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**Summary**

ADAMTS13 dysfunction has been involved in the pathogenesis of Thrombotic Thrombocytopenic Purpura. This disorder occurs more frequently in women and, in 13% of them, is associated with pregnancy. However, there is little information on the protease behaviour in normal pregnancy. We studied von Willebrand factor and ADAMTS13 activity changes in normal non-pregnant, pregnant and post-delivery women. Fifty-five non-pregnant women, normal blood bank donors, who were not taking contraceptive pills were included as controls. A prospective cross-sectional study of 270 normal pregnant and post-delivery women was carried out. ADAMTS13 activity decreased progressively as from the period of 12-16 weeks up to the end of early puerperium (mean 52%, range 22-89, $p < 0.0001$), to increase slightly thereafter. Nulliparous presented mildly lower levels of ADAMTS13 activity than parous women (65% vs. 83 %, p=0.0003), and primigravidae than multigravidae between 6-11 weeks up to 17-23 weeks of pregnancy (69% vs. 80%, p=0.005). Although in all women the protease levels were the same by blood groups, the O blood group non-pregnant women showed a higher mean of ADAMTS13 activity than those non-O (78% vs. 69%, p= 0.064). Our results suggest that the changing levels of protease activity during pregnancy and puerperium, induced by unidentified mechanisms, could render the peripartum time more vulnerable to developed thrombotic microangiopathies.

**Keywords**

ADAMTS13 activity, normal women, pregnancy, puerperium

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## Introduction

Until recently, the pathogenic mechanisms of Thrombotic Thrombocytopenic Purpura (TTP) were poorly described. In 1982, Moake et al. described the presence of unusually large multimers of von Willebrand factor (ULVWF) in plasma of patients with chronic relapsing TTP (1), suggested to play a pathogenic role in the microvascular thrombosis of this disorder. In 1996, a major breakthrough in the understanding of VWF multimer regulation occurred with the discovery of proteolytic activity against VWF in normal plasma (2-3). The protease, which cleaves the peptide bond between Tyr-842 and Met-843 of the mature VWF subunit (4), prevents spontaneous interaction of the largest multimers with platelets. Levy et al.

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provided the first direct evidence of an etiologic role for ADAMTS13 and its genetic deficiency in the pathogenesis of congenital TTP (5).

Adult patients with TTP are most frequently women, 66 to 78%, with 12-25% of episodes associated with pregnancy (6-11). Reports describing sisters from families with congenital TTP, suffering a first TTP crisis during their first pregnancies (12-14), sustain this relationship. Furthermore, in another case, the hormonal dependence of TTP was postulated (15).

In normal pregnancy, Mannucci et al. (16) described a moderate decrease of the protease in the second and third trimester, although no puerperium patients were included. The authors considered that an oestrogen regulation could be one of the mechanisms involved in the ADAMTS13 changes.

The aim of the present study was to determine the ADAMTS13 activity in normal non-pregnant, pregnant and puerperium women, to describe the changes induced by such states and to analyse the relationship between ADAMTS13 activity and clinical features.

# Patients, materials and methods

## Patients

Three hundred and twenty five healthy women were recruited. None of them had preeclampsia, or personal/family history of miscarriage (except induced abortion), bleeding/thromboembolism or hypertension. The Hospital Ethics Committee approved the study and the women provided informed consent. A haematologist and an obstetrician thoroughly interviewed all women to identify haemostatic disorders and gynaecologic/obstetric history. Demographic data (height, weight, blood pressure) and personal habits (cigarette smoking, alcohol/drug intake) were recorded on entry to the study.

Fifty-five healthy non-pregnant women, blood bank donors, of childbearing age (mean 30.4 years, range 20-40), who were not taking oral contraceptive pills, were included as non-pregnant group (41.8% parous women, range 1-9). ABO blood group typing in these women resulted in: 27 (49%) type O, 17 (31%) type A, 8 (14.5%) type B and 3 (5.5%) type AB blood group.

A cohort of 270 consecutive patients (mean age 24.8 years, range 14-41), attending the routine antenatal and postnatal care, from August 2000 to January 2002, was studied. Women were either primigravidae (n = 124, 45.9%) or multigravidae (second pregnancy or more, n = 146, 54.1%) with previous pregnancies free of complications. They were examined once during pregnancy (n = 202) or post-delivery (n = 68). Following delivery, records were checked to confirm that pregnancy and puerperium had been uneventful. In relation to ABO blood group, the distribution of these women was: 158 (58.5%) were O blood group, 85 (31.5%) were A blood group, 19 (7%) were B blood group and 8 (3%) women were AB blood group. Most post-delivery women underwent vaginal delivery, without any anaesthesia, except 5 women from early puerperium and 4 from late puerperium. These women underwent caesarean section for obstetric reasons, all of whom received local anaesthesia. No infections, haemorrhagic or thrombotic complications were recorded during post-delivery periods.

## Materials

Type I collagen from calf skin (C3511), aprotinin, bovine serum albumin (BSA), Tween 20, polyethylene glycol (PEG) 10000, n-ethylmaleimide (n-NEM) (E1271), and 10-mm flat width dialysis tubing (D9277) were from Sigma (St. Louis, MO). Rabbit antibodies to human: VWF (A0082), avidin-biotin-peroxidase complex (ABComplex/HRP, K377) and biotinylated antiserum to rabbit immunoglobulin (E353) were from Dako Corp (Carpinteria, CA). Covalink microtiter plates were from Nunc (Roskilde, Denmark). Remaining reagents were of analytical grade.

## Methods

### Platelet blood count

The platelet blood count (normal value: 150 $10^9$/L–450 $10^9$/L) was determined for each woman at the time of the ADAMTS13 and VWF measurements. Platelets were counted by a Thrombocounter (Coulter Electronics Inc).

### Plasma preparation

Plasma for ADAMTS13 activity evaluation was collected from whole human blood anticoagulated with 12.9 mM sodium citrate. For VWF methods, blood was collected in polypropylene tubes with 129 mM trisodium citrate, 50 mM EDTA, 60 mM n-NEM and 2,000 KIU/ml aprotinin (1 : 10 v/v) (17). Samples were centrifuged by using two-step centrifugation (2,500 × g, 20 min) at 20°C. Samples were aliquoted and frozen at -80°C until analyzed.

A pool of healthy women (n = 26) – non-pregnant, and not taking contraceptives pills – and healthy men (n = 22) were used as control (pool normal plasma: PNP). Control subjects were different from those who comprised the healthy population under study. The standard pool was calibrated for VWF:Ag and VWF:Rco against the International Reference Preparation for Factor FVIII related activities in plasma (IRP), National Institute for Biological Standards and Control, London, UK.

### VWF methods

VWF:Ag was measured by immunoelectrophoresis technique (18). Comparison of results obtained by this method for VWF:Ag and by ELISA assay closely correlated (17).

## Purified VWF

VWF was isolated from fresh frozen plasma of normal volunteers following the procedures described by Thorell and

Blombäck (19). The cryoprecipitate obtained was dissolved in 55 mM sodium citrate buffer, 5 mM EDTA, pH 7.4, at room temperature, and purified by gel filtration with Sephacryl S-1000. Elution was performed with 55 mM sodium citrate buffer, 5 mM EDTA, pH 7.4. Fractions were collected at a flow rate of 3 ml/h. $cm^2$. Eluted fractions containing VWF were concentrated using 20% PEG 10,000 in 150 mM NaCl (pH 8.5). The substrate was then dialyzed against 150 mM NaCl, to eliminate EDTA.

### Assay of ADAMTS13

Plasma samples for ADAMTS13 assay were diluted (1 : 5, 1 : 10) with 5 mM Tris-HCl buffer, 150 mM NaCl (pH 8.3) and full protease activation of 120-µl aliquots achieved by preincubation for 30 min at 37°C with 10 mM $BaCl_2$. PNP aliquots of 120 µl diluted from 1/10 to 1/320 in 5 mM Tris-HCl buffer, 150 mM NaCl (pH 8.3) (also incubated with $BaCl_2$) were used for the calibration curve. Purified VWF (0.5 IU/ml) containing 10 mM $BaCl_2$ was added to each sample, and then incubated overnight at 37°C in dialysis tubing against 1500 mM urea, in 5 mM Tris-HCl buffer, pH 8.3 (20). After dialysis for 120 min against 150 mM NaCl, digestion was stopped by addition of 20 mM EDTA (final concentration).

Sample collagen binding activity was determined by the method of Gerritsen et al. (21) with some minor modifications. We used anti-VWF (30 µg/ml), biotinylated antiserum to rabbit immunoglobulin (1 µg/ml) and avidin-biotin peroxidase complex (22) instead of peroxidase conjugated anti-VWF. The collagen type I from calf skin was used because Hubbard et al. (23) and Neugebauer et al. (24) suggested that pepsin-digested collagen (human type III, used by Gerritsen) revealed a higher affinity for low and medium VWF multimers.

An absorbance curve (492 nm) vs. % ADAMTS13 of PNP dilutions with unfolded purified VWF was plotted to interpolate sample absorbance values and extrapolate ADAMTS13 percentage. The activity of ADAMTS13 in a PNP dilution of 1/10 was arbitrarily defined as 100%. Inter-assay variation, expressed as between-run coefficient of variation (CV) was determined using a normal and a chronic relapsing TTP samples, frozen in aliquots and subsequently thawed and tested repeatedly on different days. Intra-assay variation (also expressed as within-run CV) was determined using those samples, tested repeatedly on the same day. The between-run CV (n = 8) was: normal 7.8% and TTP patient 15.4%. The within-run CV (n = 8) was: normal 4.5% and TTP patient 14.2%.

### Data analysis

Pregnant and post-delivery women were classified into 8 periods (25), 6 during gestation (weeks 6-11, 12-16, 17-23, 24-28, 29-35, 36-40) and 2 during early and late puerperium, to identify any significant differences in protease levels and to describe normal changes in ADAMTS13 activity. Samples from early puerperium were taken during 1-3 days post-delivery (mean = 1.5), while the women were inpatients in maternity unit. Samples from late puerperium were taken at 7-21 days post-delivery (mean = 15.6), during the first outpatient visit for post-delivery control. For descriptive purposes the values of each measurement are given as means ± standard deviation (SD) and ranges for each subgroup. The correlations coefficients (2-tailed) were determined to evaluate the relationship between quantitative variables. One-way analysis of variance was performed to ascertain the relationship between ADAMTS13 means of different groups of women (during pregnancy and puerperium). Mann-Whitney test was carried out to compare means of protease activity (non-pregnant *vs.* each period of pregnancy and puerperium, O *vs.* non-O blood group, nulliparous *vs.* parous women, primigravidae vs. multigravidae in each period of pregnancy, and smokers *vs.* non-smokers). Statistical significance was set at $p < 0.05$. All analyses were performed with the SPSS 10.0 package for Windows.

## Results

The study included 325 women (55 non-pregnant, 202 pregnant, 68 puerperium). Mean, ranges and SD of ADAMTS13 activity and platelet count for each period are shown in Table 1. Mean ADAMTS13 activity decreased slightly and progressively during pregnancy, from the 12-16 week period up to the end of the early postnatal period ($p < 0.0001$, one-way analysis of

**Table 1: ADAMTS13 activity and platelet blood count in non-pregnant, pregnant and post-delivery women.** Mean (%), range and standard deviation (SD) for each studied period.

| Period | N | ADAMTS13 (%) Mean and range | SD | Platelet Count ($10^9$/L) Mean and range | SD |
|---|---|---|---|---|---|
| **Non-pregnant** | 55 | 74<br>36-114 | 19 | 282<br>165-461 | 75.4 |
| **6-11 weeks** | 30 | 87<br>58-123 | 18 | 282<br>157-447 | 78.5 |
| **12-16 weeks** | 43 | 72<br>30-116 | 18 | 255<br>83-537 | 102 |
| **17-23 weeks** | 30 | 65<br>28-88 | 15 | 252<br>80-584 | 109 |
| **24-28 weeks** | 28 | 61<br>30-88 | 14 | 271<br>140-541 | 113 |
| **29-35 weeks** | 43 | 65<br>28-95 | 16 | 285<br>156-559 | 77 |
| **36-40 weeks** | 28 | 58<br>29-87 | 13 | 277<br>182-444 | 75.3 |
| **Early puerperium** | 31 | 52<br>22-89 | 16 | 292<br>97-507 | 90.2 |
| **Late puerperium** | 37 | 102<br>67-161 | 25 | 301<br>138-592 | 94.1 |

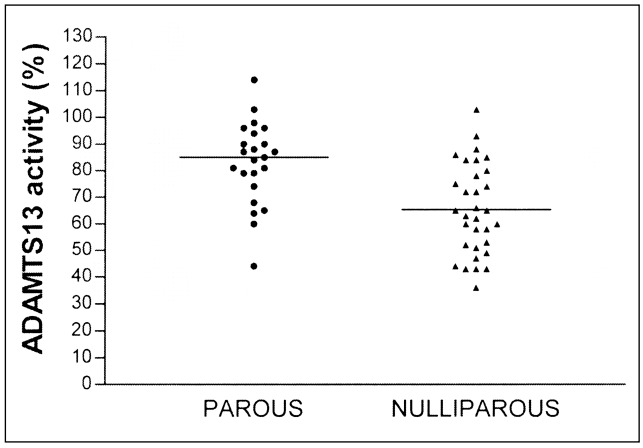


**Figure 1: ADAMTS13 activity in non-pregnant women.** ADAMTS13 activity (%) in nulliparous and parous non-pregnant women.

variance). In a paired comparison between the non-pregnant state and the 6-11 week period of pregnancy, there was no statistical difference in the levels of ADAMTS13 activity ($p = 0.1$, Mann-Whitney test), but starting from the 12-16 week, group differences became significant ($p = 0.013$, Mann-Whitney test). The late postpartum showed the highest level of protease activity, including in the non-pregnant state ($p < 0.0001$, Mann-Whitney test). In non-pregnant women, protease activity was significantly lower in nulliparous (mean 65%, range 36-103) compared to parous women (mean 83%, range 44-114) ($p = 0.0003$, Mann-Whitney test) (Fig. 1). In pregnant and post-delivery women, mean ADAMTS13 activity was slightly lower in primigravidae than in multigravidae (68% *vs.* 74%, $p = 0.192$, Mann-Whitney test), although not significantly (Fig. 2). As it appears in figure 2, the relationship between parity and protease levels was strong in the 3 first periods of gestation: between

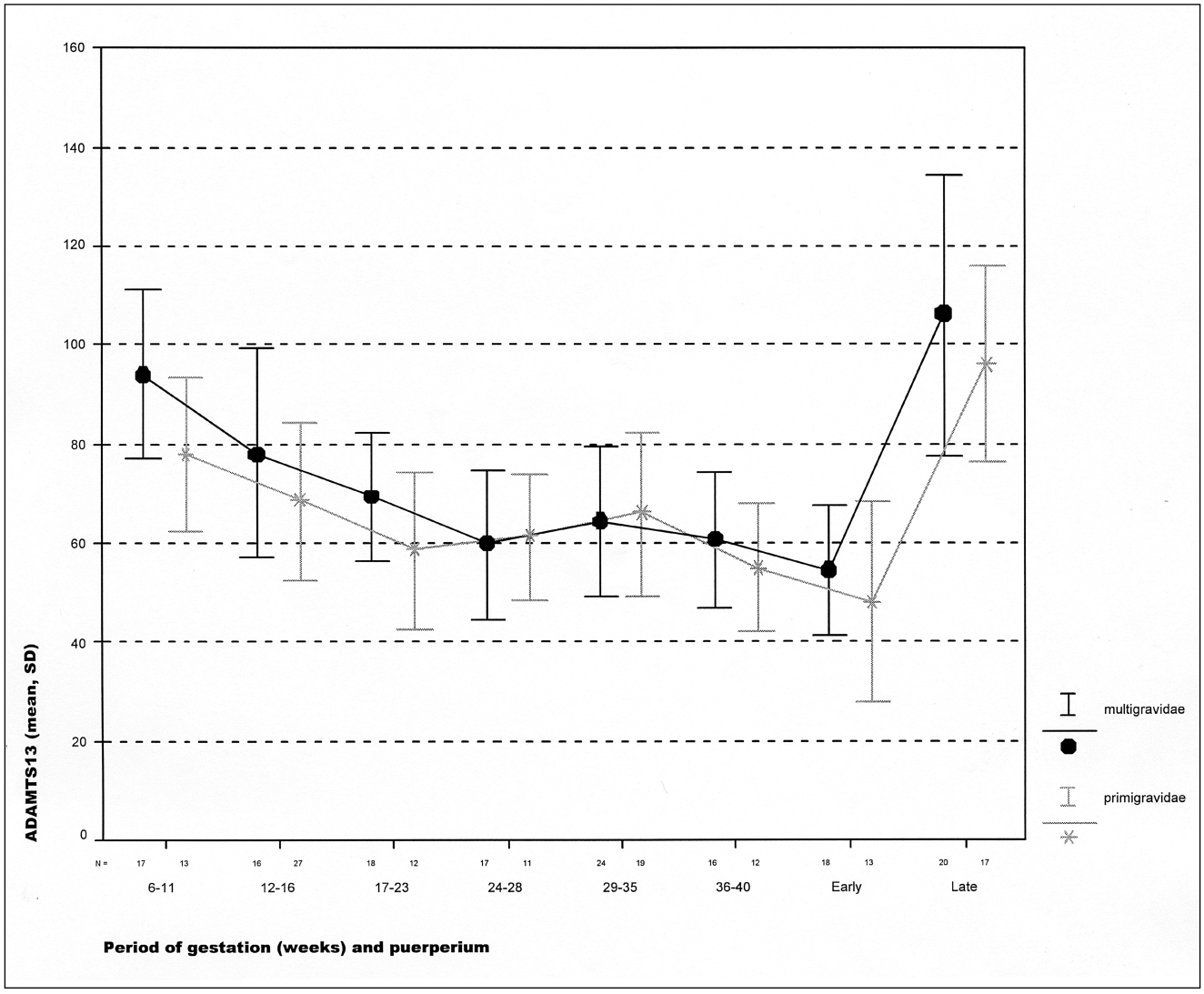


**Figure 2: ADAMTS13 activity in pregnant women.** ADAMTS13 activity (%) in primigravidae and multigravidae pregnant women.

6-11 weeks up to 17-23 weeks of gestation, primigravidae women showed lower ADAMTS13 levels than multigravidae (69% *vs.* 80%, p = 0.005, Mann-Whitney test). VWF:Ag was no different according to parity in the non-pregnant women (nulliparous *vs.* parous, p = 0.91, Mann-Whitney test) and the pregnant/post-delivery women (primigravidae *vs.* multigravidae, p = 0.96, Mann-Whitney test) (data not shown).

A significant correlation between higher levels of VWF:Ag with lower ADAMTS13 activity was observed during gestation (CC -0.217, p = 0.0003, Pearson's correlation) as in non-pregnant women (CC -0.342, p = 0.039, Pearson's correlation) (data not shown).

On evaluating the influence of blood group, ADAMTS13 activity in non-pregnant women was higher in the O (80%) than in the non-O group (69%), though the difference was not significant (p = 0.064, Mann-Whitney test). The data of ADAMTS13 activity in non-pregnant women by blood group are delineated in table 2. In pregnant and post-delivery women, there was no difference in ADAMTS13 activity for the different blood groups, in every period of gestation.

The platelet count remained unchanging throughout pregnancy and puerperium, and it was not different from non-pregnant state (p = 0.318, One Way ANOVA test). Besides, no correlation between protease activity and platelet count was found (CC 0.03, p = 0.626, Pearson's correlation). Thirteen pregnant and post-delivery women (4.8%) presented mild thrombocytopenia (platelet count below 150 $10^9$/L, range 83-146 $10^9$/L): 6 were from 12-16 weeks period, 2 from 17-23 weeks, 3 from 24-28 weeks and one was from early and late puerperium each one. There were no significant causes for thrombocytopenia in this group of women. ADAMTS13 activity of women with thrombocytopenia was no different than the mean from each correspondent period.

There were no correlations between ADAMTS13 activity and age or height (CC = 0.008, p = 0.9 and CC = -0.053, p = 0.42, respectively) in all groups of women (data not shown). A significant correlation between higher weight with lower ADAMTS13 activity was observed (-0.157, p = 0.011, Partial correlation, controlling for the different periods). In pregnant women, the protease activity was significant higher in smokers (n = 30) women than in no-smokers (n = 240) (79% ± 25 vs. 70% ± 23, p = 0.045, Mann-Whitney test).

**Table 2: ADAMTS13 activity in non-pregnant women by ABO blood group.**

| ABO Blood Group | n | Mean ADAMTS13 activity (%) | SD |
|---|---|---|---|
| O | 27 | 77 | 18.6 |
| A | 17 | 66.5 | 15 |
| B | 8 | 67 | 16 |
| AB | 3 | 84 | 33 |

## Discussion

TTP has been an intriguing disease, with unknown pathogenic mechanisms, and frequently fatal outcome, up to the introduction of plasma therapy. Although in 1982, the presence of ULVWF plasmatic multimers in TTP patients was described (1), just recently a new model of disease has been presented (26). In this model, the severe congenital or autoimmune-mediated dysfunction of ADAMTS13 prevents normal VWF proteolysis, resulting in circulating ULVWF. These multimers, because of their ability to induce platelet aggregation at high shear stress, could be responsible for the microvascular thrombosis.

Adults with TTP are frequently women, particularly in the third trimester of pregnancy and postpartum (6-12, 27). However, there is scanty information on ADAMTS13 in normal pregnancy. We present data in a large and homogeneous group of healthy non-pregnant, pregnant, and post-delivery women. In the first report on VWFCP activity during normal pregnancy, Mannucci et al. (16) found lower levels of protease activity in second and third trimesters compared to the first. However, no women were evaluated during puerperium. Recently, Lattuada et al. (28) reported lower ADAMTS13 activity in acute phase of patients with HELLP syndrome compared to normal pregnant women (third trimester) and normal non-pregnant women.

In our series, ADAMTS13 activity showed mild but progressive decreasing levels from the 12-16 weeks period of pregnancy up to the end of the early postpartum period, when the lowest levels were observed. Protease activity proved normal in the first period of pregnancy, 6-11 weeks, and the late puerperium period, when reaching values slightly higher than those of non-pregnant women. Mannucci et al. (16) and Lattuada et al. (28) have ascribed the progressive decrease in protease activity during gestation to higher VWF levels, as a mechanism of protease consume by the higher substrate levels. Besides, the inverse relationship between protease activity and VWF level was recently reported in healthy centenarians (29). Another setting featuring an acute increase in VWF levels, but in the short term, occurs secondary to desmopressin infusion, when ADAMTS13 activity decreases to about half initial value (30), although authors failed to specify whether the excess of substrate exhausted the ADAMTS13 or if it was taken up and cleared from plasma. Our results, according to these previous observations, showed a strong relation between VWF and ADAMTS13 activity.

Alternatively, the feasibility of oestrogen control over protease levels has been postulated (16). One of the most remarkable findings in our series, was that the parity affected ADAMTS13 activity, not only in non-pregnant but also in preg-

nant women. In non-pregnant women we found significantly lower levels of protease activity in nulliparous compared to those of parous women. Moreover, in the nulliparous group, ADAMTS13 activity reached values as low as 36%, but except for a single parous woman, with a protease activity of 44%, it remained over 60% in the rest of this group. The primigravidae presented lower protease levels than the multigravidae, from the 6-11 weeks up to the 17-23 weeks periods of gestation. Previous studies (31-32) had shown that maternal oestradiol levels were significantly affected by parity, it has been higher in the nulliparous and first pregnancy than in the parous women and second pregnancy, respectively. As shown in a previous study on VWF in normal pregnancy from our group (25), the VWF levels in all women were no different by parity (data not shown). Although, the oestrogen levels were not evaluated in our present study, and not only oestrogen is affected by parity, the hormonal changes or one major hormone are attractive factors that deserve to be explored.

The ADAMTS13 activity, in pregnant and post-delivery women, was no different between O and non-O blood groups. Although in non-pregnant women there appeared to be higher protease activity in O blood group women, numbers were too small to be statistically meaningful. Bowen reported (33) that the rate of VWF proteolysis by ADAMTS13 was greater for VWF from O blood group than for non-O VWF. As it has been suggested (34), exploring the relation between VWF, ABO blood group and ADAMTS13 in a normal population may be useful to better understanding the mechanism that regulates VWF variability.

Normal pregnancy is associated with a mild, and generally unappreciated decrease in the circulating platelet count, by an average of 10%. Most of this decrease occurs during the third trimester (35). The thrombocytopenia may reflect the effects of haemodilution or accelerated platelet clearance. In our series, the platelet count was stable throughout all groups, and no relationship between platelet count and ADAMTS13 was observed, even in the women with thrombocytopenia.

The individual risk to developing TMA is not predictable, including the risk associated with pregnancy. A recent study on a cohort of 105 patients with classical TTP (36), found that obesity was a risk factor (odds ratio: 7.6). Our finding, that a higher weight was associated to lower protease levels could explain this association. Women who smoked appeared to have a higher ADAMTS13 levels; however, the number of these women was too small to be clinically meaningful. Exploring the relation between smoking and ADAMTS13 activity may be helpful in better understanding the controversial protective effect of tobacco against preeclampsia.

As it was reviewed by George JN (27), pregnancy is a precipitating event for TTP acute crisis. Most acute episodes of TTP-HUS during pregnancy have been described near term or postpartum (27). In our cohort, the 36-40 weeks of gestation and early puerperium periods were characterized by the lowest ADAMTS13 activity, so that it could be speculated that the protease changes may be a predisposing factor for the higher occurrence of TTP acute episodes near the term of pregnancy.

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