

## A Subset of African-American Type 2 Diabetics has Markedly Elevated Factor VIII Levels

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A Subset of African-American Type 2 Diabetics  
has Markedly Elevated Factor VIII Levels

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## Abstract

**Aims** - We have previously found that otherwise healthy African-American type 2 diabetics have shorter activated partial thromboplastin times (aPTT) and higher plasma prothrombin levels than non-diabetic subjects recruited from the same community. The current study was designed to assess what parameters were associated with a shortened aPTT in the study population.

**Methods** – Stored aliquots of previously assayed specimens (n=27 diabetics and 13 non-diabetics) were assayed for additional parameters of endothelial, inflammatory and procoagulant relevance. The results were evaluated using a statistical technique for analysis of high dimension, low sample size data.

**Results** – We found that in the majority of diabetics, the level of coagulation factor VIII (FVIII) was moderately elevated and was correlated with the prothrombin level. Six of the 27 diabetic subjects had extremely high FVIII levels. The variables that most effectively discriminated between these two groups were serum iron, HDL cholesterol (negative), total plasma protein, aPTT (negative), plasma folate (negative), and plasma homocysteine. Elevated FVIII was not likely to be due to a generalized inflammatory state, since other acute phase reactants, fibrinogen and CRP, were not higher in this subset.

**Conclusions/interpretation** – Both elevated prothrombin and FVIII likely contribute to short aPTTs in diabetics. A short aPTT and elevated FVIII are each associated with thrombosis. Thus, a subset of diabetics with greatly elevated FVIII may be a particular risk of thrombosis. We speculate that this could be a reflection of oxidant stress related to increased iron availability.

**Keywords** – *blood coagulation, oxidant stress, factor VIII, partial thromboplastin time, type 2 diabetes, iron*

## Abbreviations

coagulation factor VIII - FVIII; activated partial thromboplastin time – aPTT; C-reactive protein – CRP; alanine amino transferase - ALT; body mass index – BMI; glycated hemoglobin - HbA<sub>1c</sub>; complete blood count – CBC;

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**Introduction**

We have previously reported that African-American subjects with type 2 diabetes have shortened activated partial thromboplastin times (aPTT) and elevated plasma prothrombin levels [1]. However, we found no differences in antithrombin, fibrinogen and plasminogen activator inhibitor-1 (PAI-1) levels between subjects with and without diabetes. A shortened aPTT as been associated with the risk of thrombosis [2]. Therefore, we felt that short aPTTs in the non-hospitalized diabetics in our study likely reflected an increased risk of thrombotic events, and the mechanism of the shortened clotting times deserved further study. While elevated prothrombin alone could shorten the aPTT and predispose to thrombosis, we wanted to identify other variables that might be associated with a short aPTT or contribute to the risk of thrombosis in otherwise healthy diabetics.

**Methods**

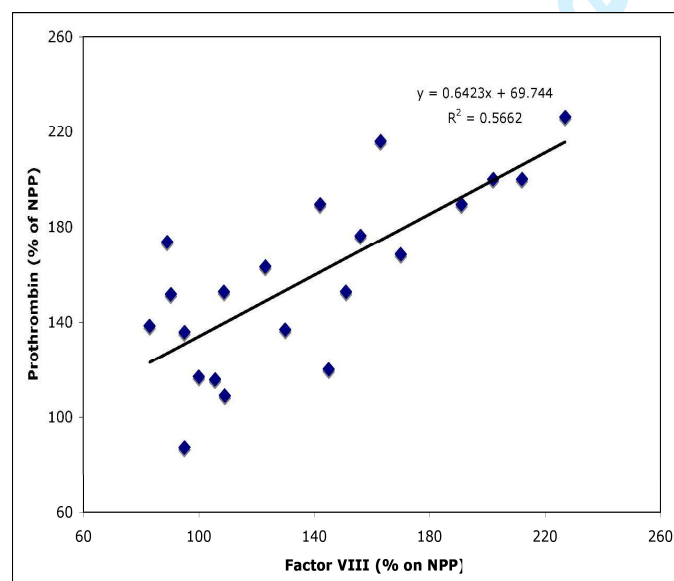
The samples used in this study were from the first 40 of 81 subjects recruited into a trial on the effects of muscadine grape juice on glycemic control. Subjects were African-Americans recruited from local medical clinics, community centers and churches. This study was conducted under a protocol approved by the Institutional Review Board of North Carolina State University. Details of this study have been published previously [3]. Blood samples were collected before and after a period during which some subjects consumed wine or grape juice daily. Blood samples collected before the dietary intervention were used for our study. The classification of subjects as diabetic was initially by self reporting and verified based on fasting glucose, insulin and glycated hemoglobin (HbA<sub>1</sub>C) levels.

Samples were subjected to a battery of clinical laboratory tests, including a complete blood count (CBC), chemistry panel, plasma homocysteine, plasma folate, plasma vitamin B<sub>12</sub>, C reactive protein (CRP), and a lipid panel, at the time of collection. These assays were run in the Clinical Hematology and Chemistry Laboratories of the Durham VA Medical Center, Durham, North Carolina in a manner identical to usual patient samples. Residual plasma and serum samples were frozen and stored at -80°C for later assays.

Factor VIII was measured by ELISA (Affinity Biologicals, Ontario, Canada), as was plasma thrombomodulin (Cell Sciences, Canton, MA). Thrombin generation was assayed using the calibrated automated thrombogram (CAT assay, Diagnostica Stago).

## Results

Initial analysis was done to assess the FVIII levels, since the FVIII has been associated with an increased risk of thrombosis in the general population [4]. We found that, indeed, FVIII levels were significantly higher in diabetic than non-diabetic subjects;  $173 \pm 44$  vs  $99.1 \pm 40$  % of the value in normal pooled plasma ( $p < 0.003$ ). In addition, 6 of the 27 diabetics had remarkably high FVIII levels ( $\geq 299\%$ ). This was the maximum level detectable in the assay. Because of the limited amount of sample available, serial dilutions could not be performed to precisely determine the FVIII levels in these subjects. None of the control subjects had a FVIII level this high, and only one of the 13 controls had a FVIII level above 155%. The aPTT was shorter in the extremely high FVIII group at  $22.6 \pm 2.9$  compared to  $25.8 \pm 3.0$  seconds in the rest of the diabetic



**Figure 1. Relationship between prothrombin and FVIII levels in diabetics.** There was a significant correlation between FVIII and prothrombin levels in diabetic subjects that did not have extremely elevated FVIII levels ( $R=0.75$ , Pearson correlation coefficient). The values are expressed relative to the level in normal pooled plasma (=100%)

subjects. This led us to speculate that the subjects with exceptionally high FVIII levels might be at particularly great risk for thrombosis. It also led us to search for a correlation between an exceptionally high FVIII level and any of the other analytes assayed.

We found a significant correlation between prothrombin and FVIII levels in the diabetic subjects, as shown in figure 1. This finding suggests that similar mechanisms control both FVIII and prothrombin levels in most diabetics. However, the subset of diabetics with extremely high FVIII did not have similarly high prothrombin

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levels. In fact, the average prothrombin level was identical for diabetics with exceptionally high FVIII and with moderate FVIII ( $158\pm 21$  vs  $158\pm 44$  %). There was also no correlation between prothrombin and FVIII in control subjects. This suggests that a different stimulus – one that does not affect the prothrombin level - is responsible for the extremely high FVIII levels.

We used statistical analysis as a hypothesis-generating technique to try to identify parameters that might be associated with the extremely high FVIII levels is a subset of diabetic subjects.

As a first step, the data set was subjected to multivariate analysis using Distance Weighted Discrimination (DWD) [5]. This technique allows the analysis of high dimensional and low sample size data. DWD focuses on two-class discrimination (in this case extremely high FVIII vs moderate FVIII classes) in the multidimensional space formed by the data. The objective is to find a direction, a loading vector, that maximizes the separation between the two groups. Each component of the loading vector corresponds to one variable. The variables used in this analysis were: Abdominal girth, alkaline phosphatase, alanine amino transferase (ALT), Amylase, aPTT, antithrombin, diastolic and systolic blood pressure, Body Mass Index (BMI), blood urea nitrogen (BUN), total cholesterol, creatinine, fibrinogen, folate, hemoglobin A1c, hematocrit, homocysteine, HDL cholesterol, insulin, iron, lactate dehydrogenase (LDH), LDL cholesterol, lipase, magnesium, monocyte count, platelet count, PT, RBC count, total plasma protein, plasma thrombomodulin, triglycerides, uric acid, vitamin B<sub>12</sub>, vonWillebrand Factor and parameters of thrombin generation. Some of the parameters were log transformed to produce a more normal distribution of values. The DWD technique does not assign a *p*-value to individual variables. Loading vectors were obtained for 39 variables; with those of greatest magnitude being best able to separate the high and moderate FVIII groups.

The 6 parameters from this analysis with the loading vectors of the greatest magnitude were: serum iron, HDL cholesterol (negative), total plasma protein, aPTT (negative), plasma folate (negative), and plasma homocysteine. A positive loading value indicates that the subjects with High FVIII have higher levels for the given variable; a

negative loading indicates a lower value.

The second step was to project the assay results on the DWD vector to obtain a two sample  $t$ -statistic. The Direction Projection Permutation (DiProPerm) test is used to assess the significance of the statistic [6]. All of the data from each assay and each subject were randomly relabeled into two classes. This permutation was performed 100 times, and each time produced a new  $t$ -statistic. The empirical  $p$ -value corresponds to the proportion of the  $t$ -statistics of the permuted data at, or above, the  $t$ -statistic for the actual data set. (<http://www.stat.colostate.edu/~chihoon/FDAratPMdata.pdf>). The  $t$ -statistic was not significant at  $p=0.2$ . However, one subject appeared to be a clear outlier on graphical representations of the data. On review, this subject had listed an antibiotic as one of her medications, suggested she suffered from an infection at the time of the blood draws. If true, she should have been excluded on that basis. When this subject was removed from the analysis the  $t$ -statistic was significant at  $p=0.05$ .

We also re-evaluated the data from all of the subjects using only the nine variables of greatest importance in distinguishing the high and moderate FVIII groups in the initial DWD analysis: folate, total plasma protein, magnesium, endogenous thrombin potential, log homocysteine, hematocrit, HDL, aPTT and serum iron. The DiProPerm analysis of that data set revealed the  $t$ -statistic to be significant at  $p=0.05$ .

**TABLE 1 Values of Selected Analytes in Diabetic Subjects**

	<b>High FVIII group</b> n=6	<b>Moderate FVIII group</b> n=21
<b>FVIII (%)</b>	$\geq 296$	$137 \pm 44$
<b>Prothrombin (%)</b>	$157 \pm 21$	$158 \pm 37$
<b>Iron (mg/dL)</b>	$74.3 \pm 34$	$65.1 \pm 16$
<b>Folate (ng/mL)</b>	$9.1 \pm 3.6$	$12.7 \pm 4.8$
<b>Homocysteine (uM)</b>	$14.9 \pm 5.7$	$11.8 \pm 3.8$
<b>aPTT (seconds)</b>	$22.6 \pm 2.9$	$25.8 \pm 3.0$
<b>Total protein (g/dL)</b>	$7.4 \pm 0.5$	$6.7 \pm 1.0$

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Table 1 summarizes key parameters that distinguished the two groups. There was no difference in HbA1c, insulin and fasting glucose between the groups. However, there were potential differences in iron, folate, total protein, and homocysteine. The elevated total protein likely reflects increased immunoglobulins, since the level of albumin (the other bulk plasma protein) did not differ. However, acute phase reactants (fibrinogen and CRP) were not different between the groups.

We had expected that high FVIII levels might reflect endothelial damage. However, thrombomodulin (TM), a marker of endothelial injury, was not different between groups.

**Discussion**

Mounting evidence suggests that iron plays a role in the pathogenesis of type 2 diabetes [7]. Elevated body iron stores have been linked to inflammation and to atherothrombotic events and mortality [8]. This leads us to speculate that high iron stores or a high level of plasma iron may be an underlying cause of the abnormalities in the high FVIII subgroup of diabetics. Iron can catalyze electron transfer reactions to produce potent oxidants and other free radicals. Oxidants can inactivate folates [9]. Low folate levels lead, in turn, to elevated homocysteine. Elevated homocysteine has been linked to elevated FVIII levels [10]. While we cannot draw any conclusions on causation, our results suggest that further studies are indicated to examine the link between iron and markers of thrombotic risk in diabetics.

We conclude that in our study population of type 2 diabetics: 1) FVIII and prothrombin are elevated; 2) FVIII and prothrombin levels are highly correlated in diabetics and may be driven by a common mediator; 3) both FVIII and prothrombin contribute to a shortened aPTT; 4) a subset of diabetics has extremely high FVIII, which may put them at especially high risk of thrombosis; 5) extremely high FVIII levels are associated with high serum iron, homocysteine, and total plasma protein, and low folate. We hypothesize that this constellation of findings may be associated with iron-related oxidant stress.



## ***Acknowledgements***

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