Investigating a bleeder?

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Making the diagnosis

- Investigating the right patients
- Appropriate laboratory staff and facilities
- Selecting the right tests
- Controlling the tests
- Referral network for complex cases

Von Willebrands Disease (VWD)

- Most common inherited bleeding disorder.
- Arises from deficiencies or defects in von Willebrand factor (VWF).
- VWF has two primary functions/roles:
  - –'carries' FVIII, and protects/stabilises FVIII:C function.
  - –permits adhesion of platelets to sites of vascular damage.

Clinical picture

- Physical examination - bruising

–Bleeding history (spontaneous/surgery)

–Family history (siblings/parents/grandparents)

–Age, gender, Pr analyticals eg stress, exercise etc

Bleeding scores in VWD

EU study (mainly adults) – Tosetto et al JTH 2006:4:766-773

- 195 controls score median <1 and all but 1 <4
- Index cases – median score 9
- Affected family members – median 4

Biss et al JTH 2010:8; 950-956 paediatric scoring

- VWD – median score 7, control group 0

VWD - Classification

- *Type 1:* **Partial quantitative deficiency (reduced levels of functionally normal VWF).**
- *Type 2:* **Qualitative defect (absolute levels of VWF low or normal, but VWF 'function' diminished).**
- *Type 3:* **Total quantitative deficiency (VWF 'absent').**

Type 1 VWD diagnosis
(ISTH/SSC - Sadler & Rodeghiero 2005)

- Significant mucocutaneous bleeding
- Laboratory tests compatible with type 1 VWD
- Either positive family history or appropriate VWF mutation

- ALL 3 CRITERIA REQUIRED

Laboratory Investigation - VWD

- Screening tests (APTT, [PT], FBC/platelet count/Hct, bleeding time or PFA-100
- Primary 'Diagnostic' assays (FVIII:C, VWF:Ag, VWF:CB, VWF: RCo).
- Secondary 'Confirmatory/VWD-subtype assisting' assays (2A, 2B, 2M - RIPA, VWF:Multimers; 2N - VWF:FVIII binding assay).

Minimal diagnostic criteria

FVIII:C

At least one functional VWF assay (VWF:CB, and/or VWF:RCo)

VWF:Ag

Further investigation

– repeat of initial tests for confirmation

– additional confirmatory/subtype assisting tests (RIPA, VWF:Multimers, VWF:FVIII binding assay).

Samples should not be stored at 4°C

- 39 normal subjects.
- 3.5 hours at 4°C or 22°C before centrifugation
- FVIII, VWFAg and VWF:CB significantly lower lower in 4°C
- Half could be falsely classified as VWD

Favaloro et al 2004

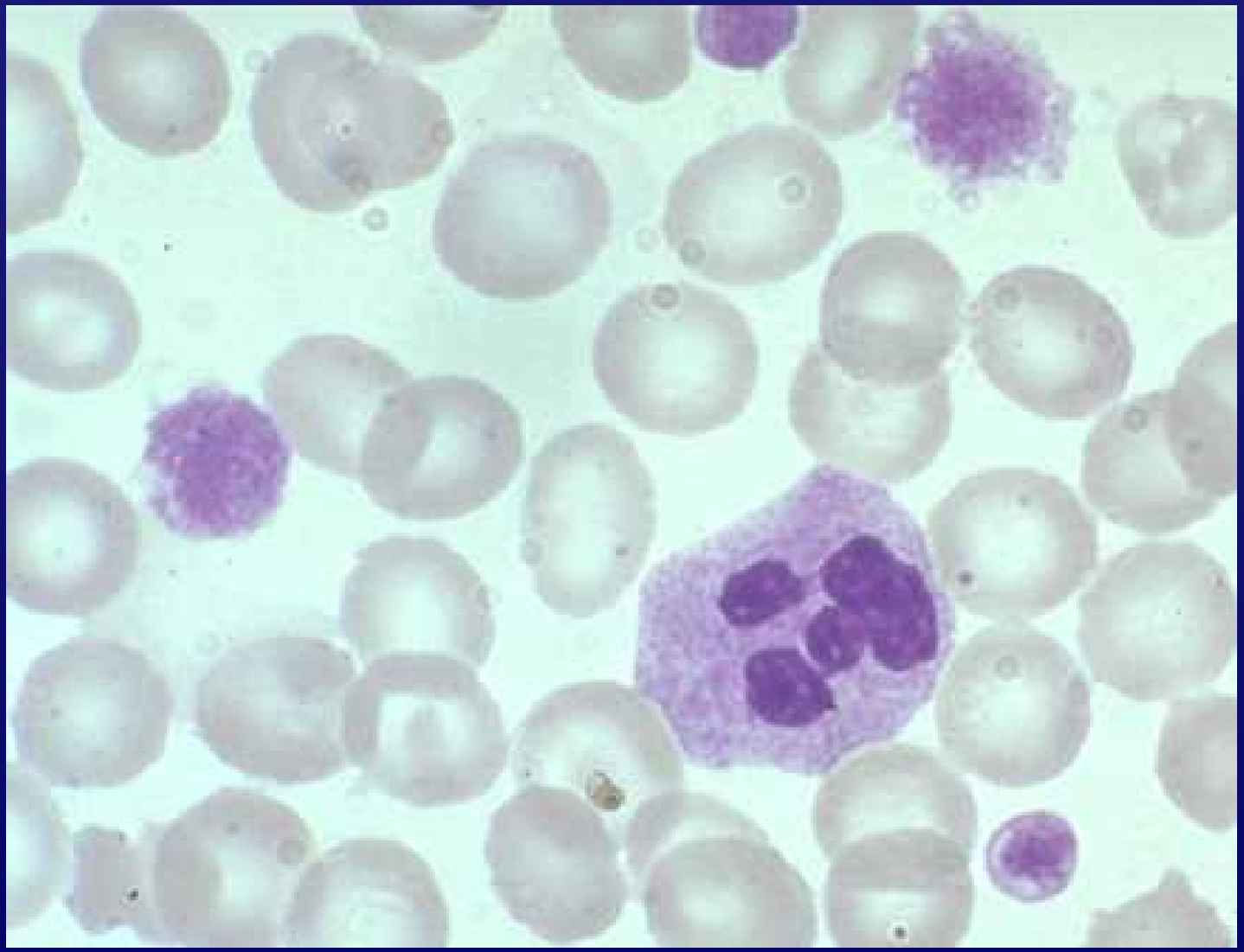
Platelet Investigations

- Clinical History
  - Personal
    - Skin and mucous membrane bleeding (petechiae, ecchymoses)
    - Purpura
    - Recurrent epistaxis
    - GI haemorrhage
    - Menorrhagia
    - Post-operative bleeding, eg post-dental extraction
    - Drug history
  - Family
    - ? Congenital defect

Platelet Investigations
"Screening tests"

- Platelet count – thrombocytopaenia, thrombocytosis
- Platelet Size – beware rbc fragments, platelet clumps, giant platelets
- Platelet Morphology

Giant platelets in Bernard Soulier Syndrome



Platelet Investigations
"Further investigations"

- Platelet Aggregation
- Platelet Nucleotides
- Platelet Glycoproteins
- Thromboxane B2 assay
- Platelet Activation Markers

- Platelet Adhesion
- Clot Retraction
- Electron Microscopy

Methods of platelet Aggregation

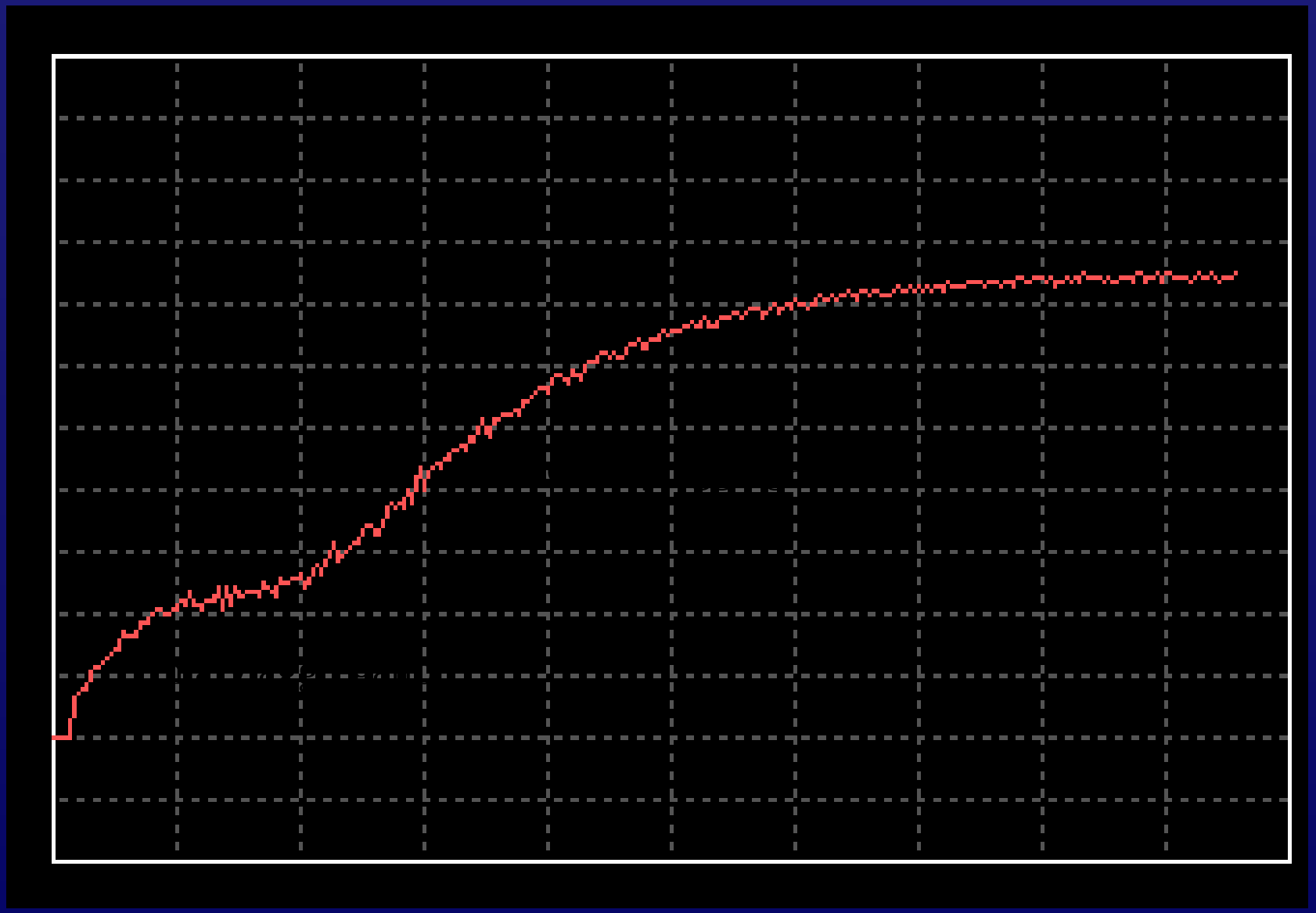
- Light transmission

- Supernatant Platelet count
- Platelet Aggregation Ratio
- Fresh blood film
- Visual assessment
- Electrical Impedance
- Filtration Pressure

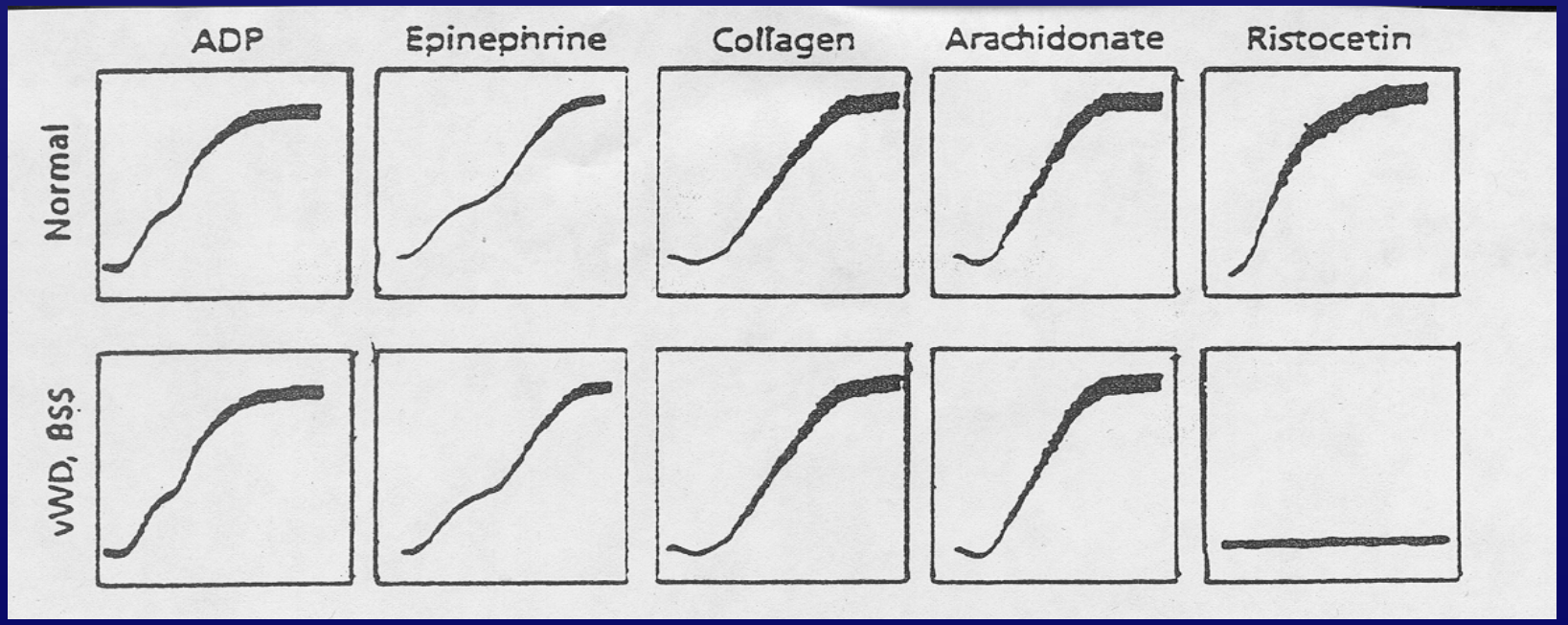
Platelet Aggregation –Pretest variables

- Blood collection (clean venepuncture, .106M citrate, RT°)
- Time of testing (>20min, <2hrs)
- Centrifugation (PRP: 200 x g, 10-15min)
- Platelet count of PRP (~300 x 10^9/l)
- pH (7.7-8.0)
- Mixing/stir speeds (800-1000rpm)
- PCV (citrate may need adjusting)
- Temperature (storage RT, testing 37°)
- Lipaemia (reduces measured response)
- Red cells/granulocytes in PRP (affects measured response)

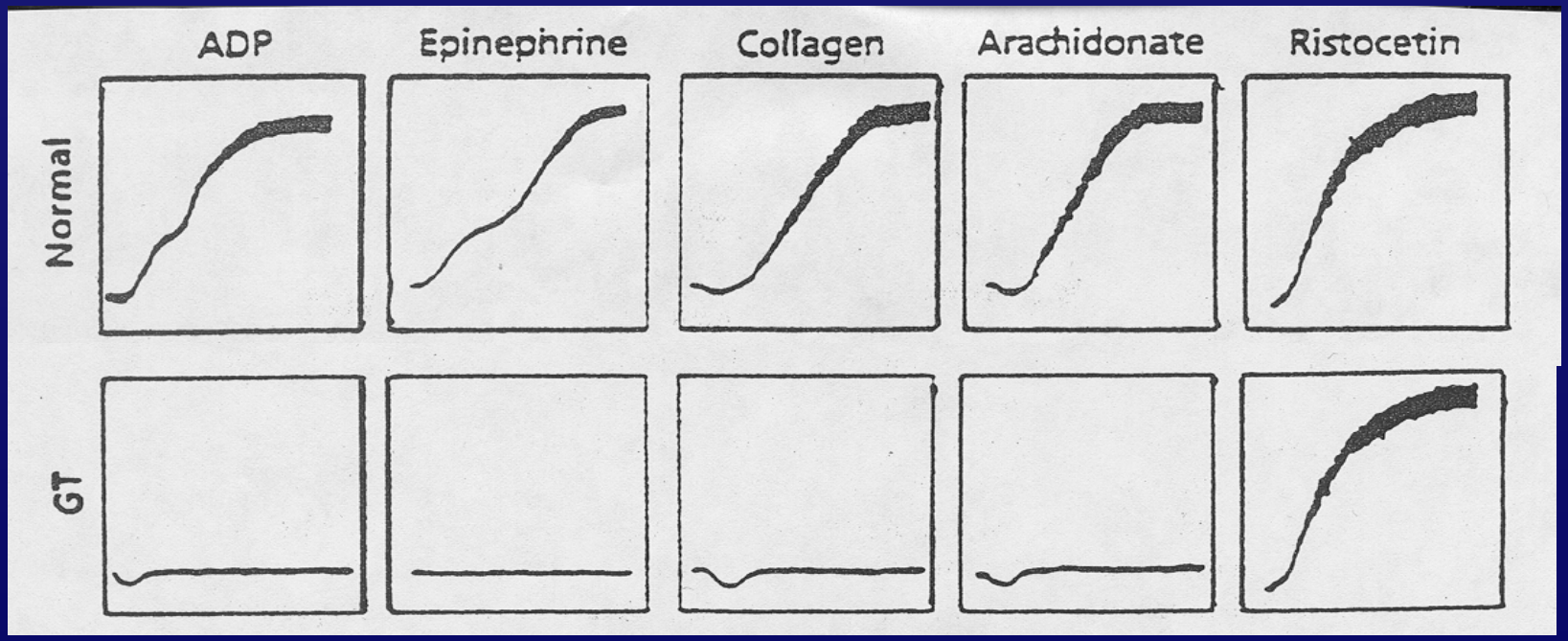
Biphasic aggregation curve



Bernard-Soulier Syndrome



Glanzmann's Thrombasthenia



Storage pool disorders

- More common than BSS or GT
- Storage pool defects
  - Dense body defects: storage/release of ADP, Serotonin
  - "Grey platelet syndrome" – congenital or acquired a-granule defect
- Signal processing defects
  - Particularly acquired defects, drug-induced
- Primary aggregation responses, particularly with ADP
- Confirmation with further platelet investigations

Platelet nucleotides

HPLC

Bioluminescence

- ATP + luciferin + luciferase = light emission
- ADP converted to ATP
- Low levels (nmol/plt) or altered ATP/ADP ratio can indicate SPD

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LABORATORY MANUAL

DIAGNOSIS OF HAEMOPHILIA AND OTHER BLEEDING DISORDERS

A LABORATORY MANUAL

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Prepared for
THE WORLD FEDERATION OF HEMOPHILIA
LABORATORY SCIENCES COMMITTEE

WORLD FEDERATION OF HEMOPHILIA
FÉDÉRATION MONDIALE DE L'HÉMOPHILIE
FEDERACIÓN MUNDIAL DE HEMOFILIA

WFH Laboratory manual
Diagnosis of Bleeding Disorders

- Sample collection & handling
- Reference Ranges
- Quality Control and Assurance
- Screening Tests
- Factor Assay Design
- Reference or Standard plasmas
- Diagnosis of VWD and other disorders

Anticoagulation of Blood

- 9 parts blood decalcified with 1 part anticoagulant
- Trisodium citrate (dihydrate)
- 0.109M (3.2%)
- 0.129M (3.8%)
- 0.109M recommended by WFH, WHO, CLSI
- Both continue to be used

Sample underfilling - Effect of citrate concentration

- Mimimum fill volume

0.109M citrate

- PT (Innovin) 60%
- APTT (Actin FS) 70%

0.129M citrate

- PT 70%
- APTT 90%

Adcock et al 1998

Other Sample variables

- Anaemia and polycythaemia
- Under (over) filling
- Composition (glass, plastic etc)
- Storage time and temperature
- Centrifugation conditions
- Air space in sample (heparin control)

Changing Tubes ?

- Review Scientific literature
- Review manufacturers data
- Assess locally where necessary
- Pay particular attention to screening tests
- Effects may be reagent specific
- Expect differences unless evidence to contrary
- Consider new local normal range

Reference ranges - 1

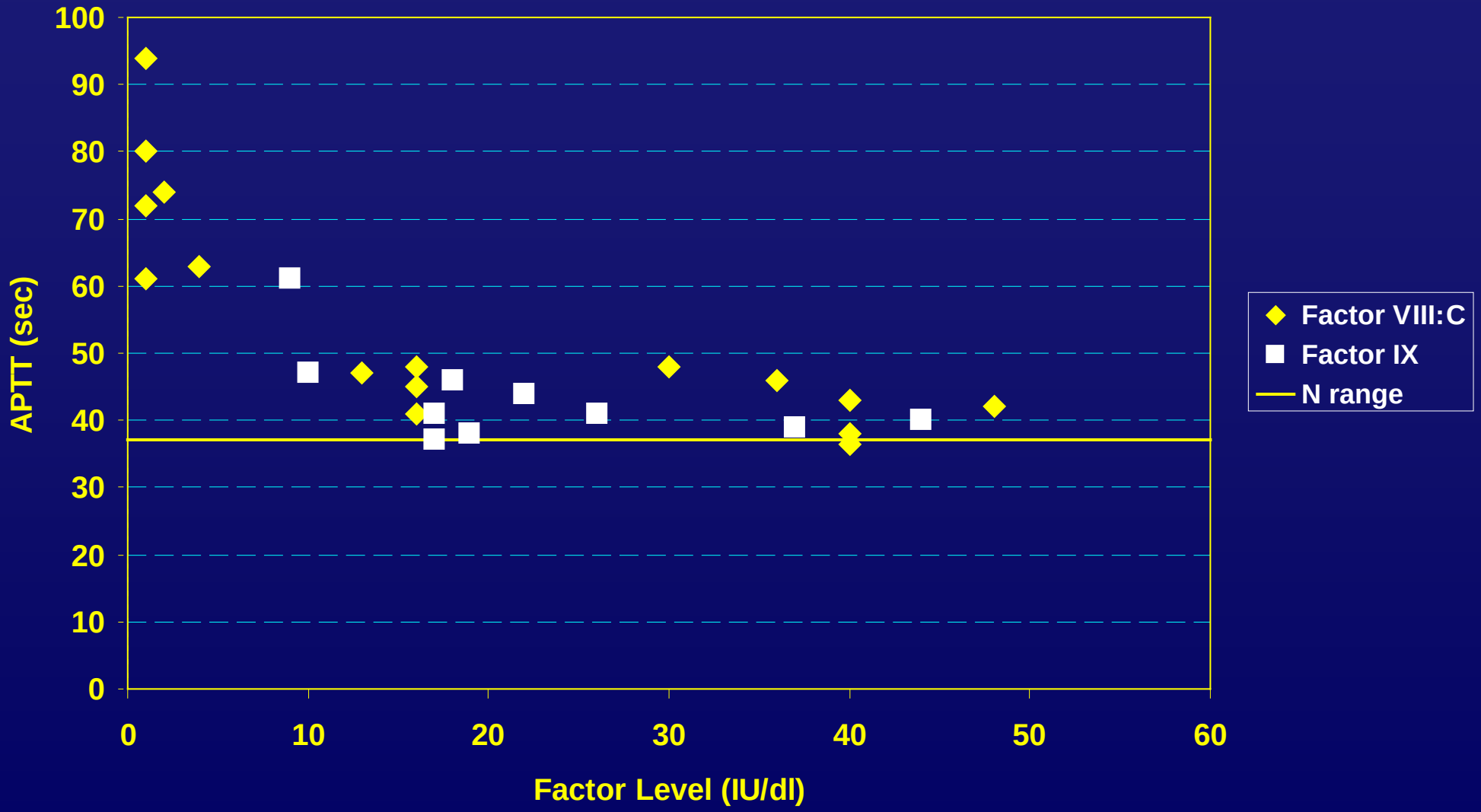
- Establish locally
- Use literature as a guide
- Normal subjects
- Identical collection, processing and analysis as patient samples
- Assess when introducing or altering a method
- Screening Tests – with each new lot number

Reference ranges - 2

- N = 25-30 is adequate for most tests in diagnosis of bleeding disorders
- Inspect data in a graphical form
- Clear/statistical outliers can be excluded
- Mean +/- 2 sd if a normal distribution
- Alternatives- log normal, exclusion of extreme 2.5% from either end
- Normal range is only a guide

Sensitivity of APTT to deficiency of FVIII or IX

Synthasil/Sysmex CA series



Limitations of APTT as a screening test

- Obligatory carrier of Haem B, FIX- 35 IU/dl
- APTT – 32 sec (Normal range 25-35sec)
- Previously prolonged APTT three times with same reagent
- FVIII:C – 280 IU/dl
- Acute phase related increase in FVIII can normalise APTT when FIX (or FXI reduced)

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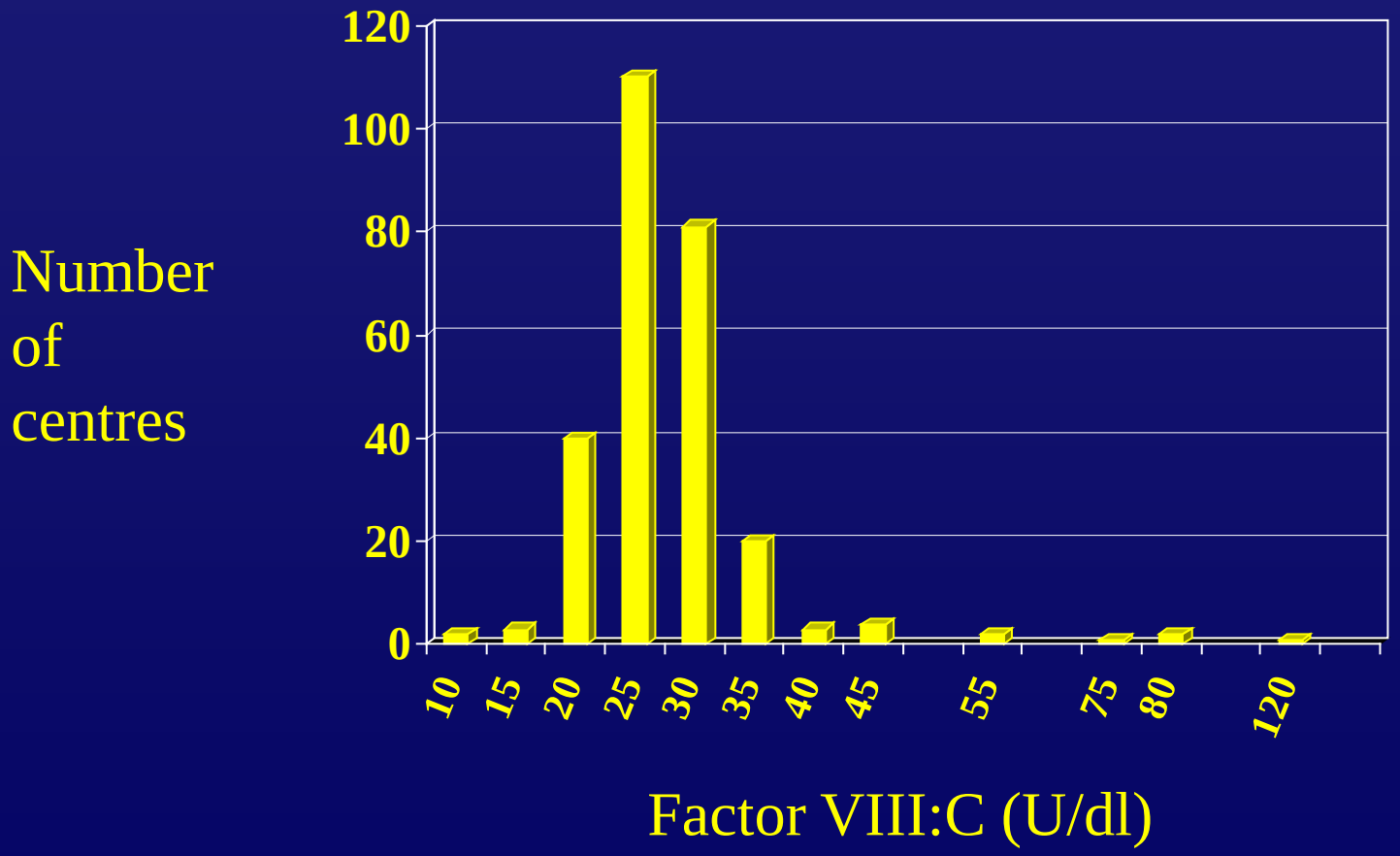
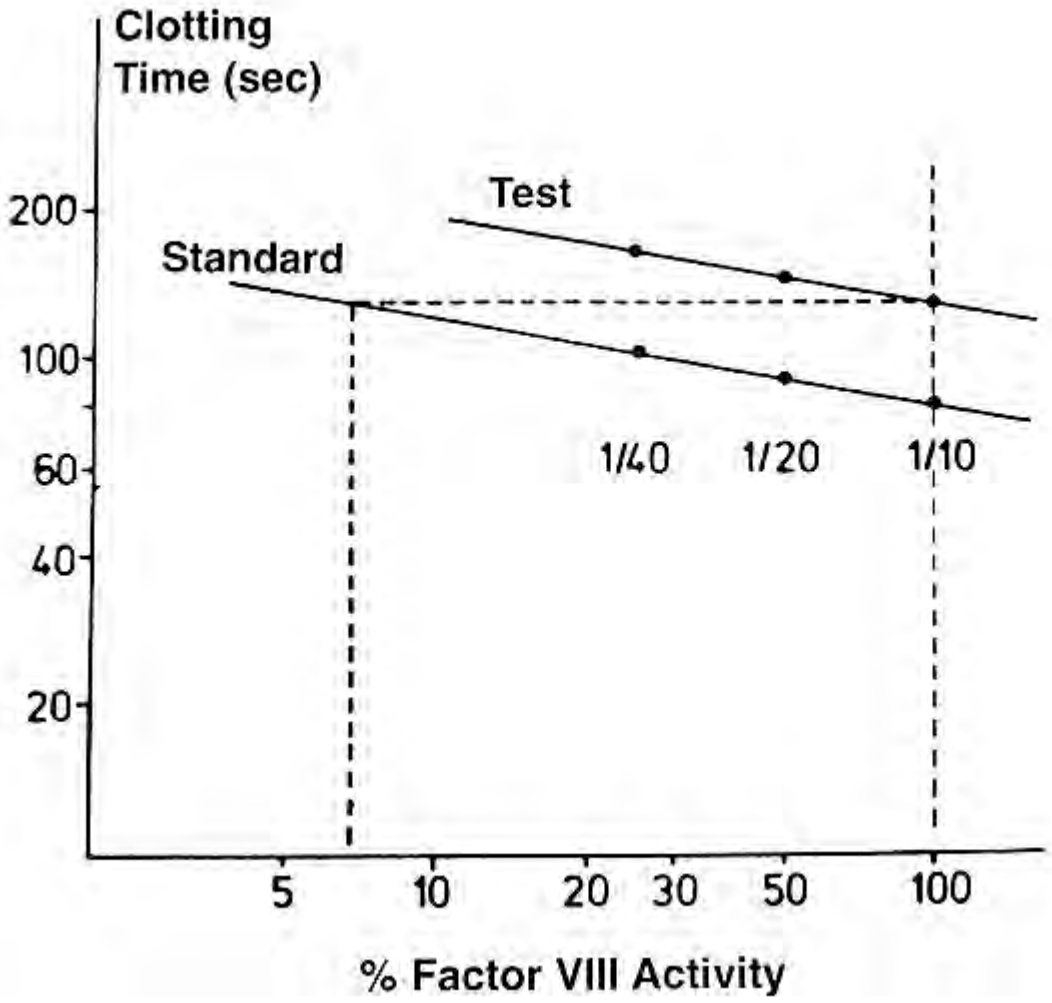
Factor VIII:C results in Different centres

256 centres (UK NEQAS 1999)



5 centres < 15 u/dl

6 centres > 50 u/dl


Clotting
Time (sec)
200
100
60
40
20
Test
Standard
1/40
1/20
1/10
5
10
20
30
50
100
% Factor VIII Activity

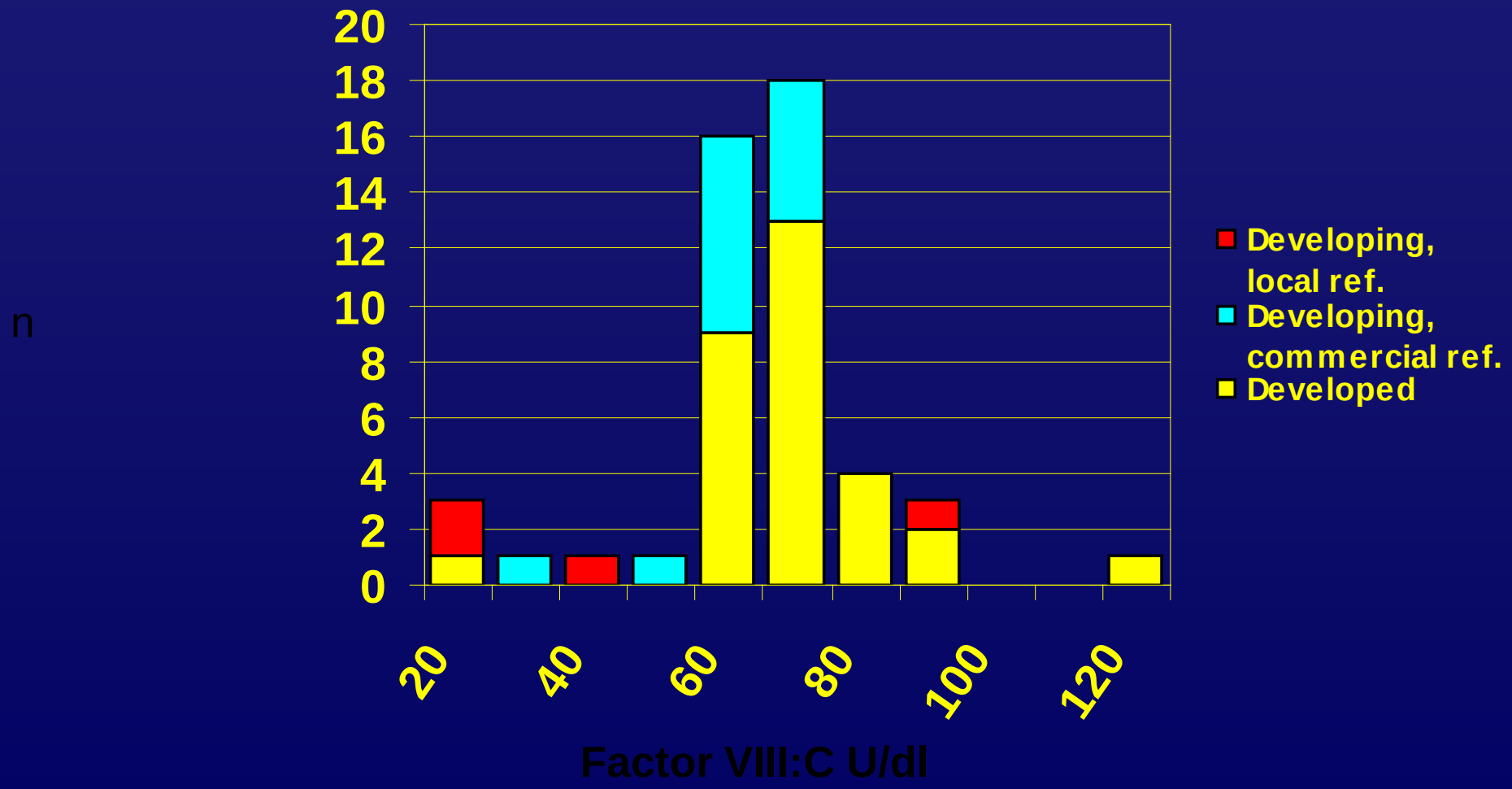
Factor assays - Why 3 test dilutions?

FIX supplementary exercise 2003

Test dilutions	n	Mean FIX U/dl	CV %
1	22	6.3	54%
2	17	6.5	29%
3	42	6.0	23%
ANOVA		ns	P = 0.03

WFH Survey 4, 2005: FVIII:C
(UK NEQAS median 75.0 IU/dl)

Developing Country results by source of reference plasma



Factor VIII:C – NEQAS survey

Commercial reference plasmas (n>10)

Source	n	Median (u/dl)
1	46	86
2	81	76
3	86	76
4	14	72
5	12	73
6	10	75
All	299	77

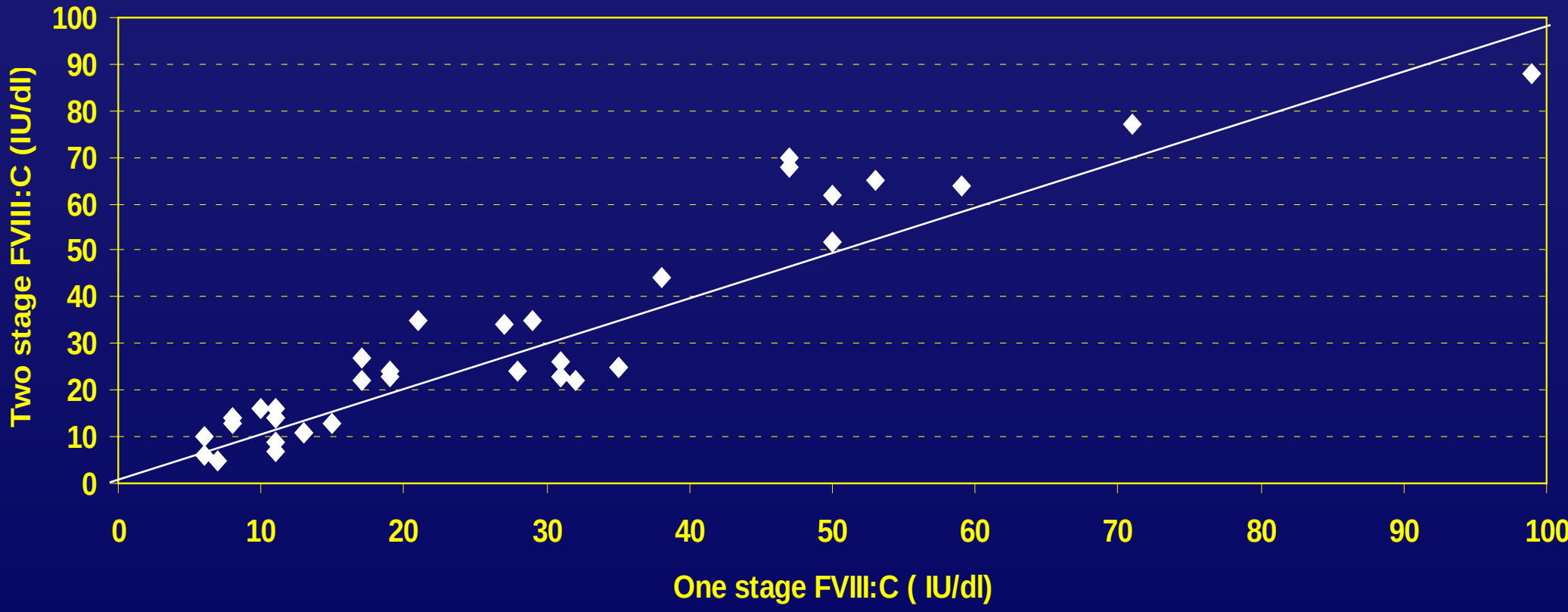
Factor VIII:C

Commercial deficient plasmas (S149 2005)

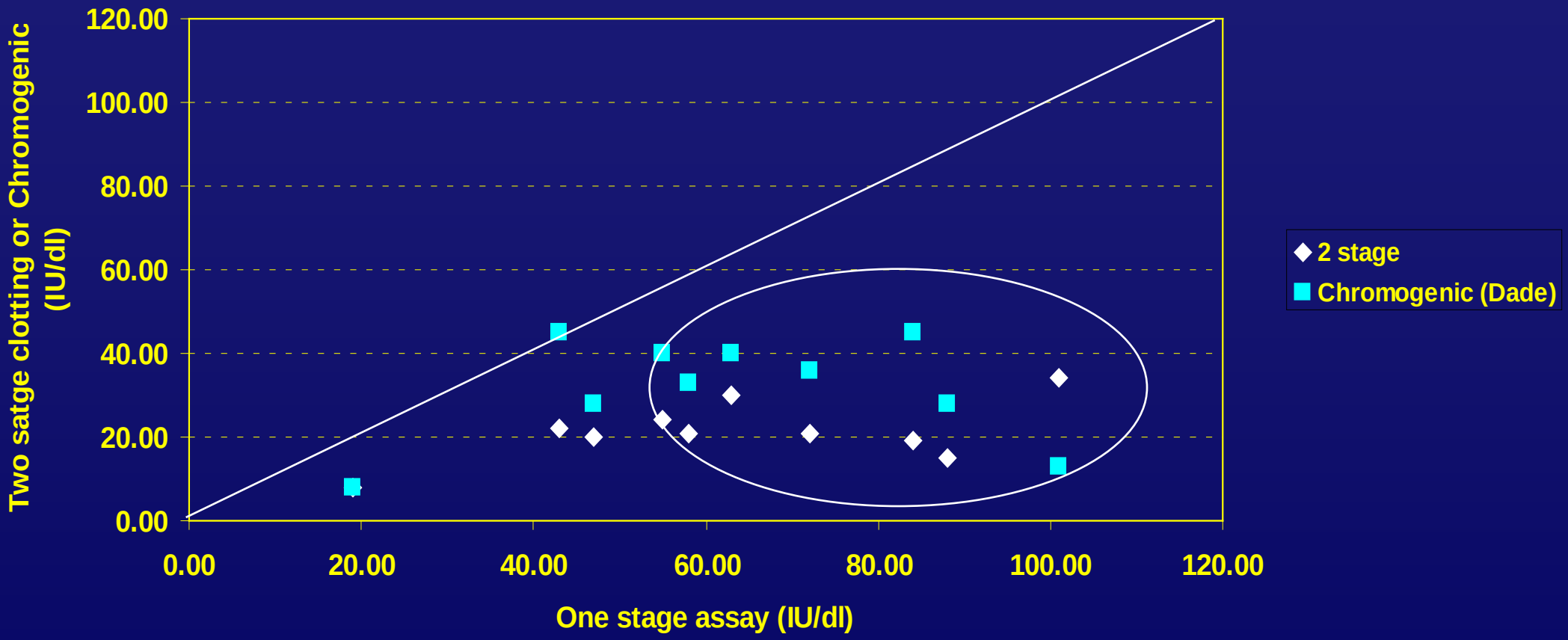
Source	n	Median (u/dl)
A	32	13.0
B	82	15.0
C	7	30.0
D	47	15.0
E	84	17.0
F	18	12.6
All	327	15.0

C – FVIII< 1 U/dl, FV = 3 U/dl, other factors normal

One Stage and Two stage (clotting) FVIII:C results in previously diagnosed mild Haemophilia A (75-80% of patients)



Factor VIII assays in Mild Haemophilia A
7/68 patients have normal 1 stage, reduced 2 stage



Normal One stage FVIII and Normal APTT in ~ 10% Sheffield cases

Common discrepancy

Bleeding symptoms consistent with mild haemophilia

(ie consistent with the lower result)

How common is this?
(Rodgers et al 2008)

- 97 mild Haem A (many genetically confirmed)
- 39 have discrepant phenotype
- 8 (8%) have normal one stage FVIII:C
- Bleeding consistent with mild haemophilia

Common discrepancy

	Patients screened	Discrepancy	Normal 1 stage	
France	73 mild/mod	11 (15%)	0	Parquet-Gernez, et al 1988
Australia	97 mild	39 (40%)	8	Rodgers et al 2008
UK	68 mild	10 (15%)	7	Sheffield data
Spain	163 mild	24 (15%)	0	Cid et al 2008
Denmark	92 mild	36% of families	0	Poulsen et al 2009

Different chromogenic assays in discrepant patients

	Mean IU/dl	Range IU/dl	Incubation time
Coamatic	26	17 - 34	150 sec
Dade-Behring	41	33 - 47	100 sec
Hyphen	22	13 - 31	300 sec
One stage	39	32 - 55	-
Two stage	10	6 - 14	-

Rodgers et al 2008.

Conclusions – Common Discrepancy

- Occurs in 10-35% of mild haemophilia A
- Totally normal FVIII:C by one stage in ~ 5 -10% of mild Haem A
- APTT totally and consistently normal in these 5-10%
- Bleeding consistent with mild haemophilia

Recommendations - 1

1. Use a single citrate concentration and sample type where possible
2. Determine local normal ranges using same sample collection and processing as for patients.
3. Consider the performance characteristics & limitations of the test.

Recommendations 2 – Assays

- Reference plasma traceable to WHO standards where available
- Calibration curve with each group of tests
- 3 dilutions of test plasma
- QC sample with each group of tests
- EQA where available
- Two types of FVIII assay to detect all cases

Convincing History but nothing identified?

- Platelet disorder (eg storage pool disease) with normal initial aggregation
- Mild Haemophilia A with normal One satge FVIII
- Factor XIII deficiency
- Type 2M VWD with normal VWF:RCo and reduced CBA
- Fibrinolytic problem (eg alpha 2 Antiplasmin)

2M VWD with normal VWF:RCo

(Keeling et al Haemophilia 2011

- 17 year old girl, menorrhagia, epostaxis, Bleeding score 5
- FVIII:C 107 IU/dl , VWF:Ag 73 IU/dl
- VWF:RCo 88 IU/dl
- CBA (Corgenix, Equine type III) – 10, 27 IU/dl (on 2 visits)
- CBA (2nd visit) –(Technoclone, pepsin digested Human type III) 66 IU/dl

Laboratory tests and assays should be used in conjunction with a careful personal and family history.