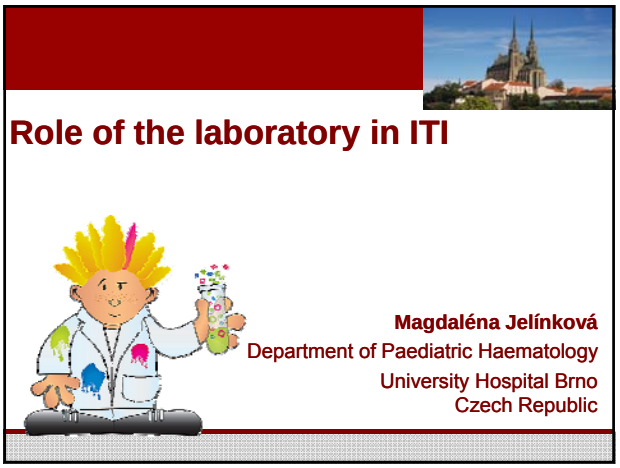

Role of the laboratory in ITI

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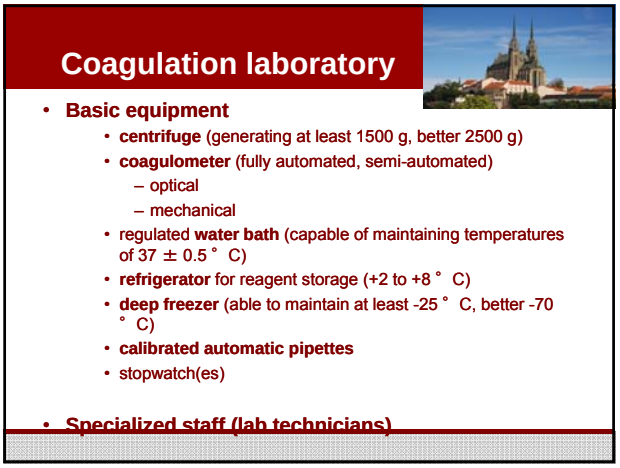
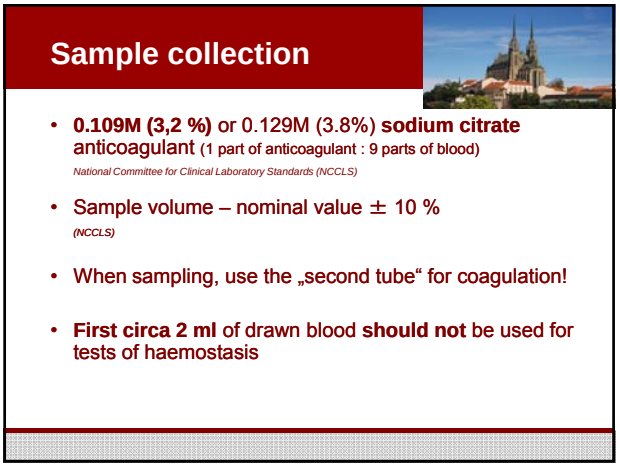


Coagulation laboratory

- **Basic equipment**
  - **centrifuge** (generating at least 1500 g, better 2500 g)
  - **coagulometer** (fully automated, semi-automated)
    - optical
    - mechanical
  - regulated **water bath** (capable of maintaining temperatures of 37 ± 0.5 ° C)
  - **refrigerator** for reagent storage (+2 to +8 ° C)
  - **deep freezer** (able to maintain at least -25 ° C, better -70 ° C)
  - **calibrated automatic pipettes**
  - stopwatch(es)
- **Specialized staff (lab technicians)**



Sample collection

- **0.109M (3,2 %)** or 0.129M (3.8%) **sodium citrate** anticoagulant (1 part of anticoagulant : 9 parts of blood)
  National Committee for Clinical Laboratory Standards (NCCLS)
- Sample volume – nominal value ± 10 %
  (NCCLS)
- When sampling, use the „second tube“ for coagulation!
- **First circa 2 ml** of drawn blood **should not** be used for tests of haemostasis

Sample collection

- **Haematocrit > 0.60** and < 0.25 ⟶ the volume of anticoagulant is specifically calculated from the formula:

$$V\,(ml)\ \underline{citrate} = \frac{100 - haematocrit(\%)}{595 - haematocrit(\%)}\ xV\,(ml)blood$$

Transport of the sample

- Up to **2 hours** at room temperature +15 to +25 ° C for whole blood samples
- Frozen plasma samples at -20 ° C and below can be stored/transported much longer

Pre-analytical variables

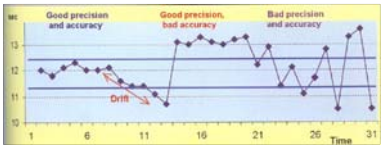
- **Centrifugation**
  - **PPP** (platelet-poor-plasma) - at minimum 1500 g for at least 10 min at room temperature +15 to +25 ° C
    - Cold activates FVII and system of kalikreins
    - Refrigerated centrifuge (< +15 ° C) – specific samples only (e.g.anti Xa, PAI,…)
  - Some test procedures require the plasma to be centrifuged twice (e.g. LA)
- **Testing of the sample**
  - Samples should be tested within 4 hours of sample collection when possible (it depends on the test procedure – e.g. aPTT within 2 hours, samples with heparin within 1 hour)

Pre-analytical variables

- **Deep-frozen plasma samples**
  - Better to freeze plasma at -196 ° C („shock-freezing“ in liquid nitrogen)
  - Storage at -70 ° C or lower is preferred (some clotting factors can be then tested even after 6 months of storage)
  - Storage at -20 ° C (max 1 month)
  - Thawing for 5-10 min at +37 ° C , then mix by reversing the tube gently

Quality Control

- **Internal quality control (IQC)**
  - Intermediate precision (results ± 2 SD, mean, CV,…)
  - Reproducibility
  - Commercial QC with target range of acceptable values
  - Quality control between instruments



Quality Control

- **External quality assessment (EQA)**
  - Is used to identify the degree of agreement between one laboratory`s results and those obtained by other centres
- **ISO (certification, accreditation of the laboratory)**

Normal reference range

- **Respect age, sex, ABO group**! (F VIII, vWF)
- Follow **national/international recommendation**
- Check **the literature** ☺
- Respect **manufacturer`s information/recommendation**

- You may also rely on your **own laboratory reference range** (healthy normal subjects - reference range should include the central 95 % of values)

Activated partial thromboplastin time (aPTT)

- **Screening aPTT reagent:**
  - sensitive to defect of factors
  - sesitive to heparin (UFH)
  - sensitive to LA

- **aPTT reagent for determination of factors, inhibitors:**
  - non-sensitive to LA
  - sensitive to defects of factors

Carefully select your aPTT reagent !

aPTT

- **Calibration**
  - aPTT time for „normal plasma“ in seconds (mean of repeated measurements)
  - calibrator = „normal plasma“ (pooled normal plasma (PNP) or commercially provided normal plasma)

- **Results**
  - time (seconds)
  - Ratio (R)

R = (t_{pac}/t_{norm})

aPTT

- **Normal reference range**

Age	aPTT - R
0 – 1 m	0,8 – 1,5
1 m – 1 y	0,8 – 1,3
> 1 y	0,8 – 1,2

  - **Time in seconds is significantly dependent on reagent and instrument used!**

- **Clinical relevance**
  - Hypo coagulation (prolonged coagulation time)
  - Hyper coagulation is not relevantly mirrored in aPTT!
    - shortened aPTT times – low sensitivity – no clinical relevance (except sampling errors)

aPTT

- **Prolonged aPTT:**
  - Defect of factors: VIII, IX, XI, XII, PK, HMWK
  - Defect of factors V, X, II (the common pathway, PT longer too)
  - VWD
  - Dys- or afibrinogenemia
  - Presence of inhibitors (specific, non-specific)
  - Presence of heparin (UFH)
  - Presence of FDP
  - Physiologically - newborns (R = 0,8 – 1,6)
    - children up to age 1 year (R = 0,8 – 1,3)
  - Sampling errors

Correction (mixture) tests

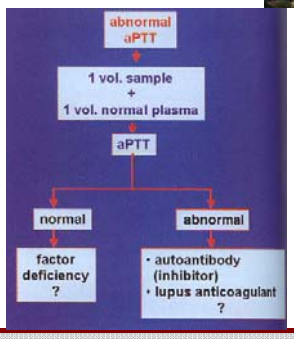
- For further investigation of abnormal aPTT (when PT is normal) or PT (when aPTT is normal)
- **N** (normal plasma)
  P (patient`s plasma)
  M (mix = N+P (1+1))
- Determination of aPTT/PT

Correction (mixture) tests

- **Is the defect in patient`s plasma corrected adding normal plasma ?**
  - **YES** ⟶ aPTT /PT correction of more than 50 % ⟶ **factor deficiency** is very likely
  - **poor correction or no correction** ⟶ **inhibitor** to one of the clotting factors (specific inhibitor) or non-specific inhibitor (e.g. LA) ⟶ circulating anticoagulant

Correction (mixture) tests

- normal PT



Circulating anticoagulant

- Identification of time-dependent / time-independent inhibitor
- Specific inhibitor (against coagulation factor)
- Assays based on aPTT/PT

- Tubes: **N, P, 4N+1P, 1N+1P, 1N+4P**
- Determination of aPTT/PT
- Incubation for **2 hours at 37 ° C**
- Determination of aPTT/PT

Circulating anticoagulant

Sample	defect of the factor	time-dependent inhibitor	immediate-acting inhibitor
normal plasma	37	37	37
patient`s plasma	90	90	90
P+N (1:1) before incubation	41	45	70
P+N (1:1) after incubation	43	70	72

Time (s)

Incubation for 2 hours at +37 ° C

Circulating anticoagulant

Sample	defect of the factor	time-dependent inhibitor	immediate-acting inhibitor
normal plasma	37	37	37
patient`s plasma	90	90	90
P+N (1:1) before incubation	41	45	70
P+N (1:1) after incubation	43	70	72

Time (s)

Incubation for 2 hours at +37 ° C

Circulating anticoagulant

- **Calculate:**

  4N+1P: 4 x time (N) + 1 x time (P) / 5

  1P+1N: 1 x time (P) + 1 x time (N) / 2

  1N+4P: 1 x time (N) + 4 x time (P) / 5

  time.....time of aPTT (PT) in seconds

Identification of inhibitor:

Addition of 1/5 pacient`s plasma to 4/5 of normal plasma causes markedly prolonged time of normal plasma sample.

F VIII (one-stage assay)

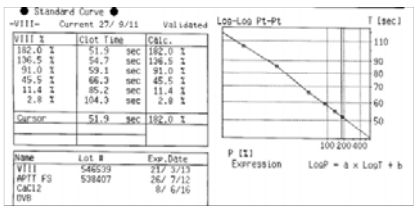
- Activity of the F VIII is determined by modified aPTT test using correction of aPTT in FVIII-deficient plasma by FVIII present in patient's plasma
- **FVIII-deficient plasma**
  - available commercially
  - very low or undetectable level of the FVIII (FVIII < 1 %)
  - high activity of all other factors
  - no anti-FVIII antibodies

F VIII (one-stage assay)

- Measurement of the FVIII:
  - 1 part of diluted patient`s plasma (1:10 with buffer OVB)
  - 1 part of FVIII deficient plasma
  - 1 part of aPTT reagent, incubation
  - 1 part of $CaCl_2$
- Determination of aPTT(in seconds)
- Derive % FVIII from the calibration curve

F VIII – calibration

- Calibrator (calibrated against WHO standard, when possible) with defined factor activity
- The calibration curve (min. 4 dilutions) – double logarithmic plot

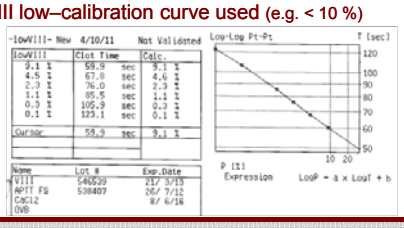


F VIII:C

- **Note!**
  - Not all combinations of deficiency plasma and aPTT reagent work equally well !
- **Significant role**
  - Quality of the deficient plasma
  - The sensitivity of the aPTT reagent to factor deficiency
- **F IX**
  - The same procedure but with F IX deficient plasma

F VIII:C – test for low levels

- FVIII < 10 % (with standard dilution of the sample 1:10)
  ⟶ use lower dilution 1:5 (or 1:2,…) depending on the instrument and the calibration curve used
- FVIII low–calibration curve used (e.g. < 10 %)



F VIII:C – test for low levels

- **FVIII low - calibration curve** (e.g. < 10 %)
  - Dilution of the standard plasma with the FVIII deficient plasma (recommended for optical coagulometers)
- **Quality control**
  - Commercial lyophilized human plasmas with defined factor activity (2 levels: N and P)

Normal reference range

Age	FVIII (%)	FIX (%)
0 - 1d	60 – 140	20 – 75
1d – 1m	60 – 125	65 – 110
1m – 1y	55 – 100	50 – 125
1y – 6y	75 – 150	50 – 110
6y – 18y	50 – 150	60 – 150
> 18y	50 – 150	50 – 150

(Reccomendation of the Czech Haematology Society, 2013)

Defects of F VIII

- Congenital
  - Hemophilia A
- Acquired
  - Due to acquired inhibitors (antibodies)

Chromogenic F VIII assay

- More than 20 % of **mild** hemophilia A patients show discrepancy between activity of F VIII determined by **one –stage** assay and **chromogenic** assay
- Patients with normal aPTT, normal F VIII (one-stage assay) but with **personal or family history** with mild hemophilia ⟶ use **chromogenic** FVIII assay

Quantitative measurement of FVIII inhibitors

- Time-dependent inhibitors
- Show linear inhibition kinetics (type I)
- The presence of an inhibitor might be suspected from a reduced half-life and recovery of FVIII
- Bethesda (BA), Nijmegen, Oxford assay

Quantitative measurement of FVIII inhibitors

One **Bethesda unit** (BU) is defined as the amount of an inhibitor that will neutralize 50 % of FVIII present in normal plasma after 2 hours of incubation at +37 ° C.

Quantitative measurement of FVIII inhibitors (BA)

- Different dilutions of the plasma **sample** with imidazole buffer (1:2,1:4,1:8,1:16,…1:256)
  - in case of strong inhibitor, use more titres (1:512, 1:1024,…)
- FVIII inhibitor **positive controls** (QC)
- **Control mixture** (imidazole buffer)
- Mix the sample with the equal part of **normal plasma** (pooled plasma)
- **Incubation for 2 hours at +37 ° C**
- Inhibitors inactivate FVIII present in the normal plasma

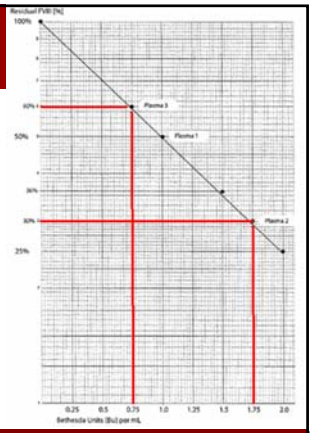
Quantitative measurement of FVIII inhibitors (BA)

- After incubation at +37 ° C the activity of the FVIII in patients' samples and control mixtures is determined
- Calculate **residual activity** of the factor (%):

$$residual\_activity = \frac{activity\_of\_the\_factor\_in\_the\_sample}{activity\_of\_the\_factor\_in\_control\_mixture} x100$$

- Calculate separately for patient's samples and positive controls
- Dilution of the patient's plasma that gives a **residual FVIII nearest to 50 %** but within the range **30 - 60 %** (event. 25 – 70 %) is chosen for calculation of the inhibitor

- The inhibitor activity is calculated from a **graph of residual FVIII activity versus inhibitor units (BU)**
- Derive the inhibitor titre from the graph and **multiply by the dilution** to give the final titre



Quantitative measurement of FVIII inhibitors

- **Normal values:**
  < 0,6 BU/ml
- **Inhibitor:**
  < 5 BU/ml Low responder
  > 5 BU/ml High responder

The Nijmegen modification

- Use **buffering** the normal plasma with 0.1M imidazole buffer at pH 7.4
- Use **imunodepleted FVIII deficient plasma** in the control mixture and for dilution of the patient samples

Advantage:

- Is better at **low inhibitor titres** (<1 BU) than classical Bethesda assay (Bethesda assay can result in false positivity)
- ***ISTH recommendation (at least for CCC)***

The Oxford modification

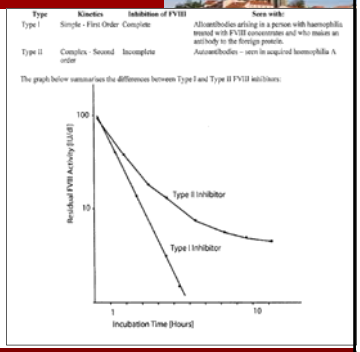
- Use a **concentrate of the FVIII** instead of normal plasma
- Use **4 hours** incubation at +37 ° C
- Not often used these days…

Quantitative measurement of FVIII inhibitors

- **Potential problems (troubleshooting)**
  - Determination of the inhibitor during IT treatment – interference of the residual FVIII in the sample – **false low titre** of the inhibitor or **negativity** of the inhibitor
- **Possible resolution**
  - Heat the test plasma **at +58 ° C for 90 min** (will destroy all the clotting factors including FVIII, but not the inhibitor – it is heat resistant)

FVIII inhibitors

- Type of the inhibitors (**type I and II)** depends on the **kinetics** of the inhibitor



Recovery and half-life

- The sampling points:
  - **0 (baseline),** 0.25, **0.5, 1**, **3, 6**, 9**, 12, 24**, 28, 32, **48, 72** h
  - at least 5 time-points: patient ≤ 6 years old
- *ISTH recommendation*

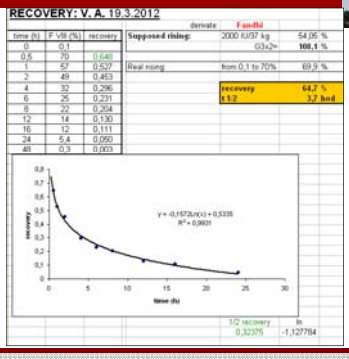
Recovery and half-life

- **Recovery**
  - Ratio of desired and real increase of FVIII/FIX (IU/dl) in patient's plasma after the dose of injected factor (in IU/kg)
    - Given in percentage
    - **1 IU/kg of FVIII** should increase the plasma level **in 2 %,** for **FIX in 1 %**
  - Is often calculated from the highest measured FVIII/FIX plasma concentration of FVIII/FIX within the first hour post infusion
  - Recovery shall be **more than 66 %** (hemophilia A)

Recovery and half-life

- **Half-life**
  - The period of time required for the concentration or amount of drug in the body to be reduced to exactly one-half of a given concentration or amount
  - Half-life **greater than 7 hours** (hemophilia A)

Recovery and half-life



Thank you for your attention.
Questions welcome! ☺

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