

Lack of correlation between factor VIII related antigen multimeric analysis pattern and parallel or non-parallel dose response curves in an ELISA factor VIII related antigen assay

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SUMMARY Factor VIII related antigen has been measured and epitope distribution has been explored by testing the degree of parallelism between standard and test plasma dose response curves using an enzyme immunoassay. Normal plasma, plasma fractions, and plasma from patients with haemophilia and von Willebrand's disease have been tested. All showed parallelism except for plasma from patients with the variant type IIA von Willebrand's disease, of which 10 had parallel and five had non-parallel dose response curves when compared with that of normal plasma. In one family plasma from seven members showed parallelism but from four others did not. An unrelated patient was tested on three occasions, and although the samples were parallel to each other, no sample was parallel to the standard. No correlation was found between parallelism as shown by the enzyme immunoassay and differences in factor VIII related antigen multimeric pattern, including triplet configuration, seen in the type IIA patients.

Von Willebrand's disease occurs as either a quantitative or qualitative abnormality of factor VIII related antigen (VIII:AG).¹⁻³ The patients with qualitative abnormalities have been subclassified into types IIA and IIB.⁴ VIII:AG from type IIA patients has an increased anodal migration by crossed immunoelectrophoresis⁵⁻⁷ and lacks high and intermediate molecular weight multimers.⁸ More recently, differences in degree of deletion of the multimers and differences in triplet configuration have been reported.⁹

Measurement of VIII:AG by immunoelectrophoresis is inaccurate in type IIA patients.¹⁰ Immunoradiometric assays have been described but have not given parallel dose response curves for all patients.¹¹⁻¹³ Five patients with atypical von Willebrand's disease were studied by an immunoradiometric assay and their VIII:AG showed reduced

antigenic activity and non-parallel dose response curves when compared with normal plasma.¹¹ Lack of parallelism was also shown in five of 14 variant patients by an immunoradiometric assay using rabbit antihuman VIII:AG,¹² and in some other variants by an immunoradiometric assay using antiserum and anticonformation antibodies.¹³

We have previously reported a simple enzyme linked immunosorbent assay (ELISA) which is rapid, sensitive, and reproducible within and between batches (coefficient of variation 4.1% and 4.0% respectively).¹⁴ This assay has now been used to investigate the binding of pooled polyclonal antisera with VIII:AG from normal plasmas, plasma fractions, and patients with haemophilia and classical and type IIA von Willebrand's disease. Crossed immunoelectrophoresis and multimeric analysis have also been performed to ascertain if these correlate with normal or abnormal antibody binding patterns.

Material and methods

SUBJECTS

Venous blood was obtained from eight normal adult

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men, seven haemophiliacs, 13 patients with classical von Willebrand's disease, and 15 patients with type IIA von Willebrand's disease. Eleven of the group with type IIA von Willebrand's disease were members of one family,¹⁵ while the four others were CB,⁷ a patient described by Peake *et al.*,⁶ a patient described by Kernoff *et al.*,⁵ and another unreported patient HW.

PLASMA AND PLASMA FRACTIONS

Nine parts of blood were added to one part of 0.11 mol/l trisodium citrate. Platelet poor plasma was prepared by centrifugation at 1500 g for 15 min at 4°C. Separated plasma was used immediately for VIII:C assays, but stored at -20°C and thawed at 37°C immediately before assaying other factor VIII parameters. Cryoprecipitate was prepared as follows: 10 ml of platelet poor plasma was snap frozen by immersing the centrifuge tube in a mixture of dry ice and ethanol, followed by thawing at 4°C, before centrifugation at 1700 g for 15 min. The supernatant was removed and the cryoprecipitate was washed four times with 0.9 g/l saline solution at 0°C before dissolving it in 1 ml of saline at 37°C.

CONTROL PLASMAS

The ninth British standard for blood coagulation factor VIII human plasma (NIBSC, London) was used as the VIII:C standard and as an additional standard for Laurell and ELISA assays. A pool of equal volumes of plasma from 10 normal adult men was used as the standard for VIIIIR:WF assays and for Laurell and ELISA assays.

ANTISERA

A commercial rabbit antihuman VIIIIR:AG (DAKO Immunoglobulins, Mercia Brocades Ltd) consisting of a pool of sera from 10 immunised rabbits was used. The immunoglobulin fraction had been partially purified. This antiserum was used for the Laurell assay and the first stage of the ELISA assay and conjugated with horseradish peroxidase for use in the end stage of the same assay. The same antisera labelled with ¹²⁵I as previously described⁹ was used to identify VIIIIR:AG multimers by autoradiography after multimeric analysis.

A different rabbit antihuman VIIIIR:AG (Behringwerke AG) was used in the second dimension of crossed immunoelectrophoresis.

ASSAYS

VIII:C was measured by a two stage technique using a Diagen kit (Diagnostic Reagents Ltd).

VON WILLEBRAND'S FACTOR

VIIIIR:WF was assayed in the patients using a fresh

washed platelet assay as previously described⁷ and in the plasma fractions, normal subjects, and haemophiliacs using fixed platelets as described by Brinkhous and Read.¹⁶

VIIIIR:AG

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was done as previously described by Enayat and Hill.¹⁷

VIIIIR:AG was measured by the modified Laurell technique of immunoelectrophoresis¹⁸ and by ELISA.¹⁴ Serial doubling dilutions of plasma were assayed by both techniques, and the rocket height (Laurell) and optical density (ELISA) were plotted against log reciprocal of plasma dilution to determine dose response curves and obtain, by extrapolation, VIIIIR:AG values.

A *t* test was used to compare the slopes of the least squares regression lines obtained from the dose response curves.¹⁹ The curves were parallel if there was no significant difference in slopes.

MULTIMERIC ANALYSIS OF VIIIIR:AG

Multimeric analysis of VIIIIR:AG was done by a modification of the method of Ruggeri and Zimmerman,⁸ as described by Enayat and Hill,⁹ using a discontinuous buffer system and a 2.5% acrylamide (with 5% crosslinking) 0.5% agarose gel with sodium dodecyl sulphate. Multimeric analysis of cryoprecipitate, cryosupernatant, and the type IIA patients' plasma was performed.

Results

NORMAL SUBJECTS AND PATIENTS WITH HAEMOPHILIA, AND CLASSICAL VON WILLEBRAND'S DISEASE

The ranges of factor VIII values are shown in Table 1, and the parallel dose response curves for VIIIIR:AG for normal subjects and for patients with classical von Willebrand's disease are shown in Fig. 1. The plasma from haemophiliacs gave parallel dose response curves but these are not shown. The coefficient of correlation between VIIIIR:AG determined by Laurell and ELISA for these subjects was 0.92 ($p < 0.001$); crossed immunoelectrophoresis produced normal precipitin arcs and migration indices when VIIIIR:AG values were greater than 0.15 U/ml.

NORMAL CRYOPRECIPITATE AND CRYOSUPERNATANT

VIIIIR:AG was measured on cryoprecipitate and cryosupernatant prepared from the plasma of six normal adult men. VIIIIR:AG concentration was increased in each cryoprecipitate and parallel dose

Table 1 Ranges of factor VIII values, migration indexes and *t* values comparing parallelism of dose response curves of patient and control groups

Group	<i>n</i>	VIII:C u/ml	VIII:WF (u/ml)	2DCIEP MI	VIII:AG EIA (u/ml)	<i>t</i> EIA	VIII:AG ELISA (u/ml)	<i>t</i> ELISA
Normal subjects	8	0.65-1.30	0.64-1.22	0.99 ± 0.11	0.64-1.30	<2.3	0.64-1.30	<2.3
Haemophiliacs	7	<0.01-0.10	—	0.99 ± 0.11	0.68-5.00	<2.3	0.50-4.00	<2.3
Patients with classical von Willebrand's disease	13	<0.01-0.25	<0.10-0.28	0.99 ± 0.11	<0.10-0.30	<2.3	<0.01-0.46	<2.3

A *t* value of <2.3 indicates no significant difference between test and control dose response curve.

2DCIEP = Crossed immunoelectrophoresis.

EIA = immunoelectrophoresis.

MI = migration index.

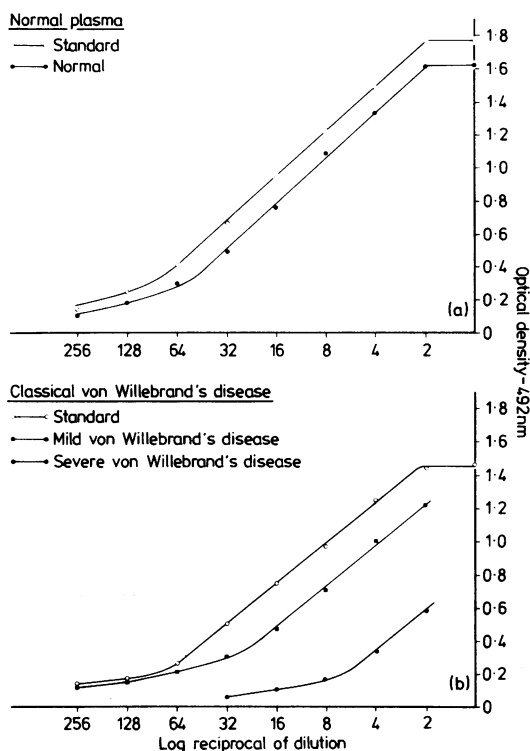


Fig. 1 Parallel dose response curves as determined in an ELISA assay for normal plasma and plasma from patients with classical von Willebrand's disease, compared with a standard.

response curves were obtained. Anodal migration by crossed immunoelectrophoresis and multimeric analysis patterns were normal. Cryosupernatants showed reduced concentrations of VIII:AG, but the dose response curves in the ELISA assay were all parallel to the standard (Fig. 2). Crossed immunoelectrophoresis of cryosupernatants showed an increased anodal migration and multimeric analysis showed lack of high and intermediate molecular weight VIII:AG forms. Triplet struc-

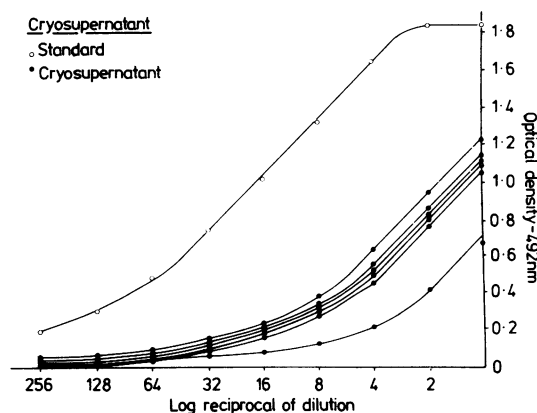


Fig. 2 Parallel dose response curves as determined in an ELISA assay for normal cryosupernatants compared with a standard.

ture, however, was maintained and the pattern was that seen in the triplets of normal plasma (Fig. 4).

PATIENTS WITH TYPE IIA VON WILLEBRAND'S DISEASE

Factor VIII values and the *t* values testing parallelism between test and control plasma by immunoelectrophoresis and ELISA are shown in Table 2. Except for three patients (KR, TR and HW) VIII:AG concentration was consistently lower by ELISA than immunoelectrophoresis. The correlation coefficient for VIII:AG in type IIA patients measured by immunoelectrophoresis and ELISA was only 0.49. All patients had increased anodal migration of VIII:AG by crossed immunoelectrophoresis. Ten of the 15 patients gave dose response curves parallel to the standard (not shown), whereas five others were always non-parallel. Of the family tested, seven of 11 had parallel dose response curves, two others (SB and IM) were non-parallel to the standard but parallel to each other, while a further two (EL Jr and SL) were non-parallel to the standard and other family members but parallel to each other (see Fig. 3).

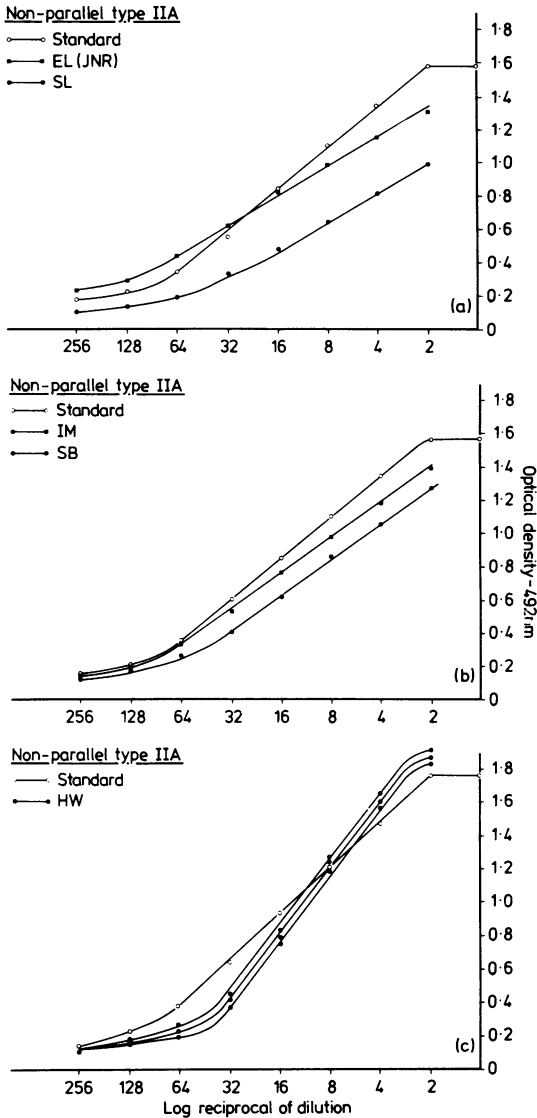


Fig. 3 Non-parallel dose response curves as determined in an ELISA assay for five patients with type IIA von Willebrand's disease (EL Jr, SL, IM, SB and HW).

An unrelated patient, HW, was tested on three separate occasions. Non-parallel dose response curves when compared with standard were always obtained, but the curves were parallel to each other. The other unusual characteristic of this patient's dose response curves was that greater than normal amounts of antibody were bound in the presence of antigen excess, while less than normal amounts of antibody were bound when antibody was present in excess (Fig. 3). In contrast, in patient EL Jr VIII:AG bound more antibody than normal

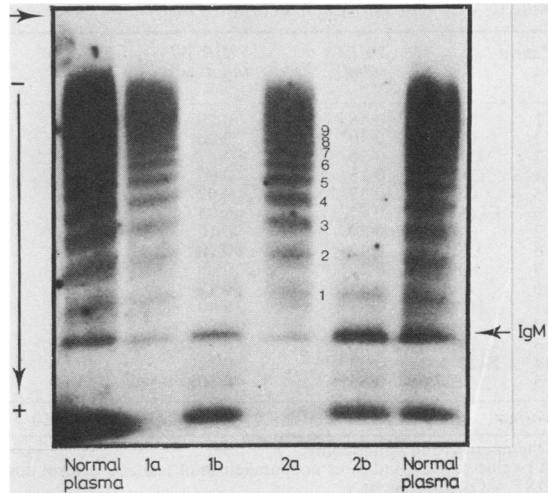


Fig. 4 Multimeric analysis patterns for cryoprecipitate and cryosupernatant. 1 and 2 = different individuals; a = cryoprecipitate, b = cryosupernatant.

VIII:AG at low antigen concentrations, but bound less antibody than normal VIII:AG at higher antigen concentrations.

Multimeric analysis patterns on normal subjects and some of the type IIA patients (HW, CB, Kernoff's original patient (designated Oxford), and Peake's patient (designated Cardiff) are shown in Fig. 5, while selected members of the family are shown in Fig. 6. Although differences in degree of deletion of the high and intermediate VIII:AG forms and differences in triplet configuration can be seen, there does not appear to be any one multimer pattern that is associated with parallelism or non-parallelism in the ELISA technique. The differences in the triplet configuration are shown by different intensities of staining between the central band and the a and b bands.

Discussion

Qualitative defects or differences of VIII:AG have not been shown in plasma from normal subjects or patients with haemophilia or classical von Willebrand's disease by crossed immunoelectrophoresis, ELISA dose response curves, or multimeric analysis. Cryosupernatant had altered anodal migration by crossed immunoelectrophoresis and abnormal multimeric analysis patterns, but parallel dose response curves were obtained with the ELISA assay.

Type IIA patients had qualitatively abnormal VIII:AG as shown by crossed immunoelectrophoresis, absent VIII:WF, and abnormal mul-

Table 2 Factor VIII values, migration indexes and *t* values comparing parallelism of dose response curves with control in patients with type IIA von Willebrand's disease

Patient no.		VIII:C (u/ml)	VIII:WF (u/ml)	2DCIEP MI	VIII:AG EIA (u/ml)	<i>t</i> EIA	VIII:AG ELISA (u/ml)	<i>t</i> ELISA
1	SD*	0.48	0.18	1.33	1.55	2.22	0.80	1.00
2	CB	0.40	0.06	1.33	0.62	2.72	0.49	0.84
3	OXF	0.30	—	1.40	0.32	2.92	0.26	1.26
4	CAR	0.15	<0.05	1.30	1.00	3.63	0.61	0.62
5	GL*	0.37	<0.02	1.50	1.00	2.32	0.84	0.46
6	EL(S)*	0.75	<0.05	1.50	2.00	2.92	0.90	0.35
7	ER*	0.30	<0.10	1.62	2.00	5.66	1.07	0.00
8	EM*	0.42	<0.10	1.68	1.00	4.34	0.76	0.43
9	KR*	0.26	0.08	1.50	0.60	12.55	0.72	0.93
10	TR*	0.38	0.19	1.37	0.74	2.49	1.14	1.57
11	HW	0.83	0.40	1.30	0.60	2.45	1.40	4.92
12	SB*	0.39	<0.10	1.62	1.25	2.75	0.47	5.00
13	IM*	0.06	<0.10	1.56	2.60	3.77	1.52	6.82
14	EL(J)*	0.38	0.22	1.40	1.00	3.47	0.48	6.06
15	SL*	0.36	<0.10	1.66	1.60	3.66	0.80	4.34
Normal		0.50-1.50	0.50-1.50	0.88-1.10	0.50-1.50	<2.3	0.50-1.50	<2.3

*Members of the same family.

A *t* value of <2.3 indicates nonparallelism of test and control dose response curves.

OXF = Oxford patient.

CAR = Cardiff patient.

For other abbreviations, see table 1.

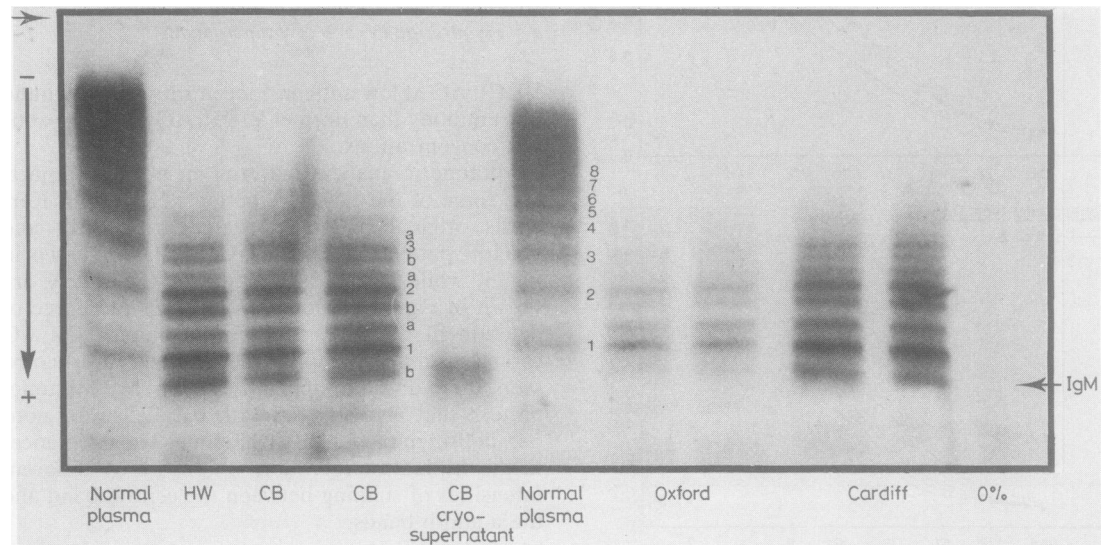


Fig. 5 Multimeric analysis patterns for normal plasma (NP), plasma from four type IIA von Willebrand's disease variants (HW, CB, Oxford and Cardiff), cryosupernatant of CB, and plasma from a patient with severe homozygous von Willebrand's disease (0%).

timeric analysis. In 15 type IIA patients no parallelism was obtained by immunoelectrophoresis, but in 10 of these patients parallelism was obtained in the ELISA assay. Immunoelectrophoresis is affected by electrophoretic charge and molecular size, as well as antigen antibody reaction, whereas the ELISA assay is mostly dependent on antigen antibody reaction. This may explain why parallelism was obtained in two thirds of the patients using the ELISA assay. The complicated binding pattern seen in HW suggests that this may be an oversimplification.

In multimeric analysis stability of the SH bonds of VIII:AG in the presence of sodium dodecyl sulphate was evaluated. Although the type IIA patients displayed differences in multimer deletion and triplet configuration, there was no correlation between multimer pattern and degree of parallelism in the ELISA assay. This was emphasised by the family study, in which 11 showed parallelism while the remaining four showed two different patterns of non-parallelism, although the multimeric analysis pattern was identical for all affected individuals.

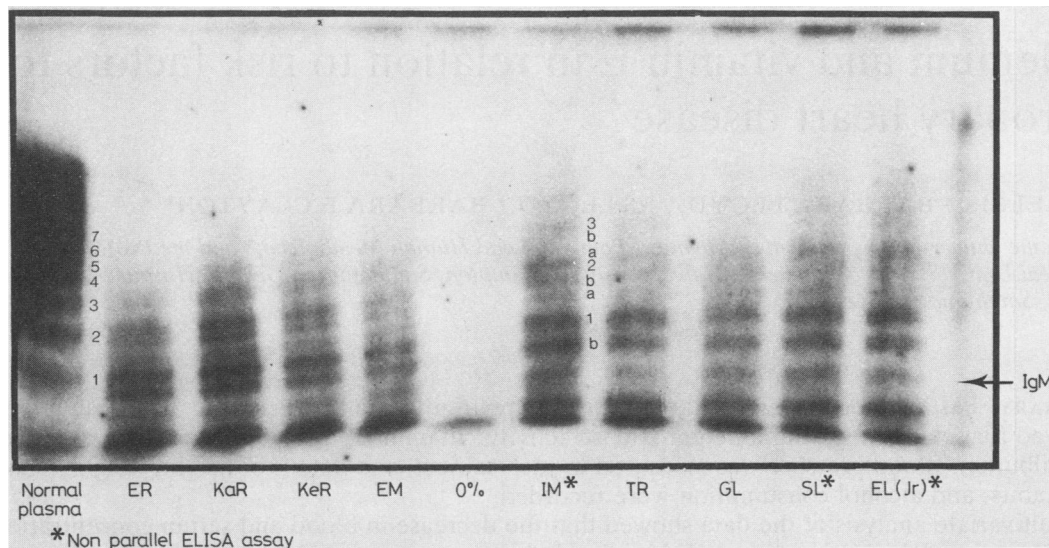


Fig. 6 Multimeric analysis patterns for nine members of a family with type IIA von Willebrand's disease (ER, KaR, KeR, EM, IM, TR, GL, SL and EL Jr) and normal plasma and a patient with severe homozygous von Willebrand's disease (0%).

Determining parallelism by the ELISA assay may be a useful way of assessing antibody binding to epitopes and the kinetics of this reaction.

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References

- ¹ Bouma BN, Sixma JJ, de Graff S, Wiegierinck Y, van Mourik TA, Mochtar IA. Factor VIII antigen and platelet retention in a glass bead column. *Br J Haematol* 1973;**25**:645-56.
- ² Holmberg L, Nilsson IM. Two genetic variants of von Willebrand's disease. *N Engl J Med* 1973;**288**:595-8.
- ³ Thomson C, Forbes CD, Prentice CRM. Evidence for a qualitative defect in factor VIII related antigen in von Willebrand's disease. *Lancet* 1974;*i*:594-6.
- ⁴ Ruggeri ZM, Zimmerman TS. Classification of variant von Willebrand's disease subtypes by analysis of functional characteristics and multimeric composition of factor VIII/von Willebrand factor. *Ann NY Acad Sci* 1981;**370**:205-9.
- ⁵ Kernoff PBA, Gruson R, Rizza CR. A variant of factor VIII related antigen. *Br J Haematol* 1974;**26**:435-44.
- ⁶ Peake IR, Bloom AL, Giddings JC. Inherited variants of factor VIII related protein in von Willebrand's disease. *N Engl J Med* 1974;**291**:113-7.
- ⁷ Hill FGH, Chan MCK, Hardisty RM. Von Willebrand's syndrome. Studies on a variant factor VIII. *Haemostasis* 1976;**5**:276-84.
- ⁸ Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease. Characterisations of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J Clin Invest* 1980;**65**:1318-25.
- ⁹ Enayat MS, Hill FGH. Analysis of the complexity of the multimeric structure of factor VIII related antigen/von Willebrand protein using a modified electrophoretic technique. *J Clin Pathol* 1983;**36**:915-9.
- ¹⁰ Over J, Vlooswijk HAA, Sixma JJ. Assay of FVIII related antigen in a variant of von Willebrand's disease. *Thromb Haemostas* 1977;**37**:367-70.
- ¹¹ Peake IR, Bloom AL. The use of an immunoradiometric assay for factor VIII related antigen in the study of atypical von Willebrand's disease. *Thromb Res* 1977;**10**:27-32.
- ¹² Ardaillou N, Girma JP, Meyer D, Lavergne JM, Shoa'i I, Larrieu MJ. "Variants" of von Willebrand's disease. Demonstration of a decreased antigenic reactivity by immunoradiometric assay. *Thromb Res* 1978;**5**:817-30.
- ¹³ Girma JP, Pietu G, Lavergne JM, Meyer D, Larrieu MJ. Abnormal antigenic reactivity of factor VIII/von Willebrand factor subunit in variants of von Willebrand's disease. *J Lab Clin Med* 1982;**39**:351-5.
- ¹⁴ Short PE, Williams CE, Picken AM, Hill FGH. Factor VIII related antigen: an improved enzyme immunoassay. *Med Lab Sci* 1982;**39**:351-5.
- ¹⁵ Hill FGH, Enayat MS, George AJ. Investigation including VIII:AG multimeric analysis of a large kindred with type IIA von Willebrand's disease. *Thromb Haemostas* 1983;**50**:735-9.
- ¹⁶ Brinkhous KM, Read MS. Preservation of platelet receptors for platelet aggregating factor by air drying, freezing or lyophilization: new stable platelet preparations for von Willebrand factor assays. *Thromb Res* 1978;**13**:591.
- ¹⁷ Enayat MS, Hill FGH. Qualitative VIII:AG function screening of multiple samples by two-dimensional crossed immunoelectrophoretic technique. *Med Lab Sci* 1982;**39**:357-62.
- ¹⁸ Zimmerman TS, Ratnoff OD, Powell AE. Immunologic differentiation of classic haemophilia (factor VIII deficiency) and von Willebrand's disease with observations on combined deficiencies of anti-haemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against haemophilic factor. *J Clin Invest* 1971;**50**:224-54.
- ¹⁹ Armitage P. *Statistical methods in medical research*. London: Blackwell Scientific Publications, 1971.