This is an author produced version of a paper published in Journal of thrombosis and haemostasis. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:
Lethagen, Stefan.
"Genetic analysis of 31 Swedish type 1 von Willebrand disease families reveals incomplete linkage to the von Willebrand factor gene and a high frequency of a certain disease haplotype. "
Journal of thrombosis and haemostasis, 2005, Vol: 3, Issue: 12,
pp. 2656-63.

http://dx.doi.org/10.1111/j.1538-7836.2005.01631.x

Access to the published version may require journal subscription.
Published with permission from: Blackwell

Genetic Analysis of 31 Swedish Type 1 von Willebrand Disease Families Reveals Incomplete Linkage to the von Willebrand Factor Gene and a High Frequency of a Certain Disease Haplotype

Running Head: Genetic Analysis of von Willebrand Disease

E. LANKE,* A. M. JOHANSSON,† C. HALLDÉN,§ S. LETHAGEN*

*Department for Coagulation Disorders, University Hospital, Malmö, Sweden; †Department of Cell and Organism Biology, Lund University, Sweden; §Department of Clinical Chemistry, University Hospital, Malmö, Sweden

Correspondence: Stefan Lethagen, Department for Coagulation Disorders, Malmö University Hospital, SE-205 02 Malmö, Sweden. Tel.:+46 40 332374; fax: +46 40 336255; e-mail: Stefan.Lethagen@med.lu.se

Summary

Background: The most common type of von Willebrand disease (VWD), type 1, has in only a few cases been explained by an identified causative mutation in the von Willebrand Factor (VWF) gene. The ABO blood group and other modifier loci outside the VWF gene may contribute to the development of type 1 VWD. *Objectives and Methods:* Our aim was to determine whether there was genetic linkage to the VWF gene in 31 Swedish type 1 VWD families. Stringent diagnostic criteria in accordance with ISTH guidelines were used. Genetic linkage was investigated by using two highly informative dinucleotide microsatellite markers, which we have recently identified, located in introns 6 and 15 of the VWF gene. We also investigated the existence of common disease haplotypes and the relation between type 1 VWD and ABO blood group. *Results:* We found genetic linkage to the VWF gene in 27 (87%) of the families. However, in 4 (13%) of the families there was clearly no genetic linkage. We found the 4751A>G (Tyr1584Cys) sequence variation in exon 28, which is a common mutation in the Canadian VWD population (14.3%), in only 1 of the 31 families (3.2%). A possible common mutation was identified in 6 of the 27 (22%) families with genetic linkage. Blood group O was over-represented among type 1 VWD patients. *Conclusion:* We conclude that there is linkage between the VWF gene and hereditary type 1 VWD in a majority of families.

Keywords: ABO**,** coagulation, genetic linkage, microsatellite marker, von Willebrand disease

Introduction

VWD is a congenital bleeding disorder, which can be manifested phenotypically in different ways. The classification divides VWD into three categories: type 1, 2 and 3 [1]. Type 1, which is inherited as an autosomal dominant trait and accounts for approximately 70-80% of all cases, is characterized by mild to moderate bleeding symptoms and a quantitative reduction of the VWF. This type of VWD is inherited with variable expressivity and penetrance; in one study up to 50% of obligatory carriers of type 1 VWD were found to be completely asymptomatic [2].

Even though type 1 VWD is the most prevalent subtype, few mutations have so far been identified in type 1 patients. There are several explanations for this shortcoming. The size of the gene − it is approximately 178 kb long and contains 52 exons [3], the existence of a pseudogene [4], incomplete and variable penetrance, and clinical heterogeneity are factors that all lead to difficulties in correctly diagnosing this type of VWD and are part of the explanation for the low number of identified causative mutations. In addition, defects in the VWF gene may not always be the sole cause of the disease; modifier genes are thought to exist [5,6]. It is a well-known fact that ABO blood group significantly affects VWF plasma levels [7,8]. Mean VWF antigen levels for type O individuals are approximately 25% below and for type AB individuals 25% above the level for a pool of normal donor plasma [9]. Recently the metalloprotease ADAMTS13 was suggested to be involved in this phenomenon [10,11]. It has been suggested that ABO blood group is more influential in families with incomplete penetrance and mild phenotypes than in families with complete penetrance [12]. However it is still unclear whether the ABO influence can explain all of these so far unexplained cases, or whether additional loci are involved [13]. In addition, age, the inflammatory response, environmental factors including physical and psychological stress,

and hormonal changes such as during the menstrual cycle and pregnancy, influence VWF levels [14,15]. All of these factors contribute to the difficulty of properly diagnosing type 1 VWD. Obviously, different VWF mutations and the allelic composition of other loci, one or several, together determine the phenotypic effect at the level of the individual. It is therefore of great interest to identify and more closely study non-co-segregating families since this may allow the identification of additional factors affecting the VWF level. Another interesting feature of type 1 VWD is the existence of certain disease-causing mutations present at high frequency among affected individuals. A common mutation, the 4751A>G (Tyr1584Cys) sequence variation, appeared in 14.3% of the investigated families of a Canadian population [16].

This study investigates 31 families from a Swedish population for (1) co-segregation between VWF gene haplotypes and type 1 VWD, (2) the existence of common disease-associated haplotypes and (3) the relation between type 1 VWD and ABO blood group.

Materials and Methods

Patients

All patients diagnosed with type 1 VWD at Malmö University Hospital are included in a register. The current study includes families from the register where the index case fulfils diagnostic criteria according to ISTH [17] − with low levels of VWF in plasma, with a normal multimeric VWF pattern, significant bleeding symptoms, and with at least one more family member diagnosed with type 1 VWD or having significant bleeding symptoms. In families with only two affected members these should not have a direct parent-offspring relationship.

We used historical VWF levels usually determined at the time of the original diagnostic work-up. There were no further analyses of VWF levels in this study. Therefore, different phenotypical methods have been used. VWF activity was measured with the traditional VWF:RCo method based on aggregation of formalin-fixed platelets, or an automated VWF:RCo assay based on the BCS coagulation analyser using the BC von Willebrand reagent (Dade Behring Inc., Newark DE) containing lyophilised platelets, ristocetin and EDTA. VWF antigen levels (VWF:Ag) were measured with Electroimmunoassay (Laurell) and IRMA, ELISA or LIA. We included 31 families (Table 1), with 5-26 members per family and a mean family size of 10.5, who fulfilled our inclusion criteria. All individuals responded to a questionnaire, which was used mainly to identify family members and their relationship to each other, and to extend the pedigrees. The questionnaires also included questions about bleeding symptoms. In total 325 individuals were included. All individuals were re-evaluated and only those that had either low VWF levels or significant bleeding symptoms according to ISTH criteria [17] or both, were classified as "affected", in total 127 individuals. All other individuals were classified as "others". Blood samples were taken at the patients´ regular hospital and sent to us. Samples were stored frozen. The ethical committee of Lund University and the Swedish Data Inspection Board approved the study and informed consent was obtained from all participating individuals.

Microsatellite marker analysis

Genomic DNA was extracted from blood collected in EDTA using QIAamp 96 DNA Blood Kit (Qiagen, Germany). DNA concentrations were determined by fluorometry using Pico Green. For segregation analysis, two dinucleotide microsatellite markers located in introns 6 and 15 of the VWF gene were analysed. The microsatellites correspond to nucleotides 2787-

2840 in intron 6 and 1059-1095 in intron 15. The following primers were used to analyse these markers: 6F: 5′-HEX-TTTGGGATGGAGACTTAACAT-3′, 6R: 5′-ATGCCCAGCTAGTTAGTGAGT-3′, 15F: 5′-FAM-GTGAGTTATGATCGCACCA-3′, and 15R: 5′-GGACCCAAAATAGTAGCACTT-3′. The two markers were selected from a set of four microsatellite markers that were identified in the VWF gene region using a search for tandem repeats by Tandem Repeat Finder [18]. Expected heterozygosity for the two markers was calculated to be 0.94 and 0.85 respectively. The allele sizes seen using the reported primers were 220-300 and 220-250 bp, respectively, and the repeat sequences and the range of number of repeat units seen were $CA_{(16-56)}$ and $TG_{(16.5-31.5)}$, respectively. The microsatellite markers were amplified using PCR conditions as recommended by the manufacturer of the Taq polymerase (Applied Biosystems, USA) and an annealing temperature of 58 ºC. PCR products were resolved using capillary electrophoresis run on an ABI PRISM™ 3730 sequencer employing GeneMapper software (Applied Biosystems, USA). The allele sizes of the microsatellite markers were determined in relation to a size marker, GeneScan-500™ ROX (Applied Biosystems, USA). All participating individuals were successfully genotyped for both markers; hence there were no missing values.

SNP genotyping

Genotypes were determined using the Sequenom MassARRAY MALDI-TOF system. The system analyses allele-specific primer extension products using mass spectrometry. Two polymorphisms were studied: the O-genotype of the ABO blood group system (rs8176719) and the 4751A>G (Tyr1584Cys) polymorphism of the VWF gene (rs1800386). Assay design was made using the SpectroDESIGNER software (Sequenom Inc, USA). The following primers were used to analyse the ABO blood group and the 4751A>G polymorphism: F: 5′-

TCCCAGACAATGGGAGCCAG-3′, R: 5′-TCTCCATGTGCAGTAGGAAG-3′, E: 5′-GGAAGGATGTCCTCGTGGT-3′ and F: 5′-AACAGGACCAACACTGGGCT-3′, R: 5′-TGGCTGACCAAGAAGCTGTG-3′, E: 5′-AGCTGTGGTCAGAGAGG-3′, respectively. The analyses were then carried out as described on http://www.sequenom.com/publications/scientific_applicationnotes.php.

Genetic analysis

The pedigrees were constructed using Progeny (Progeny Software LLC, USA). Haplotypes for all individuals were reconstructed manually. The frequency of the disease allele was estimated to be 0.005, which derives from the common assumption that 1% of the population is affected by type 1 VWD. Microsatellite marker allele frequencies were calculated from 59 healthy unrelated individuals sampled from the same population as the investigated families. Genotype inconsistencies were identified manually and using the program PedCheck [19]. Linkage analysis was performed using Genehunter [20].

Results

Co-segregation of VWF haplotypes and type 1 VWD

A total of 325 individuals from 31 type 1 VWD families were genotyped using two microsatellite markers in the VWF gene. Of these, 127 individuals were classified as "affected". In 27 of the 31 families marker haplotypes co-segregated with type 1 VWD, whereas in the remaining four families there was clearly no co-segregation (Table 1). Co-segregation in the current study is defined as all affected individuals in a given family having

a specific haplotype in common, but allows for non-penetrant individuals sharing the same haplotype. Non-co-segregating families are therefore defined as those where not all affected individuals share the same haplotype. As an example of a co-segregating family with complete penetrance, the pedigree of family number 143 is shown in Fig. 1(A). The haplotype **262:242** is present in all affected type 1 VWD individuals and is absent in all other individuals in this pedigree. The pedigree of family number 244 (Fig. 1B) is an example of a non-co-segregating family. The four non-co-segregating families originate from different parts of Sweden.

Single-point LOD-scores for linkage between type 1 VWD and the two microsatellite markers were calculated for all families (Table 1). In the families where VWD co-segregated with VWF marker haplotypes, LOD-scores varied between -0.02 and 2.65. In all cases, the low LOD-scores are directly due to the small number of affected individuals in the families. In addition: (1) because of the conservative approach for assigning individuals as "affected" rather few individuals were given disease status and (2) since the penetrance is incomplete and varies between families, all individuals non-affected by type 1 VWD have been classified as "others"; hence no individuals were classified as "healthy". When LOD-scores are summed over all families, the resulting LOD-scores are 10.53 and 8.63 for the intron 6 and intron 15 markers respectively, clearly establishing linkage between the VWF locus and the VWD phenotype in the families treated as a group. When LOD-scores are summed over the 27 co-segregating families, the resulting LOD-scores are even higher, 15.79 and 12.84 respectively. The families that do not show a co-segregating disease haplotype all have negative LOD-scores (-0.87 to -2.05), confirming that these families do not show co-segregation (Table 1). Again there is a good correlation between the number of affected individuals in the families and the size of the LOD-scores. In the families with stated co-segregation we found 30-40 individuals who carried the disease-associated haplotype but

were classified as “others” (Table 1); hence these individuals were non-penetrant. As an example of a co-segregating family with incomplete penetrance, the pedigree of family number 261 is shown in Fig. 1(C). The haplotype **240:232** is present in all affected type 1 VWD individuals in this pedigree in addition to individual II:3 who is classified as ”others”. Phenotypically, the co-segregating and the non-co-segregating groups do not differ from each other (Table 2). The two groups have VWF:RCo/VWF:Ag ratios of 1.1 and 1.2, respectively.

Common disease haplotypes of the VWF gene

Six of the families showing co-segregation shared the same disease haplotype. The frequency of this haplotype among the disease haplotypes was 0.22 (Table 3) and its frequency in all the 31 investigated families was 0.19. When the frequency of this disease haplotype in the families with co-segregation (0.22) was compared with the frequency of the haplotype among normal unrelated individuals (0.06) it was found to be much higher in the former group; a chi-square value of 7.14 (p=0.0075) was obtained, which is highly statistically significant. This suggests a founder effect. The six families are not apparently related; however four of them come from the same part of Sweden. We found a slight difference in VWF levels in the six families with a common haplotype as compared to the 25 families without this common haplotype (Table 2). The VWF:RCo/VWF:Ag ratios were 0.5 and 1.2, respectively. Three other disease haplotypes occurred in two families each. These indicate the possibility that more than one common disease haplotype exists for type 1 VWD in Sweden.

The 4751A>G (Tyr1584Cys) sequence variation in exon 28 was identified in 1 of the 31 families (3.2%). Of 285 individuals analysed for this polymorphism, four presented the variation in a heterozygous state (Fig. 2). Two of the heterozygous individuals were blood group O, of whom one was an affected individual, and two were non-O. This family was one

of the families with stated co-segregation (number 525); however, the Tyr1584Cys variation did not co-segregate with the disease-associated haplotype.

Relation between VWD and ABO blood group

The number of O alleles was successfully determined in 110 of the affected individuals and in 180 individuals belonging to the "others" group (Table 4). When these two groups were compared, blood group O was found to be more frequent in the patient group; a chi-square value of 10.96 (p=0.0009) was obtained, which is highly statistically significant. When the two groups were compared regarding the number of O alleles, a chi-square value of 11.80 (p=0.0027) was obtained, which is also highly significant. When each of the two groups was compared with the general population (where the frequency of blood group O is 38%; information from the Blood Transfusion Centre, Lund, Sweden) in a two-sided binomial test, the patient group obtained a p-value of 0.0095, whereas the "others" group obtained a p-value of 0.040. One should note that the differences are in opposite directions; compared with the general population blood group O is over-represented in the patient group whereas it is slightly under-represented in the "others" group (Table 4). Comparing the co-segregating and the non-co-segregating groups, we did not find any difference in relation between blood group O and VWD (data not shown). In the four non-co-segregating families, the frequency of blood group O varied widely; e.g., in families 244 and 283, all affected individuals had blood group O, whereas in families 216 and 296, the corresponding figures were only 3/7 and 0/3. However, the frequency of blood group O in the four families varied accordingly, such that individuals classified as "others" also carried the blood group O at similar frequencies as the affected in each family.

Discussion

The genetic background to the most common subtype of VWD, type 1, has not yet been satisfactorily explained. Only a small number of type 1 mutations have been identified in the VWF gene. This is in contrast to type 2 and 3 VWD, in which mutations have been found in many cases. The current definition of VWD, set by ISTH, involves a genetic defect in the VWF gene locus [1]. This has been disputed in the case of type 1 VWD. Considering the complexity of VWF biosynthesis, secretion, and function, the existence of genes outside the VWF gene locus affecting VWF levels seems likely. Modifier genes which influence the VWF levels, such as the ABO gene locus, have been suggested as the cause of incomplete linkage [12,21]. Several studies have been conducted in order to establish/exclude linkage between VWD and the VWF gene, with results ranging from complete linkage [22,23] to incomplete linkage [12,21]. One study attempted to develop linkage analysis in Swedish VWD families; however, due to insufficiently informative markers this attempt was not successful [24]. The varying degrees of established linkage can possibly be explained by the limited number of investigated families in some of these studies, inclusion of individuals that do not fulfil stringent diagnostic criteria, and different diagnostic criteria resulting in some included type 2 VWD families. The aim of our study was to determine whether there is co-segregation between haplotypes of the VWF gene and cases of inherited type 1 VWD. We have analysed a large number of type 1 VWD families and we have used a conservative approach to identify affected individuals.

We identified all tandem repeats in the VWF gene region, selected four for further investigation and by using the two most informative microsatellite markers we were able to establish/exclude genetic linkage in the 31 families. Expected heterozygosity for the two microsatellite markers was 0.94 and 0.85 respectively; hence they can be regarded as

highly informative markers. This can be compared with the commonly used microsatellites in the promoter and in intron 40 of the VWF gene, which are less informative [12]. The very informative markers used in the current study provided good tools for analysing putative co-segregation and can beneficially be used in future VWD studies.

Our results show that the type 1 VWD families we have investigated can be divided into two groups: those that have a disease-associated microsatellite marker haplotype of the VWF gene and those that clearly do not. The fact that no causative mutation has been identified in some type 1 VWD families in earlier studies can to some extent, but not completely, be explained by our findings. By using our inclusion criteria, with exclusion of all families with only two affected members with a direct parent-offspring relationship, we have selected for larger families with more affected individuals and this may have introduced bias towards positive linkage. Therefore, our study may not be fully comparable to other studies that have applied different inclusion criteria. Phenotypically, the co-segregating and the non-co-segregating groups in this study are comparable (Table 2). The two groups have roughly the same VWF levels and VWF:RCo/VWF:Ag ratios of 1.1 and 1.2, respectively. In the families with stated co-segregation a search for causative mutations can be focused on the VWF gene region. In these families we found 30-40 individuals who carried the disease haplotype but were classified as “others” (Table 1); hence these individuals were non-penetrant. This illustrates the diagnostic problem in type 1 VWD. Thirteen percent of the investigated families failed to show linkage to the VWF gene locus. These non-co-segregating families come from different parts of Sweden. Additional genetic modifiers have been suggested as part of the explanation for the lack of genetic linkage in some VWD families [13]. Further studies are required of the non-co-segregating families to ascertain whether a locus, or possibly several different loci, other than the VWF gene are responsible for the

development of type 1 VWD, or whether several defects of the VWF gene interact to cause the type 1 VWD phenotype.

We have also analysed the data for the presence of haplotypes with a possible common origin. A common disease-associated microsatellite haplotype was identified in six of the families with co-segregation. When the frequency of this disease haplotype among the families with co-segregation (0.22) was compared with the frequency among normal unrelated individuals (0.06) a chi-square value of 7.14 (p=0.0075) was obtained, which is highly statistically significant. This suggests a founder effect and it is interesting in the context of the evolutionary history of the mutations causing type 1 VWD. The six families seem to be unrelated; however four of them come from the same part of Sweden. Phenotypically, the six families seem to differ from the 25 families without this common haplotype (Table 2). The two groups have VWF:RCo/VWF:Ag ratios of 0.5 and 1.2, respectively. Therefore, it may be suggested that the group with the six families represent a subgroup compared to the rest of the investigated families. However, this should be interpreted carefully due to the small sample size. Further, the 4751A>G (Tyr1584Cys) sequence variation was identified in 1 of 31 (3.2%) of the investigated families. This variation has been identified in 10 of 70 (14.3%) Canadian families and in 8 of 30 (27%) UK families with type 1 VWD [16,25]. Thus, this variation is present at much higher frequencies among families with VWD in Canada and UK compared to Sweden. The variation has been recorded as a polymorphism with a frequency of 0-2% [16,26]. The original publication of the non-conservative amino acid substitution, described it to be a sequence variation without harmful consequences [26]. In contrast, the substitution has been shown to result in increased intracellular retention of the VWF in vitro [16]. Furthermore, a recent publication demonstrated the substitution to increase the susceptibility of the VWF to proteolysis by ADAMTS13 [27]. It is noteworthy that three of the four individuals in our study with this

sequence variation (285 individuals tested) were not diagnosed with type 1 VWD; hence this mutation does not have complete penetrance. Interestingly, in the family where the Tyr1584Cys variation was identified, it did not co-segregate with the disease-associated haplotype, since the affected individual with this variation has inherited the Tyr1584Cys variation from his father and the disease-associated haplotype (**236:230**) from his mother (Fig. 2). In addition to these two alleles, the affected individual has blood group O.

Information collected on the number of O alleles in the ABO blood group system clearly shows that blood group O is over-represented among type 1 VWD patients; a chi-square value of 10.96 and a p value of 0.0009 reveal the statistical significance of the difference between the "affected" and the "others" groups. We have also shown that the phenotype (absence or presence of blood group O) matters rather than the number of O alleles, i.e. there does not seem to be a dosage effect. Interestingly, the relation between blood group O and VWD did not seem to differ between the co-segregating and the non-co-segregating families. Thus, blood group O cannot on itself explain the presence of disease in the non-co-segregating families. Therefore, in the families where VWD is not due to a dominantly inherited mutation in the VWF gene, a locus or several loci different from the VWF gene *and* the ABO locus may be involved. These families definitely represent a valuable resource when searching for modifier genes. Alternatively, several defects within the VWF gene may interact to give the type 1 VWD phenotype in certain individuals in these families. This remains to be elucidated.

In conclusion, we have shown that the VWF gene locus is responsible for inherited type 1 VWD in a majority of families in our population. We have also identified a possible founder effect: a disease-associated haplotype whose frequency was 22% among the disease haplotypes in the investigated families. In the co-segregating families, molecular genetic testing will facilitate future investigations of additional family members. In addition,

we have confirmed that blood group O is over-represented among type 1 VWD patients. Our findings may also contribute to the present discussion about the definition of VWD. One issue is if the diagnosis should include all cases with a VWD phenotype, irrespective of the locus of the genetic defect. Alternatively, as has been suggested, low VWF levels may in many cases be regarded as an epidemiological risk factor for bleeding, not a disease [7,28,29].

Acknowledgements

The study was supported by research grants from Lund University, Region Skåne, Malmö University Hospital, The Arosenius fund, ZLB Behring and Baxter.

Figure Legends

Formaterat

Fig. 1A. Pedigree demonstrating co-segregation between type 1 VWD and the haplotype **262:242** of the VWF gene in family number 143. Filled symbols indicate affected individuals. We have had access to blood samples only of individuals with haplotypes indicated below.

Fig. 1B. Pedigree demonstrating absence of co-segregation between type 1 VWD and the VWF gene in family number 244.

Fig. 1C. Pedigree demonstrating co-segregation with incomplete penetrance between type 1 VWD and the haplotype **240:232** of the VWF gene in family number 261. In addition to all affected individuals, individual II:3, who is classified as ”others”, presents this haplotype.

Fig. 2. Pedigree demonstrating the 4751A>G (Y1584C) sequence variation in a heterozygous state in four individuals in family number 525. This family was one of the families with stated co-segregation; however, the Y1584C variation did not co-segregate with the disease-associated haplotype **236:230**. *Y1584C. **Not genotyped for Y1584C. ***Not genotyped for blood group O/non-O.

References

1 Sadler JE. A revised classification of von Willebrand disease. *Thromb Haemost* 1994; **71**: 520-5.

2 Miller CH, Graham JB, Goldin LR, Elston RC. Genetics of classic von Willebrand's disease. I. Phenotypic variation within families. *Blood* 1979; **54**: 117-36.

3 Mancuso DJ, Tuley EA, Westfield LA, Worrall NK, Shelton-Inloes BB, Sorace JM, Alevy YG, Sadler JE. Structure of the gene for human von Willebrand factor. *J Biol Chem* 1989; **264**: 19514-27.
4 Mancuso DJ, Tuley EA, Westfield LA, Lester-Mancuso TL, Le Beau MM, Sorace JM, Sadler JE. Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. *Biochem* 1991; **30**: 253-69.
5 Rodeghiero F, Castaman G. Congenital von Willebrand disease type I: definition, phenotypes, clinical and laboratory assessment. *Best Pract Res Clin Haematol* 2001; **14**: 321-35.
6 Rodeghiero F. von Willebrand disease: still an intriguing disorder in the era of molecular medicine. *Haemophilia* 2002; **8**: 292-300.
7 Mohlke KL, Nichols WC, Ginsburg D. The molecular basis of von Willebrand disease. *Int J Clin Lab Res* 1999; **29**: 1-7.
8 Nitu-Whalley IC, Lee CA, Griffioen A, Jenkins PV, Pasi KJ. Type 1 von Willebrand disease - a clinical retrospective study of the diagnosis, the influence of the ABO blood group and the role of the bleeding history. *Br J Haemat* 2000; **108**: 259-64.
9 Gill JC, Endres-Brooks J, Bauer PJ, Marks WJJ, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood* 1987; **69**: 1691-5.
10 Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, Tamai H, Konno M, Kamide K, Kawano Y, Miyata T, Fujimura Y. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Nat Acad Sci U.S.A.* 2002; **99**: 11902-7.
11 Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost* 2003; **1**: 33-40.
12 Casaña P, Martínez F, Haya S, Espinós C, Aznar JA. Significant linkage and non-linkage of type 1 von Willebrand disease to the von Willebrand factor gene. *Br J Haemat* 2001; **115**: 692-700.
13 Souto JC, Almasy L, Soria JM, Buil A, Stone W, Lathrop M, Blangero J, Fontcuberta J. Genome-wide linkage analysis of von Willebrand factor plasma levels: results from the GAIT project. *Thromb Haemost* 2003; **89**: 468-74.
14 Zhang Z, Lindstedt M, Blombäck M, Anvret M. Effects of the mutant von Willebrand factor gene in von Willebrand disease. *Hum Genet* 1995; **96**: 388-94.
15 Goodeve AC. Laboratory methods for the genetic diagnosis of bleeding disorders. *Clin Lab Haem* 1998; **20**: 3-19.
16 O'Brien LA, James PD, Othman M, Berber E, Cameron C, Notley CRP, Hegadorn CA, Sutherland JJ, Hough C, Rivard GE, O'Shaunessey D, Lillicrap D. Founder von Willebrand factor haplotype associated with type 1 von Willebrand disease. *Blood* 2003; **102**: 549-57.
17 Sadler JE, Rodeghiero F. Provisional criteria for the diagnosis of VWD type 1. *J Thromb Haemost* 2005; **3**: 775-7.
18 Benson G. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res* 1999; **27**: 573-80.
19 O'Connell JR, Weeks DE. A program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 1998; **63**: 259-66.
20 Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES. Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am J Hum Genet* 1996; **58**: 1347-63.
21 Castaman G, Eikenboom JCJ, Bertina RM, Rodeghiero F. Inconsistency of association between type 1 von Willebrand disease phenotype and genotype in families identified in an epidemiological investigation. *Thromb Haemost* 1999; **82**: 1065-70.

22 Cumming AM, Armstrong JG, Pendry K, Burn AM, Wensley RT. Polymerase chain reaction amplification of two polymorphic simple repeat sequences within the von Willebrand factor gene: application to family studies in von Willebrand disease. *Hum Genet* 1992; **89**: 194-8.

23 Inbal A, Kornbrot N, Zivelin A, Shaklai M, Seligsohn UT. The inheritance of type I and type III von Willebrand's disease in Israel: linkage analysis, carrier detection and prenatal diagnosis using three intragenic restriction fragment length polymorphisms. *Blood Coagul Fibrinolysis* 1992; **3**: 167-77.

24 Anvret M, Blombäck M, Lindstedt M, Söderlind E, Tapper-Persson M, Thelander A-C. Genetic and blood coagulation characterization of "Swedish" families with von Willebrand's disease types I and III: New aspects of heredity. *Hum Genet* 1992; **89**: 147-54.

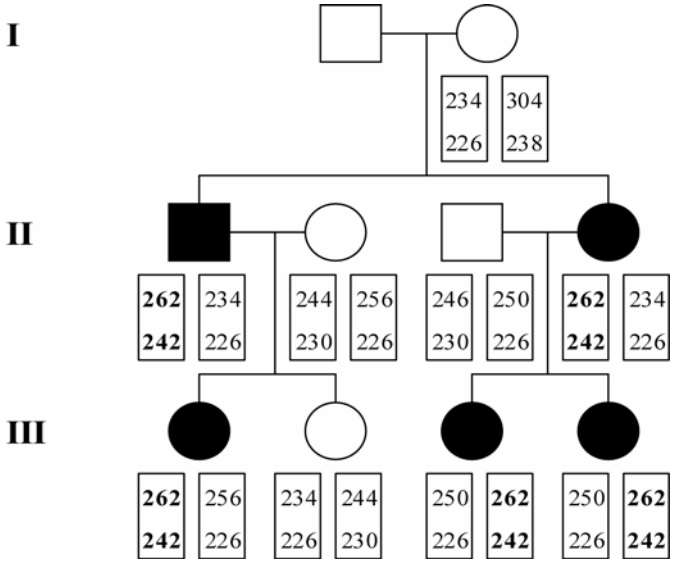
25 Bowen DJ, Collins PW, Lester W, Cumming AM, Keeney S, Grundy P, Enayat SM, Bolton-Maggs PHB, Keeling DM, Khair K, Tait RC, Wilde JT, Pasi KJ, Hill FGH. The prevalence of the cysteine1584 variant of von Willebrand factor is increased in type 1 von Willebrand disease: co-segregation with increased susceptibility to ADAMTS13 proteolysis but not clinical phenotype. *Br J Haemat* 2005; **128**: 830-6.

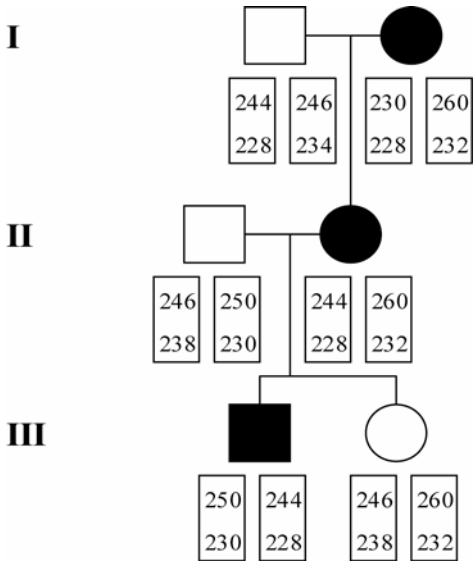
26 Donnér M, Kristoffersson AC, Berntorp E, Scheibel E, Thorsen S, Dahlbäck B, Nilsson IM, Holmberg L. Two new candidate mutations in type IIA von Willebrand's disease (ARG834->GLY, GLY846->ARG) and one polymorphism (TYR821->CYS) in the A2 region of the von Willebrand factor. *Eur J Haematol* 1993; **51**: 38-44.

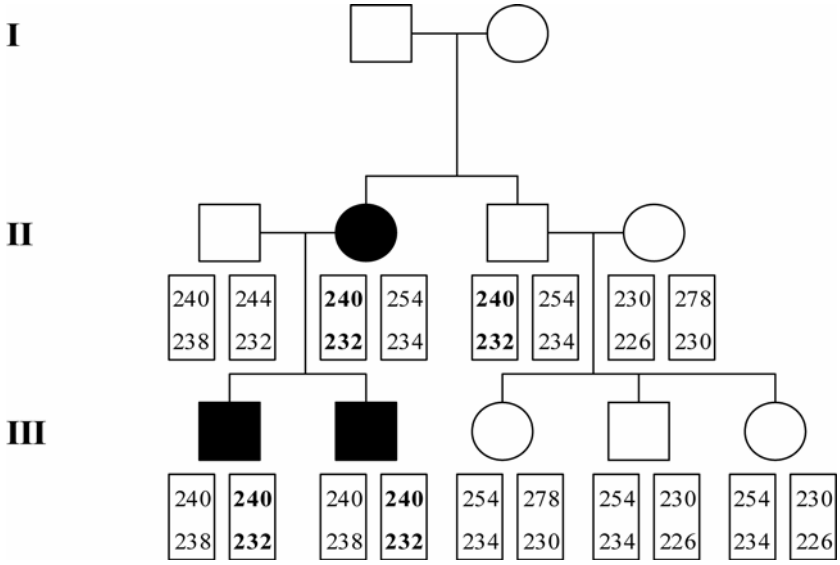
27 Bowen DJ, Collins PW. An amino acid polymorphism in von Willebrand factor correlates with increased susceptibility to proteolysis by ADAMTS13. *Blood* 2004; **103**: 941-7.

28 Sadler JE. Von Willebrand disease type 1:a diagnosis in search of a disease. *Blood* 2003; **101**: 2089-93.

29 Sadler JE. Slippery criteria for von Willebrand disease type 1. *J Thromb Haemost* 2004; **2**: 1720-3.


I
234
226
304
238
II
262
242
234
226
244
230
256
226
246
230
250
226
262
242
234
226
III
262
242
256
226
234
226
244
230
250
226
262
242
250
226
262
242


I
244
228
246
234
230
228
260
232
II
246
238
250
230
244
228
260
232
III
250
230
244
228
246
238
260
232


I
II
III
240 238
244 232
240 232
254 234
240 232
254 234
230 226
278 230
240 238
240 232
240 238
240 232
254 234
278 230
254 234
230 226
254 234
230 226

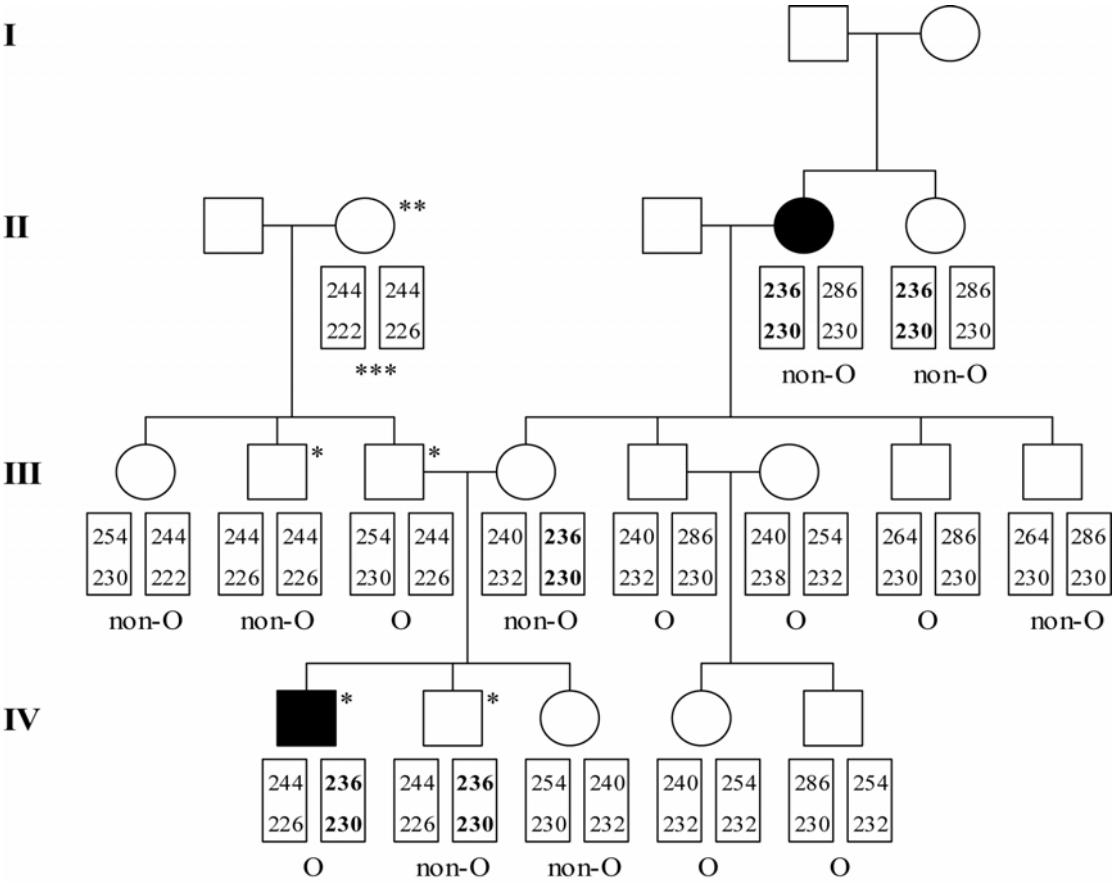

I
II
III
IV
**
***
244 222
244 226
236 230
286 230
236 230
286 230
non-O
non-O
*
*
254 230
244 222
244 226
244 226
254 230
244 226
240 232
236 230
240 232
286 230
240 238
254 232
264 230
286 230
264 230
286 230
non-O
non-O
O
non-O
O
O
O
non-O
*
*
244 226
236 230
244 226
236 230
254 230
240 232
240 232
254 232
286 230
254 232
O
non-O
non-O
O
O

Table 1 Information on the 31 families investigated for co-segregation

Family number	Number of individuals	Number of type 1 VWD individuals	Number of "others" with disease haplotype	Co-segregating disease haplotype	LOD-scores at theta 0	
					Intron 6 marker	Intron 15 marker
18	13	2	1	236:228	0.44	0.35
89	6	3	1	240:230	0.57	0.57
101	16	8	2	234:226	1.62	1.43
102	7	2	1	250:232	-0.01	-0.02
143	9	5	0	262:242	0.86	0.86
173	7	3	1	240:232	0.25	0.25
191	24	8	0-7***	238:230	0.84	0.11
192	11	7	0-1***	238:230	1.21	0.63
196	11	3	0	248:242	0.28	0.28
198	5	3	0	288:226	0.28	0.28
216	15	7	—	—	-2.05	-0.93
228	7	4	0	242:230	0.58	0.58
244	6	3	—	—	-1.17	-1.17
248	8	2	2	258:230	0.28	0.00
259	26	10	3	238:230	2.22	2.65
261	9	3	1	240:232	0.29	0.29
265	19	3	4	238:230	0.29	0.29
271	7	3	1	240:226	0.56	-0.01
283	5	3	—	—	-0.87	-0.94
284	11	3	0	256:230	0.29	0.29
292	14	6	4	234:224	1.16	1.13
296	12	3	—	—	-1.17	-1.17
305	8	4	1	260:236	0.58	0.58
512	9	4	1-3***	238:230	0.24	0.44
515	5	2	1	242:232/244:230*	0.28	0.28
517	6	3	0	234:226	0.01	0.29
525	16	2	3	236:230	0.27	0.27
528	9	4	1	238:230	0.40	0.86
549	9	6	0	254:230/238**	1.13	-1.11
553	8	5	1	234:224	0.58	0.98
565	7	3	1	246:230	0.29	0.29
Sum	325	127	30-40		10.53	8.63

*The two affected individuals share the same genotype, i.e. both haplotypes are co-segregating with the disease but the exact disease haplotype is uncertain.**Recombination event between the two markers. ***Two identical haplotypes appear in the pedigree and it cannot be ascertained which one is disease-associated.

Table 2 Phenotypic evaluation of all index cases in the investigated families (VWF levels given as IU/dL)

		Median	Min	Max	Number of patients (n)*
All 31 investigated families	VWF:Ag	36	12	63	31
	VWF:RCo	41	<4	71	20
The 27 familes with co-segregation	VWF:Ag	36	12	63	27
	VWF:RCo	38	<4	71	17
The 4 families with no co-segregation	VWF:Ag	40	29	59	4
	VWF:RCo	47	31	49	3
The 6 families with a common disease-associated haplotype	VWF:Ag	25	18	35	6
	VWF:RCo	12	<4	12	3
The 25 families without a common disease-associated haplotype	VWF:Ag	37	12	63	25
	VWF:RCo	43	<4	51	15

*We used historical phenotypic VWF data. Some older index cases had not been tested with VWF:RCo.

Table 3 Disease-associated haplotypes in the 27 co-segregating families and in normal individuals

Disease haplotype	Number of families with a certain disease haplotype (frequency among disease haplotypes)	Disease haplotype frequency among normal unrelated individuals
238:230	6 (0.22)	0.06
234:224	2 (0.07)	0
234:226	2 (0.07)	0.04
240:232	2 (0.07)	0.02
236:228	1 (0.04)	0
236:230	1 (0.04)	0
240:226	1 (0.04)	0.02
240:230	1 (0.04)	0
242:230	1 (0.04)	0.02
246:230	1 (0.04)	0
248:242	1 (0.04)	0
250:232	1 (0.04)	0
256:230	1 (0.04)	0
258:230	1 (0.04)	0
260:236	1 (0.04)	0
262:242	1 (0.04)	0
288:226	1 (0.04)	0

The haplotypes of families 515 and 549 are not listed in the table since the exact disease haplotypes are uncertain in these cases.

Table 4 ABO blood group testing

Number of O alleles	Number of affected individuals	Number of "others" individuals	Sum
0	16 (15%)	28 (16%)	44
1	39 (35%)	97 (54%)	136
2	55 (50%)	55 (31%)	110
Sum	110	180	290

Blood group O is found to be more frequent in the affected than the "others" individuals; when comparing these groups a chi-square value of 10.96 (p=0.0009) was obtained, which is highly statistically significant.

Stefan Lethagen

Professor, Dr Med

Copenhagen University Hospital (Rigshospitalet)
Copenhagen Hemophilia Center

Blegdamsvej 9
Copenhagen
DK-2100
DENMARK

+45-3545 4816 (Fax)
+45-3545 1359 (Arbete)

Namn

Efternamn: Lethagen
Förnamn: Stefan
Mellaninitial:
Prefix:
Suffix:

Formaterat namn

Stefan Lethagen

Telefonnummer (Fax)

+45-3545 4816

Telefonnummer (Arbete)

+45-3545 1359

Företag

Copenhagen University Hospital (Rigshospitalet)
Copenhagen Hemophilia Center

Adress

Postadress:
Utökad adress:
Gatuadress: Blegdamsvej 9
Plats: Copenhagen
Region:
Postnummer: DK-2100
Land: DENMARK

Version

2.1

Befattning

Professor, Dr Med