Laboratory assays for the measurement of Factor VIII and FIX

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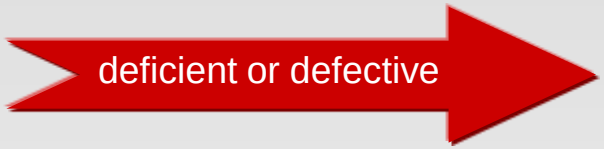
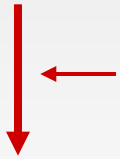
Haemophilia A
Haemophilia B



Factor VIII
Factor IX

clinical severity of haemophilia A/B



circulating activity
of FVIII/IX

severe (FVIII/IX:C <1.0 IU/dl)
moderate (FVIII/IX:C 1.0–5.0 IU/dl)
mild (FVIII/IX:C >5.0 IU/dl)

FVIII/IX activity measurement

Diagnosis

- accurate diagnosis of FVIII/IX deficiency
- assessment of clinical severity
- identification of patients with sub-haemophilia
- identification carriers of haemophilia

Treatment

- monitoring of on-demand and prophylactic therapy
- prerequisite for patient-tailored treatment strategy
- monitoring postinfusion pharmacokinetics of concentrates

Manufacturers

- potency labelling of clotting factors concentrates

Laboratory analyses of FVIII/IX deficiency

First-line coagulation testing

aPTT - simple, rapid and highly reproducible → provide only a little information

Second-line coagulation testing

One-stage clotting assays
Two-stage clotting assays → Fibrin formation as the endpoint of the test

Chromogenic assays → records the enzymatic activity of activated factor X (FXa)

Alternative coagulation tests

Global hemostasis assays

Thrombin generation test (TGT)
Thromboelastography (TEG)
aPTT waveform analysis → kinetics of clot formation

Ideal Laboratory FVIII/IX activity measuring assays

- **High Specificity** - to assess unequivocally FVIII/IX in the presence of components which may be expected to be present
- **High Accuracy** -the closeness between an accepted reference value and the value found.
- **High Precision** –close results between a series of measurements obtained from multiple sampling of the same homogeneous sample
  - **Repeatability**
  - ***Reproducibility***
- **Detection Limit** -lowest amount of FVIII/IX in a sample which can be detected but not necessarily quantitated as an exact value (<1 IU/dL)
- **Quantitation Limit** -is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy
- **Calibration** (traceability to International Standard)
- **Compliance with international guidelines**

Current State

FVIII/IX content
in plasma or treatment concentrates

evaluated by activity-based assays

one-stage activated partial thromboplastin time (APTT) based clotting assay

chromogenic assay

One-stage vs Chromogenic Assay

One-stage assay

- Plasma based system with native concentrations of coagulation factors
- Based on measuring the activated partial thromboplastin aPTT) - endpoint: clot formation
- Wide heterogeneity of available methods (reagents, alysers,deficient plasmas)
- High inter- and intra laboratory variability

Chromogenic assay

- Artificial system employing highly diluted plasma with high thrombin concentrations
- Two sequential reactions : 1st – FXa forming ; 2nd –measurement of FXa by chromogenic substrate
- Independence of clotting-specific reagents, easy to automate, only several commercial kits available
- Less inter- and intra laboratory variability

Disadvantage

inability to measure very low levels of clotting factor activity

Variables influencing assays

32–68% of all laboratory errors occur in the preanalytical phase

Bonini et al 2002

- Pre-analytical Variables
  - sample collection- phlebotomy, blood collection systems,
  - sample handling- transport, exposure to heat, overall time to delivery, haemolysis, icterus and lipaemia, storage
- Analytical
  - ✓quality of the reagents
    - Reference plasma
    - Factor VIII/IX-deficient plasma -the absolute absence of FVIII/IX, excess of all factors except FVIII,presence of VWF
    - aPTT reagent
  - ✓alterations in the procedures used in different laboratories
  - ✓Detection system employed
  - ✓experience and precision of the personnel

one-stage activated clotting assay
chromogenic assay

generally a good correlation between factor levels and clinical phenotype

Dicrepancies between measured factor VIII/IX levels and clinical phenotype

Discrepancies

Decreased assay sensitivity at very low VIII/IX levels of activity (<1IU/dl)

Discrepancy between chromogenic and clotting assays VIII levels

Clinical impact

- heterogeneity in bleeding pattern among patients with severe hemophilia
- effect of FVIII replacement therapy in the absence of FVIII activity response
- haemophilia B gene therapy – therapeutic effect already at FIX level of <1 IU/dL
- no standardized assays to assess patient response to bypassing agents

- Misdiagnose haemophilia with one of the methods

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Mild haemophilia A patients with specific mutations

- Potency labeling of newly developed recombinant products

Heterogeneity in bleeding pattern among patients with severe hemophilia

10–15% of severe hemophiliacs (factor activity<1 IU/ dL) differ considerably in their bleeding tendency possibly due to:

E.Santagostino et al 2010;van den Berg M et al 2008

Individual

prothrombotic mutations -FV Leiden or prothrombin G20210A

inter-individual variance in the pharmacokinetics of FVIII

von Willebrand antigen level (ABO blood group -patients with blood group O, have a 30% lower von Willebrand antigen level)

Gene defects

Contradictory data, more studies are needed

Laboratory

variations in FVIII/IX activity below detection limits (FVIII/IX<1IU/dl)

Standard clotting and chromogenic assays

precise low levels of FVIII/IX:C (<0.2 IU/dl)
more consistent with severe clinical phenotype

Shima et al 2008

Can this problem be solved?

Application of two standard curves covering the lower and higher ranges of clotting factor activity

Optimised chromogenic assay -method based on commercially available reagents that claims a detection limit of 0.15 IU /dL

Yatuv R et al 2006

Can this problem be solved?

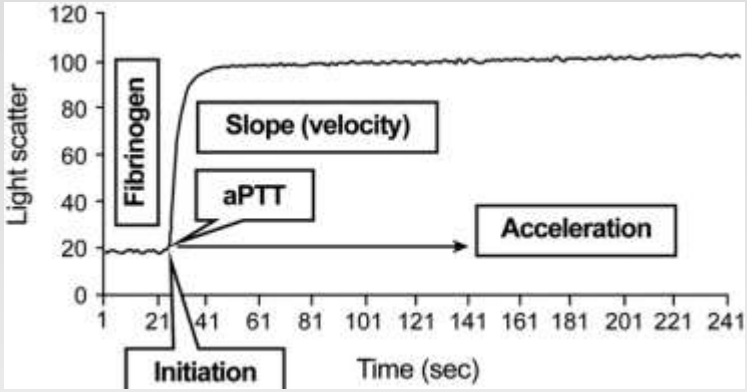
Global hemostasis assays

Clot waveform analysis

Thrombin generation

Thromboelastography

Clot waveform analysis



detect the optical profile of clot formation during performance of the aPTT

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provides information on velocity and amount of the formed fibrin

- discriminate between different levels of FVIII:C in severe HA (FVIII:C <1.0 IU/dl by conventional assays)

•Shima M, et al 2002, Matsumoto T,et al 2006

- detect variability in coagulation activity in individual patients

Nair SC et al 2010

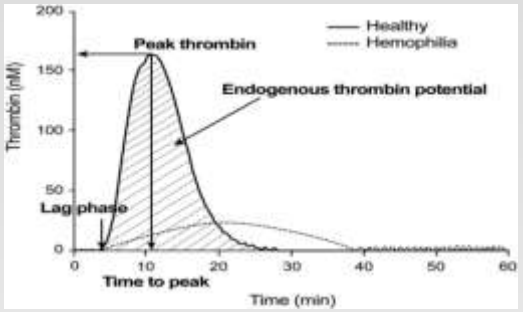
- monitoring the haemostatic effect of FVIII concentrate during ITI therapy

Kasuda S, et al 2004

- assessment of clotting function in acquired haemophilia A

Matsumoto T, et al 2012

Thrombin generation test



Dynamic assay – real time measurement of thrombin generation in PPP and PRP

Can TGT discriminate FVIII/IX levels <1IU/dl?

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little information is available on the use of the TGT for assessing very low levels of FVIII:C

- good correlation between clinical severity and PeakTh in hemophilia A, the TF-TGT did not offer any advantages for the evaluation of clinical severity

Beltran-Miranda 2005

- a modified TF/ELG-TGT is sensitive to very low levels of FVIII:C (app.0.2 IU/dl)

Matsumoto et al 2009

Thrombin generation test

- individually assess bleeding risk in hemophilia patients or to define the phenotypic heterogeneity seen in these patients

Dargaud Y et al 2005

- monitor bleeding risk and response to treatment in acquired hemophilia

Spiezia L et al 2009

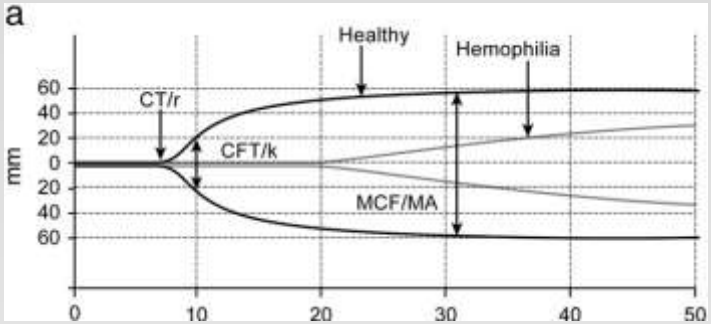
- show inter- and intra-patient variability in response to treatment

Kennet G et al 2010

- corelates with bleeding risk and could predict dosing of bypassing agents for hemophilia patients with high-titer inhibitors

Chitlur M 2012 Dargaud Y et al 2010;

Thromboelastography



TEG or ROTEM - continuous assessment of the viscoelastic properties of a forming clot

- Correlation of thromboelastography with clinical phenotype

- Considerable heterogeneity in the thromboelastography patterns amongst patients with verified factor VIII levels < IU/dl

Ingerslev J,et al 2003

Less pathological clotting profiles are associated with a less severe bleeding phenotype

Chitlur M,et al 2008

- predict the clinical response to bypassing agents in patients with inhibitors

Sorensen B, Ingerslev J 2004, 2007

Global hemostasis assays in determination of low FVIII/IX levels (limitations)

- still more expensive
- labor -intensive
- require specific equipment and qualifications
- require a rigorous process of pre-analytical and analytical standardization
- the correlation between test results and FVIII concentrations are not always conclusive
- High intrapatient and interpatient variability
- a high coefficient of variation of external quality assurance samples

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limit their implementation within the routine of most clinical laboratories.

Discrepancy between chromogenic and clotting assays VIII levels chromogenic and clotting assays

Discrepancies

(1/3 of cases with non-severe haemophilia)

Poulsen et al 2009

FVIII:C one-stage > FVIII:C chromogenic

or

FVIII:C one-stage < FVIII:Chromogenic

$FVIII:C_{chr}/FVIII:C_{1st}$ or vice versa ratio ≤ 0.6

NO consensus as to which method most accurately represents the FVIII cofactor function in vivo and gives the clinically relevant FVIII:C levels.

Hereditary Phenomena

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Specific mutations in *F8*

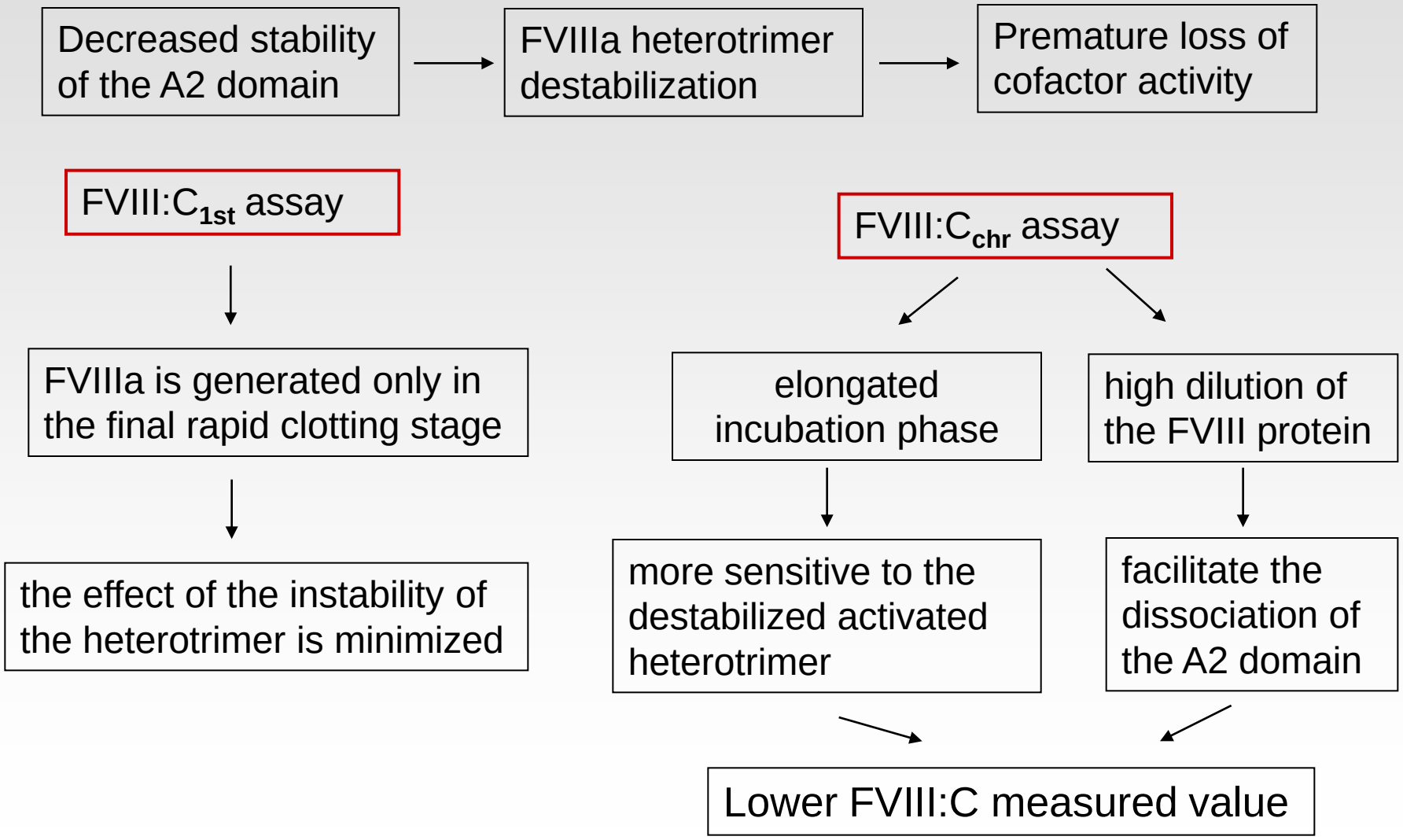
$FVIII:C_{1st} > FVIII:C_{chr}$

Mutations	FVIII: Ag (U/dl)	FVIII:C1st (IU/dl)	FVIII:Cchr (IU/dl)	Ratio FVIII:C chr/1st
Tyr114Cys	25	7	4	0,6
Thr118Ile	11	2	1	0,6
Asp203Val	8	2	1	0,6
Thr275Ile	35	4	1	0,2
Asn280Ile	15	6	3	0,5
Arg531His*	60(59-61)	26(20-31)	13(12-14)	0,5
Arg531Cys*	28(45-15)	13(11-14)	5(4-7)	0,4
Val663Ala	54	28	18	0,6
Arg1749His*	60	10	5	0,5
Pro1825ser	41(38-44)	15(14-16)	7(6-8)	0,5
Arg1941Gln	23	7	4	0,5

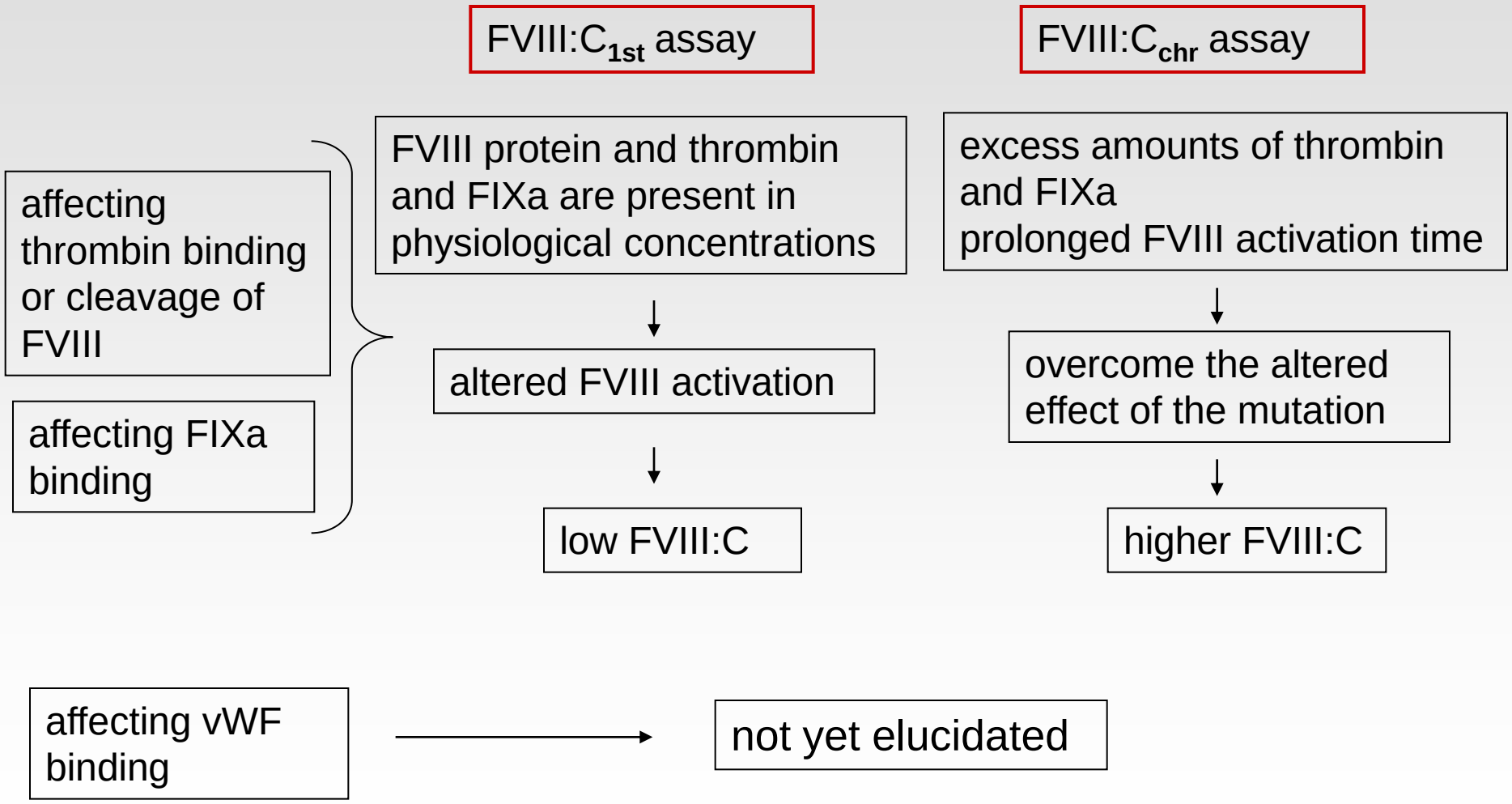
$FVIII:C_{chr} > FVIII:C_{1st}$

Mutations	FVIII: Ag (U/dl)	FVIII:C1st (IU/dl)	FVIII:Cchr (IU/dl)	Ratio FVIII:C 1st/1chr
Lys166Thr	21	9	18	0,5
The295Ala	18	9 (6-22)	17 (10-41)	0,5
Leu412Phe	18	4	10	0,4
Arg527Trp**	90 (61-138)	12 (8-23)	31 (20-52)	0,4
Val678Leu	94	39	78	0,5
Met702Leu	10	2	6	0,3
Glu720Lys*	88 (62-133)	24 (16 -35)	94 (54-120)	0,2
Tyr1680Phe	16 (10-39)	4 (2-7)	9 (4-13)	0,5
Arg1689His*	113	16	41	0,3
Leu1756Phe	17	9	19	0,5
Leu1756Val	10	3	9	0,4
Arg1781Gly	10	4	7	0,5
Ser2119Tyr	15 (10-24)	3(2-5)	7 (5 -9)	0,4

Proposed Mechanism
FVIII:C_{1st} > FVIII:C $_{chr}$ Discrepancy



Proposed Mechanism
FVIII:C_{1st} < FVIII:C $_{chr}$ Discrepancy



Conclusions (I)

- Although the difficulties in standardisation, plasma coagulation assays are still the golden standard for measuring FVIII/IX levels
- The improvement of the routine management of severe hemophiliacs requires an understanding of the clinical significance of extremely low levels of FVIII and FIX activites
- Application of global haemostatic tests for measuring FVIII/IX , especially in levels < 1IU/dl still have to be demonstrated
- Application of global haemostatic tests might help in accurate and efficient monitoring of hemostatic response to bypassing agents in tratments hemophiliac patients with inhibitors
- Better knowledge of the exact mechanism of application of different tests in hemophilic patients may improve individually tailored treatment strategies.

Conclusions (II)

- The FVIII:C one-stage and chromogenic assay discrepancies showed systematic differences
  - due to the specific missense mutation
  - due to assay procedures itself

- Mutations can be used as a model to dissect the nature and mechanisms by which the various FVIII:C assays showed systematically discrepant results

- The initial diagnoses of non-severe HA phenotypes should be based on results from both, the FVIII:C one-stage and FVIII:C chromogenic assays.

Thank you for your attention !