Molecular Testing for Coagulation Disorders

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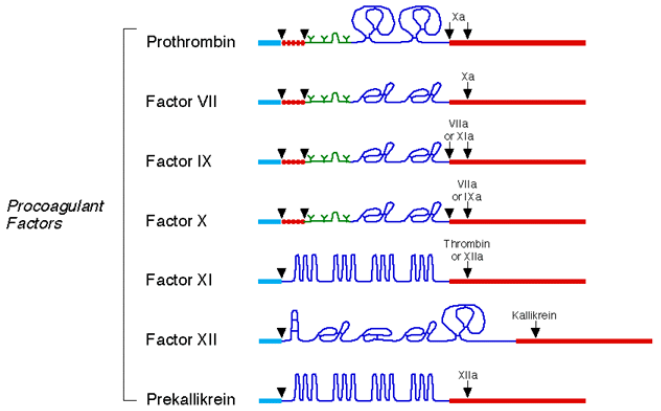


Molecular Testing for Coagulation Disorders

Inherited thrombophilias

- **Factor V Leiden Mutation**
- **Prothrombin 20210 G>A Mutation**
- **Hyperhomocysteinemia – MTHFR (Methylene-tetrahydrofolate reductase)**

Inherited bleeding disorders

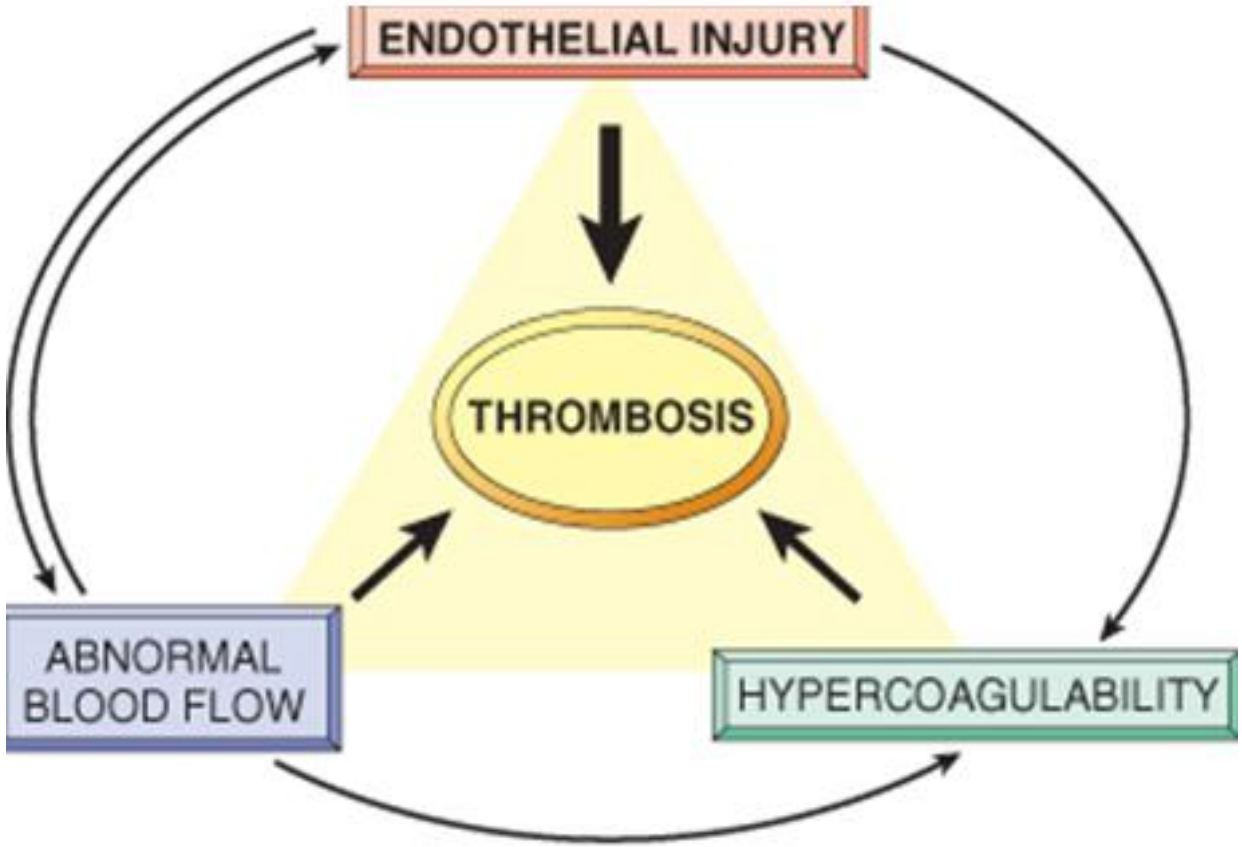
- **Hemophilia A**
- **Hemophilia B**
- **VWD**

Pharmacogenetics of anticoagulants/anti-platelet agents

- **Warfarin**
- **Clopidogrel**

Inherited Thrombophilias

The Basic Elements of Hemostasis



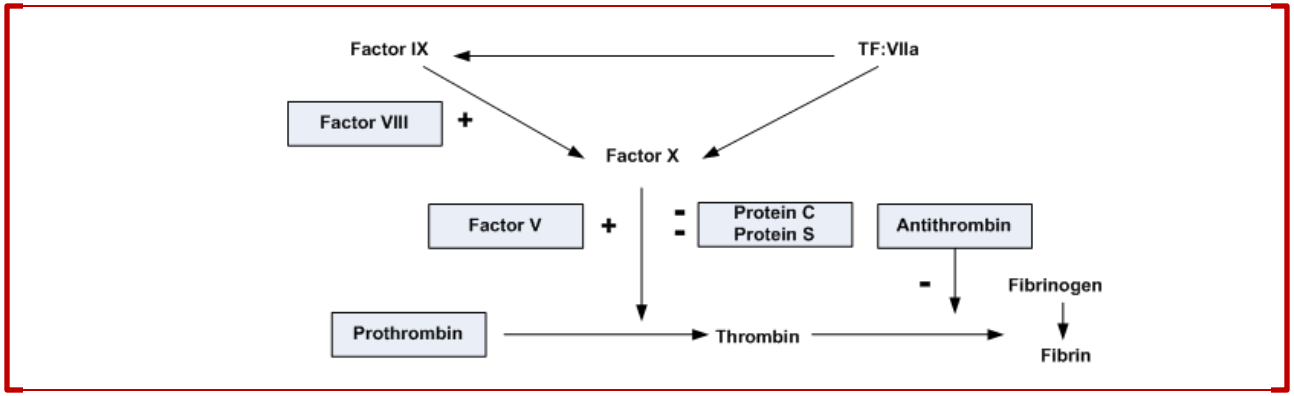
Clotting Cascade with an Emphasis on Inherited Thrombophilias

Intrinsic Pathway

1. Activated *in vitro* when measuring the activated partial thromboplastin time (aPTT)
2. *In vivo*, the activated tissue factor: factor VIIa complex (TF:VIIa) activates factor IX

Extrinsic Pathway

1. Vessel or tissue injury leads to production of tissue factor (TF)
2. TF binds to activated factor VII (FVIIa)



Overview: Both intrinsic and extrinsic pathways lead to the activation of factor X. Factor X plays a role in converting inactive prothrombin to thrombin. Thrombin converts soluble fibrinogen and fibrin, the major component of blood clots.

The protein C and antithrombin pathways are critical to maintain control of coagulation.

Factor V: Factor V is a procoagulant protein that helps promote the conversion of prothrombin to thrombin. Activated factor V is typically inactivated by a complex that forms between activated protein C and S. When factor V Leiden mutation is present, the protein C/S complex cannot break down activated factor V (Va) as efficiently, leading to a hypercoagulable state.

Prothrombin: The G20210A polymorphism results in increased production of prothrombin. This creates a hypercoagulable state since more thrombin is generated.

Antithrombin: Typically inactivates thrombin, leading to less conversion of fibrinogen to fibrin. Antithrombin deficiency reduces this inhibitory effect, creating a hypercoagulable state. Antithrombin also inactivates factors Xa, IXa and XIa.

Protein C/S: Deficiencies of either protein C or S result in decreased inhibition of activated factor V and increased thrombin generation, leading to a hypercoagulable state.

Activated protein C (ACP) resistance: Leads to a hypercoagulable state since factor Va (normally inactivated by ACP) is not inactivated as quickly. APC also inactivates factor VIIIa.

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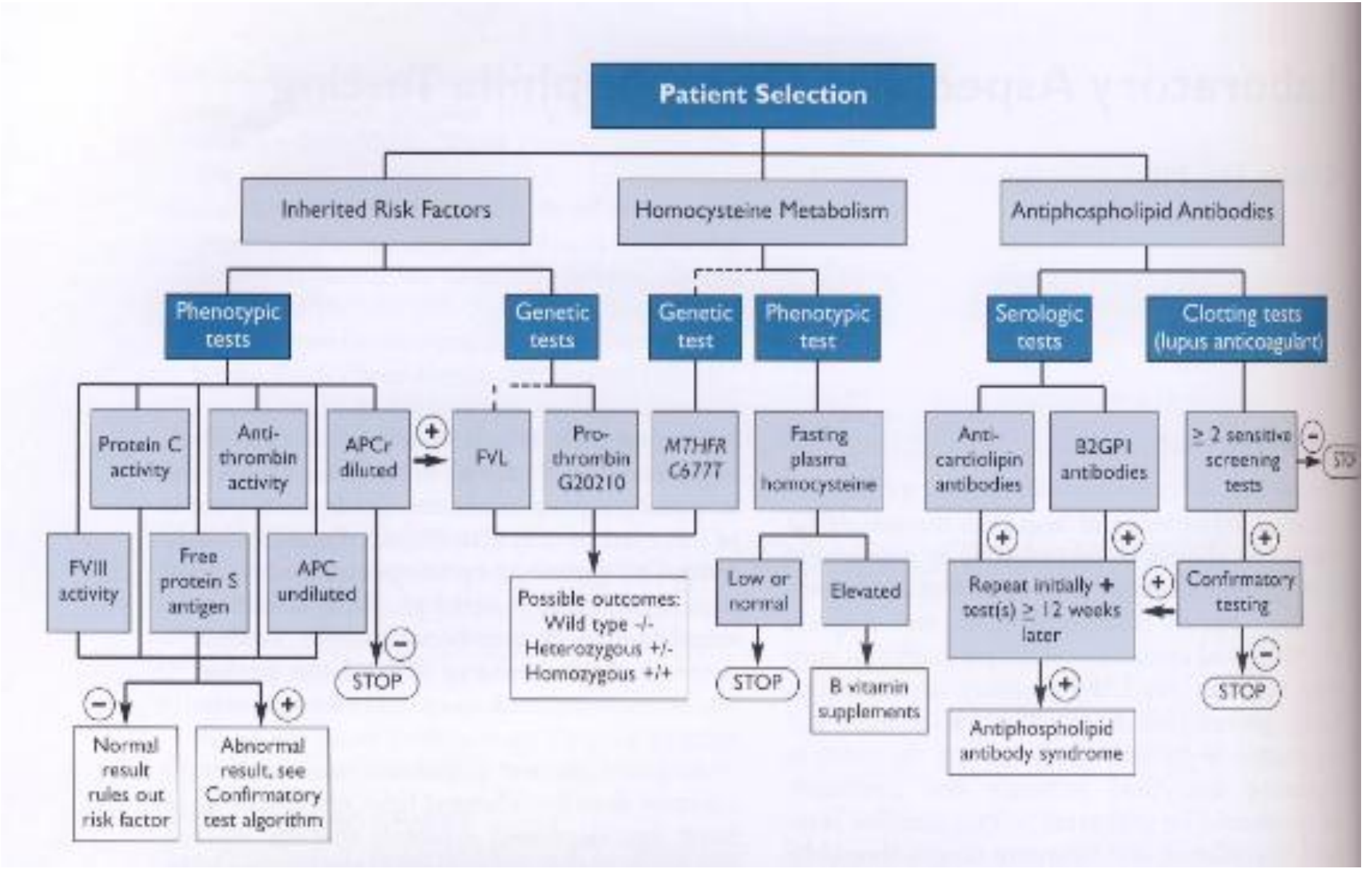

Patient Selection
Inherited Risk Factors
Homocysteine Metabolism
Antiphospholipid Antibodies
Phenotypic tests
Genetic tests
Genetic test
Phenotypic test
Serologic tests
Clotting tests (lupus anticoagulant)
Protein C activity
Anti-thrombin activity
APCr diluted
FVL
Pro-thrombin G20210
MTHFR C677T
Fasting plasma homocysteine
Anti-cardiolipin antibodies
B2GPI antibodies
≥ 2 sensitive screening tests
STOP
FVIII activity
Free protein S antigen
APC undiluted
STOP
Possible outcomes: Wild type -/- Heterozygous +/- Homozygous +/+
Low or normal
Elevated
STOP
B vitamin supplements
Repeat initially + test(s) ≥ 12 weeks later
Confirmatory testing
STOP
Antiphospholipid antibody syndrome
Normal result rules out risk factor
Abnormal result, see Confirmatory test algorithm

Prevalence of Congenital Thrombophilia

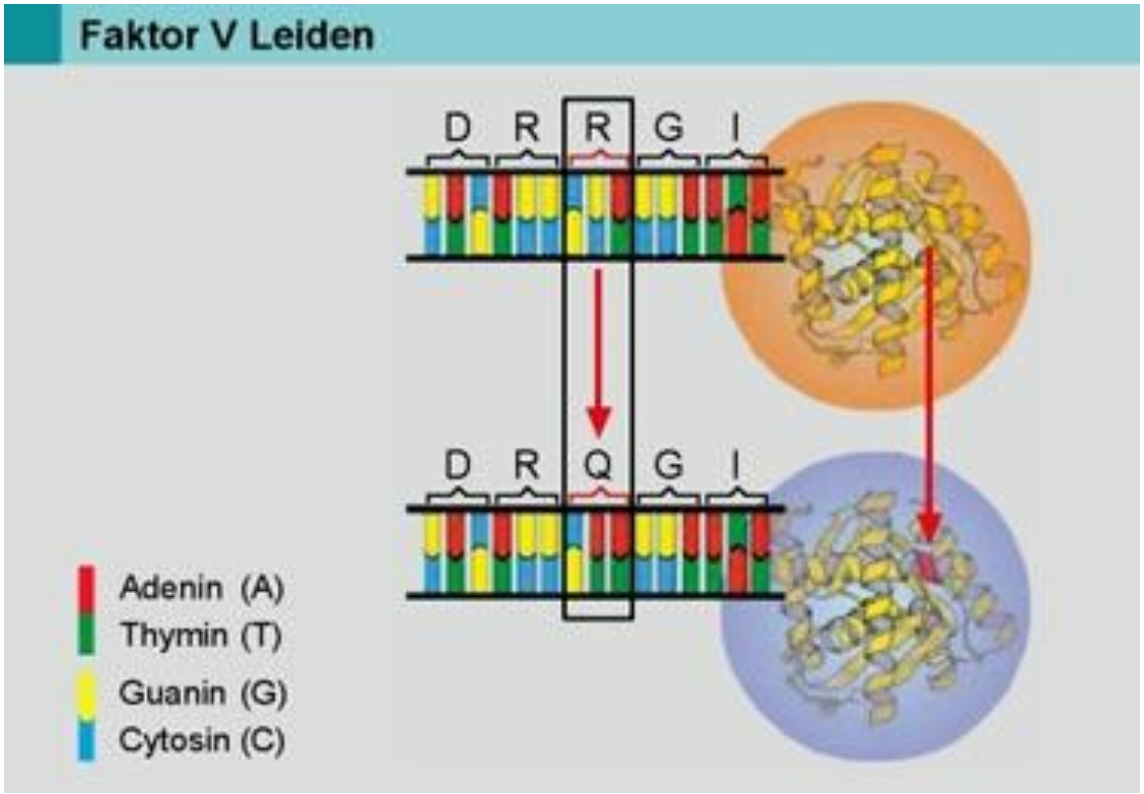
Factor	General Population	People With Thrombosis
• APCR: factor V Leiden mutation	3-8% of Caucasians	20-25%
• Prothrombin G20210A	2-3% of Caucasians	4-8%
• Antithrombin deficiency	1 in 2-5000	1-1.8%%
• Protein C deficiency	1 in 300	2.5-5.0%
• Protein S deficiency	Unknown	2.8-5.0%
• Hyperhomocysteinemia	11%	13.1-26.7%

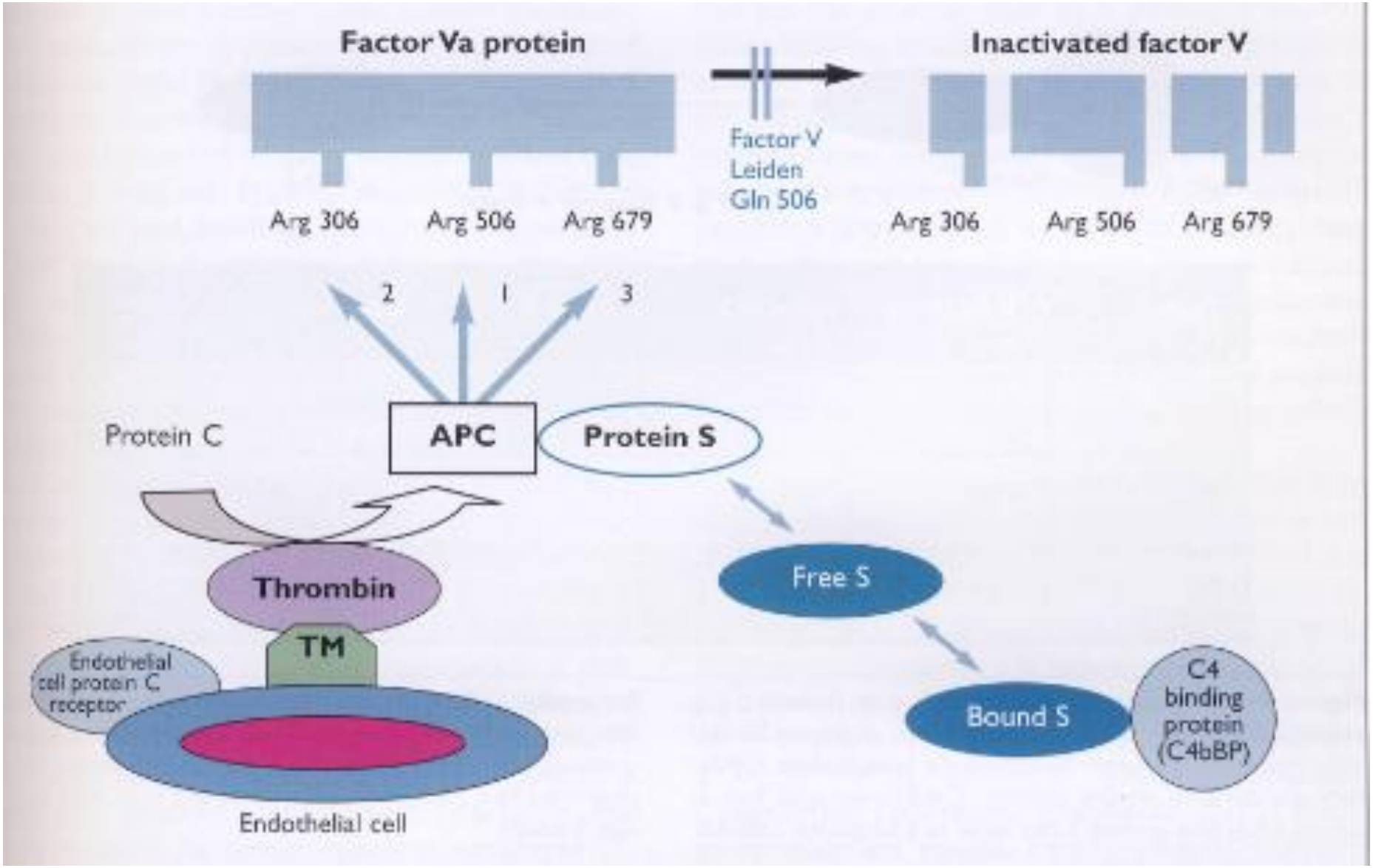
Factor V Leiden Mutation

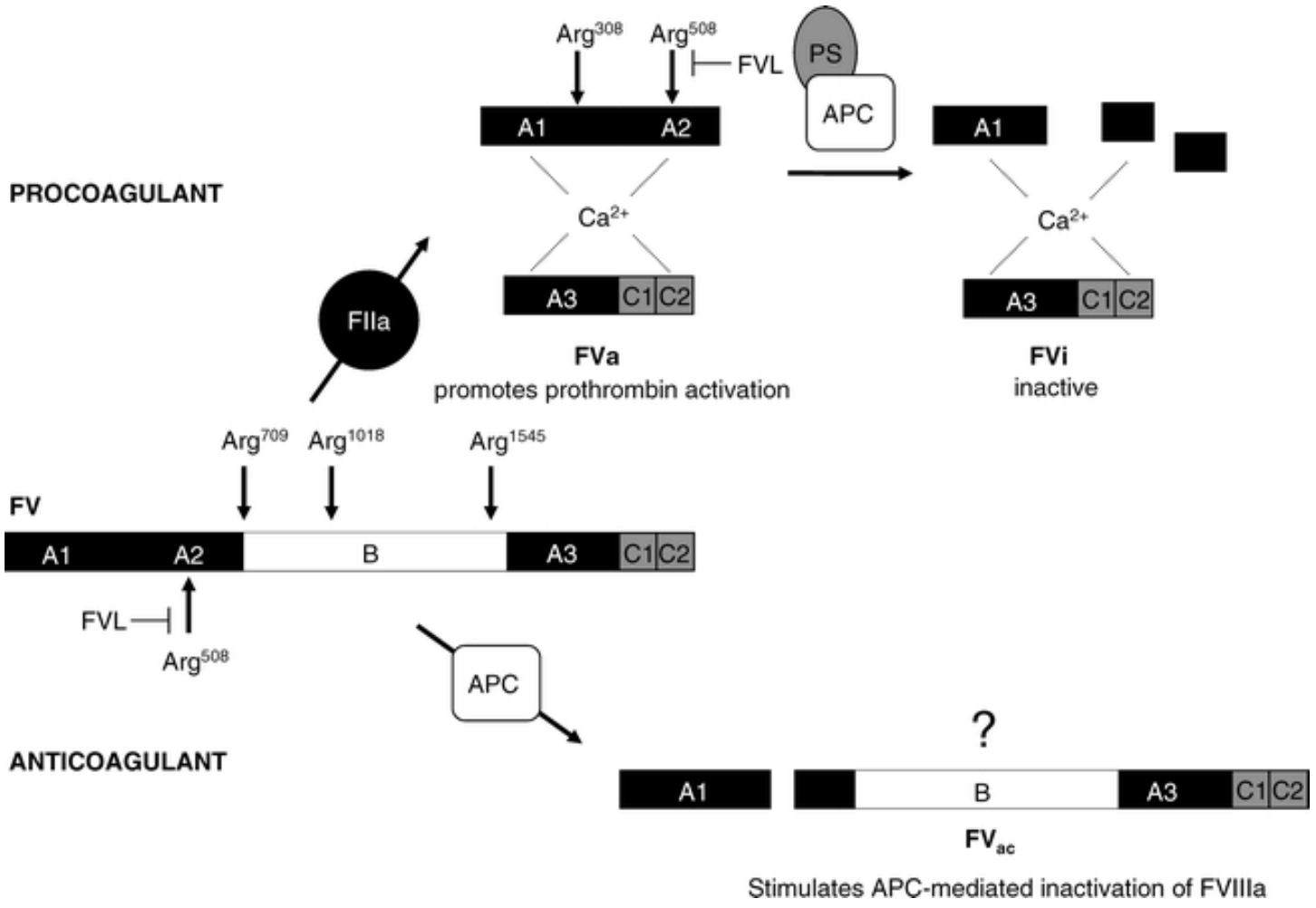
f5

1q23

1691G>A --- R506Q




Factor Va protein
Inactivated factor V
Factor V
Leiden
Gln 506
Arg 306
Arg 506
Arg 679
Arg 306
Arg 506
Arg 679
2
1
3
Protein C
APC
Protein S
Thrombin
TM
Endothelial
cell protein C
receptor
Endothelial cell
Free S
Bound S
C4
binding
protein
(C4bBP)


PROCOAGULANT
Arg308
Arg508
FVL
PS
APC
A1
A2
Ca2+
A3
C1
C2
FIIa
FVa
promotes prothrombin activation
FVi
inactive
FV
Arg709
Arg1018
Arg1545
B
ANTICOAGULANT
?
FVac
Stimulates APC-mediated inactivation of FVIIIa

Factor V Leiden (*F5*) R506Q Mutation

Characteristics: Factor V Leiden mutation is the most common cause of inherited thrombophilia and accounts for over 90 percent of activated protein C resistance. The expression of Factor V Leiden thrombophilia is impacted by coexisting genetic thrombophilic disorders, acquired thrombophilic disorders (malignancy, hyperhomocysteinemia, high factor VIII levels), and circumstances including: pregnancy, oral contraceptive use, hormone replacement therapy, selective estrogen receptor modulators, travel, central venous catheters, surgery, transplantation and advanced age.

Incidence: Approximately 5 percent of Caucasians, 2 percent of Hispanics, 1 percent of African Americans and Native Americans and 0.5 percent of Asians are heterozygous; homozygosity occurs in 1 in 5000 individuals.

Inheritance: Incomplete autosomal dominant.

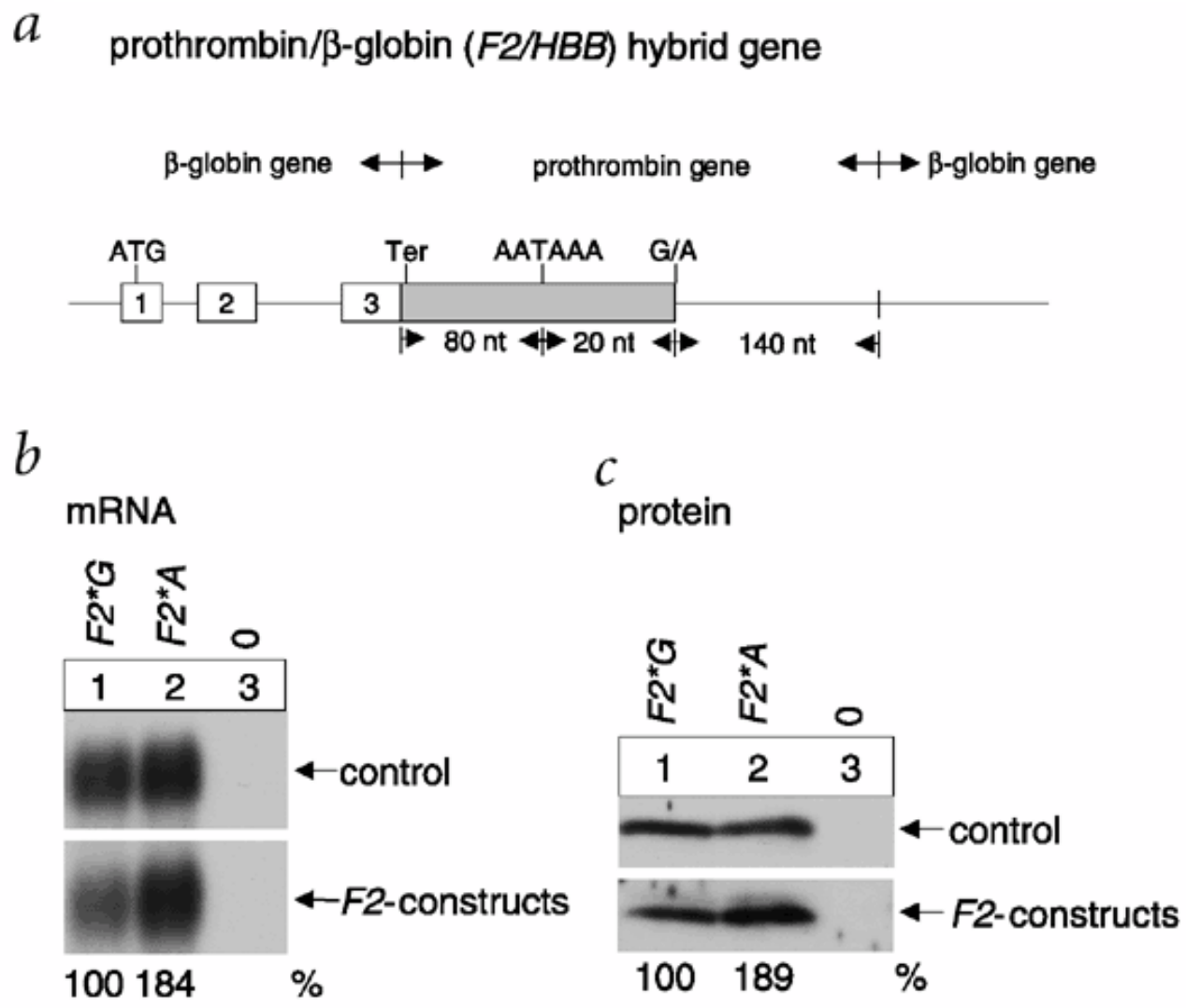
Penetrance: Lifetime risk of thrombosis is 10 percent for heterozygotes and 80 percent for homozygotes.

Prothrombin G20210A Mutation

f2

11p11 – q12

G20210A --- increased mRNA and prothrombin protein


a
prothrombin/β-globin (F2/HBB) hybrid gene
β-globin gene
prothrombin gene
β-globin gene
ATG
Ter
AATAAA
G/A
1
2
3
80 nt
20 nt
140 nt
b
mRNA
F2*G
F2*A
0
1
2
3
control
F2-constructs
100 184
%
c
protein
F2*G
F2*A
0
1
2
3
control
F2-constructs
100 189
%

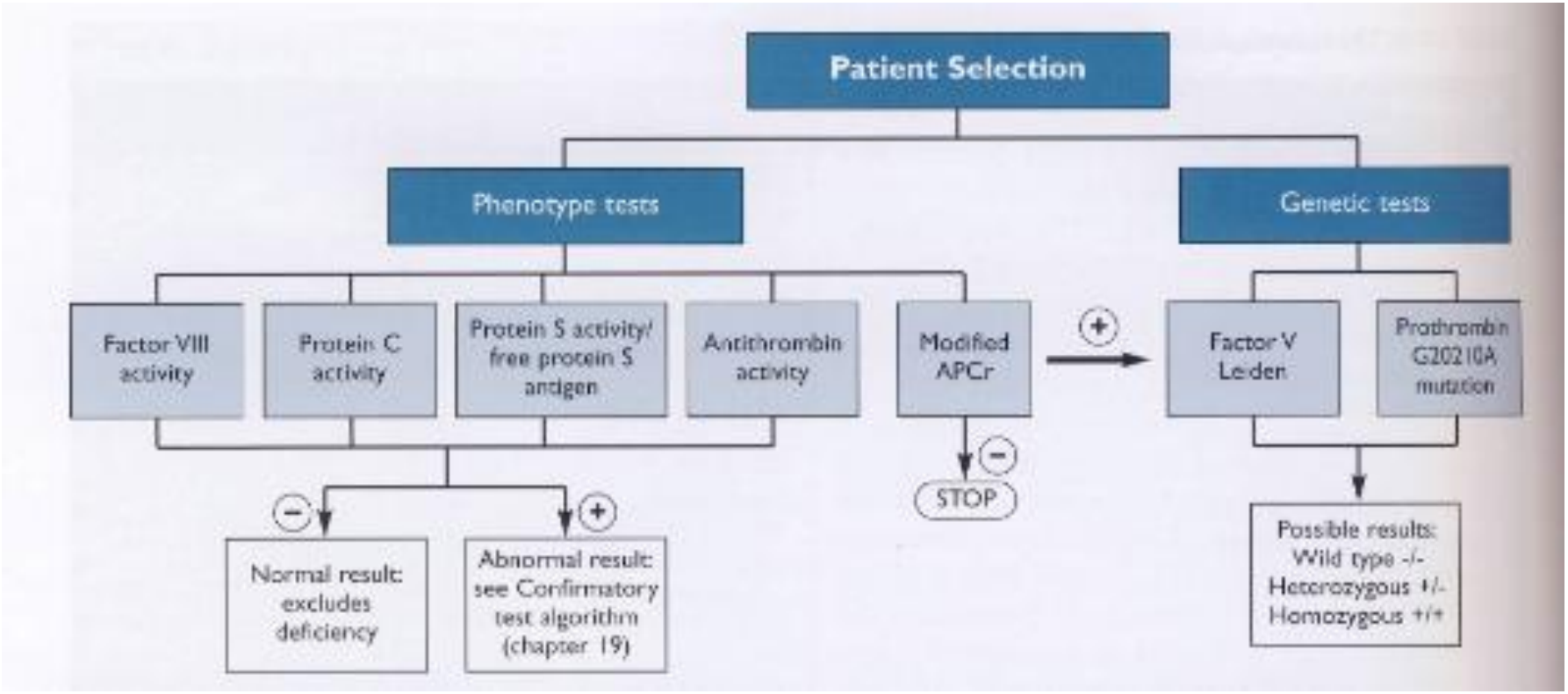
The factor II, prothrombin G20210A mutation is a common genetic risk factor for thrombosis and is associated with elevated prothrombin levels. Higher concentrations of prothrombin lead to increased rates of thrombin generation, resulting in excessive growth of fibrin clots. It is an autosomal dominant disorder, with heterozygotes being at a three- to eleven-fold greater risk for thrombosis. Although homozygosity is rare, inheritance of two G20210A mutations would increase the risk for developing thrombosis. If a patient is heterozygous for both the prothrombin G20210A and the factor V Leiden mutation, the combined heterozygosity leads to an earlier onset of thrombosis and tends to be more severe than single-gene heterozygotes.

Mutations in other genes or other mutations in the prothrombin gene that may cause elevated prothrombin and hereditary forms of venous thrombosis are not ruled out.

Relative Risk for a First Incident of Venous Thrombosis and the Incidence of Venous Thrombosis Associated with Each Risk Factor or Risk Factor Combination*

Variable	Relative Risk	Annual Incidence, %
Normal	1	0.008
Hyperhomocysteinemia[57]	2.5	0.02
Homozygous *MTHFR* C677T[58]	1	
Prothrombin G20210A[24]	2.8	0.02
Oral contraceptive use[59]	4	0.03
FVL heterozygotes[5]	7	0.06
Oral contraceptives and FVL[59]	35	0.3
FVL homozygotes[7]	80	0.5–1.0
FVL and prothrombin G20210A[60]	20	
FVL and homocystinuria[61]	21.8	

* Superscript numbers are reference citations. Modified with permission from Bauer.[62] FVL indicates factor V Leiden.


Patient Selection
Phenotype tests
Genetic tests
Factor VIII activity
Protein C activity
Protein S activity/ free protein S antigen
Antithrombin activity
Modified APCr
Factor V Leiden
Prothrombin G20210A mutation
STOP
Normal result: excludes deficiency
Abnormal result: see Confirmatory test algorithm (chapter 19)
Possible results: Wild type -/- Heterozygous +/- Homozygous +/+

ACMG Standards and Guidelines

Testing may have some utility in the following circumstances (these are the same as the general recommendations for testing any thrombophilia)[1]:

- Age < 50, any venous thrombosis;
- Venous thrombosis in unusual sites (such as portal hepatic, mesenteric, and cerebral veins);
- Recurrent venous thrombosis;
- Venous thrombosis and a strong family history of thrombotic disease;
- Venous thrombosis in pregnant women or women taking oral contraceptives;
- Myocardial infarction in female smokers under age 50.

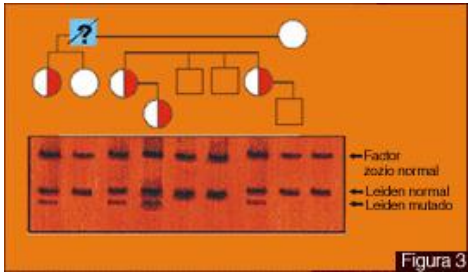


Figura 3

ACMG Standards and Guidelines

Other situations in which testing may be appropriate include the following:

- Venous thrombosis, age > 50, except when active malignancy is present;
- Asymptomatic relatives of individuals known to have factor V Leiden. Knowledge that they have factor V Leiden may influence management of pregnancy and may be a factor in decision-making regarding oral contraceptive use;
- Women with recurrent pregnancy loss or unexplained severe preeclampsia, placental abruption, intrauterine fetal growth retardation or stillbirth. Knowledge of factor V Leiden carrier status may influence management of future pregnancies. Known carriers of these mutations can be treated with anticoagulants during pregnancy to support a normal outcome.

ACMG Standards and Guidelines

Routine testing is not recommended for patients with a personal or family history of arterial thrombotic disorders (e.g., acute coronary syndromes or stroke) except for the special situation of myocardial infarction in young female smokers. Testing may be worthwhile for young patients (< 50 years of age) who develop acute arterial thrombosis in the absence of other risk factors for atherosclerotic arterial occlusive disease.

Molecular Testing Systems

FDA cleared

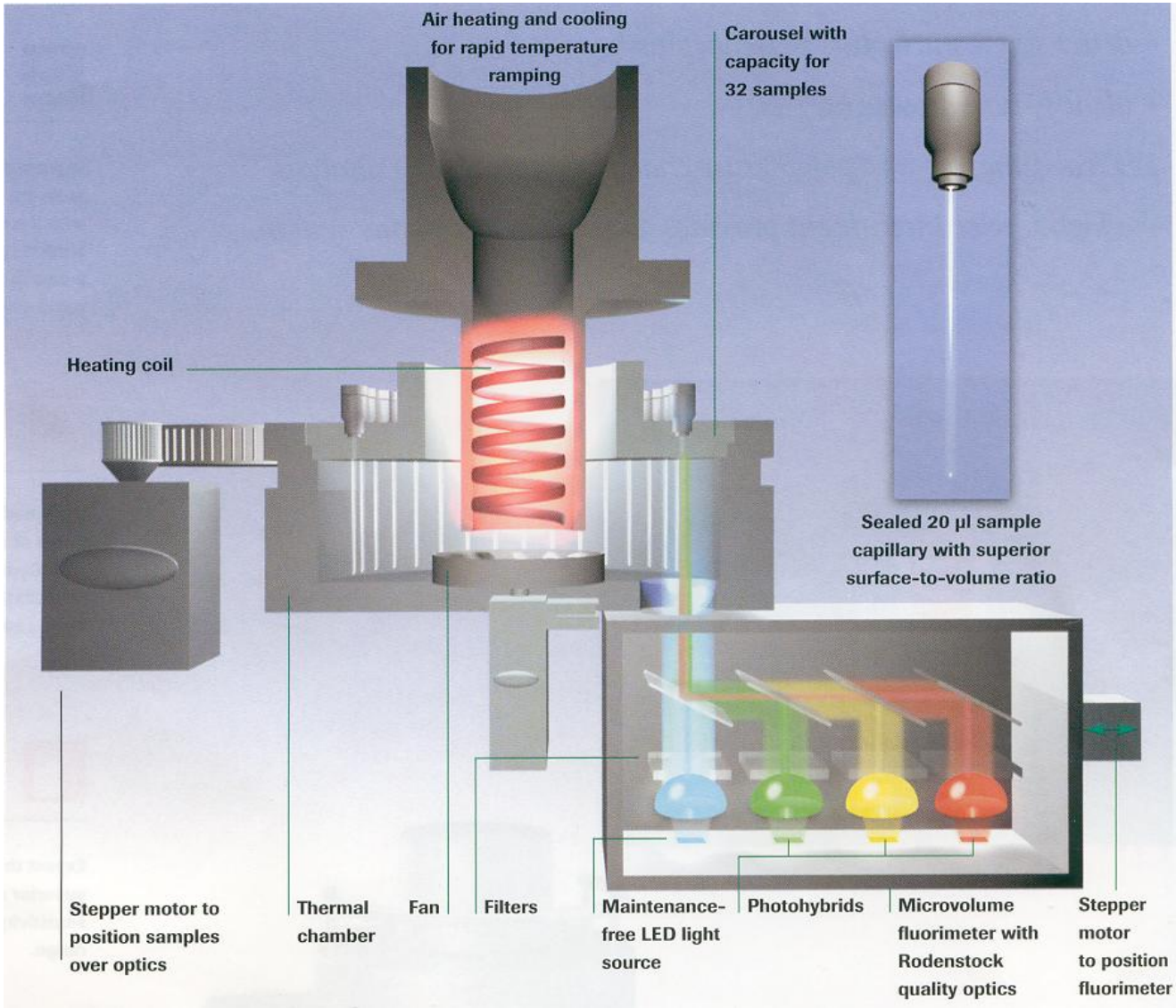
- **LightCycler – Roche Molecular Diagnostics**
- **Xpert HemoslL -- GeneXpert**

ASR

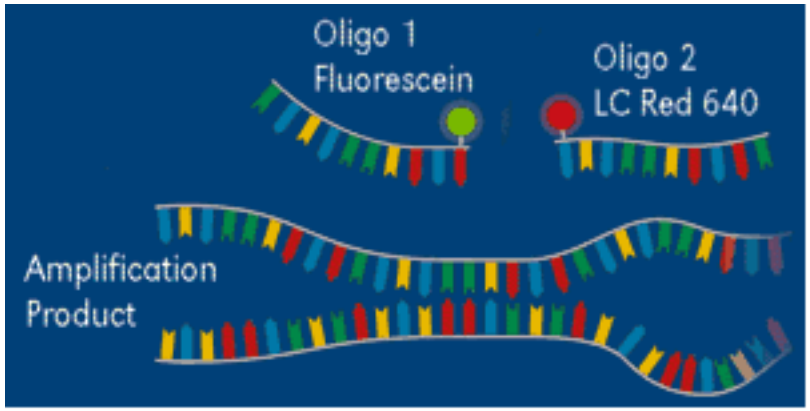
- **Many**

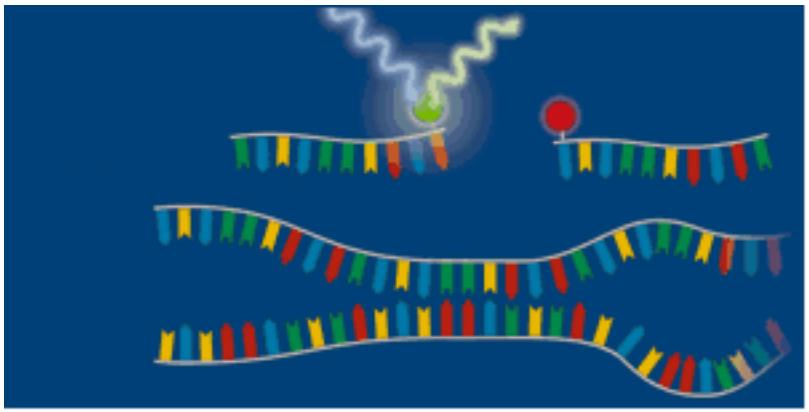
Home-brew

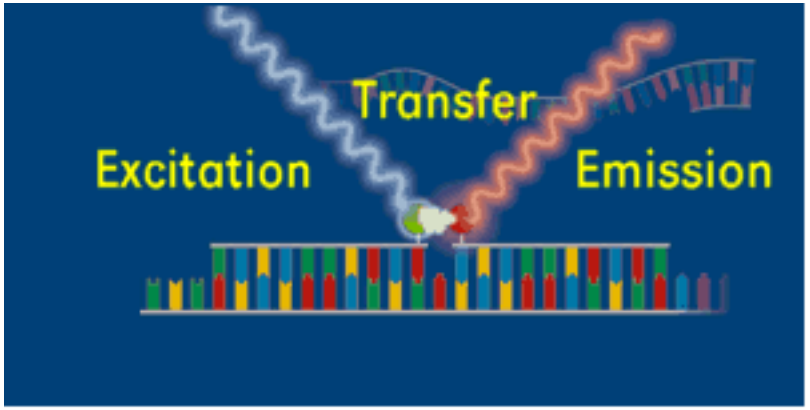
- **Restriction Fragment Length Polymorphism (RFLP)**


Air heating and cooling for rapid temperature ramping
Carousel with capacity for 32 samples
Heating coil
Sealed 20 µl sample capillary with superior surface-to-volume ratio
Stepper motor to position samples over optics
Thermal chamber
Fan
Filters
Maintenance-free LED light source
Photohybrids
Microvolume fluorimeter with Rodenstock quality optics
Stepper motor to position fluorimeter

FRET (Fluorescence Resonance Energy Transfer) Format







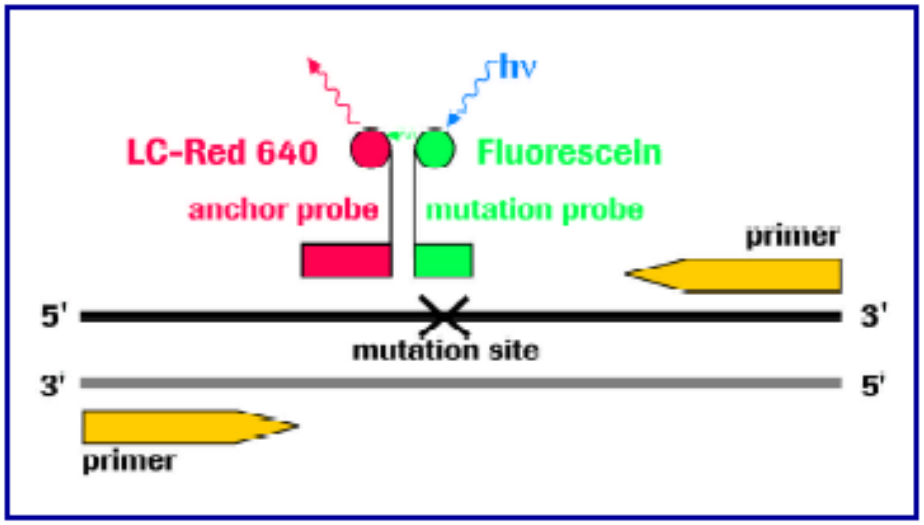


Figure 1: Genotyping experiment using the LightCycler – Factor V Leiden Mutation Detection Kit. *Schematic presentation of the PCR fragment, the orientation of the PCR primers, the anchor probe, and the mutation probe.*

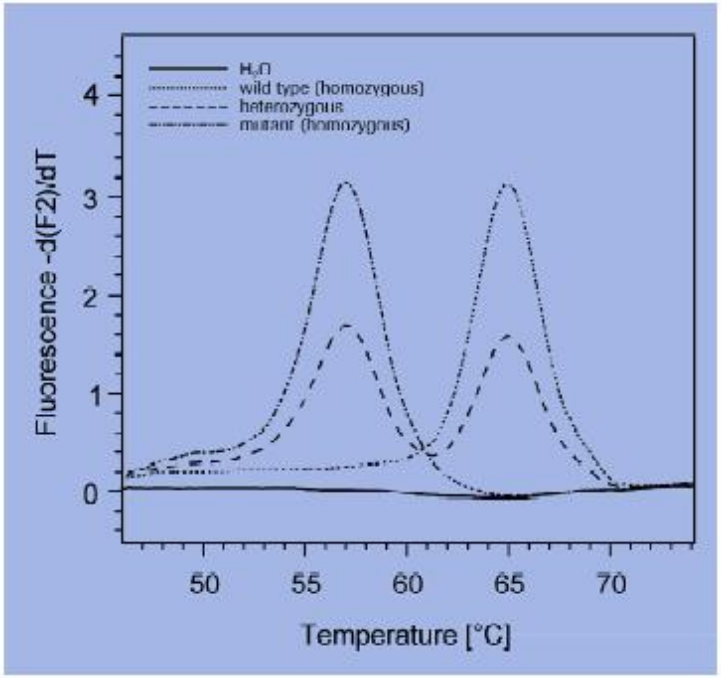
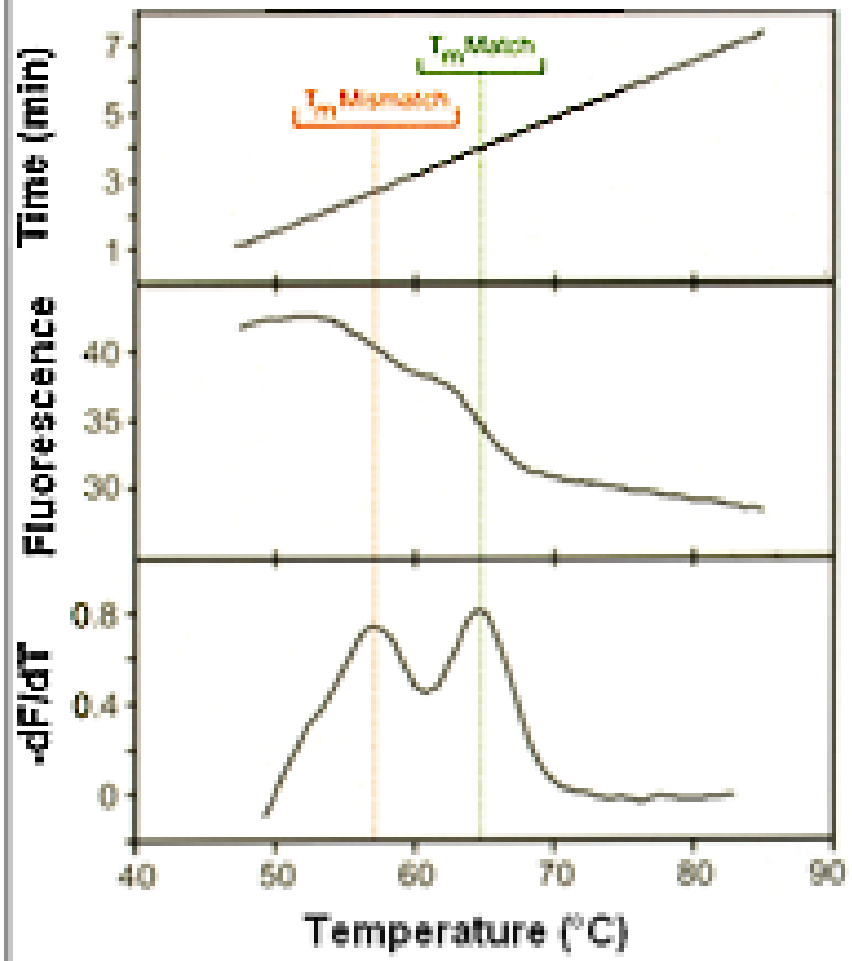
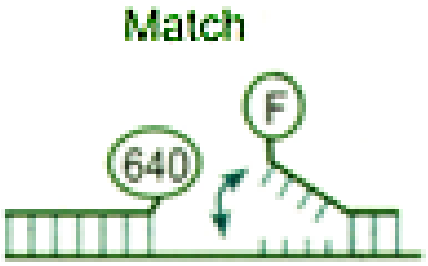
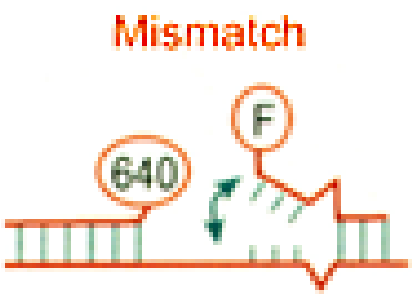


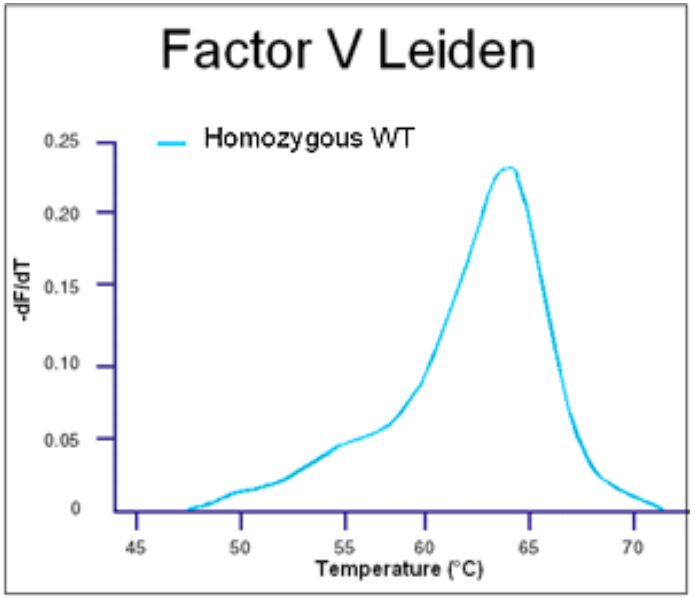
Figure 2: Melting curve analysis of the three possible genotypes of the Factor V Leiden sequence. *The first negative derivative of the fluorescence versus temperature [–d(F2)/dT] graph shows peaks with different T_m. The melting peaks indicate that the fully homologous sequence (wild type genotype) has a higher T_m than the sequence that has a mismatch with the mutation probe (mutant genotype). Samples containing both sequences (heterozygous genotype) display two peaks at exactly the same temperatures as the respective homozygous samples. As a negative control, the template DNA was replaced with PCR-grade water.*

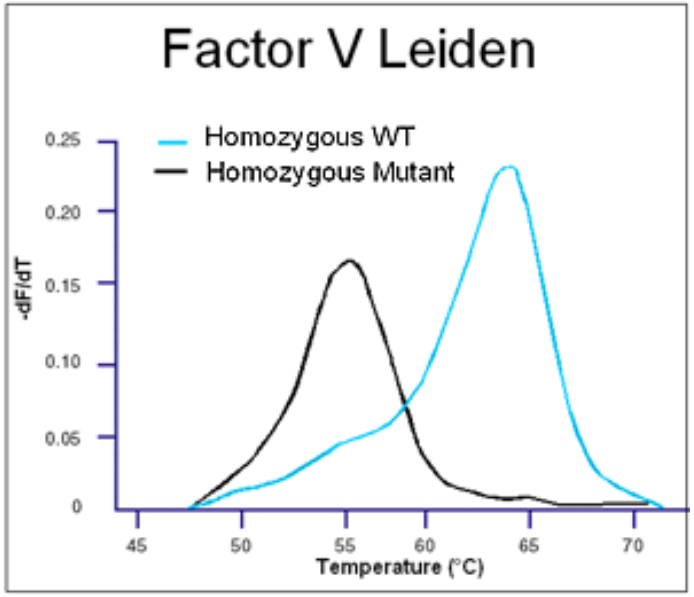
Genotyping by Melting

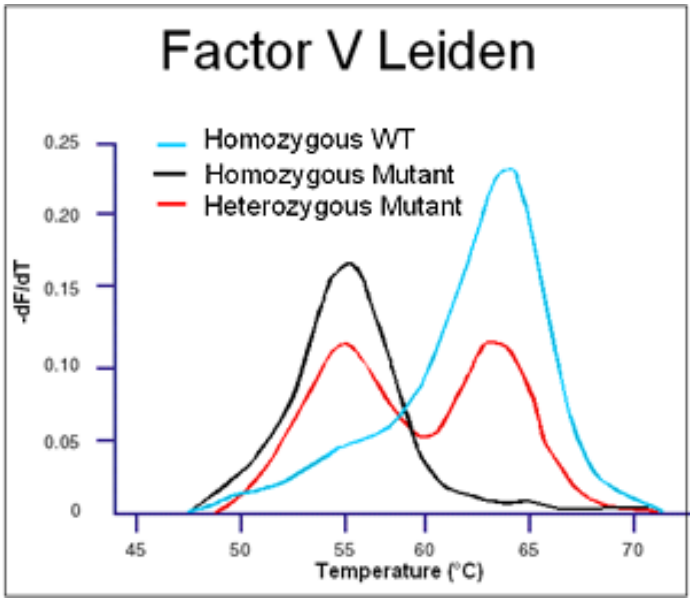


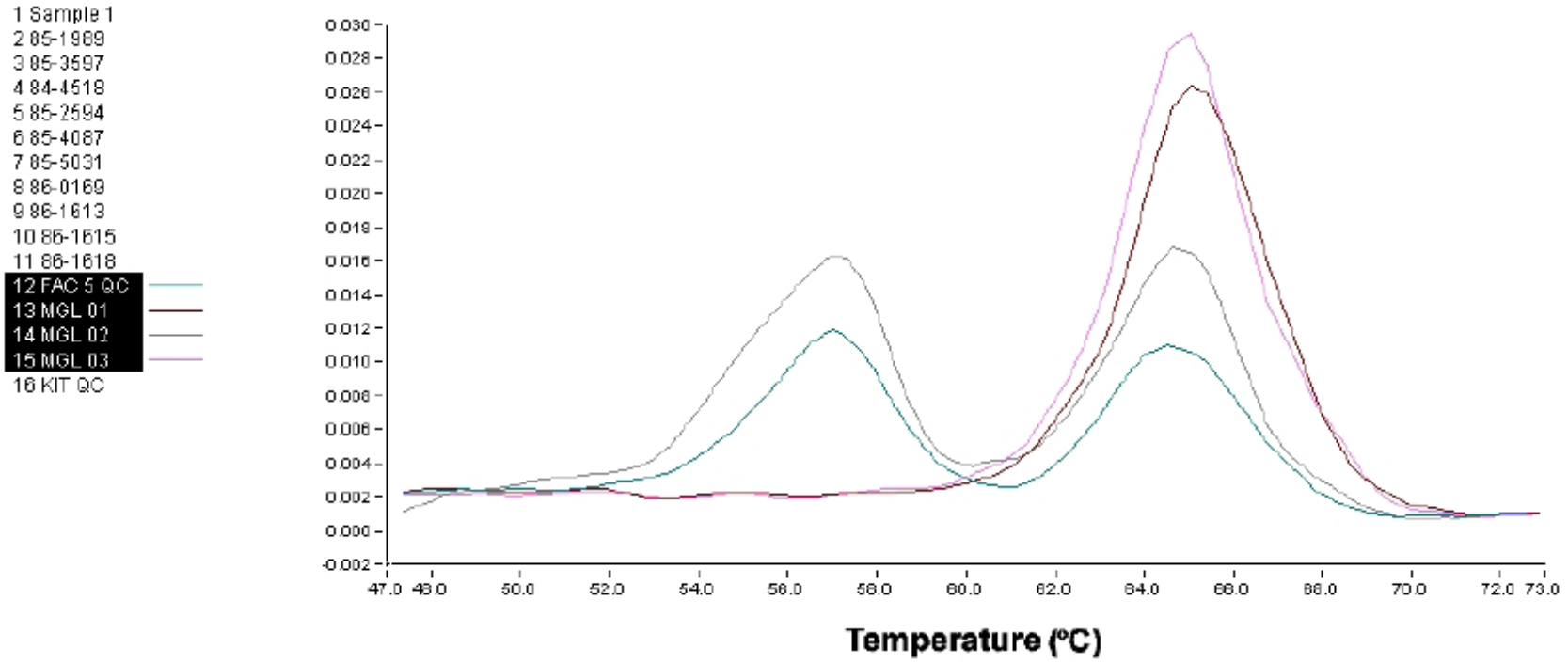




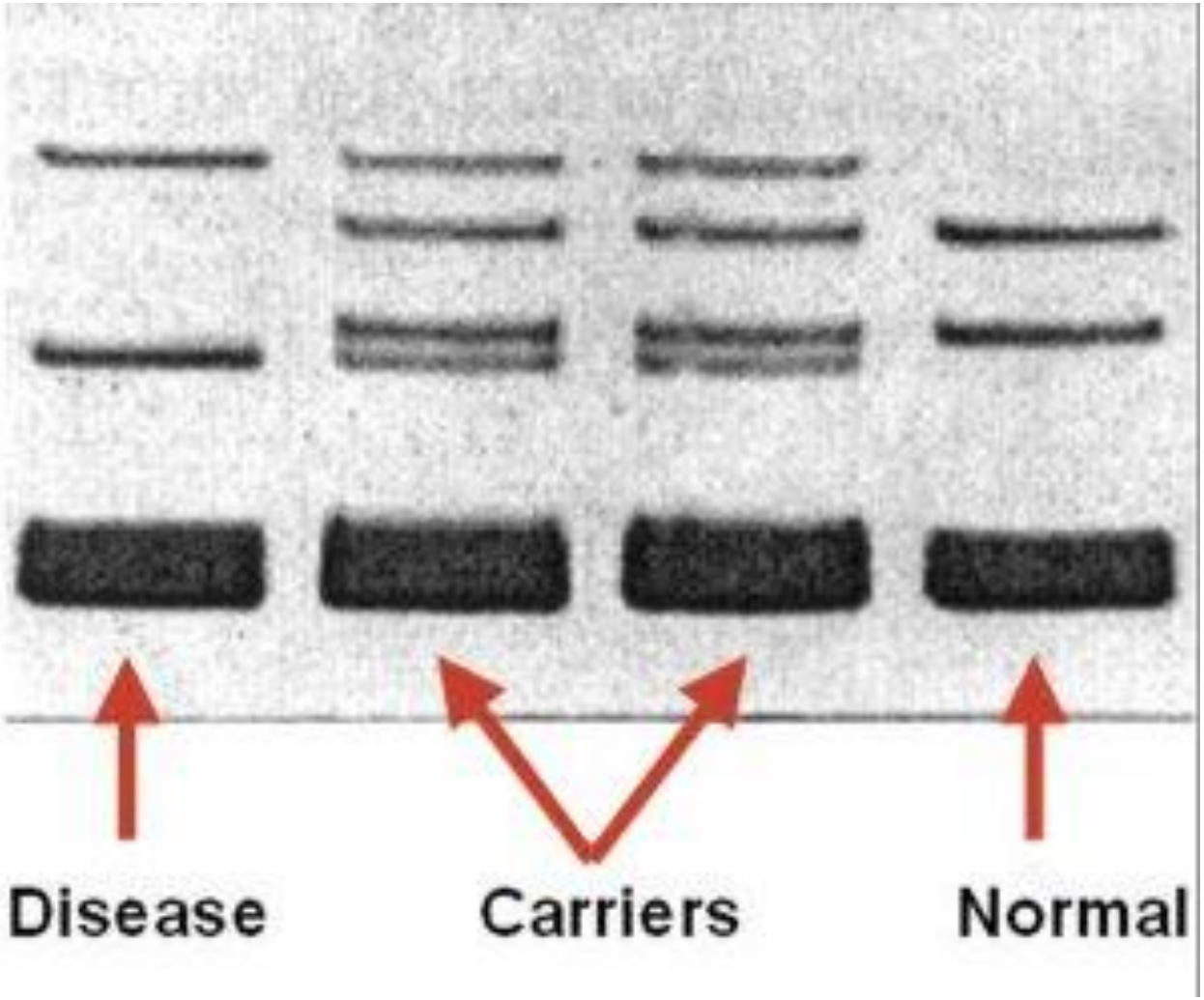

Factor V Leiden
Homozygous WT
0.25
0.20
0.15
0.10
0.05
0
-dF/dT
45
50
55
60
65
70
Temperature (°C)


Factor V Leiden
Homozygous WT
Homozygous Mutant
0.25
0.20
0.15
0.10
0.05
0
-dF/dT
45
50
55
60
65
70
Temperature (°C)


Factor V Leiden
Homozygous WT
Homozygous Mutant
Heterozygous Mutant
0.25
0.20
0.15
0.10
0.05
0
-dF/dT
45
50
55
60
65
70
Temperature (°C)



Factor V Leiden Detection on LightCycler 1.2

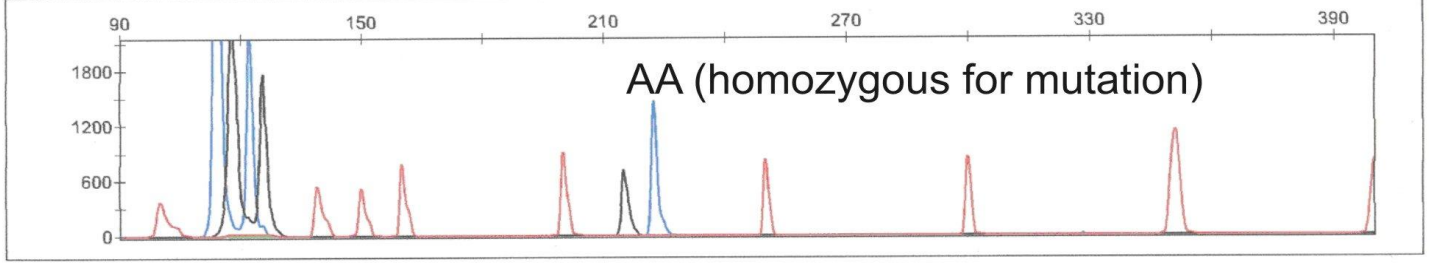

Disease
Carriers
Normal

Applied Biosystems

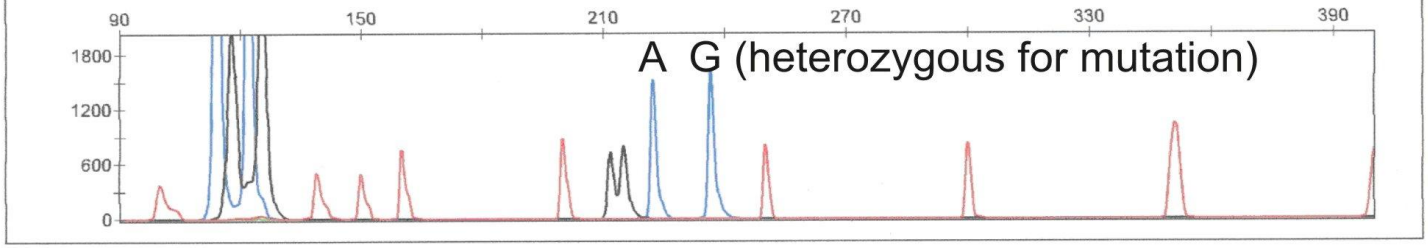
GeneMapper v3.5

jackieigh18106

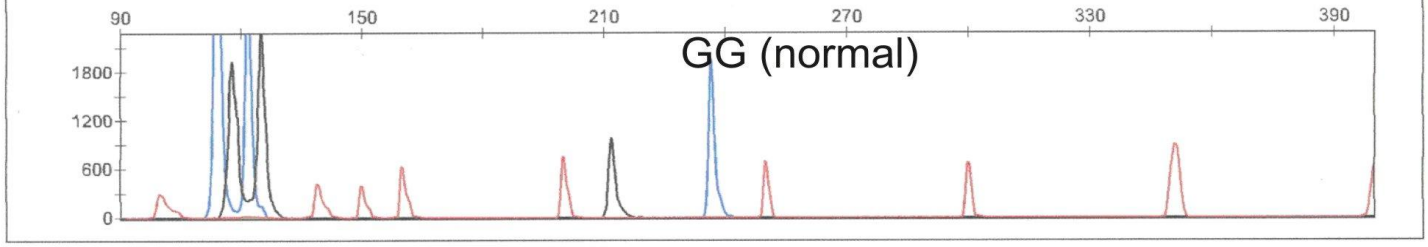
Sample File	Sample Name	Panel	SQ
frag_001_A09.fsa	FVL++25	None	



Sample File	Sample Name	Panel	SQ
frag_003_B09.fsa	FVL+-25	None	



Sample File	Sample Name	Panel	SQ
frag_005_C09.fsa	FVL--25	None	



ACMG Standards and Guidelines

July/August 2005 · Vol. 7 · No. 6

Technical standards and guidelines: Venous thromboembolism (Factor V Leiden and prothrombin 20210G>A testing): A disease-specific supplement to the standards and guidelines for clinical genetics laboratories

Elaine B. Spector, PhD[1–4], Wayne W. Grody, MD, PhD[1–3,5], Carla J. Matteson, PhD[1,6], Glenn E. Palomaki[3,7], Daniel B. Bellissimo, PhD[2,3,8], Daynna J. Wolff, PhD [3,9], Linda A. Bradley, PhD [2,3,10], Thomas W. Prior, PhD[2,3,11], Gerald Feldman, MD, PhD[2,3,12], Bradley W. Popovich, PhD[2,3,13], Michael S. Watson, PhD[3,14], and C. Sue Richards, PhD[2,3,15]

Limitation

Sensitivity and specificity

Clinical sensitivity: FV: 20-50%; FII: 5-19% -- multifactorial effects

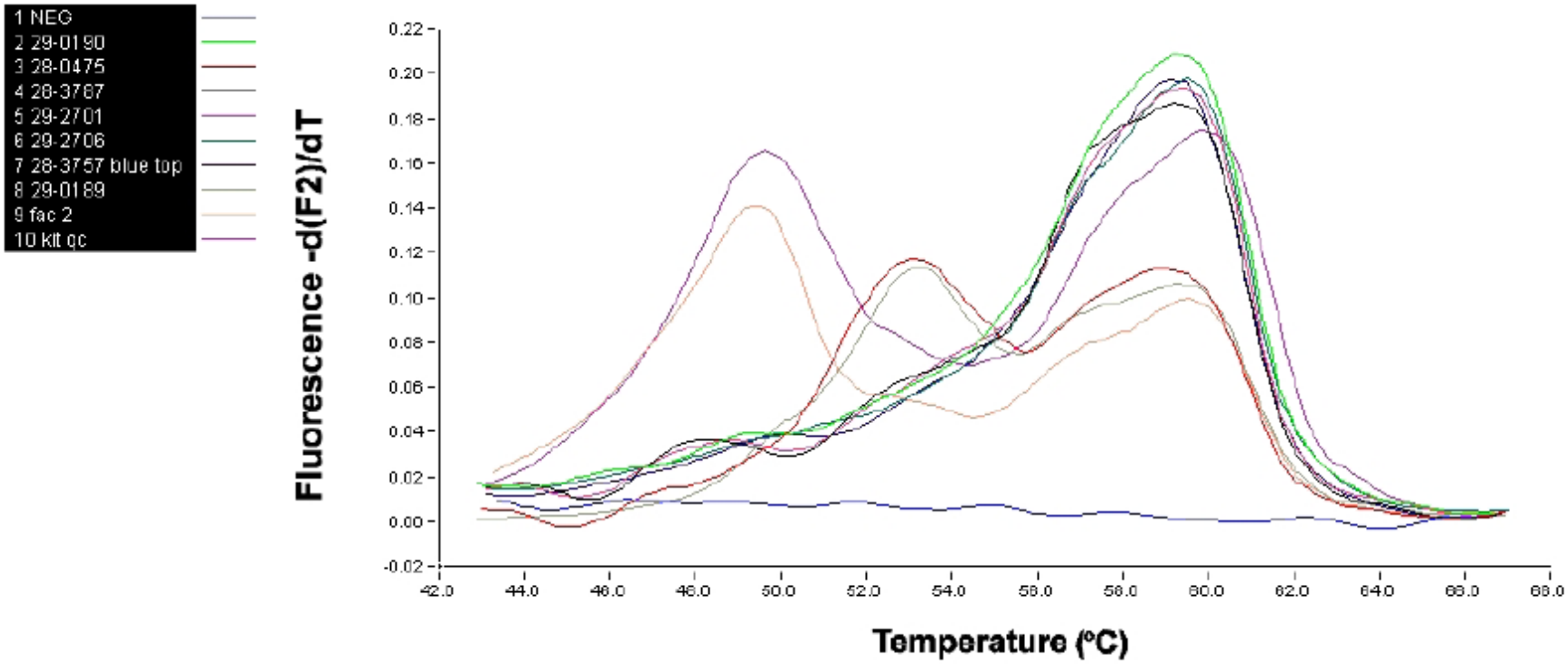
Clinical specificity: FV: >95%; FII: >98% (due to the rate of panetrance)

Other variants

FV Cambridge (R306T)
FV Hong Kong (R306C)

Polymorphism – Clinical significance?

FII C20209T has been repeatedly reported to present in patients with thrombotic and/or obstetric complications.



Factor II variants, C20209T, A20218G or C20221T

Limitation

Other causes for APC resistance

Factor V variant

R2 allele (FV H1299R) homozygotes: associated with a relative excess of the more thrombogenic FV isoform (FV_1) in plasma and reduced APC cofactor activity in FVIIIa inactivation.

Elevated FVIII levels

Low protein S levels

Lupus anticoagulant

High levels of other inhibitors, such as α1-antiprotease, direct thrombin inhibitor, etc.

Hyperhomocysteinemia

Elevated plasma homocysteine levels have been associated with atherosclerotic disease, VTE and arterial thrombosis

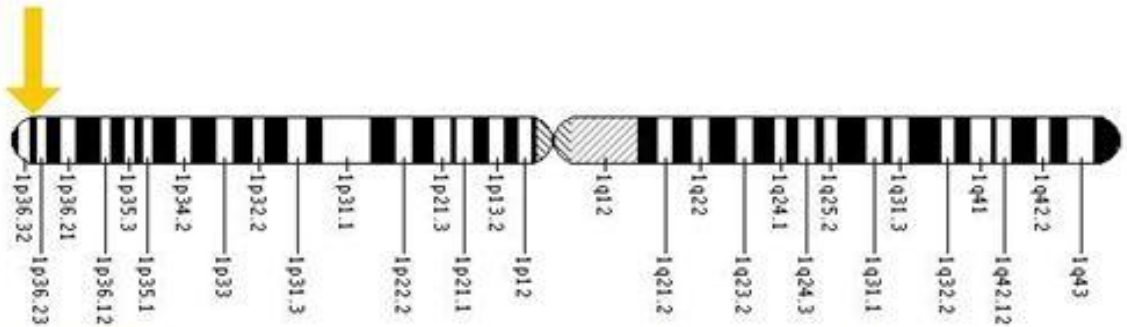
Acquired

Inherited hyperhomocysteinemia rare

Homocysteine metabolism defects caused by mutations of the *MTHFR* gene

C677T and A1298C most common

Besides these 2 mutations, at least 24 MTHFR gene mutations have already been identified in humans



The MTHFR gene is located on the short (p) arm of chromosome 1 at position 36.3.

More precisely, the MTHFR gene is located from base pair 11,769,246 to base pair 11,788,568 on chromosome 1.

Table 4. Polymorphic Mutations in 5,10-Methylenetetrahydrofolate Reductase

Mutation	Change in Amino Acid or Splice Site	Exon or Intron	Reference
677C→T	Alanine/valine	Exon 4	20
1068T/C	Serine/serine	Exon 6	21
1178+31T/C	5′ splice site	Intron 6	22
1317T/C	Phenylalanine/phenylalanine	Exon 7	25
1298A→C	Glutamine/alanine	Exon 7	23–25

Hyperhomocysteinemia

Inherited hyperhomocysteinemia

Autosomal recessive inheritance

In general population:

WT: 44%
Homozygous: 12%
Heterozygous: 24%

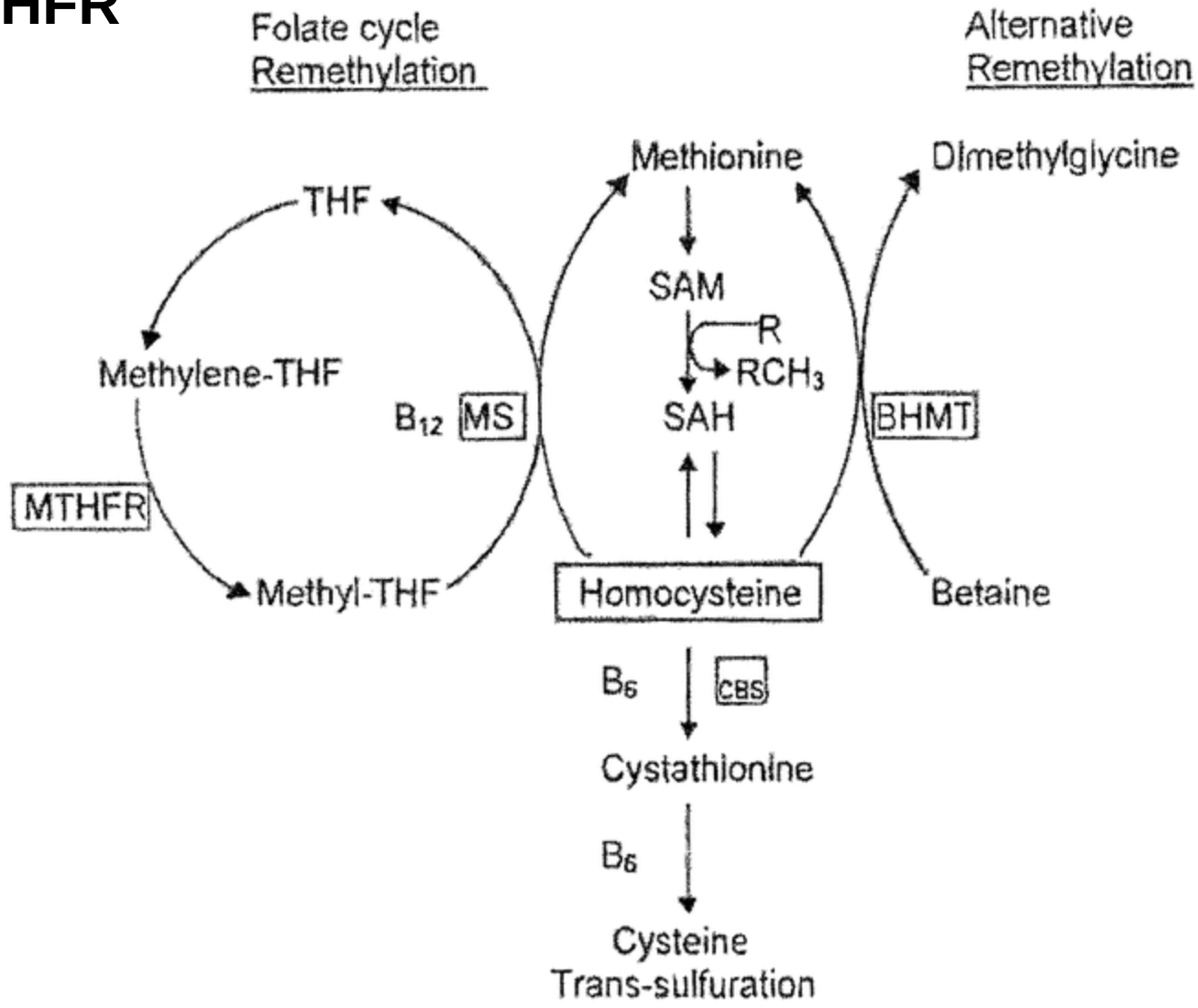
Hyperhomocysteinemia

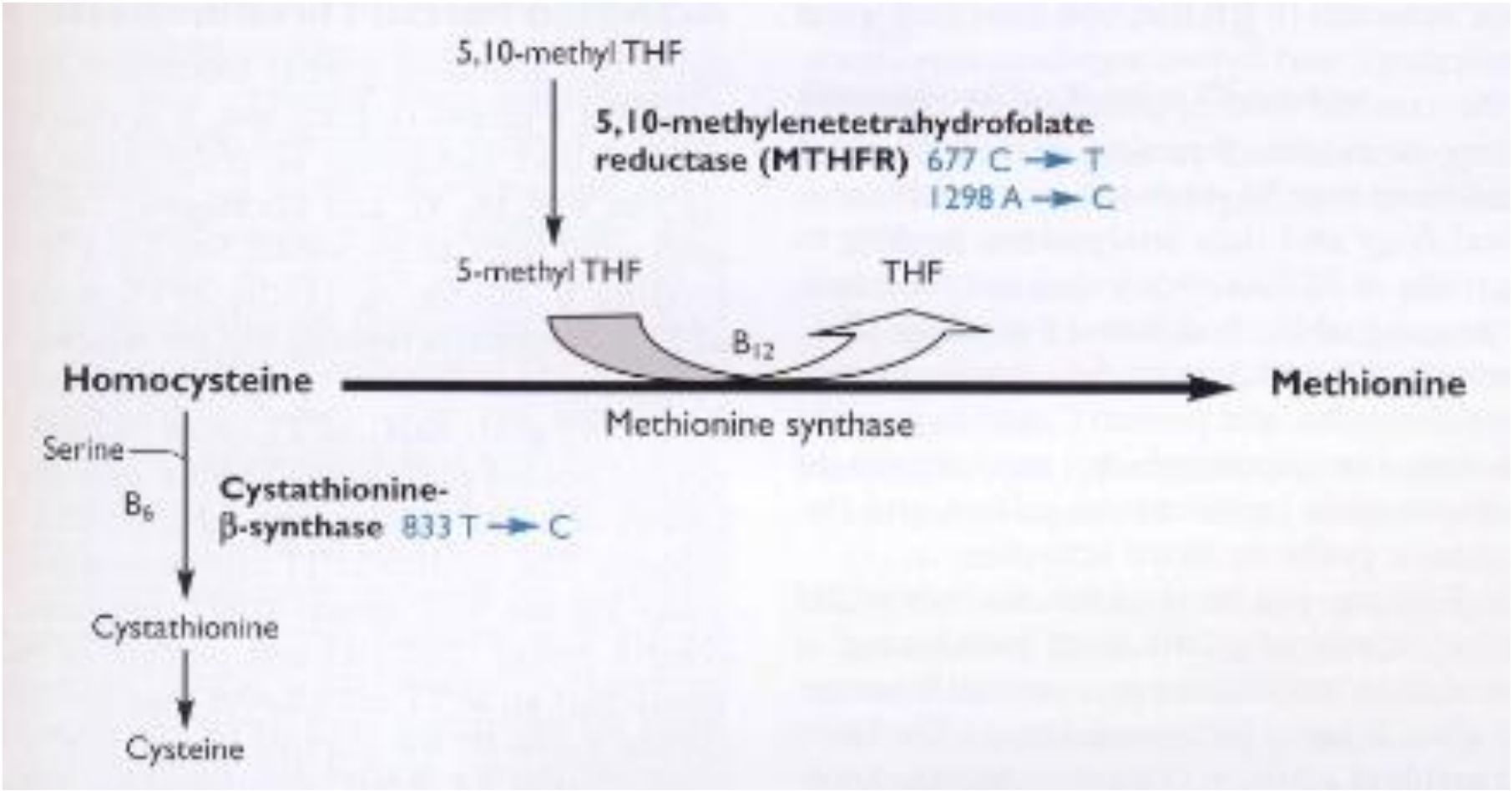
Inherited hyperhomocysteinemia

MTHFR mutations can completely inactivate the enzyme, but there are times when the defective enzyme can still function, albeit less efficiently than normal. The defective enzymes may not catch up with the body's demand for tetrahydrofolate needed for DNA synthesis during rapid cell division.

C677T results in a thermolabile variant of *MTHFR*

MTHFR


Folate cycle
Remethylation
Alternative
Remethylation
Methionine
Dimethylglycine
THF
SAM
R
RCH3
Methylene-THF
B12
MS
SAH
BHMT
MTHFR
Methyl-THF
Homocysteine
Betaine
B6
CBS
Cystathionine
B6
Cysteine
Trans-sulfuration


5,10-methyl THF
5,10-methylenetetrahydrofolate reductase (MTHFR) 677 C → T
1298 A → C
5-methyl THF
THF
B12
Homocysteine
Methionine
Methionine synthase
Serine
B6
Cystathionine-β-synthase 833 T → C
Cystathionine
Cysteine

Relative Risk for a First Incident of Venous Thrombosis and the Incidence of Venous Thrombosis Associated with Each Risk Factor or Risk Factor Combination*

Variable	Relative Risk	Annual Incidence, %
Normal	1	0.008
Hyperhomocysteinemia[57]	2.5	0.02
Homozygous *MTHFR* C677T[58]	1	
Prothrombin G20210A[24]	2.8	0.02
Oral contraceptive use[59]	4	0.03
FVL heterozygotes[5]	7	0.06
Oral contraceptives and FVL[59]	35	0.3
FVL homozygotes[7]	80	0.5–1.0
FVL and prothrombin G20210A[60]	20	
FVL and homocystinuria[61]	21.8	

* Superscript numbers are reference citations. Modified with permission from Bauer.[62] FVL indicates factor V Leiden.

Table 10. Effect of Hyperhomocysteinemia Combined With Other Thrombophilic Risk Factors	
Study	**Findings***
Fermo et al[162]	The relative risk of VT in patients with HHC combined with other thrombophilic factors was 1.6 times greater than for patients with HHC alone and patients developed first thrombotic episode at a younger age
Ridker et al[163]	10-fold increase in thrombotic risk among patients with HHC and FVL
Legnani et al[164]	• No association between HHC and thrombosis in patients with protein C, protein S, AT def, or FVL • 677C→T MTHFR did not confer additional thrombotic risk factor to the heritable thrombophilic coagulation defects
Cattaneo et al[170,171]	Coexistence of 677C→T MTHFR and FVL increased risk of VT

* VT indicates venous thrombosis; HHC, hyperhomocysteinemia; FVL, factor V Leiden; AT def, antithrombin deficiency; and MTHFR, methylenetetrahydrofolate reductase.

Table 5. Homocysteine Effect on Various Coagulation Factors		
Factor/Process*	**Effect**	**Evidence in Literature (Reference No.)**
Tissue factor expression	Increase	Suggestive (191)
Factor VII activity	Increase	Inconsistent (192–194)
Thrombin generation	Increase	Suggestive (194–196)
Factor V activation	Increase	Suggestive (197, 198)
Fibrinogen modification	Present	Suggestive (199, 200)
Thrombomodulin expression	Decrease	Inconsistent (197, 201, 202)
Inactivation of factor Va	Decrease	Inconsistent (203–205)
TFPI activity	Increase	Inconsistent (206, 207)
tPA binding	Decrease	Suggestive (208, 209)
Plasmin generation	Decrease	Suggestive (199, 209, 210)

* TFPI indicates tissue factor pathway inhibitor; tPA, tissue plasminogen activator.

Table 6. Hyperhomocysteinemia and Arterial Occlusive Disease; Studies Showing Correlation	
Study	**Findings***
Boushey et al[124]	5-μmol/L rise in total plasma HC increases relative risk of CAD, CVD, PVD
European Concerted Action Project[125]	HHC associated with increased risk of vascular disease multiplicative to other risk factors
Stampfer et al[136]	Relative risk of MI of 3.1 when HC levels were in the 95th percentile of control values
Malinow et al[137] and Voutilainen et al[127]	Increased plasma HC levels are associated with thickened carotid wall
Nygard et al[144]	Strong graded relationship between total HC and mortality
Kluijtmans et al[153] and Mudd et al[15]	677C→T MTHFR is a genetic risk for CAD

* HC indicates homocysteine; CAD, coronary artery disease; CVD, cerebrovascular disease; PVD, peripheral vascular disease; HHC, hyperhomocysteinemia; MI, myocardial infarction; and MTHFR, methylenetetrahydrofolate reductase.

Table 7. Hyperhomocysteinemia and Arterial Occlusive Disease; Studies Showing No Correlation	
Study	**Findings***
Alfthan et al[146]	No statistical difference between individuals who developed MI and those who did not
Verhoef et al[143]	No statistically significant relative risk to develop CAD, angina, and stroke
Evans et al[147]	No association between plasma HC levels and MI
Folsom et al[148]	Total HC levels correlate with CAD in women but not men
Brattström[154]	677C→T MTHFR is not a causal risk for CAD

* MI indicates myocardial infarction; CAD, coronary artery disease; HC, homocysteine; and MTHFR, methylenetetrahydrofolate reductase.

Table 8. Hyperhomocysteinemia and Venous Thrombosis; Studies Showing Correlation	
Study	**Findings***
Falcon et al[155]	High prevalence of HHC in juvenile VT
den Heijer et al[156,157]	HHC > 95th percentile of control range is a risk factor for DVT
Simioni et al[158]	Significant high prevalence of HHC in patients with DVT of upper extremities
Eichinger et al[160]	• HHC in 25% of patients with a single episode of idiopathic VT • 2.7 risk of recurrent TE in the first 24 months after discontinuation of anticoagulation
Kottke-Marchant et al[126]	Plasma HC > 13 μM is a risk factor for arterial and venous thrombosis in patients with normal coagulation profiles
Fermo et al[162]	Moderate HHC in 13.1% of patients with VT and 19.2% of patients with AOD
den Heijer et al[161]	HHC associated with a calculated pooled odds ratio of 2.6 for VTE
Arruda et al,[165] Salomon et al,[166] and Margaglione et al[167]	Evidence in support of 677C→T MTHFR being a risk factor for VT (slightly greater risk for VT in homozygous vs heterozygous genotype)
Kluijtmans et al[173]	677C→T MTHFR may be a risk factor for thrombosis in CBS-deficient patients
Lalouschek et al[174]	677C→T MTHFR increased risk of TIA or minor strokes

* HHC indicates hyperhomocysteinemia; VT, venous thrombosis; DVT, deep venous thrombosis; TE, thromboembolism; HC, homocysteine; AOD, arterial occlusive disease; VTE, venous thromboembolism; MTHFR, methylenetetrahydrofolate reductase; CBS, cystathionine β-synthase; and TIA, transient ischemic attack.

Table 9. Hyperhomocysteinemia and Venous Thrombosis; Studies Showing No Correlation	
Study	**Findings***
Martinelli et al[159]	No association of HHC and DVT of upper extremities
Trillot et al[169] and Kluijtmans et al[172]	677C→T MTHFR does not modify risk of VT
De Stefano et al[168]	• 9 case-control studies involving 2225 patients with VT and 2994 healthy controls • No significant differences in cumulative prevalence between homozygous MTHFR in cases with VT vs normal controls

* HHC indicates hyperhomocysteinemia; DVT, deep venous thrombosis; MTHFR, methylenetetrahydrofolate reductase; and VT, venous thrombosis.

-Retrospective case-control studies favor the association of hyperhomocycteinemia with arterial thrombosis and venous thromboembolism

-Many publications suggest that homocysteine is injurious to the endothelium via a variety of mechanisms

American College of Medical Genetics Consensus Statement

Oct. 2000

Homozygosity for C667T variant increases the risk for hyperhomocysteinemia, which in turn increases the risk of arterial thrombosis; but the variant by itself is not associated with arterial thrombosis in the absence of hyperhomocysteinemia, and is not associated with venous thrombosis in any case. As a simple point mutation (or point polymorphism), the C667T variant is easy to screen for using molecular methods; however, homozygosity for this mutation accounts for only about a third of cases of hyperhomocysteinemia. Therefore, many authorities feel plasma homocysteine measurement is more informative than molecular testing.

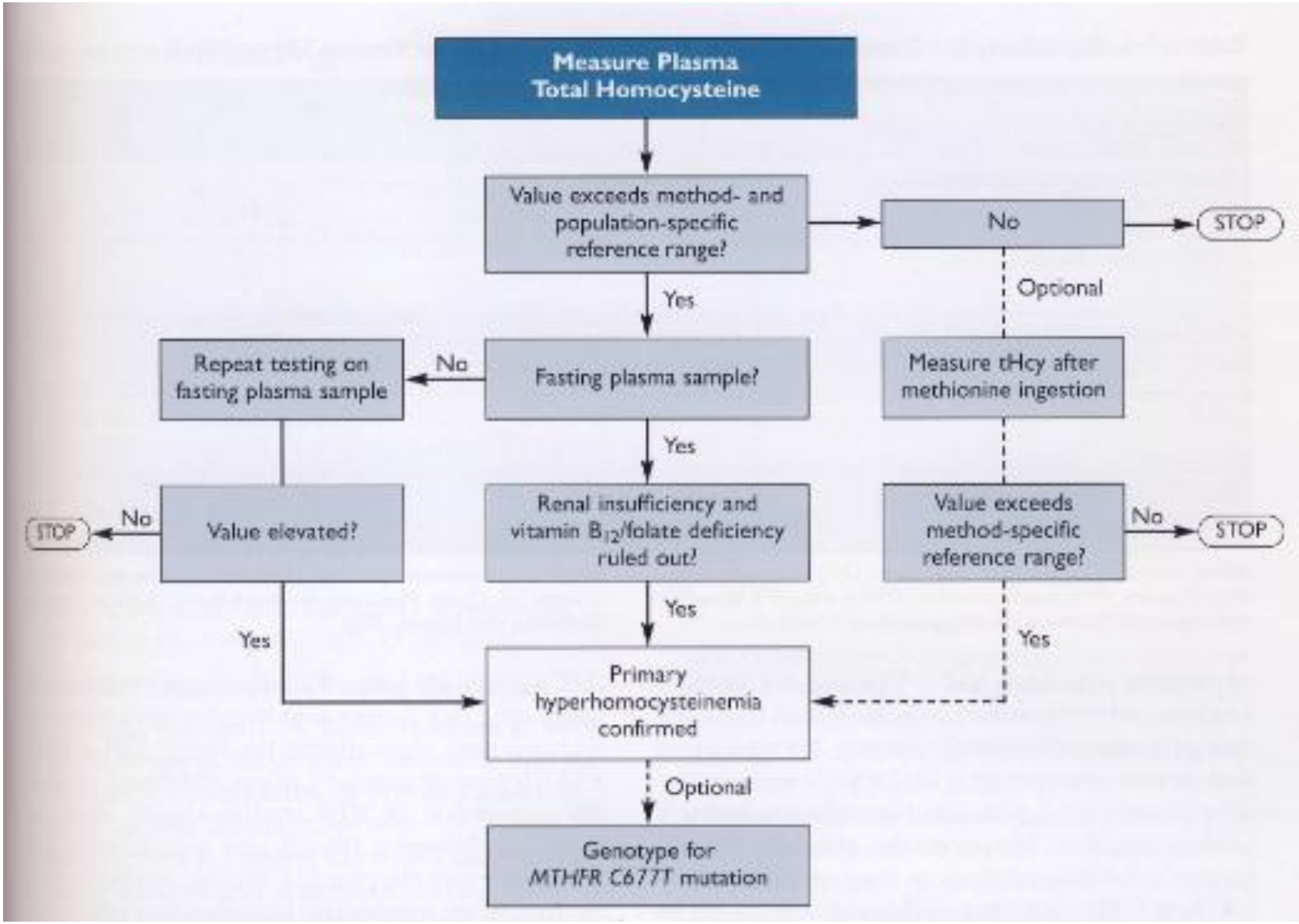

Measure Plasma Total Homocysteine
Value exceeds method- and population-specific reference range?
No
STOP
Optional
Yes
Repeat testing on fasting plasma sample
No
Fasting plasma sample?
Measure tHcy after methionine ingestion
Yes
STOP
No
Value elevated?
Renal insufficiency and vitamin B_{12}/folate deficiency ruled out?
Value exceeds method-specific reference range?
No
STOP
Yes
Yes
Yes
Primary hyperhomocysteinemia confirmed
Optional
Genotype for MTHFR C677T mutation

Table 3. Classification of Hyperhomocysteinemia	
Type	**Findings***
Severe–moderate hyperhomocysteinemia	High total homocysteine levels at all times; deficiencies in CBS, MTHFR, or in enzymes of B_{12} metabolism
Mild–moderate hyperhomocysteinemia	Moderately high total homocysteine levels under fasting conditions; reflects impaired homocysteine methylation (folate, B_{12}, or moderate enzyme defects, eg, thermolabile MTHFR)
Post-methionine load	Abnormal increase in total homocysteine after methionine load. Abnormal net increase reflects impaired homocysteine transsulfuration (heterozygous CBS deficiency, B_6 deficiency)

* CBS indicates cystathionine β-synthase; MTHFR, methylenetetrahydrofolate reductase.

PML homocysteine testing identifies a subset of individuals with normal fasting homocysteine levels but abnormal PML tests. Such patients are likely to have a heterozygous genetic defect, MTHFR polymorphisms being the most frequent and probably cause. Since PML is impractical and not routinely offered, MTHFR status may provide some insights.

It seems prudent to include measuring plasma homocysteine level and assessing MTHFR status in initial thrombophilia workup, untill such time when solid evidence against this approach is introduced in the literature.

Special coagulation Section Arch Pathol Lab Med June 2007

Cases with molecular assays are not used as the first-line tests

Antithrombin III

The gene coding for antithrombin is located to chromosome 1q23-25.

A single copy has seven exons spanning 13.4 kb of DNA. Within the introns of the gene are located nine full length and one partial Alu repeat elements. Two of these Alu5 and Alu8 within intron 4 have tails composed of ATT trinucleotide repeats that are polymorphic in copy number.

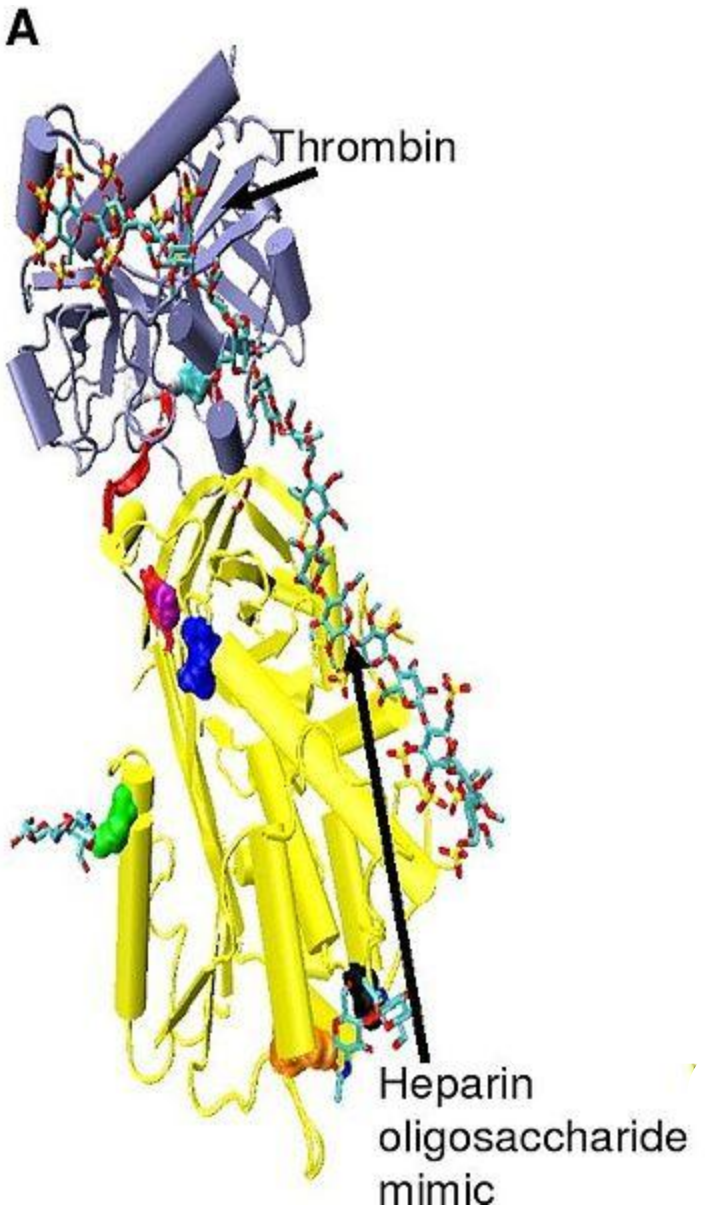
The gene codes for a protein of MW ~58 000 containing 432 amino acids, 6 of which are cysteines that form three intramolecular disulphide bonds.

Antithrombin is a member of a superfamily of proteins collectively known as serine proteinase inhibitors (serpins). Antithrombin contains a functional reactive site which participates in the inhibitory interaction with proteinases, but also a binding site for heparin and related glycosaminoglycans. Binding of heparin to the latter site induces a conformational change and accelerates its inhibition of proteinases.

Antithrombin III

Autosomal dominant inheritance

Functional assays are preferred for diagnosis



ATIII Deficiency

Type I antithrombin deficiency

A decrease in both antithrombin activity and antithrombin concentration in the blood of affected individuals.

Two subgroups

Subgroup Ia: a normal affinity for heparin

Subgroup Ib: a reduced affinity for heparin. In a revised system of classification adopted by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis, type Ib cases are now designated as type II PE, Pleiotrophic effect.

Molecular defects

Most cases of type I deficiency are due to point mutations, deletions or minor insertions within the antithrombin gene.

ATIII Dificiency

Pathogenesis

Mutations may produce unstable antithrombins that either may be not exported into the blood correctly upon completion biosynthesis or exist in the blood for a shortened period of time, e.g., the deletion of 6 base pairs in codons 106-108.

Mutations may affect mRNA processing of the antithrombin gene.

Minor insertions or deletions may lead to frame shift mutations and premature termination of the antithrombin gene.

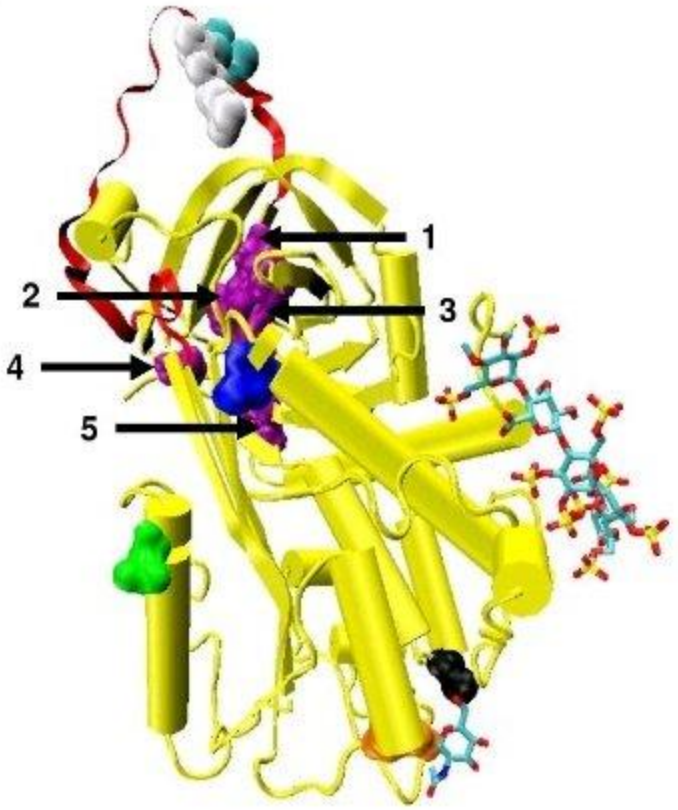
Point mutations may also result in the premature generation of a termination or stop codon, e.g. the mutation of codon 129, CGA → TGA (UGA after transcription), replaces a normal codon for arginine with a termination codon.

ATIII

Type II antithrombin deficiency

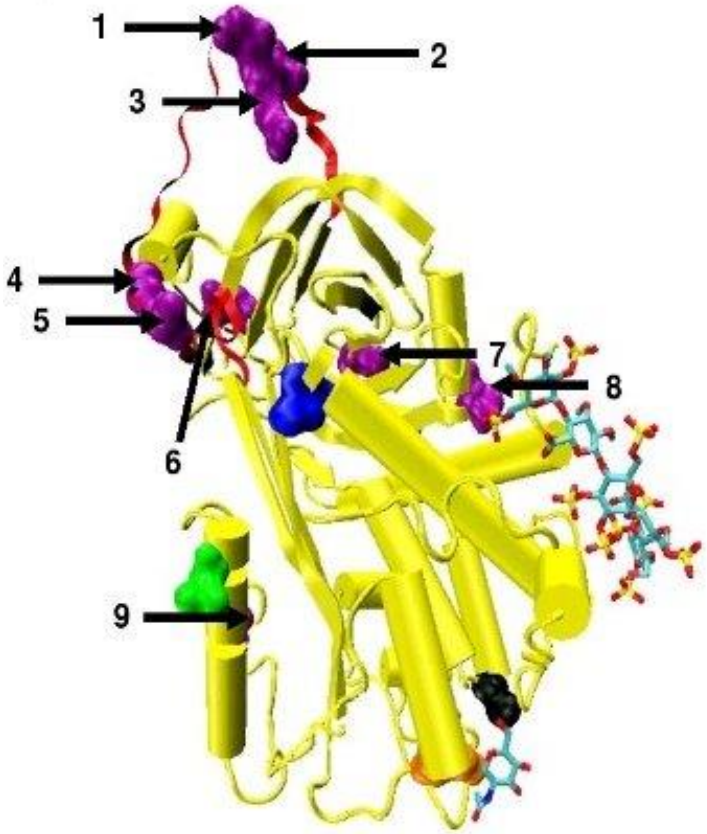
Type II antithrombin deficiency is characterised by normal antithrombin levels but reduced antithrombin activity in the blood of affected individuals. Originally it was proposed that type II deficiency be further divided into three subgroups IIa, IIb and IIc depending upon which antithrombin functional activity is reduced or retained.
Subgroup IIa - Decreased thrombin inactivation, decreased factor Xa inactivation and decreased heparin affinity.
Subgroup IIb - Decreased thrombin inactivation and normal heparin affinity.
Subgroup IIc - Normal thrombin inactivation, normal factor Xa inactivation and decreased heparin affinity.
In the revised system of classification again adopted by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis, type II antithrombin deficiency remains subdivided into three subgroups: the already mentioned type II PE, along with type II RS, were mutations effect the reactive site and type II HBS, were mutations effect the antithrombin heparin binding site. For the purposes of an antithrombin mutational database compiled by members of the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis, type IIa cases are now classified as type II PE, type IIb cases as type II RS and type IIc cases as type II HBS.

Type IIa, pleiotrophic effect mutations



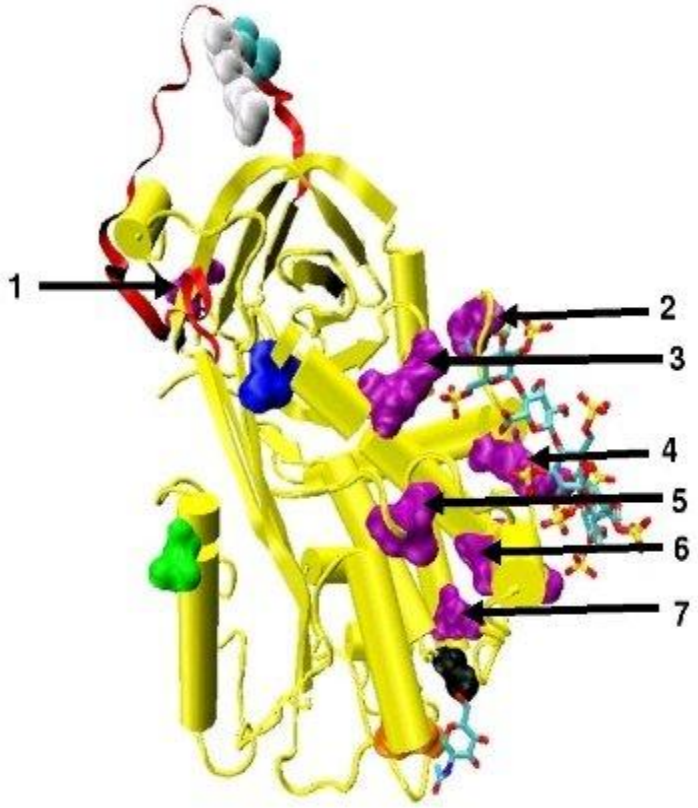
1. Phe 402, **2.** Ala 404 and Asn 405,
3. Arg 406 and Pro 407 **4.** Pro 429
5. Arg 425.

Type IIb, reactive site mutations

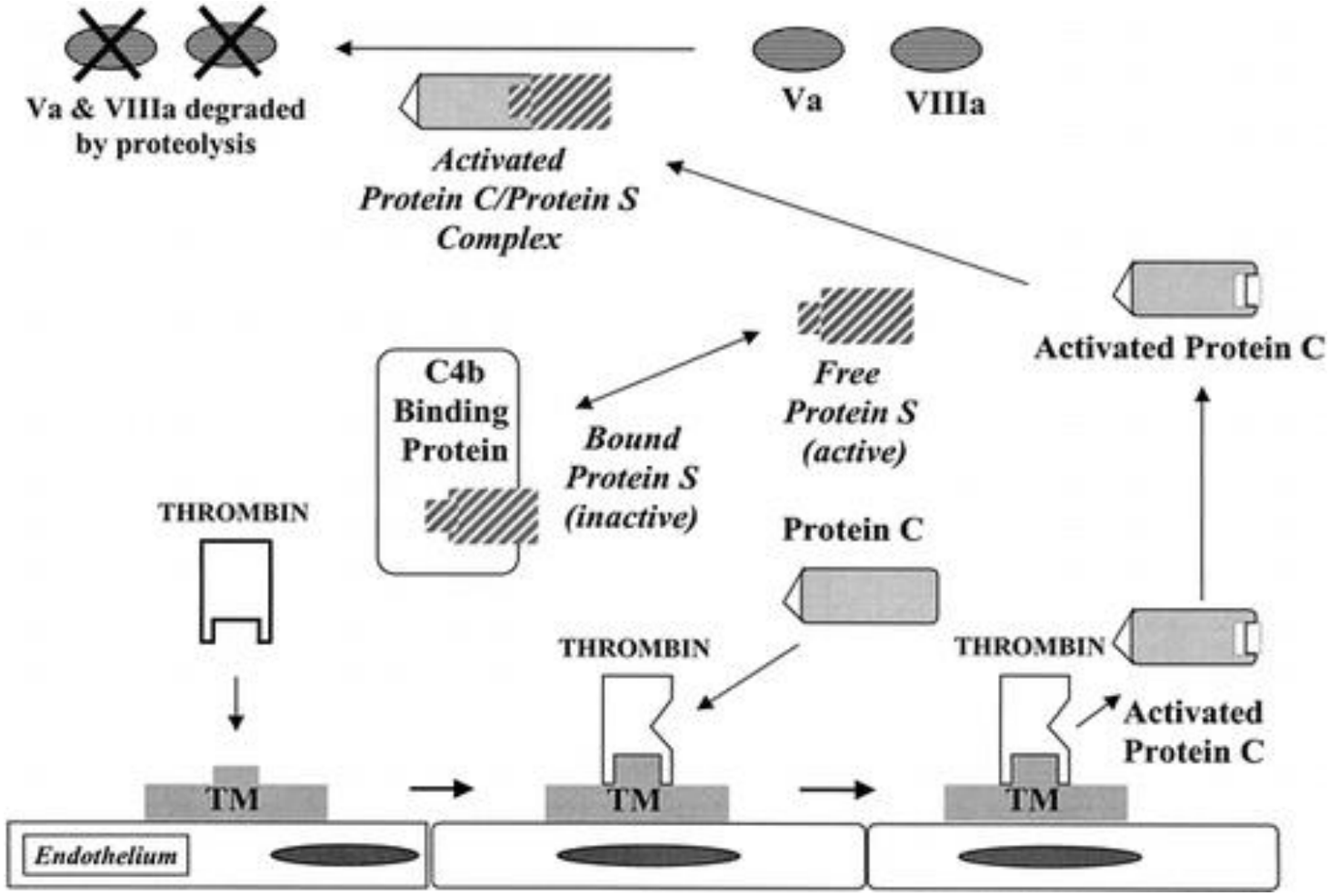


1. Gly 392, **2.** Ser 394, **3.** Arg 393,
4. Ala 384, **5.** Ala 382, **6.** Met 251,
7. Ile 284, **8.** Glu 302, **9.** Asn 187.

Type IIc, heparin binding site mutations



1. Glu 237, **2.** Pro 41, **3.** Arg 129,
4. Arg 47, **5.** Ile 7, **6.** Arg 24, Ser 116, Gln 118,
7. Leu 99.



The protein C/protein S anticoagulant pathway. Thrombin-thrombomodulin (TM) complex activates protein C. Activated protein C with its cofactor, free protein S, degrades factors Va and VIIIa. In addition, when thrombin binds thrombomodulin, thrombin loses its procoagulant functions.

Protein C

Hereditary deficiencies occur in 0.14 - 0.5% of general population (the clinically significant incidence is much lower); >160 mutations exist, either type I (76%, usually quantitative) or type II (dysfunctional protein, normal protein levels).
Causes 1-11% of cases of venous thrombosis; these patients are also at risk for warfarin-induced skin necrosis if treated with warfarin and no heparin until warfarin levels are therapeutic.

Heterozygotes have levels 35-65% of normal; first thrombotic event occurs between ages 10-50 years; only 30% have thromboembolism, increasing to 75% if coexisting factor V Leiden.

Homozygotes (1 per 500-750K births) with severely decreased levels present as newborns with DIC and purpura fulminans neonatorum, leading to death unless anticoagulation and replacement therapy with fresh frozen plasma is started.

Protein C Deficiency

Inherited protein C deficiency is uncommon

Two forms

- **Type I – quantitative**
- **Type II – qualitative**

Autosomal dominant inheritance

**** Functional assays preferred for diagnosis (rather than antigenic assays)**

Protein S

Hereditary deficiencies occur in 0.7% of general population; many mutations exist (qualitative or quantitative); much lower prevalence of thrombophilia with clustering in families; variable penetrance may be due to coexisting risk factors, such as factor V Leiden.

Causes 1-9% of cases of venous thrombosis; these patients also at risk for warfarin-induced skin necrosis if started on warfarin without the addition of heparin until warfarin levels are therapeutic.

Heterozygotes have levels 35-65% of normal; first thrombotic event occurs between ages 10-50 years; 50% risk by age 45.

Homozygotes with severely decreased levels present as newborns with DIC and purpura fulminans, leading to death unless anticoagulation and replacement therapy with fresh frozen plasma is started.

Protein S Deficiency

Type I (2/3): low free and total protein S antigen, with decreased APC cofactor activity
Type II (rare): normal free and total protein S antigen, and decreased APC cofactor activity
Type III (1/3): normal to low total protein S, low free protein S antigen, and an elevated fraction of protein S bound to C4B protein

Protein S Deficiency

Inherited protein S deficiency is uncommon

Two forms

- **Type I – quantitative**
- **Type II – qualitative**

Autosomal dominant inheritance

****Antigenic tests for free protein S preferred for diagnosis**

Inherited Abnormal Fibrinogens (dysfibrinogenemia)

The **dysfibrinogenemias** are a group of autosomal dominant disorders of qualitatively abnormal fibrinogens. There are more than 350 different fibrinogen abnormalities, each named after the place where it was discovered. Each dysfibrinogenemia is associated with slightly different effects on the thrombin time and on normal clotting. Some (25%) dysfibrinogenemias cause abnormal bleeding or even thrombosis (25%), while others have no effect on either bleeding or thrombosis.

Chapel Hill is prothrombotic dysfibrinogen with G554C in the Aα chain.

Amsterdam is a major defect, characterized by aggregation of fibrin monomers, prolonged thrombin time, and an inhibitory effect on normal clotting - but it is asymptomatic.

Detroit is a major defect, there is fibrinopeptide release, the thrombin time is prolonged, there is an inhibitory effect on normal clotting and there is abnormal bleeding.

Wiesbaden is a major defect, there is aggregation of fibrin monomers, the thrombin time is prolonged, there is an inhibitory effect on normal clotting and there is both bleeding and thrombosis.

Inherited Bleeding Disorders

Hemophilia A

X-linked recessive *F8* mutations

Penetrance – 100% in males, 10% in females

- **Female offspring of affected males are obligate carriers**
  - **10% of female carriers are symptomatic, typically with mild disease**
- **In probands with no apparent family history of hemophilia, >80% of mothers are identified as carriers; 10-15% of probands show de novo *F8* mutations**

~ 1,800 *F8* sequence variants reported to date

Hemophilia B

X-linked recessive *F9* mutations

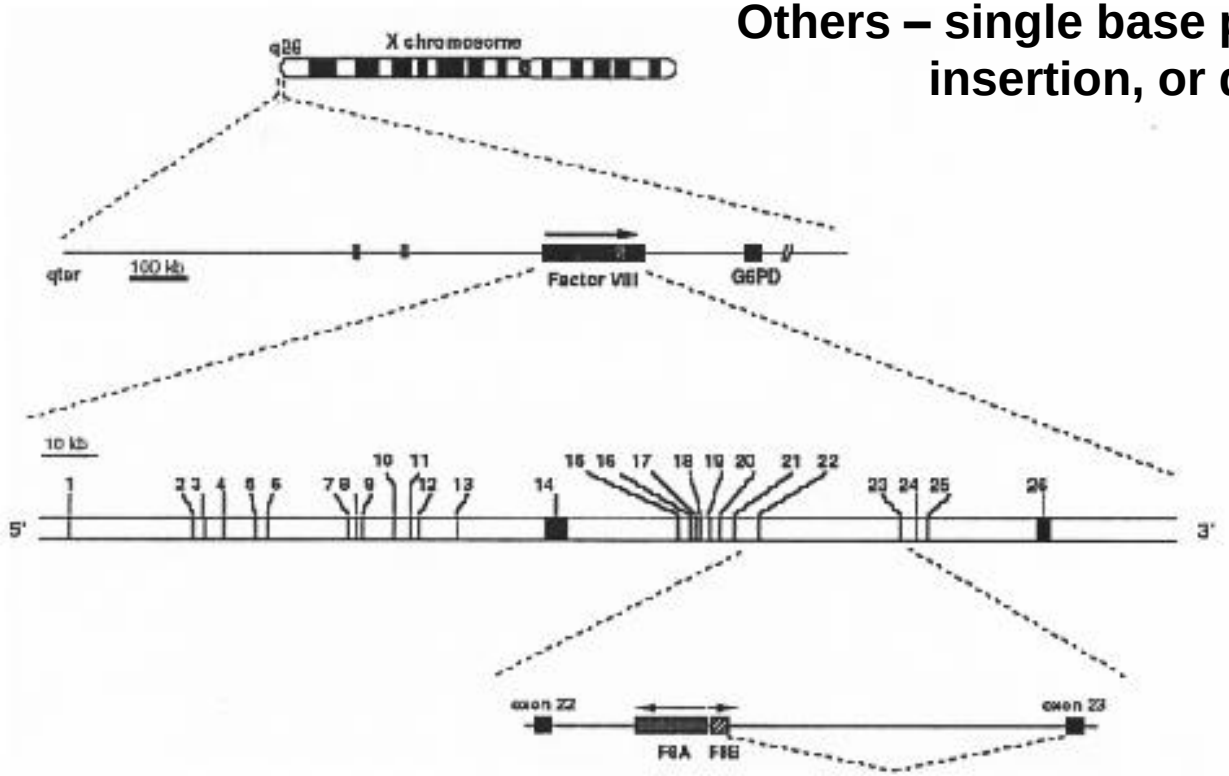
Penetrance – 100% in males, 10% in females

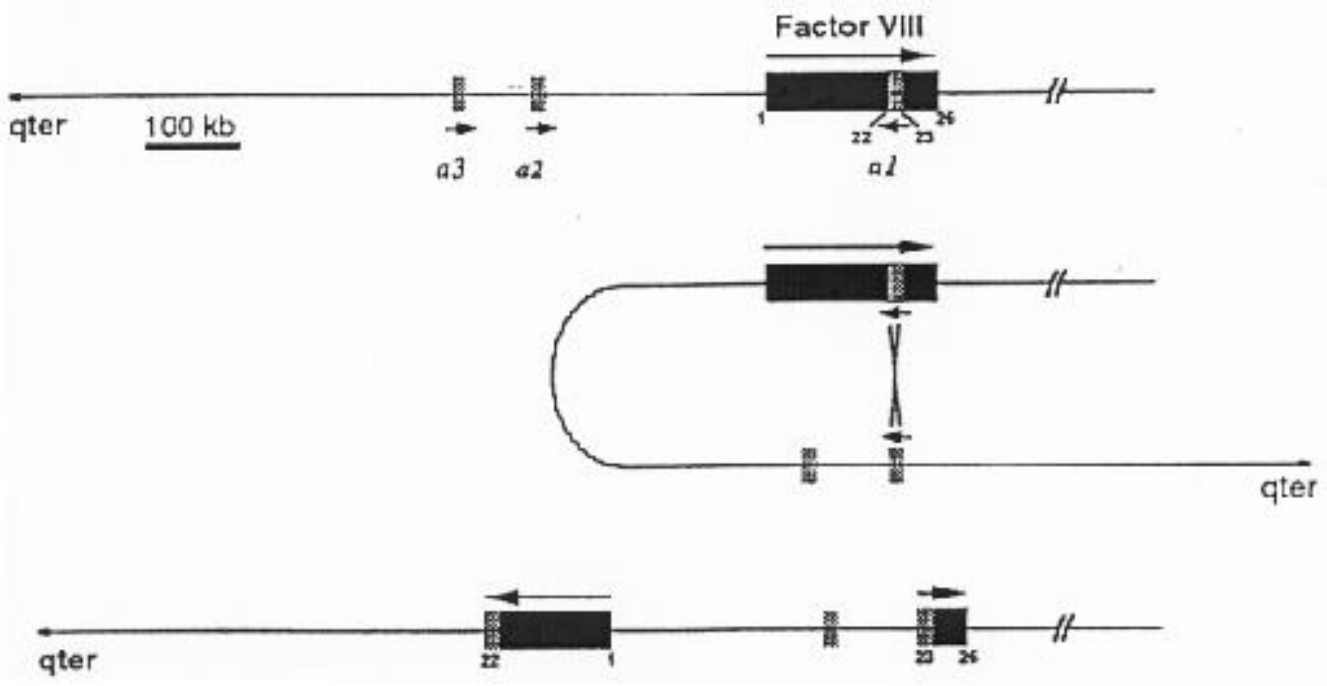
- **Female offspring of affected males are obligate carriers**
  - **10% of female carriers are symptomatic, typically with mild disease**
- **~33-50% of apparently isolated hemophilia B cases result from de novo mutations**

****~900 *F9* sequence variants reported to date**

Hemophilia A

40% - Inversion
5% - Deletion
Others – single base pair changes, insertion, or duplications




Factor VIII
qter
100 kb
a3
a2
a1
1
26
22
23
qter
qter
23
1
23
26

Clinical Utility of Molecular Testing

- **Some genotype/phenotype correlations exist for hemophilia A**
  - **Molecular causes for severe disease**
    - **Intron 22A inversion – 48%**
    - **Point mutations – 43%**
    - **Large gene deletions – 6%**
    - **Intron 1 inversion – 3%**
  - **Molecular causes for moderate to mild disease**
    - **Point mutations or small insertions/deletions – 98%**
    - **Large gene deletions – <1%**

Clinical Utility of Molecular Testing

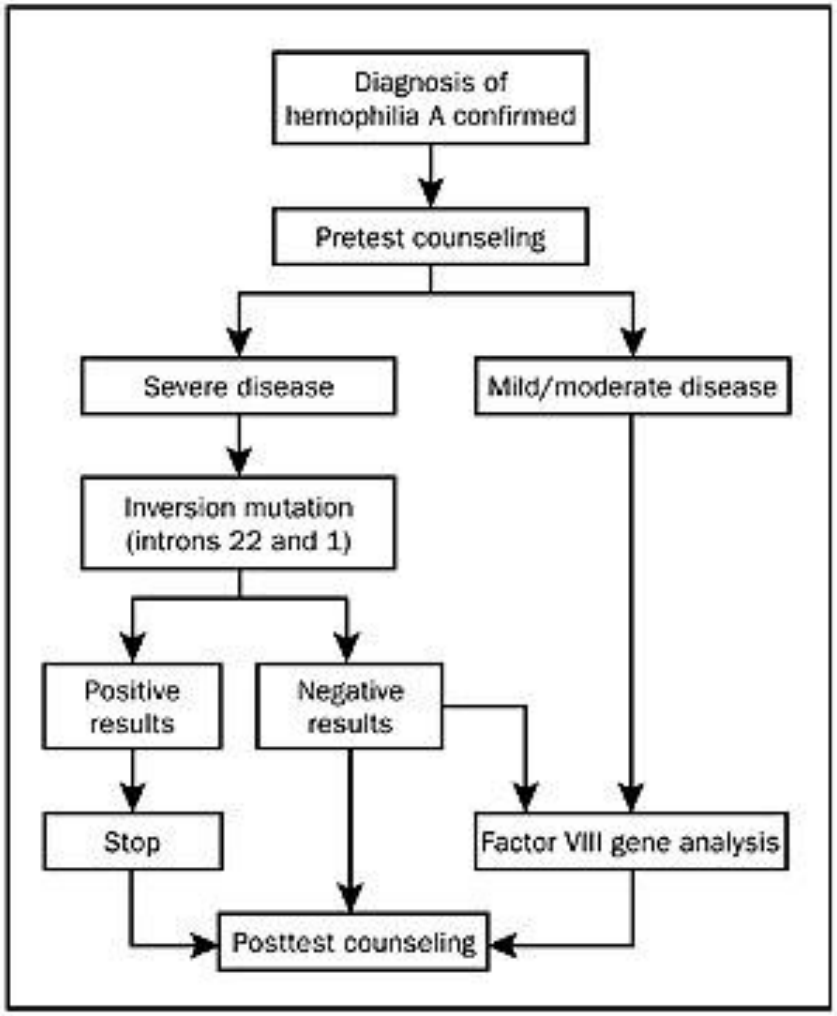
-Carrier status

A normal FVIII activity does not exclude carrier status

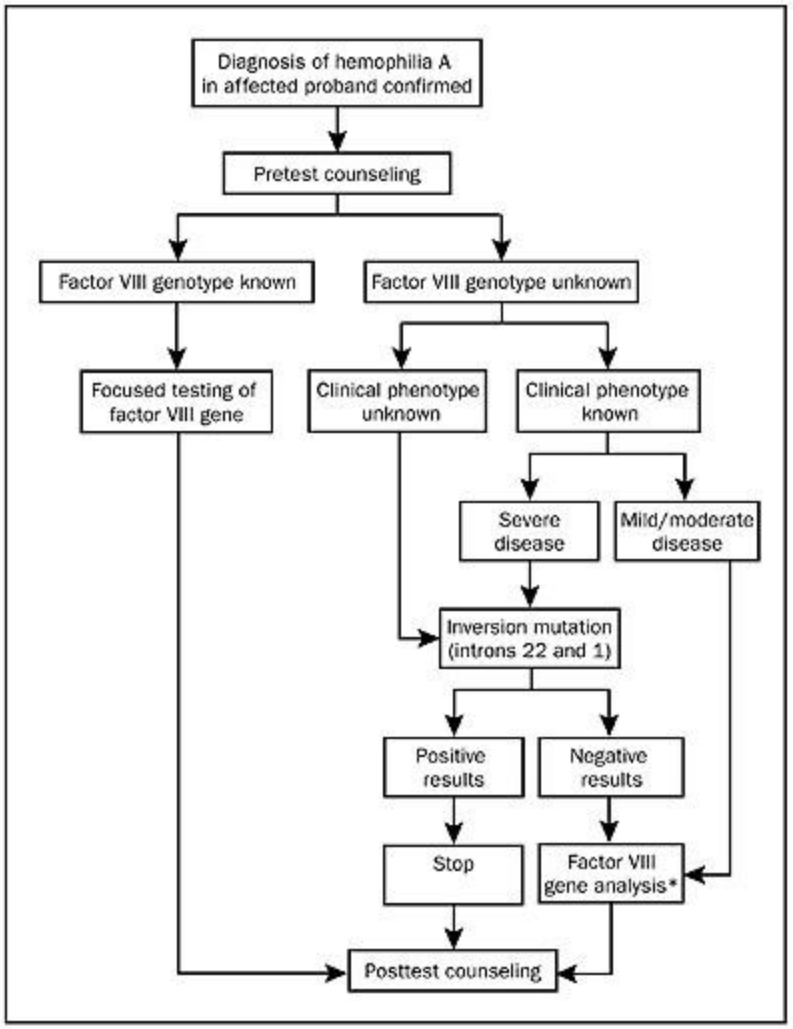
labor delivery management or pregnancy termination
carrier testing of family members
preimplantation genetic diagnosis

In probands which appear to have no family history of hemophilia, >80% of mothers are identified as carriers; the remaining 10-15% of probands show de novo *F8* mutations

-Knowledge of the causative *F8* mutation in probands may be useful in predicting the risk of developing FVIII inhibitors



Algorithm for approach to genetic testing of hemophilia A proband.



Algorithm for approach to genetic testing of at-risk hemophilia A carrier. *Refer for linkage analysis if testing detects no mutation in proband or carrier.

FVIII

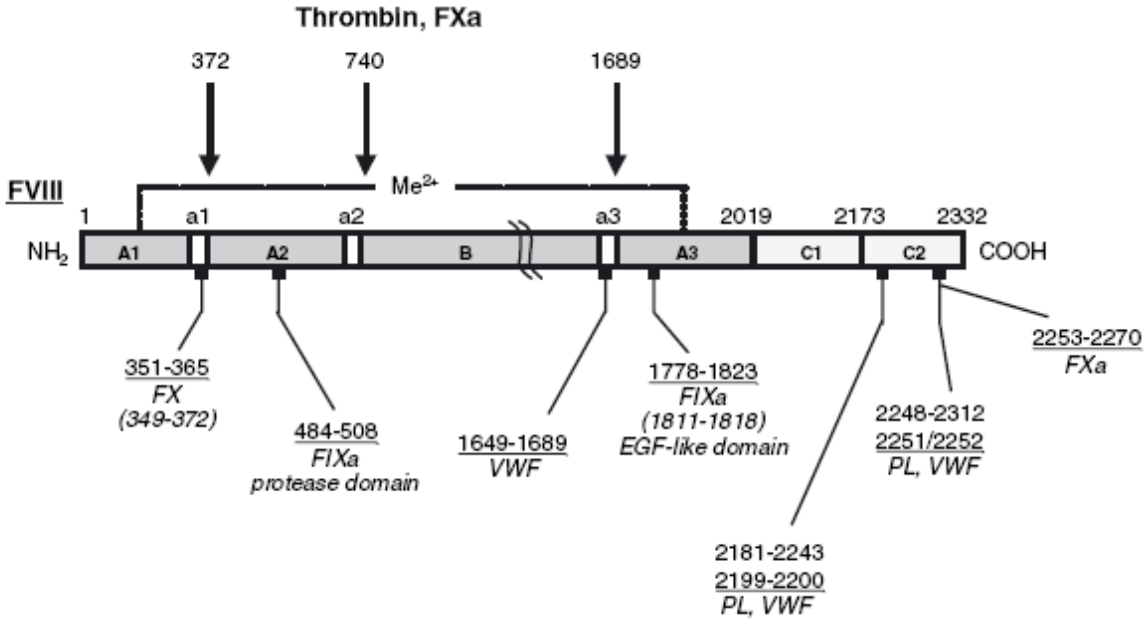
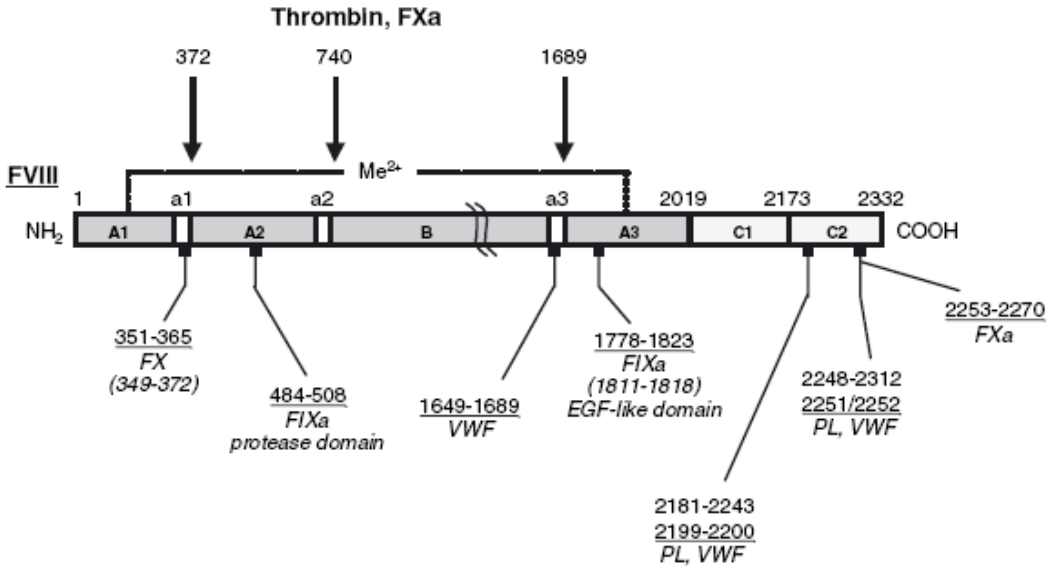


Fig 1. Factor VIII (FVIII) structure and epitopes of FVIII inhibitors. Non-activated FVIII is a heterodimer, in which the heavy chain consists of the A1 (1–336), A2 (373–740) and B domains (741–1648), and the light chain is composed of the domains A3 (1690–2019), C1 (2020–2172) and C2 (2173–2332). The A domains are flanked by acidic regions *a1* (337–372), *a2* (711–740) and *a3* (1649–1689), which contain a high number of negatively charged residues. The A1 and A3 subunits are non-covalently linked via a metal ion-mediated interaction (dotted line). Activation of FVIII (cleavage sites are shown by arrows) leads to release of the B domain and *a3*. In activated FVIII heterotrimer, the A1 and A3 domains retain the metal ion-mediated interaction, and the stable A1/A3–C1–C2 dimer is weakly associated with the A2 subunit through electrostatic interactions. The epitopes of FVIII inhibitors are indicated in bold and overlap with the binding sites (indicated in italic) for von Willebrand factor (VWF), phospholipid (PL), activated factor IX (FIXa), factor X (FX), and activated factor X (FXa). Figure and legend adapted from Ananyeva *et al* (2004); with permission from: Lippincott Williams & Wilkins.

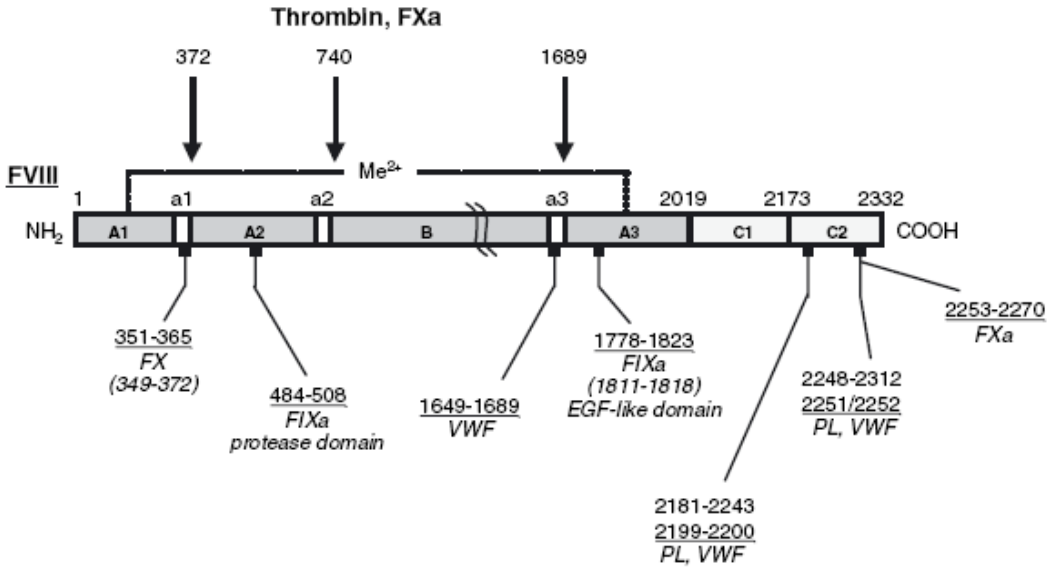


-Pts. who have a *F8* gene with inversion, large deletion and nonsense mutations show the highest inhibitor incidence

--complete deficit of any endogenous FVIII production

--wouldn't be presented to the immune system during negative selection – recognized as a foreign protein

-Deletion that affect more than one domain of FVIII have a higher risk for developing FVIII inhibitors than pts. with Gene deletions that affect one domain only



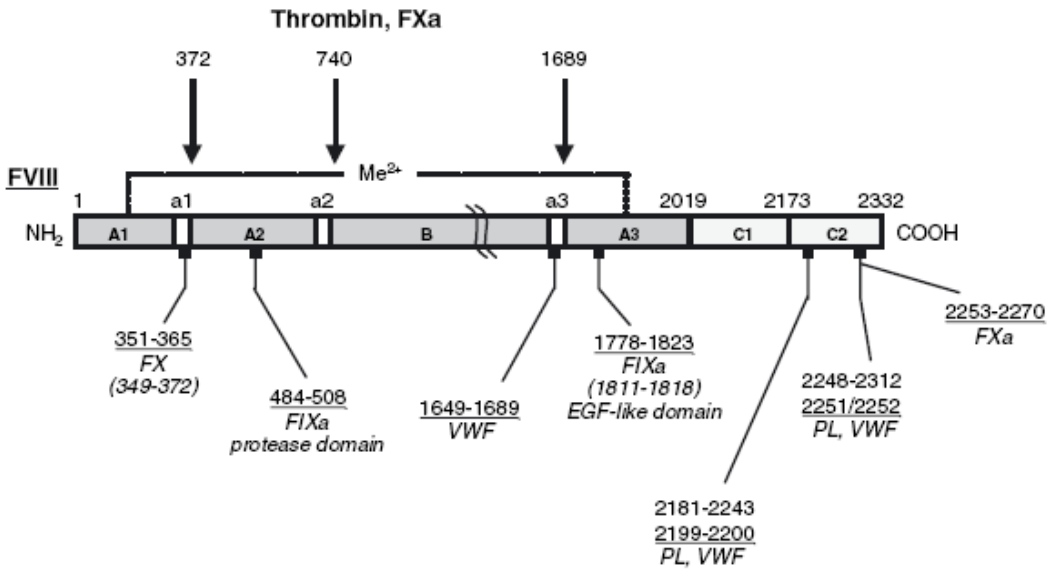
-Small deletion and missense mutations are associated with a much lower incidence of inhibitor formation

--non-functional endogenous FVIII

--the immune system tolerance – recognized as an altered self- protein

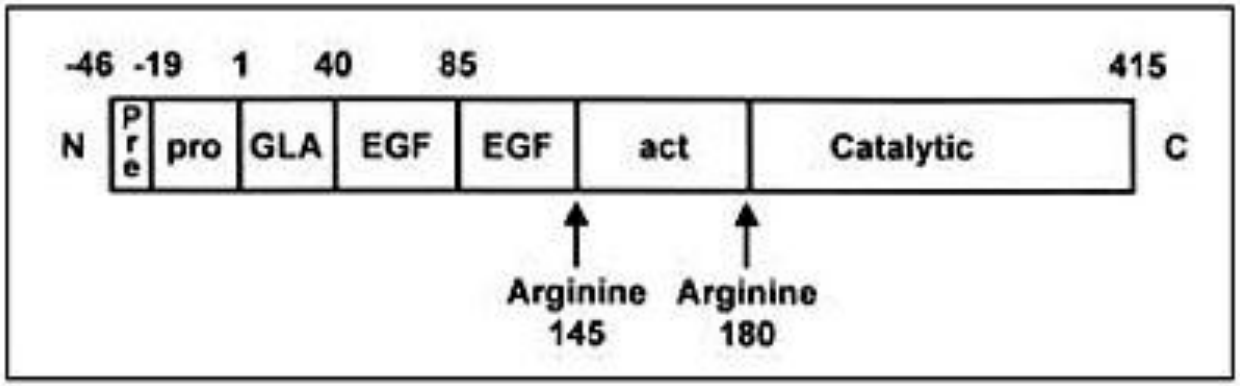
--inhibitor formation depends on the localisation of the mut.

pts. w missense mut. in the C1 and C2 have a 3x higher risk to develop FVIII inhibitors



-Inhibitor formation in mild hemophilia appears to be associated w relatively few high-risk *F8* gene mutations in the A2 or C2 domains, such as Arg593Cys, Arg2150His and Trp2229Cys

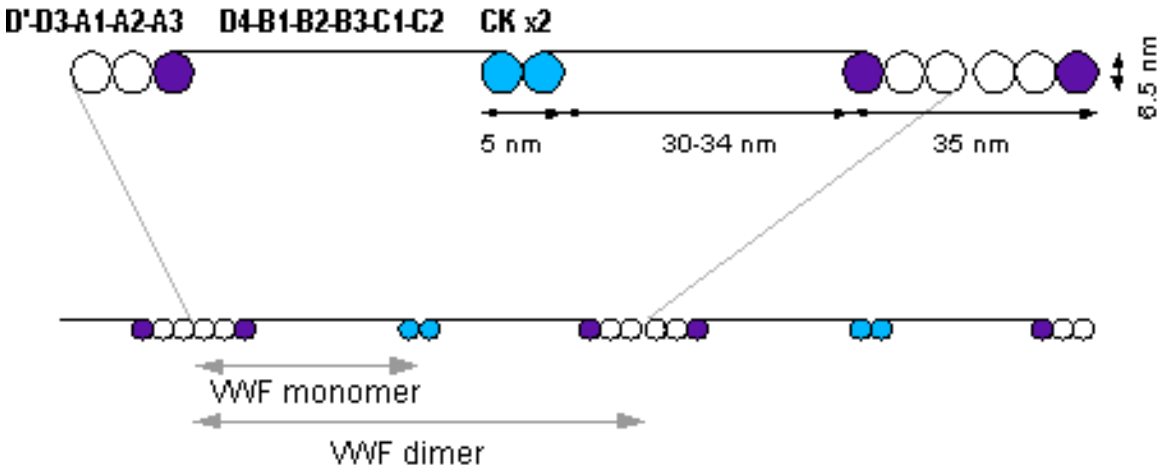
FIX



Factor IX peptide. act=activation peptide; C=carboxyl terminus; EGF=epidermal growth factor; GLA=glutamic acid rich; N=amino terminus; Pre=prepeptide; pro=propeptide.

-Some pts. experience severe allergic or anaphylactic reactions to FIX infusions simultaneously with the appearance of the inhibitor. Majority of them have large deletion or major derangement

-The majority of the F9 mutations are single base pair changes



Type 1 and 3 vWD: qualitative abnormality

Type 2 vWD: structural and functional defects

vWD

Type 1 – autosomal dominant with incomplete penetrance (60%)

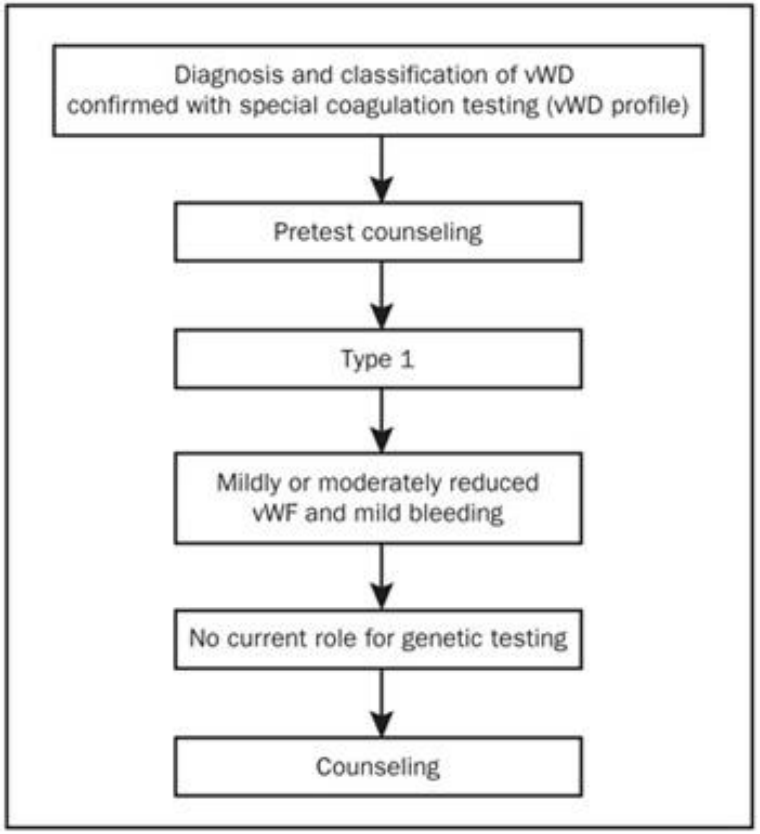
Type 2

- **2A – autosomal dominant (or recessive)**
- **2B – autosomal dominant**
- **2M – autosomal dominant (or recessive)**
- **2N – autosomal recessive**

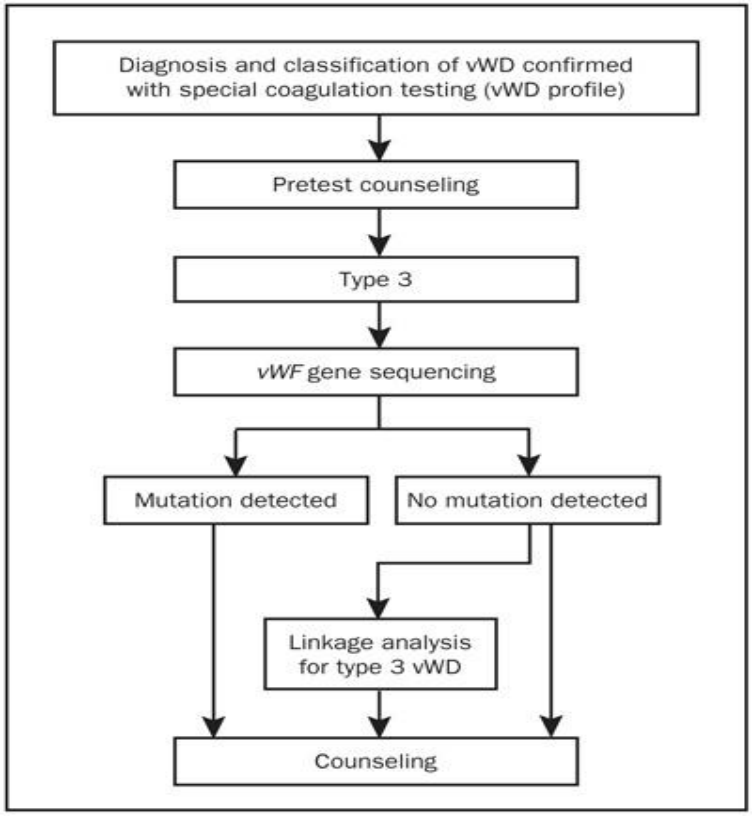
Type 3 – autosomal recessive

Platelet-type (PT-vWD) – autosomal dominant

- **Abnormality of the platelet von Willebrand receptor, glycoprotein 1b (GP1B)**

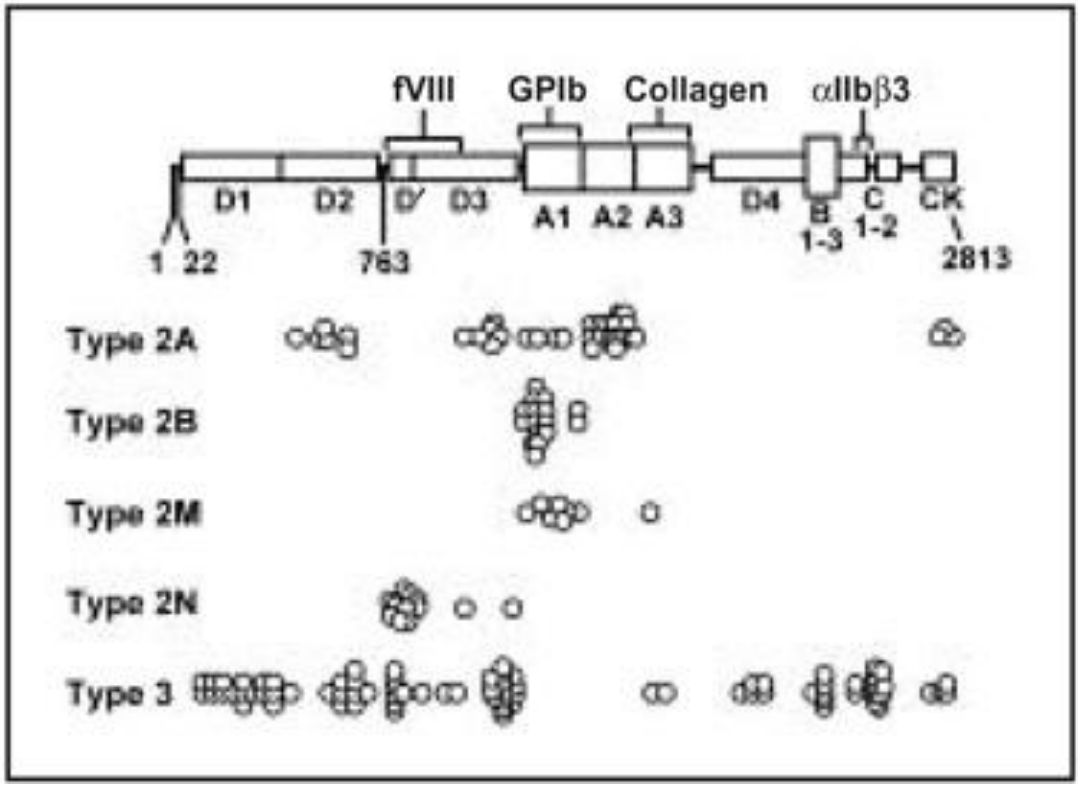


Rational approach to genetic testing in von Willebrand disease (vWD) type 1. vWF=von Willebrand factor.

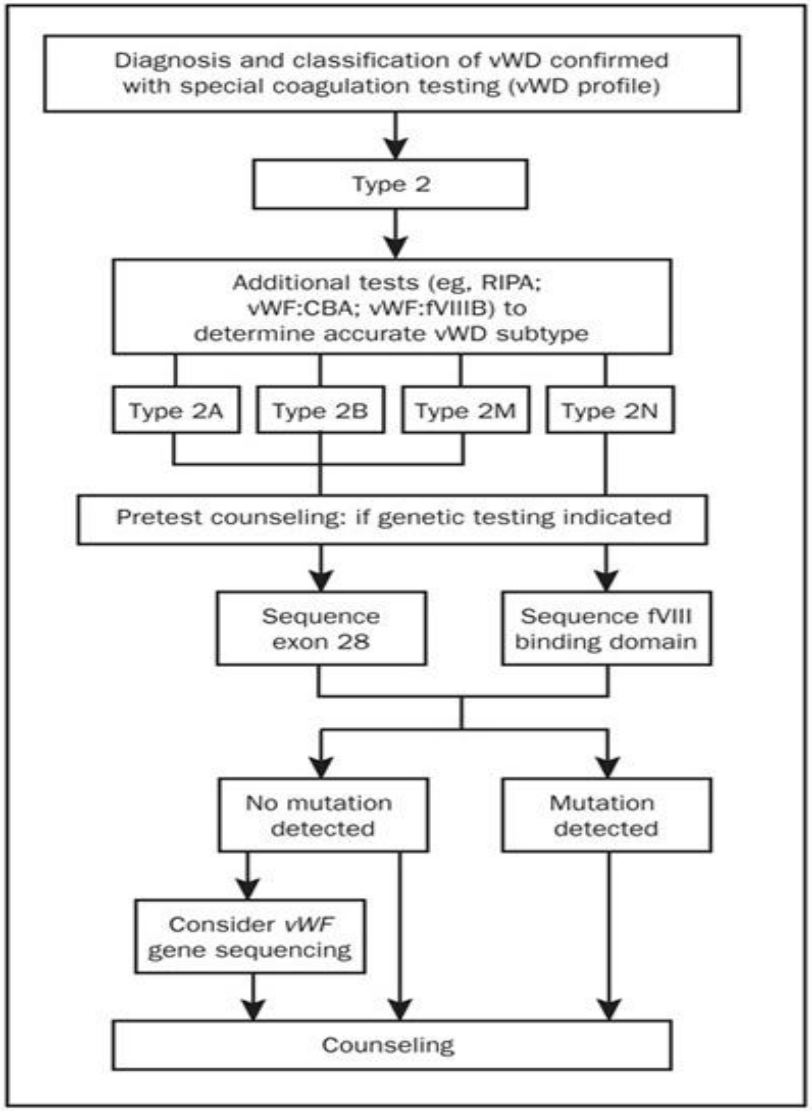


Rational approach to genetic testing in von Willebrand disease (vWD) type 3. von Willebrand factor (vWF) gene sequencing is performed if genetic testing for the family is thought to be indicated.

Type 1 vWD molecular pathogenesis: unclear; heterogenous genetic and environmental factors
Type 3 vWD mutations: frameshifts, deletions, and nonsense mutations



The von Willebrand factor peptide showing ligand-binding domains and location of mutations. fVIII=factor VIII; GP=glycoprotein.



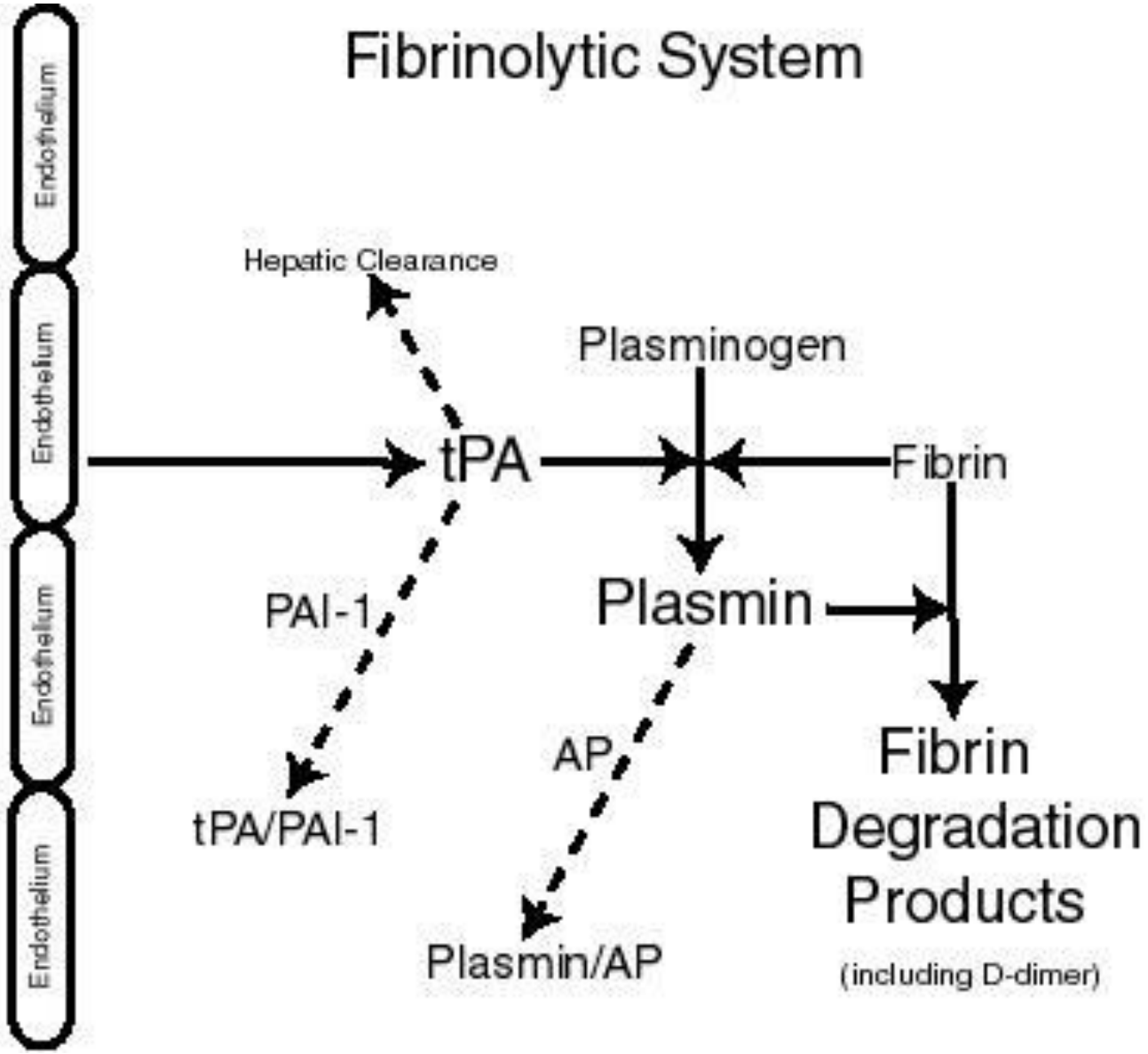
Type 2A: impaired assembly and secretion of normal vWF multimers, or increased sensitivity to proteolytic degradation

Type 2B: missense mut. in A domain may disrupt a regulatory site and result a dominant gain-of-function phenotype; mut. on plt. for the platelet-type vWD

Type 2M: mut. in A1 domain with decreased binding affinity of vWF for plt. GPIb

Type 2N: 3 mut. for 96% cases – Thr791Met, Arg816Trp, and Arg854Gln; AR pattern; can be compound heterozygotes w Type 1 vWD in pts. w low FVIII level

Rational approach to genetic testing in von Willebrand disease (vWD) type 2. von Willebrand factor (vWF) gene sequencing was considered if testing for mutations not found within exon 28. RIPA=ristocetin-induced platelet aggregation; vWF:CBA=von Willebrand factor collagen binding activity; vWF:fVIIIB=von Willebrand factor VIII binding activity.


Fibrinolytic System
Endothelium
Endothelium
Endothelium
Endothelium
Hepatic Clearance
Plasminogen
tPA
Fibrin
PAI-1
Plasmin
AP
tPA/PAI-1
Fibrin
Degradation
Products
(including D-dimer)
Plasmin/AP

PAI – 1 Deficiency

Rare congenital disorder with increased fibrinolysis

Homozygous dinucleotide insertion within exon 4 of PAI-1 gene— resulting in a premature stop codon and a truncated non-functional PAI-1 protein

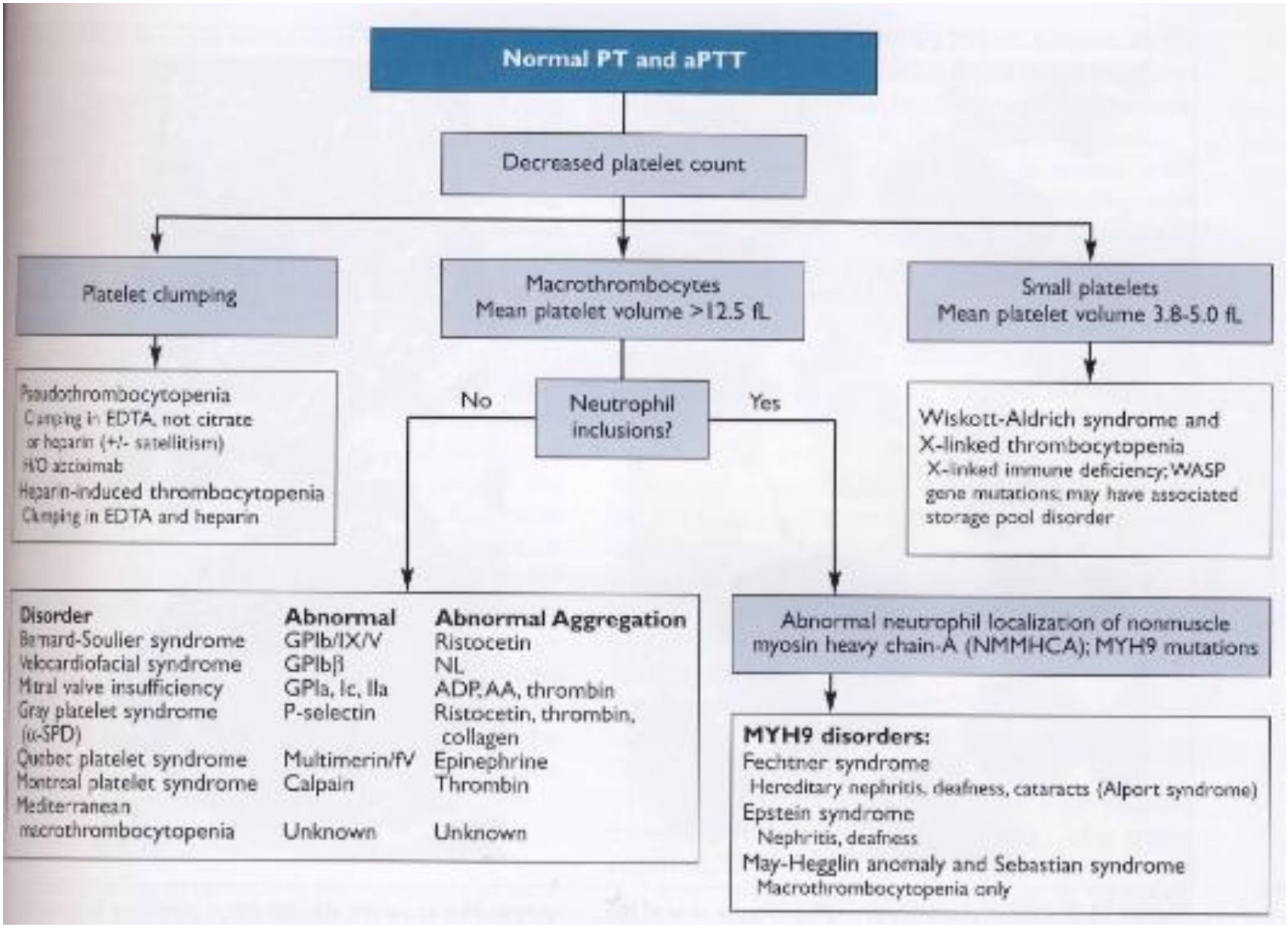
Autosomal recessive

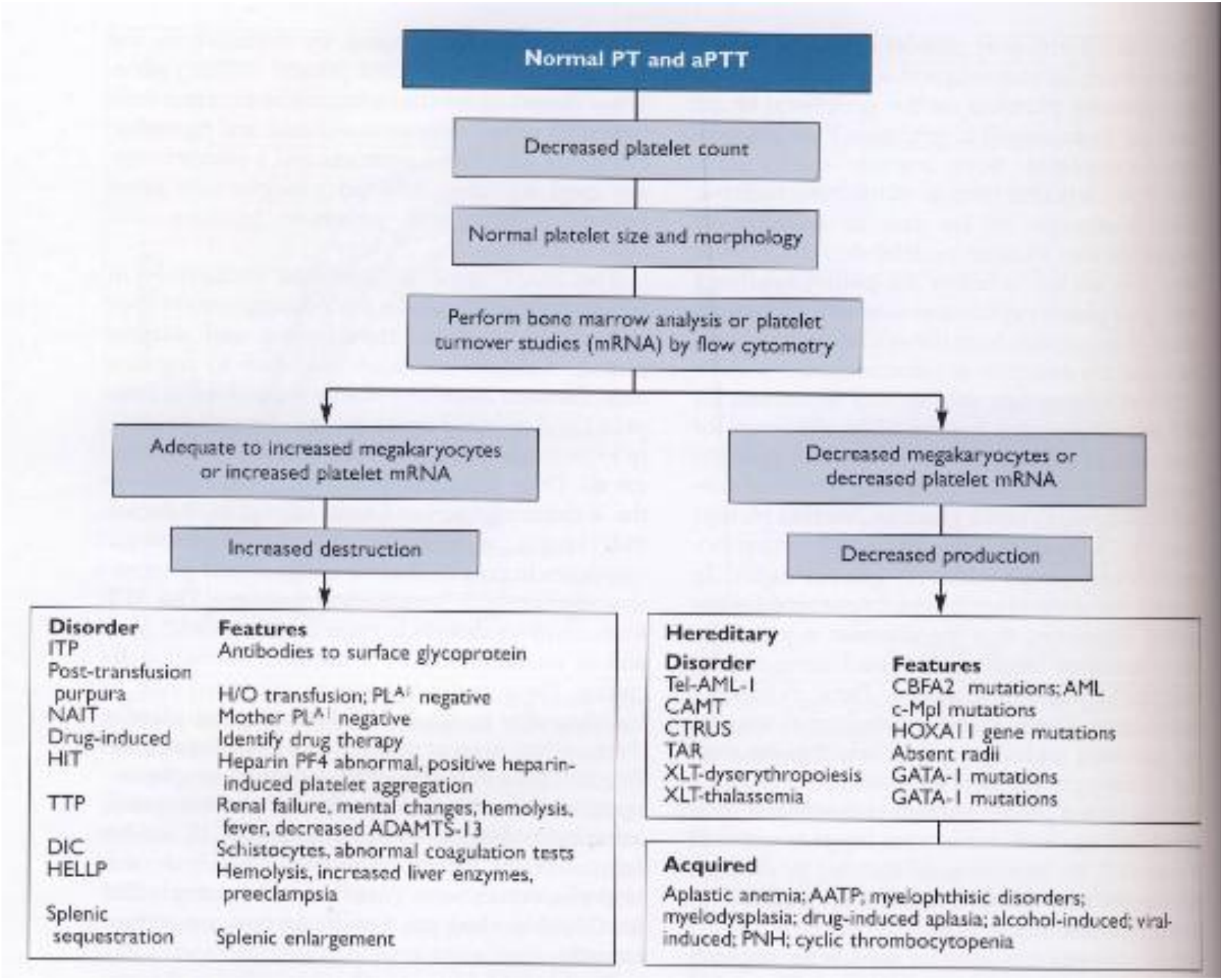
Alpha 2-Antiplasmin Deficiency

Homozygous: rare

Heterozygous: 22% patients reported to have mild bleeding
35 – 70% normal α2-antiplasmin level

Autosomal recessive


Normal PT and aPTT
Decreased platelet count
Platelet clumping
Macrothrombocytes
Mean platelet volume >12.5 fL
Small platelets
Mean platelet volume 3.8-5.0 fL
Pseudothrombocytopenia
Clumping in EDTA, not citrate or heparin (+/- satellitism)
H/O abciximab
Heparin-induced thrombocytopenia
Clumping in EDTA and heparin
No
Neutrophil inclusions?
Yes
Wiskott-Aldrich syndrome and X-linked thrombocytopenia
X-linked immune deficiency; WASP gene mutations; may have associated storage pool disorder
Disorder
Abnormal
Abnormal Aggregation
Bernard-Soulier syndrome
GPIb/IX/V
Ristocetin
Velocardiofacial syndrome
GPIbβ
NL
Mitral valve insufficiency
GPIa, Ic, IIa
ADP, AA, thrombin
Gray platelet syndrome (α-SPD)
P-selectin
Ristocetin, thrombin, collagen
Quebec platelet syndrome
Multimerin/fV
Epinephrine
Montreal platelet syndrome
Calpain
Thrombin
Mediterranean macrothrombocytopenia
Unknown
Unknown
Abnormal neutrophil localization of nonmuscle myosin heavy chain-A (NMMHCA); MYH9 mutations
MYH9 disorders:
Fechtner syndrome
Hereditary nephritis, deafness, cataracts (Alport syndrome)
Epstein syndrome
Nephritis, deafness
May-Hegglin anomaly and Sebastian syndrome
Macrothrombocytopenia only


Normal PT and aPTT
Decreased platelet count
Normal platelet size and morphology
Perform bone marrow analysis or platelet turnover studies (mRNA) by flow cytometry
Adequate to increased megakaryocytes or increased platelet mRNA
Decreased megakaryocytes or decreased platelet mRNA
Increased destruction
Decreased production
Disorder
Features
ITP
Antibodies to surface glycoprotein
Post-transfusion purpura
H/O transfusion; PLA1 negative
NAIT
Mother PLA1 negative
Drug-induced
Identify drug therapy
HIT
Heparin PF4 abnormal, positive heparin-induced platelet aggregation
TTP
Renal failure, mental changes, hemolysis, fever, decreased ADAMTS-13
DIC
Schistocytes, abnormal coagulation tests
HELLP
Hemolysis, increased liver enzymes, preeclampsia
Splenic sequestration
Splenic enlargement
Hereditary
Disorder
Features
Tel-AML-1
CBFA2 mutations; AML
CAMT
c-Mpl mutations
CTRUS
HOXA11 gene mutations
TAR
Absent radii
XLT-dyserythropoiesis
GATA-1 mutations
XLT-thalassemia
GATA-1 mutations
Acquired
Aplastic anemia; AATP; myelophthisic disorders; myelodysplasia; drug-induced aplasia; alcohol-induced; viral-induced; PNH; cyclic thrombocytopenia

Enough???

Venous thrombosis in oral contraceptive users and the presence of the JAK2V617F mutation

Elvira Grandone[1], Donatella Colaizzo[1], Giovanni L. Tiscia[1], Patrizia Vergura[1], Elena Chinni[2], Luigi Iannaccone[3], Iole Antinolfi[3], Maria A. Guardascione[4], Maurizio Margaglione[1,2]

[1]Atherosclerosis and Thrombosis Unit, I.R.C. C.S. Casa Sollievo della Sofferenza, S. Giovanni R (FG) and [2]Medical Genetics, University of Foggia, Foggia, Italy; [3]Thrombosis and Haemostasis Centre, Cardarelli Hospital, Naples, Italy; [4]Gastroenterology Department, Cardarelli Hospital, Naples, Italy

Thromb Haemost 2008; 99: 640–642

	JAK2 G1849T mutation	Age at the event	OC type	Duration of OC
Pt1*	Absent	32	EE+ gestodene Third generation	2 years
Pt2	Absent	45	EE+ gestodene Third generation	Not known
Pt3	Absent	43 (died)	EE+ gestodene Third generation	3 months
Pt4^	Absent	27 (died)	EE+ gestodene Third generation	Not known
Pt5	Absent	23	EE+cyproterone	1 month
Pt6	Present	34	EE+ gestodene Third generation	2 years
Pt7	Absent (2000) Present (2006)	38	EE+ gestodene Third generation	4 years
Pt8	Absent	26	EE+ drospirenone Fourth generation	2 years
Pt9	Absent	44	EE+desogestrel Third generation	3 years

*FV Leiden heterozygous. ^Protein C deficiency and mild hyperhomocisteinemia. EE: ethinyl estradiol.

Table 1: Features of women with portal mesenteric vein thrombosis.

The JAK2 V617F mutation frequently occurs in patients with portal and mesenteric venous thrombosis

D. COLAIZZO,* L, et al.

J Thromb Haemost 2007; 5: 55–61.

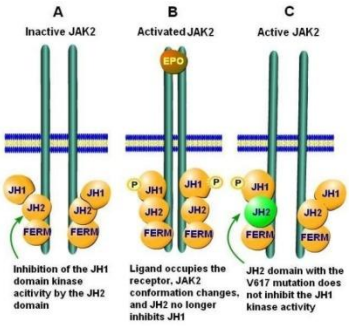
Over a 10-year period of observation, of the 99 patients presenting with PMVT, the JAK2V617F mutation was detected in heterozygous state in 17 individuals [17.2%; 95%confidence interval (95% CI) 10.9 –25.9]. None of the patients presenting with the JAK2 V617F mutation carried an inherited thrombophilic risk factor. Seven patients with (43.8%; 95% CI 19.8–70.1) and two without (2.4%; 95% CI 0.3–8.4) the JAK2 V617F mutation had a diagnosis ofMPD at the occurrence of the venous thrombotic event. After a median follow-up of 41 months (range 3–114 months), three out of the 10 patients carrying the JAK2 V617F mutation were then diagnosed as having idiopathic myelofibrosis (n ¼ 2) or polycythemia vera (n ¼ 1), whereas in seven patients a MPD was not detected. Two of the 83 patients without the JAK2 V617F mutation went on to develop MPDs.

JAK2 Mutations Across a Spectrum of Venous Thrombosis Cases

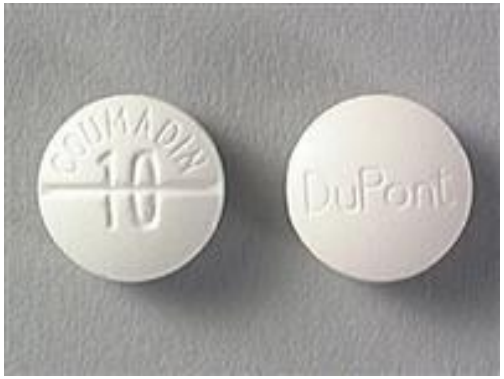
American Journal of Clinical Pathology, 2010, 134, 82-85.

Shrimati Shetty, et al.

The prevalence values for the *JAK2* mutation were 3% (1/36), 8.8% (12/137), 5% (4/78), and 3% (2/70) in DVT, BCS, PVT, and CVT, respectively.



Pharmacogenetics of Warfarin



U.S. Food and Drug Administration

U.S. Department of Health and Human Services

On Aug. 16, 2007, the FDA updated the label of warfarin to include information on pharmacogenetic testing and to encourage, but not require, the use of this information in dosing individual patients initiating warfarin therapy. The FDA completed the label update in August 2007.

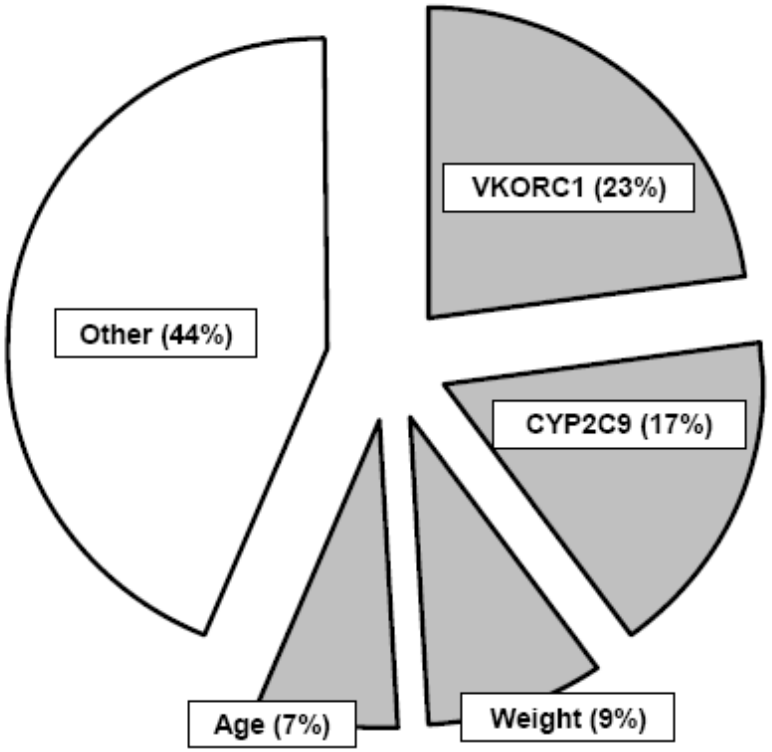
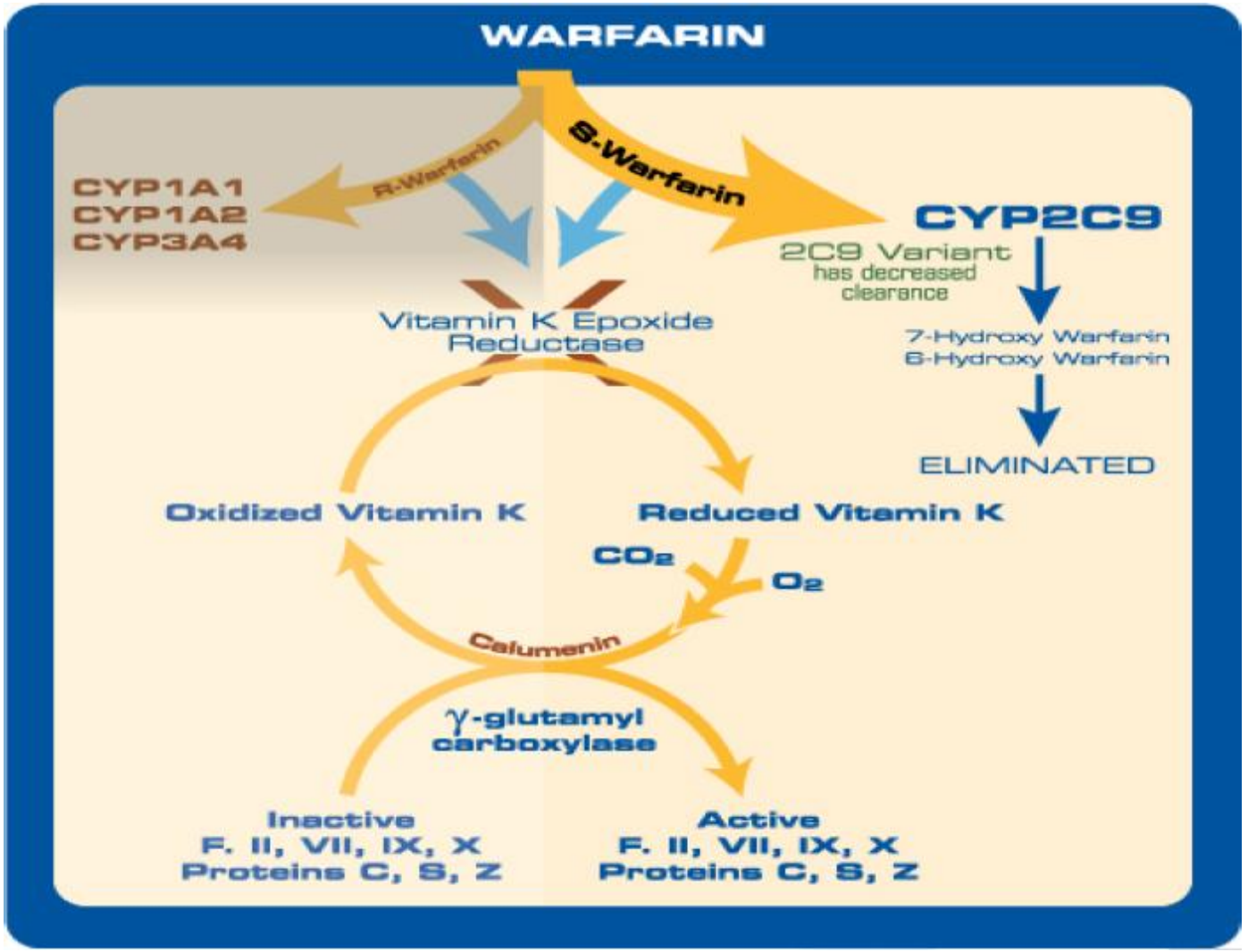


Figure 12. Pie chart showing the known sources of variability in warfarin dose needed for a stable INR. Each estimate is based on a summary analysis of partial r^2 values from multivariate regression analysis reported in six studies that included genotyping on both *CYP2C9* and *VKORC1*

Warfarin's target and metabolism



CYP2C9

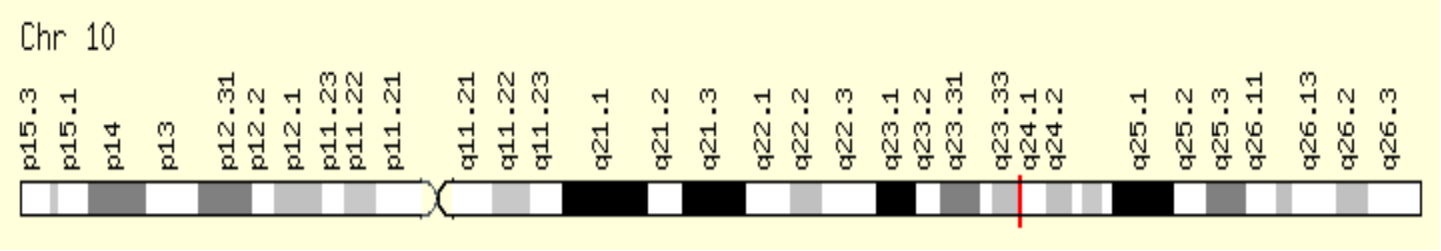
-- Located on 10q23.33 - q24

-- Encodes Cytochrome P450, family 2, subfamily C, polypeptide 9, which is involved in metabolism of many drugs, e.g., Warfarin, Phenytoin, Tolbutamide, Ibuprofen, etc.

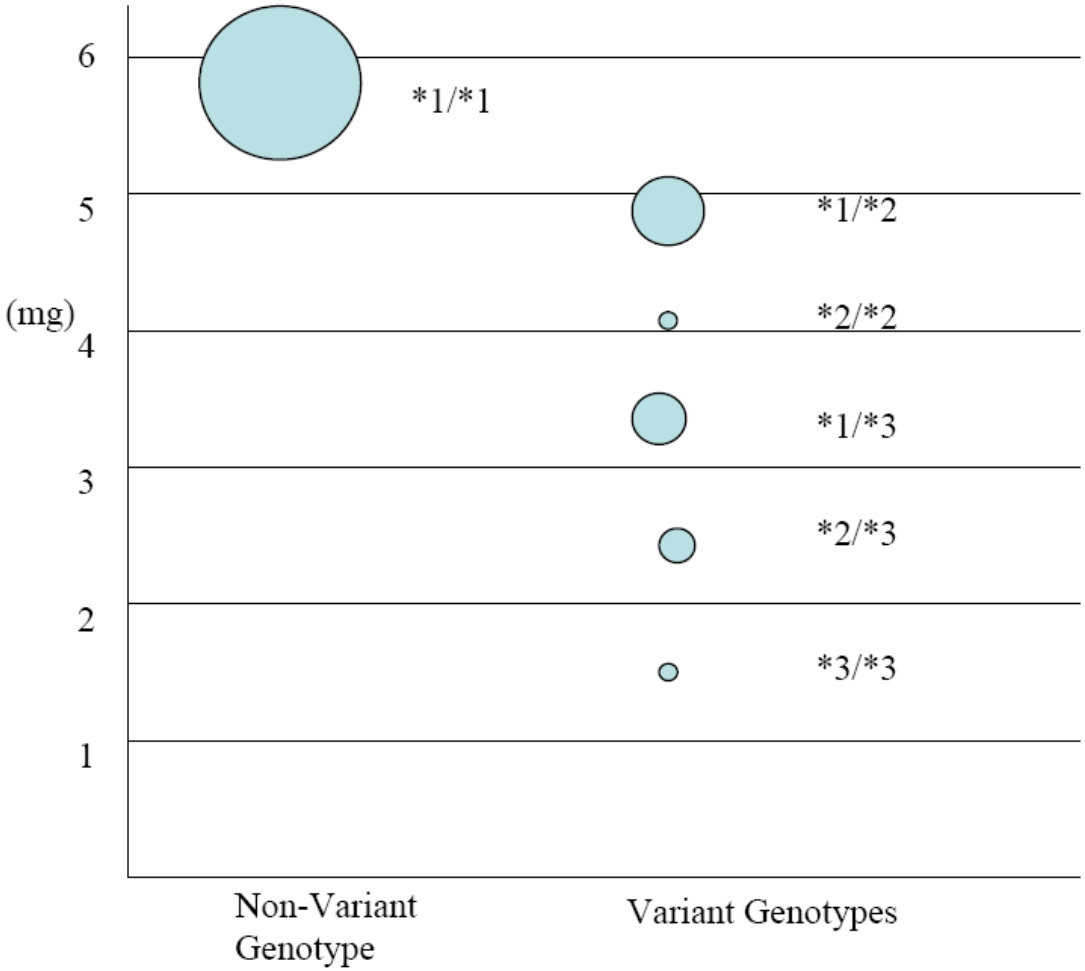
-- Contains many polymorphic variants, esp., SNPs. There are at least *CYP2C9*1 – CYP2C9*19.*

-- The variants are NOT associated with disease, but can show variable enzyme activities.

-- Patients with the allels corresponding to the proteins of reduced enzymatic activity *in vitro* have been designated as “poor metabolizers”
– REDUCED CLEARANCE, MORE ACTIVE DRUG



Genotype Prevalence and Mean Daily Maintenance Dosing for Warfarin



Genotype Prevalence and Mean Daily Maintenance Dosing for Warfarin
Circle area indicates relative population size.

CYP2C9 effect on warfarin dosing:

CYP2C9*2	**-17% per allele (-14 to -20%)**
CYP2C9*3	**-38% per allele (-21 to -49%)**

Therefore,

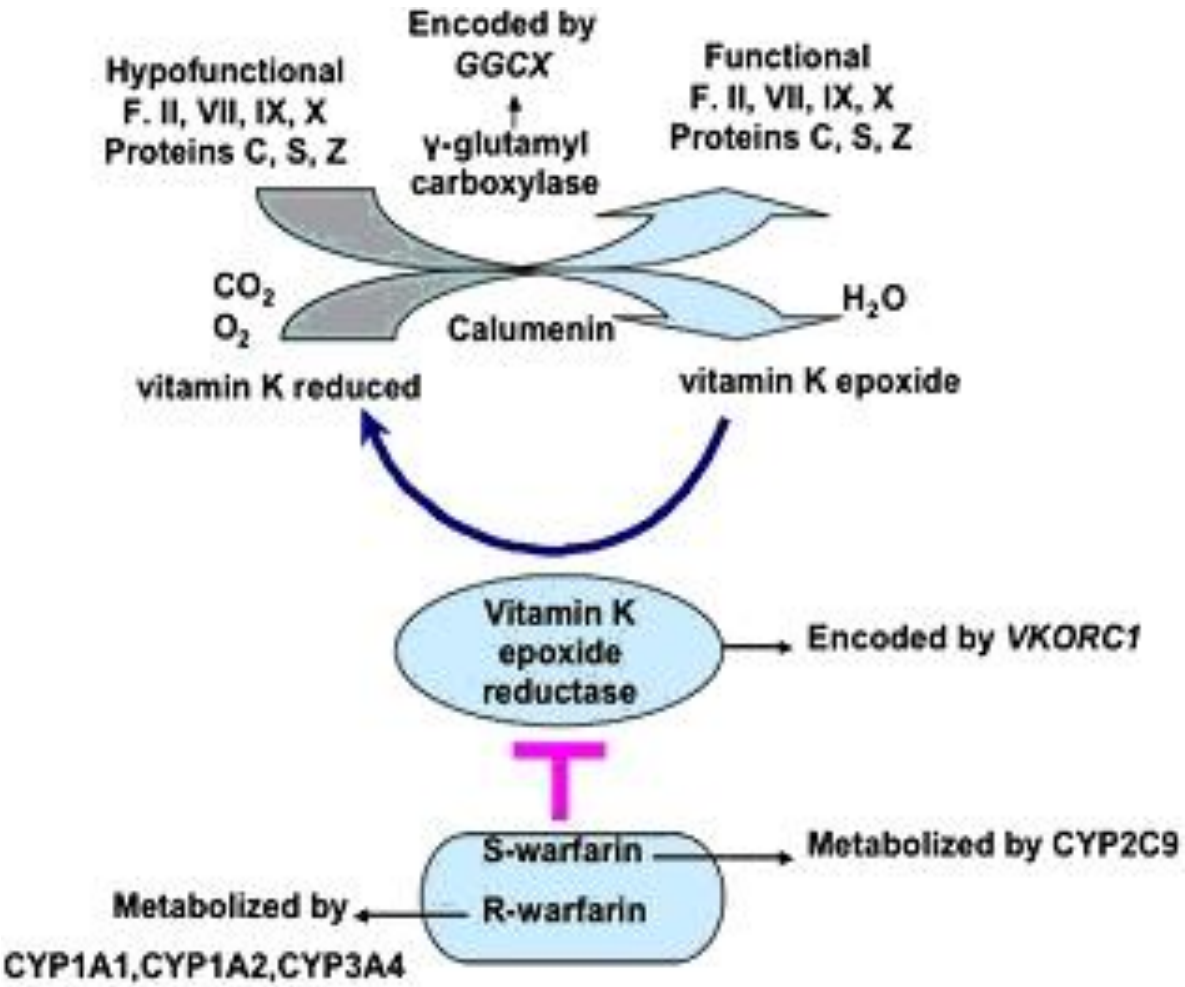
***1/*1**	**WT, original maintenance dose**
***1/*2**	**reduce maintenance dose by 20%**
***1/*3**	**reduce maintenance dose by 40%**
***2/*2**	**reduce maintenance dose by 35- 40%**
***2/*3**	**reduce maintenance dose by 55- 60%**
***3/*3**	**reduce maintenance dose by 75- 80%**

CYP2C9 allele frequencies

	*1	*2	*3
European	80.8%	12.7%	7.0%
Asian	98.2%	0%	1.8%
African-American	94.2	3.4%	1.5%

Caucasian populations

2/3	Genotype	*1/*1
1/3	Genotype	*1/*2 or *1/*3
<2.5%	Genotype	*2/*2, *2/*3 or *3/*3


Hypofunctional
F. II, VII, IX, X
Proteins C, S, Z
Encoded by
GGCX
γ-glutamyl
carboxylase
Functional
F. II, VII, IX, X
Proteins C, S, Z
CO2
O2
Calumenin
H2O
vitamin K reduced
vitamin K epoxide
Vitamin K
epoxide
reductase
Encoded by VKORC1
S-warfarin
Metabolized by CYP2C9
R-warfarin
Metabolized by
CYP1A1,CYP1A2,CYP3A4

VKROC1 polymorphism

1. Common variants

***-1639G>A*: common promoter variant**

***173+1000C>T (1173C>T)*: an intron 1 *VKROC1* variant**

-1639G>A* variant is in strong linkage disequilibrium with the intron 1 *VKROC1* variant *(173+1000C>T)

2. GG no variant
AG heterozygous for variant
AA homozygous for variant

3. *VKROC1* effect on warfarin dosing
AG -20 - 28% (95% CI: 25 -30%)
AA -40 - 50%

Table 2. Multiple regression analysis for modeling warfarin daily dose requirements based on age, gender, weight, VKORC1 (-1639G>A), and CYP 2C9 genotypes[a]

Predictor(s)	Regression equation	p-value	R^2
Age	ln (D) = 2.870 - 0.020 (Age)	0.0003	0.18
Gender	ln (D) = 1.276 + 0.415 (Gender)	0.0024	0.13
Weight	ln (D) = 0.298 + 0.006 (Weight)	<0.0001	0.28
VKORC1	ln (D) = 1.349 - 0.426 (VKORC1-AA) + 0.426 (VKORC1-GG)	0.0001	0.27
CYP2C9	ln (D) = 1.659 – 0.248 (2C9*2) - 0.625 (2C9*3)	0.0003	0.22
Full model (all variables)	ln (D) = 1.35 – 0.008 (Age) + 0.116 (Gender) + 0.004 (Weight) – 0.376 (VKORC1-AA) + 0.271 (VKORC1-GG) - 0.307 (2C9*2) - 0.318 (2C9*3)	<0.0001	0.61

[a] Age: input age in years; Gender: input 0 for female and 1 for male; Weight: input weight in pounds (lbs); ; VKORC1 (-1639AA): input 0 for GG, 0 for GA, and 1 for AA; VKORC1 (-1639GG): input 2 for GG, 0 for GA, and 0 for AA; CYP2C9: input 0, 1, or 2 for the number of CYP*2 and *3 alleles.

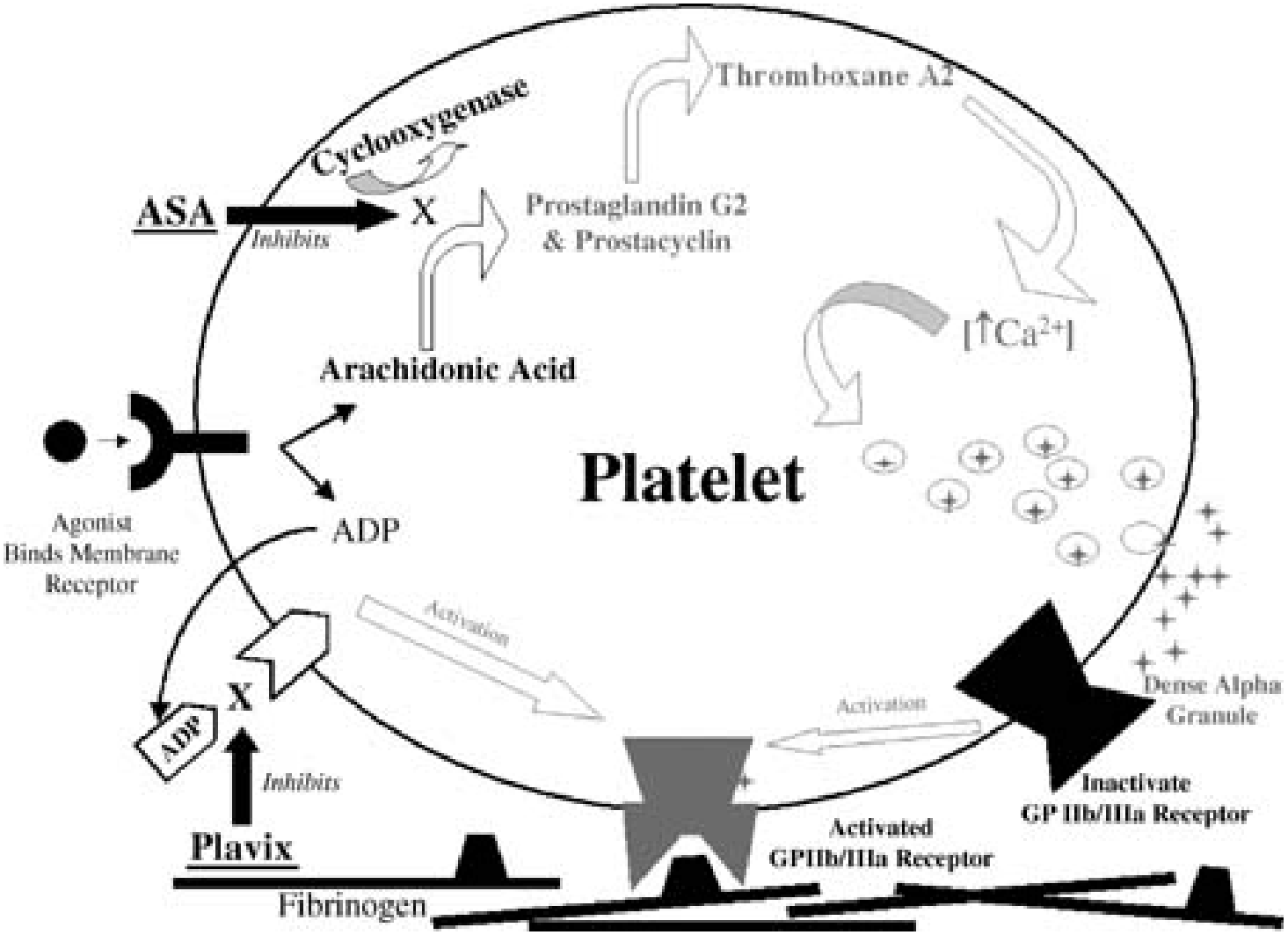
Clarification of Optimal Anticoagulation Through Genetics (COAG)

This study is currently recruiting participants.

Verified on October 2010 by National Heart, Lung, and Blood Institute (NHLBI)

Sponsor:	National Heart, Lung, and Blood Institute (NHLBI)
Collaborator:	Bristol-Myers Squibb
Information provided by:	National Heart, Lung, and Blood Institute (NHLBI)
ClinicalTrials.gov Identifier:	NCT00839657

Pharmacogenetics of Clopidogrel



Indications for Plavix

Plavix used for:

(1). Recent MI, recent stroke or established peripheral arterial disease

Plavix has been shown to induce the rate of a combined endpoint of new ischemic stroke (fetal or not), new MI (fatal or not), and other vascular death.

(2). Acute coronary syndrome (ACS)

Patients with non-ST-segment elevation ACS (unstable angina/non-Q-wave MI) including patients who are to be managed medically and those with percutaneous coronary intervention (with or without stent) or CABG, Plavix has been shown to decease the rate of a combined endpoint of cardiovascular death, MI, or stroke as well as the rate of a combined cardiovascular death, MI, stroke, or refractory ischemia.

Indications for Plavix

Plavix used for:

(2). Acute coronary syndrome (ACS)

Patients with ST-segment elevation acute MI, Plavix has been shown to reduce the rate of death from any cause and the rate of a combined death, re-infarction or stroke. This benefit is not known to pertain to patients who receive primary angioplasty.

Rationale:

It was estimated that up to 30% of patients DO NOT achieve an adequate antiplatelet effect from Plavix – probably because of significant interindividual variability in the Plavix response.

Drug-drug interactions or genetic polymorphisms in the drug metabolizing enzyme involved in metabolizing Plavix.

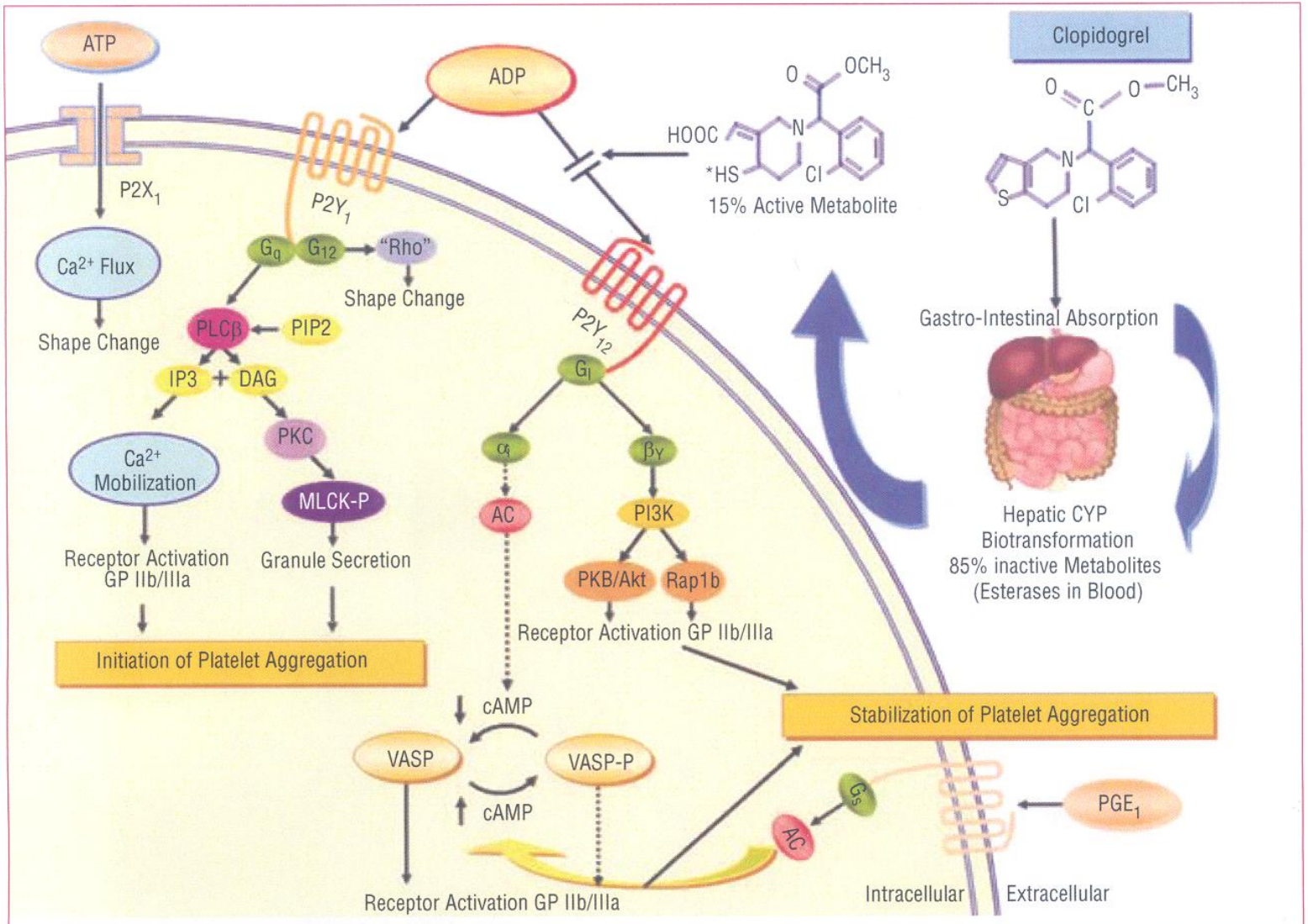


Figure 1. Purinergic receptors and mechanism of action of clopidogrel. Clopidogrel is a pro-drug of which approximately 85% is hydrolyzed by esterases

Genetic and environmental factors that can each potentially influence the generation of the active metabolite of Clopidogrel

Absorption: estimated to be at least 50% of administered dose

Metabolism:

Hydrolysis (converts ~ 85% of absorbed parent drug to clopidogrel carboxylate, an inactive metabolite)

First oxidative step (conversion of clopidogrel to 2-oxo-clopidogrel)

- ***CYP1A2* – responsible for ~36% of conversion**
- ***CYP2B6* – responsible for ~19% of conversion**
- ***CYP2C19* – responsible for ~45% of conversion**

Second oxidative step (conversion of 2-oxo-clopidogrel to the active metabolite)

- ***CYP2B6* – responsible for ~33% of conversion**
- ***CYP2C9* – responsible for ~7% of conversion**
- ***CYP2C19* – responsible for ~21% of conversion**
- ***CYP3A4* – responsible for ~40% of conversion**

Overall, ~2% of ingested Plavix ends up bound to platelet.

Circulation 2010; 121:481-3.

CYP2C19

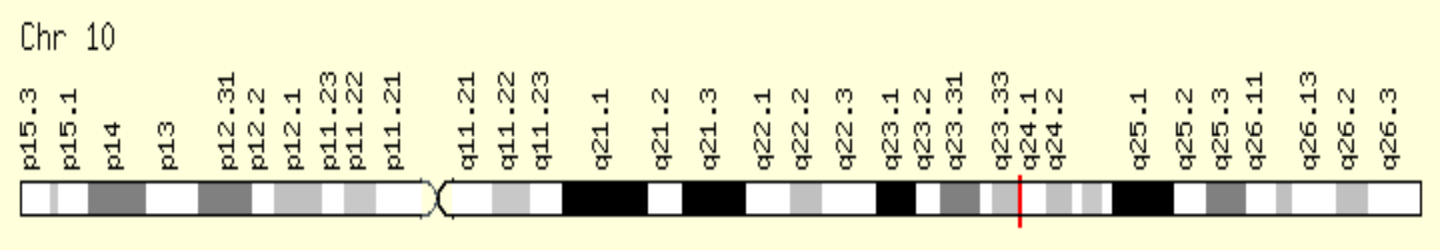
-- Located on 10q24

-- Encodes Cytochrome P450, family 2, subfamily C, polypeptide 19, which is involved in metabolism of many drugs, e.g., Clopidogrel, Mephenytoin, Opremazole, etc.

-- Contains many polymorphic variants, esp., SNPs. There are at least *CYP2C19*1 – CYP2C19*25.*

-- The variants are NOT associated with disease, but can show variable enzyme activities.

-- Subjects with the alleles corresponding to the proteins of reduced enzymatic activity *in vitro* have been designated as "poor metabolizers" – REDUCED ACTIVE FORM OF DRUG



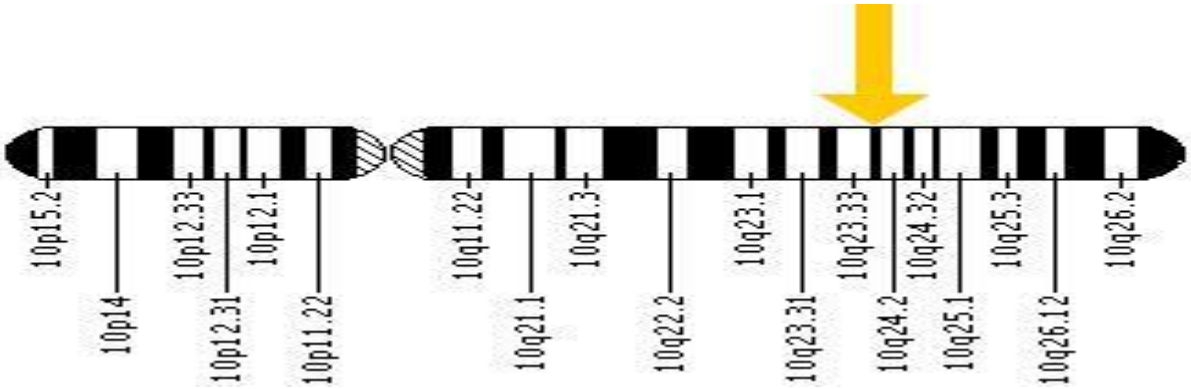
*CYP2C19*2*

-- The most common form of variants.

-- Accounts for 15 – 30% of the allelic frequency.

-- Causes a splicing defect and a complete loss in enzyme activity.

-- Presents phynotypically as a poor metabolizer.



On May 2009, the FDA updated the label of Plavix to include its pharmacogenetic information:

(1). Plavix's active metabolite pharmacokinetics and antiplatelet effects differ according to CYP2C19 genotype;
(2). Frequencies for the common CYP2C19 phenotypes and genotypes are included;
(3). Clinical trials to support the findings.

The importance of the CYP2C19 genetics in metabolizing plavix.

The FDA required the following Black Box warning for the medication on March 12, 2010.

Effectiveness of Plavix depends on activation to an active metabolite by the cytochrome P450 (CYP) system, principally CYP2C19 ;

Poor metabolizers treated with Plavix at recommended doses exhibit higher cardiovascular event rates following acute coronary syndrome (ACS) or percutaneous coronary intervention (PCI) than patients with normal CYP2C19 function;

Tests are available to identify a patient's CYP2C19 genotype and can be used as an aid in determining therapeutic strategy;

Consider alternative treatment or treatment strategies in patients identified as CYP2C19 poor metabolizers.

New Development

***1. CYP2C19*17* is associated with very rapid CYP2C19 activity.**

2. GRAVITAS trial: Gauging Responsiveness With A VerifyNow Assay-Impact On Thrombosis And Safety

This study has been completed. – Jun. 2008 – Oct. 2010

Sponsor: Accumetrics, Inc.
Information provided by: Accumetrics, Inc.
ClinicalTrials.gov Identifier: NCT00645918

Purpose The objective of the GRAVITAS trial is to determine whether tailored anti-platelet therapy using the Accumetrics VerifyNow P2Y12 assay reduces major adverse cardiovascular events after drug-eluting stent implantation.

2. GRAVITAS trial: Gauging Responsiveness With A VerifyNow Assay-Impact On Thrombosis And Safety

CONTEXT:

High platelet reactivity while receiving clopidogrel has been linked to cardiovascular events after percutaneous coronary intervention (PCI), but a treatment strategy for this issue is not well defined.

OBJECTIVE:

To evaluate the effect of high-dose compared with standard-dose clopidogrel in patients with high on-treatment platelet reactivity after PCI.

CONCLUSIONS:

Among patients with high on-treatment reactivity after PCI with drug-eluting stents, the use of high-dose clopidogrel compared with standard-dose clopidogrel did not reduce the incidence of death from cardiovascular causes, nonfatal myocardial infarction, or stent thrombosis.

Reference:

Castoldi E, et al. A highly polymorphic microsatellite in the factor V gene is an informative tool for the study of factor V-related disorders. Br. J Haematol 114 (4), 868–70, 2001

Price MJ, et al. Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial. JAMA 2011 Mar 16;305(11):1097-105.