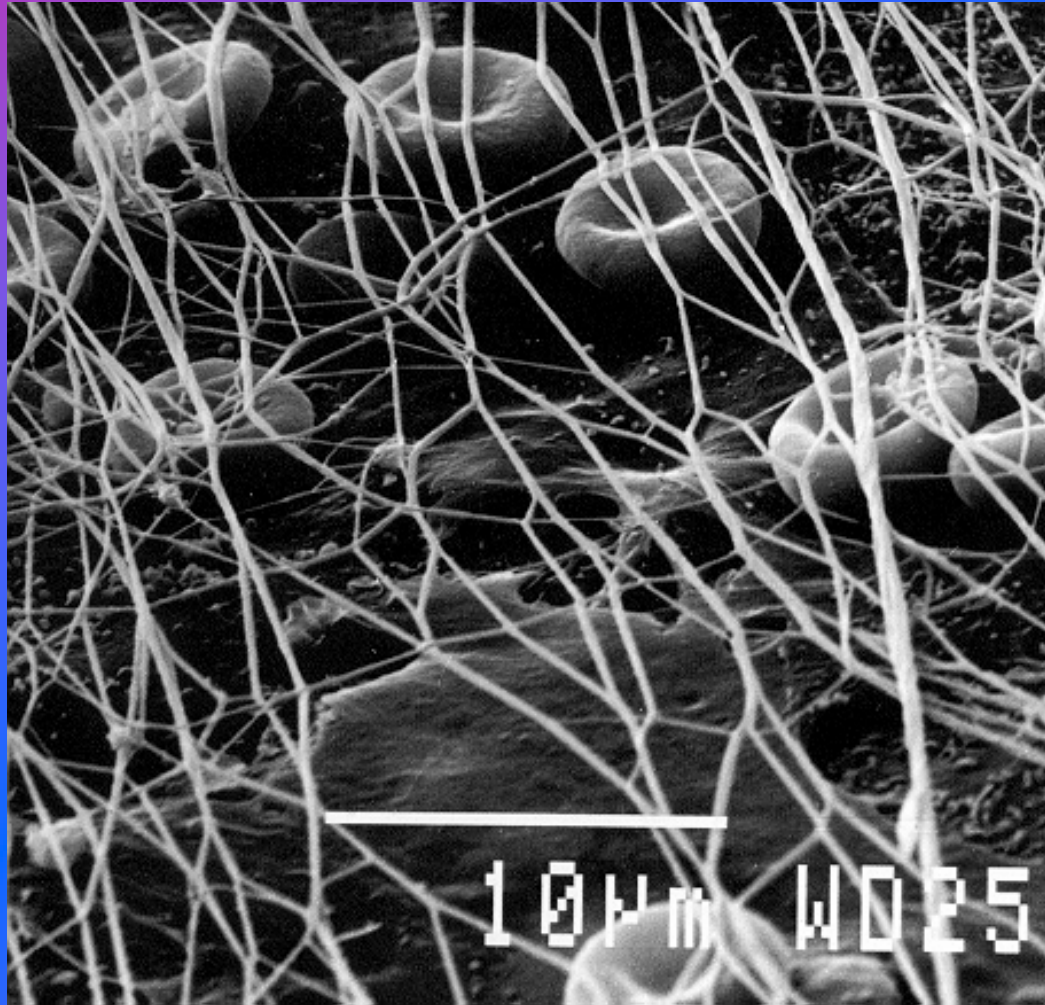


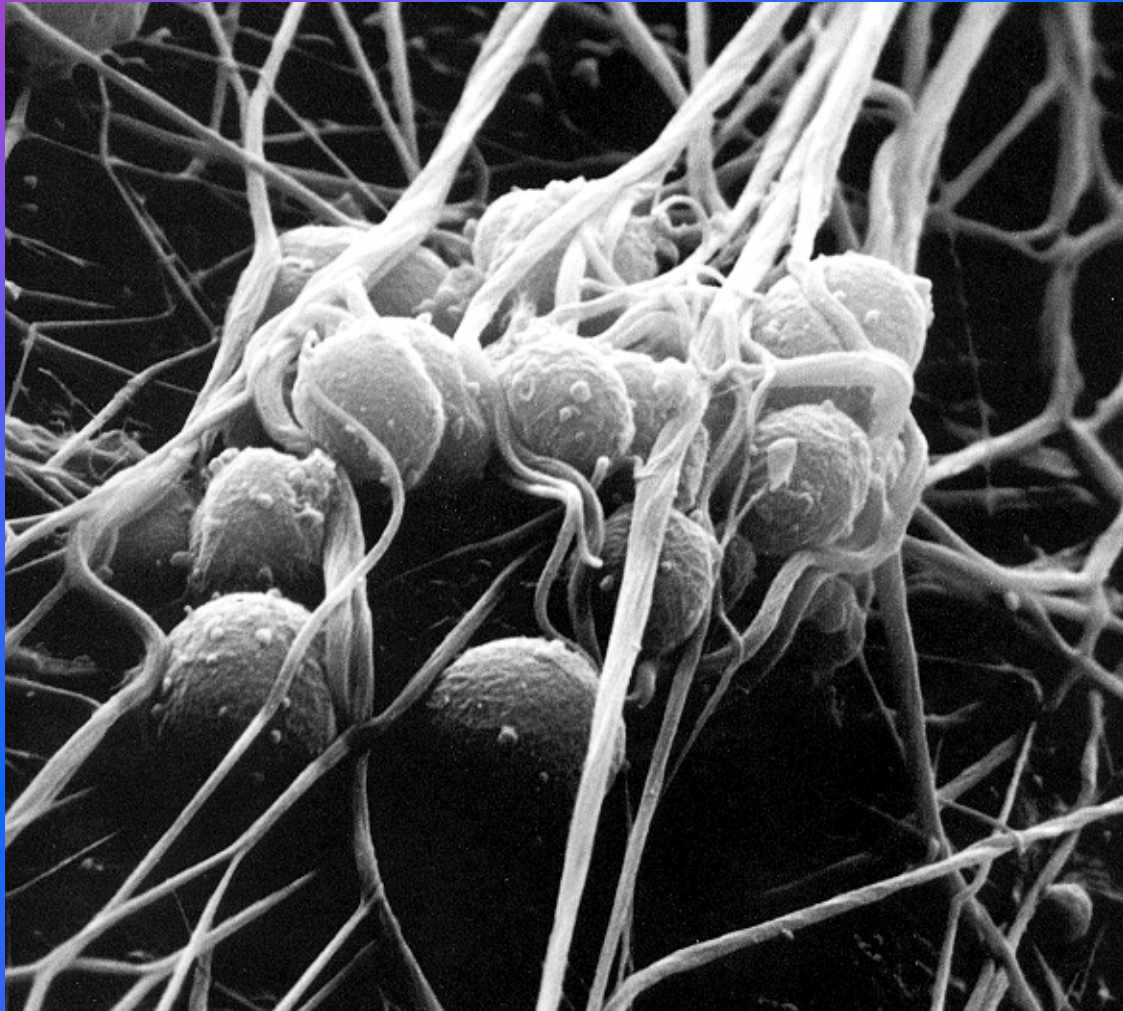
Contents

- **Introduction: biology and medicine, two separated compartments**
- **What we need to know:**
 - boring basics in DNA/RNA structure and overview of particular aspects of molecular biology techniques
 - How DNA is organized and differs in every individual
- **Molecular diagnostics of cardiovascular diseases**
 - Mutations in Factor V
 - Mutations in Factor II
 - Mutations in MTHFR gene
- **Breast cancer and BRCA1 and 2 genes**
 - Breast cancer in the industrialized countries
 - Breast cancer genes
 - sequence in selected areas
 - p53 and breast cancer
- **Pharmacogenomics: finding the right drug for a patient**
 - ADR: an emerging problem
 - structure of cytochromes
 - Example 1: TPMT-enzyme and the metabolism of azathioprine
 - Example 2: Clozapine in the treatment of psychiatric diseases
 - CYP3A4 and the metabolism of anti-coagulant drugs

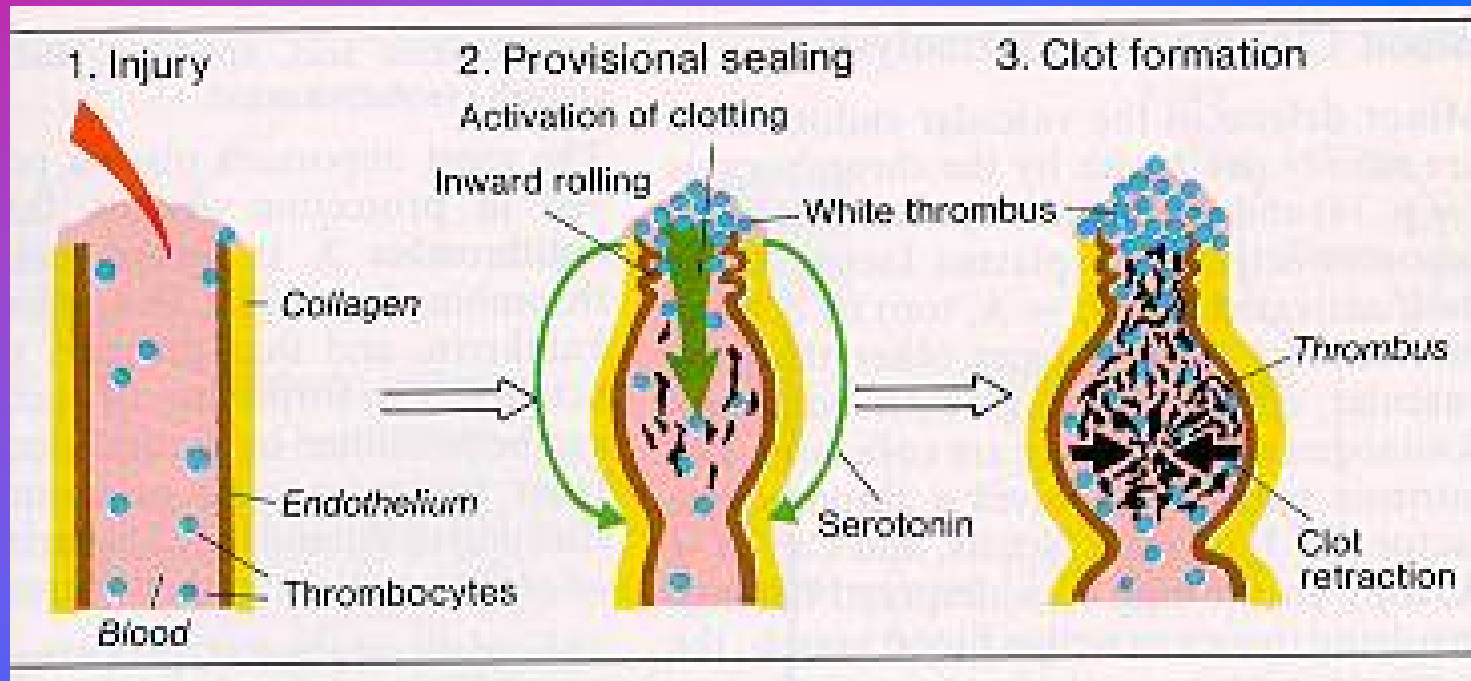
Blood clotting



Platelets emprisoned in a fibrin net.

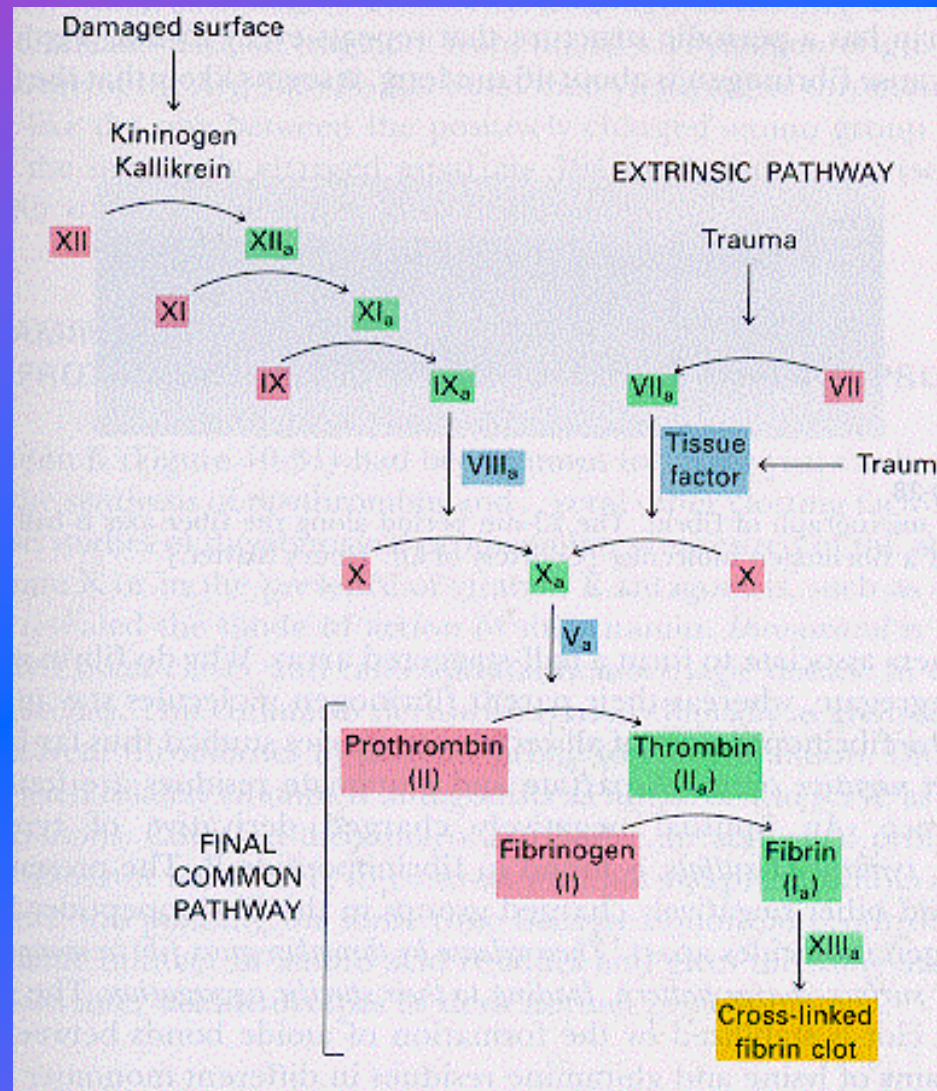


Hemostasis...how it works.



- When the endothelium is defective the blood comes into contact with collagen fibers
- Thrombocytes adhere to the site of injury (**adhesion**) and activate themselves to **secrete** substances (serotonin, PDGF, thromboxane A2 and PAF leading to **aggregation**).
- This thrombocyte plug (white thrombus) leads to a provisional stopping of the leak.

Blood clotting



Visualizing blood easily flowing through a vessel.

Doppler analysis of two parallel normal blood vessels

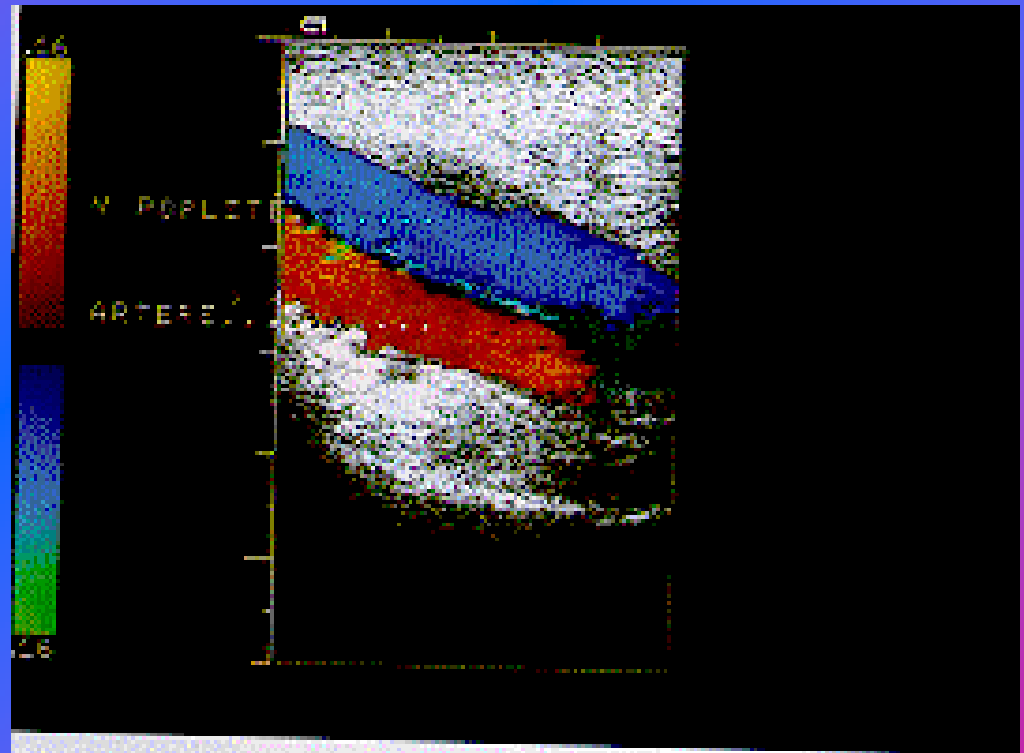


fig. 11 : artère et veine poplitées en coupe longitudinale (écho-Doppler couleur).

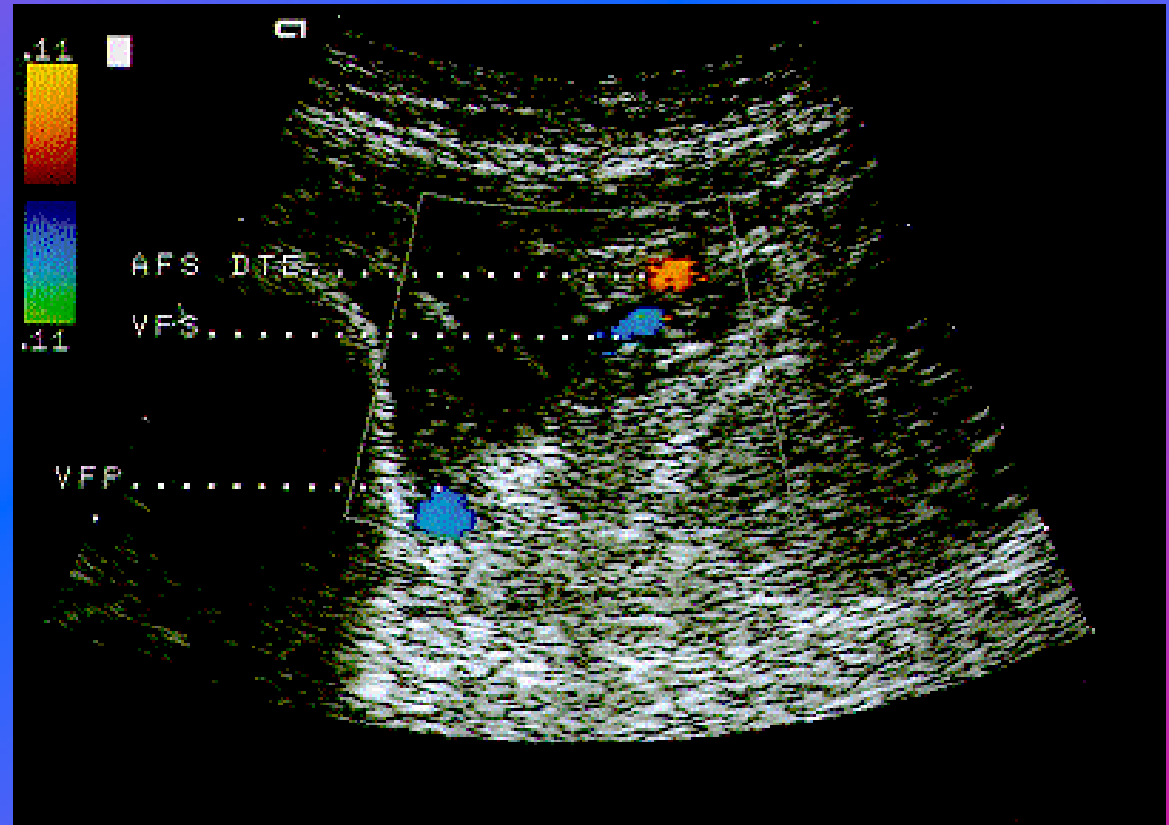
And a transversal view

Transversal cut at
1/3 of the right
leg.

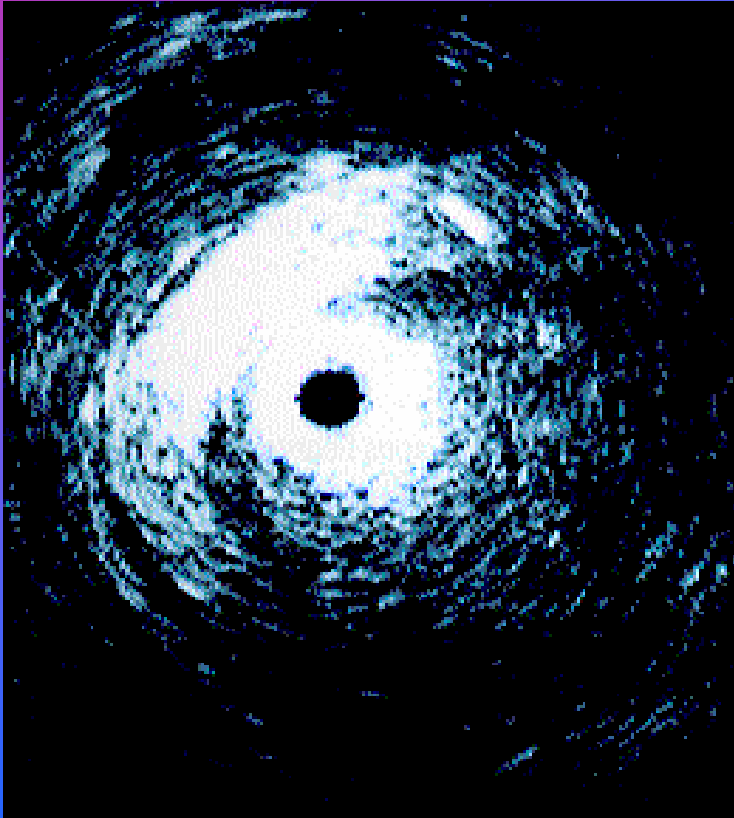
VFS: superficial
femoral vein

AFS: superficial
femoral artery

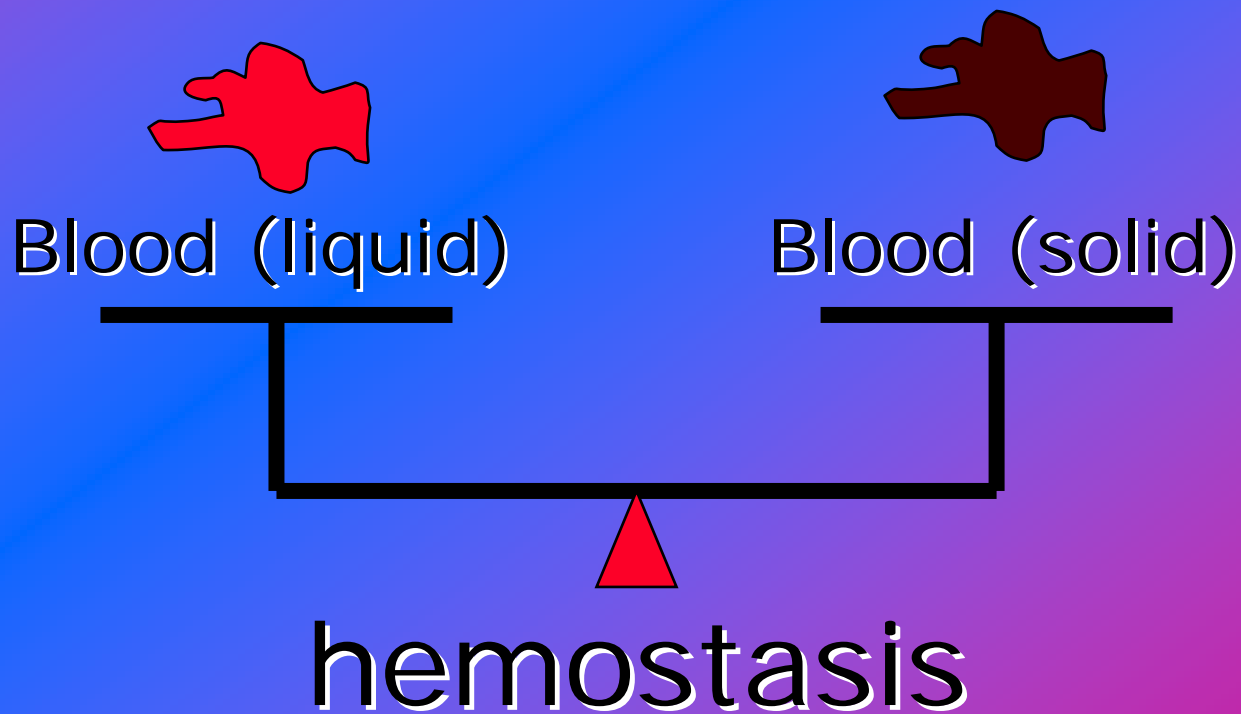
VFP: deep
femoral vein



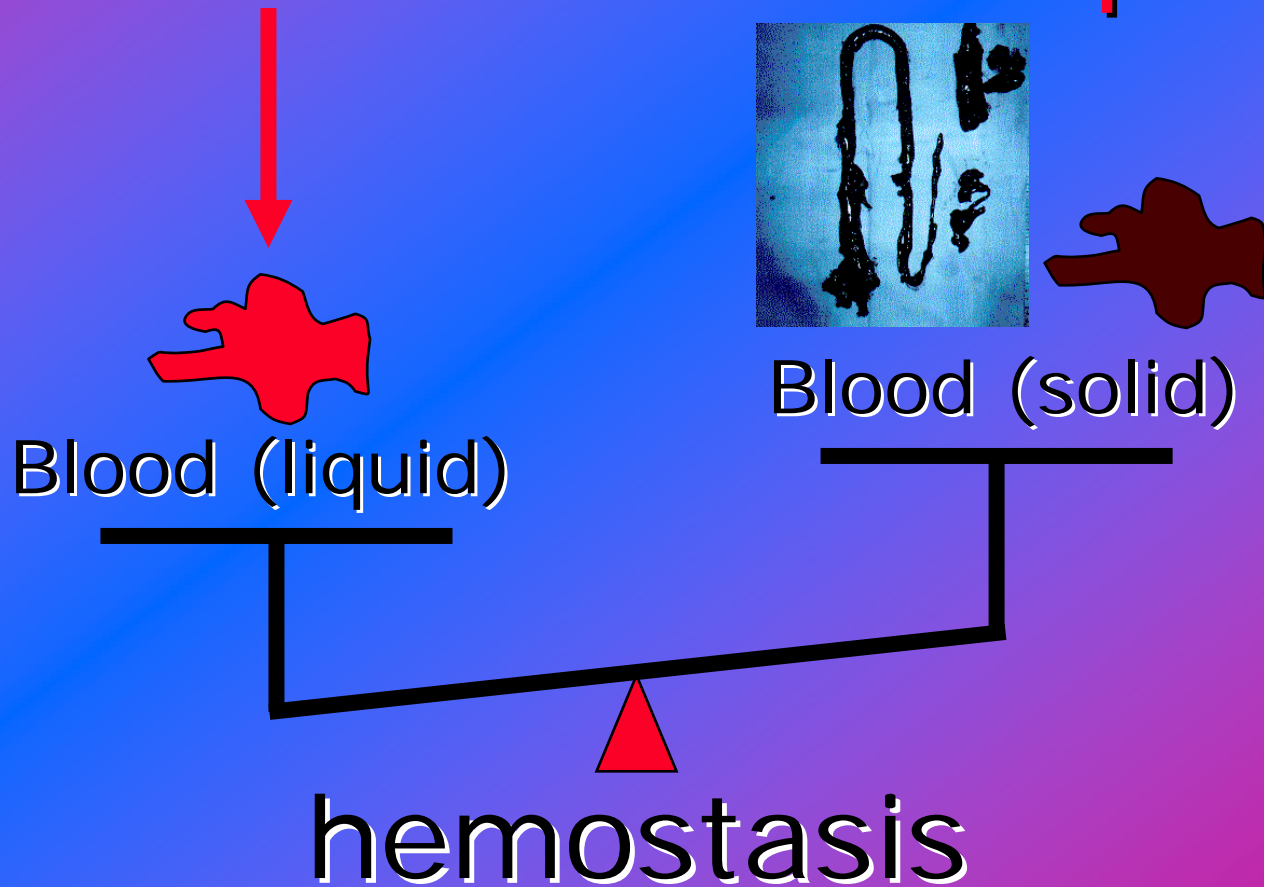
Inside view of a vein and its flow by phlebography



Blood clotting like
everything in our body, is
the result of an equilibrium



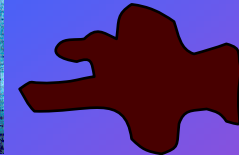
But if the equilibrium is in some way disturbed the result could be **hemophilia**



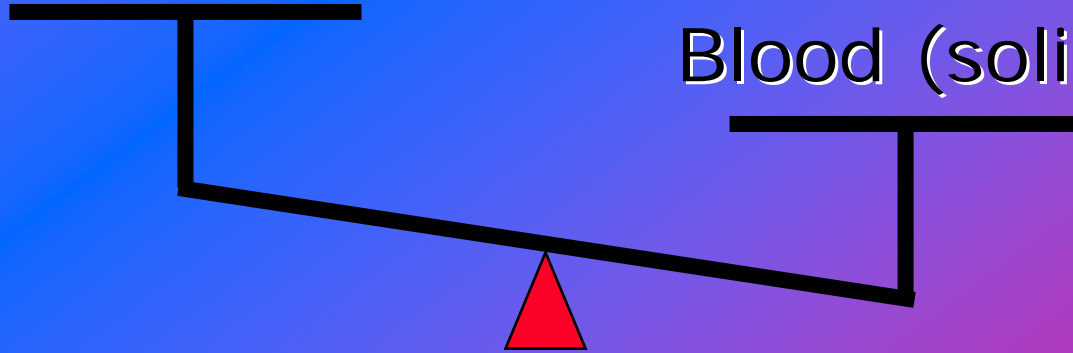
or thrombosis



Blood (liquid)

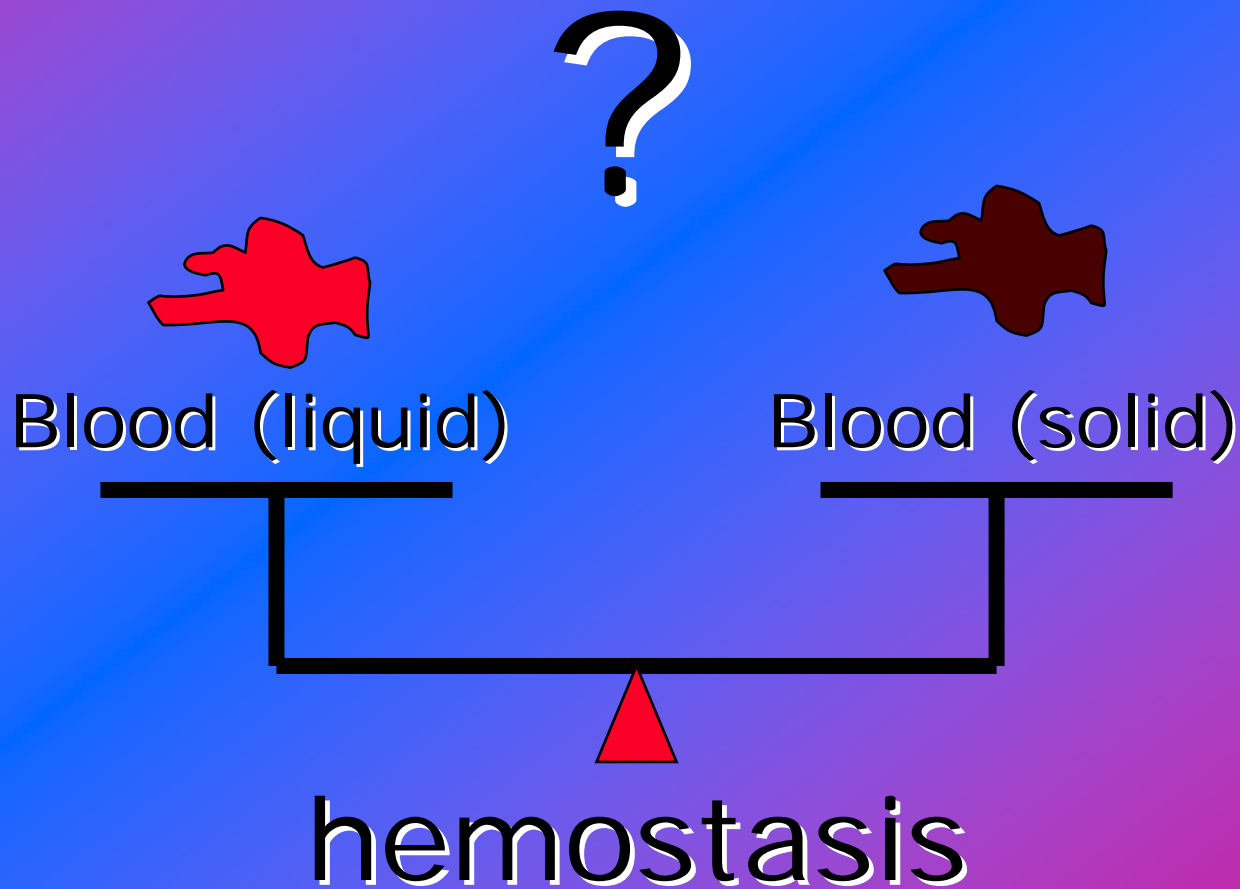


Blood (solid)

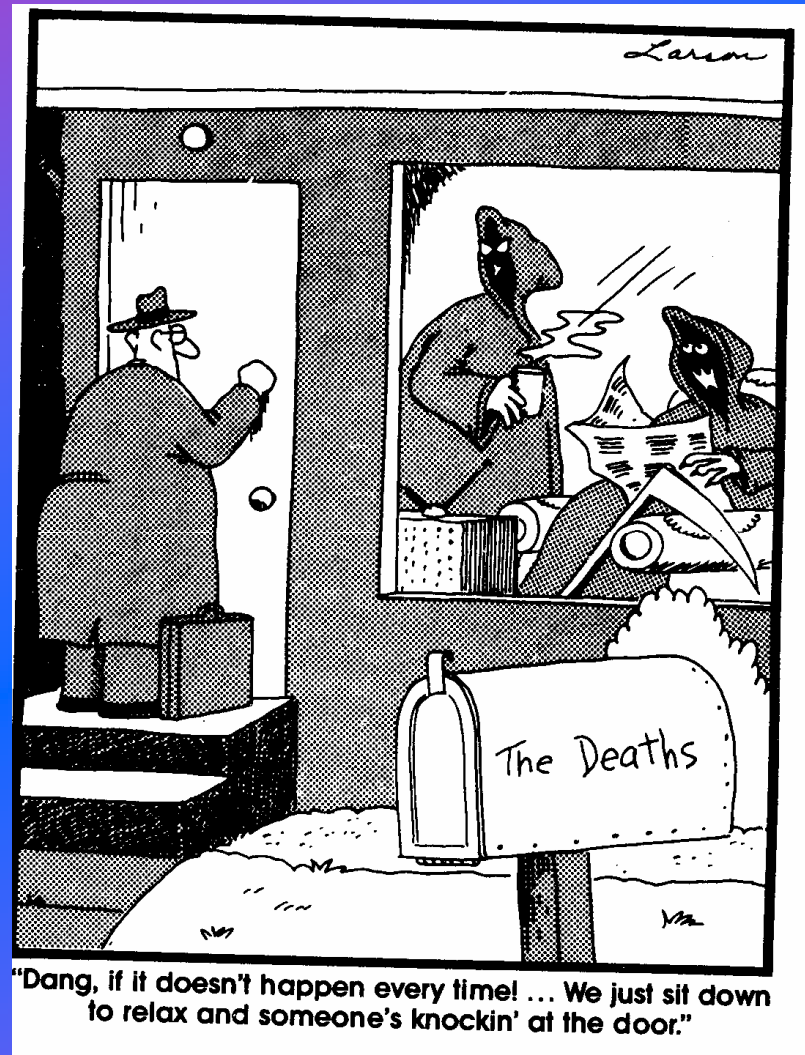


hemostasis

But how to derange this equilibrium?



In the industrialized countries the 50% of deaths are related to cardiovascular diseases



Cardiovascular risks are "classical" risks

- Smoke
- Alcohol
- Hypertension
- Cholesterol Tot/HDL
- Lpa
- Sex
- Left Ventricular hypertrophy

But also genetic risks

- Hereditary (homozygous or heterozygous; dominant or recessive);
- Confer an independent risk, different from classical
- The genetic risk is additive to the classical risks
- Generally is a loss of function or a strong reduction of function

Many genetic risks have been found in relation to cardiovascular pathologies.

Here three of them related to thrombophilia.

- The "Leiden" mutation on the **Factor V** gene of clotting
- Mutation on the **Prothrombin** gene
- Mutation on the gene coding for the enzyme Methylene tetrahydrofolate reductase (**homocysteine** metabolism)

Here is the result of a thrombotic process: the thrombus.

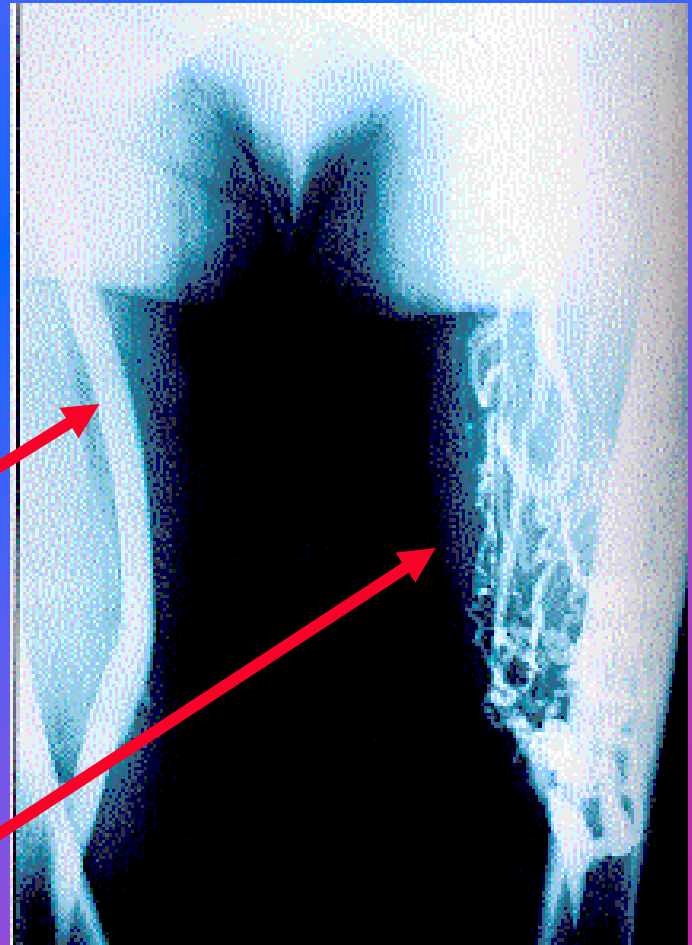


Thrombus occlude veins and stop blood flow

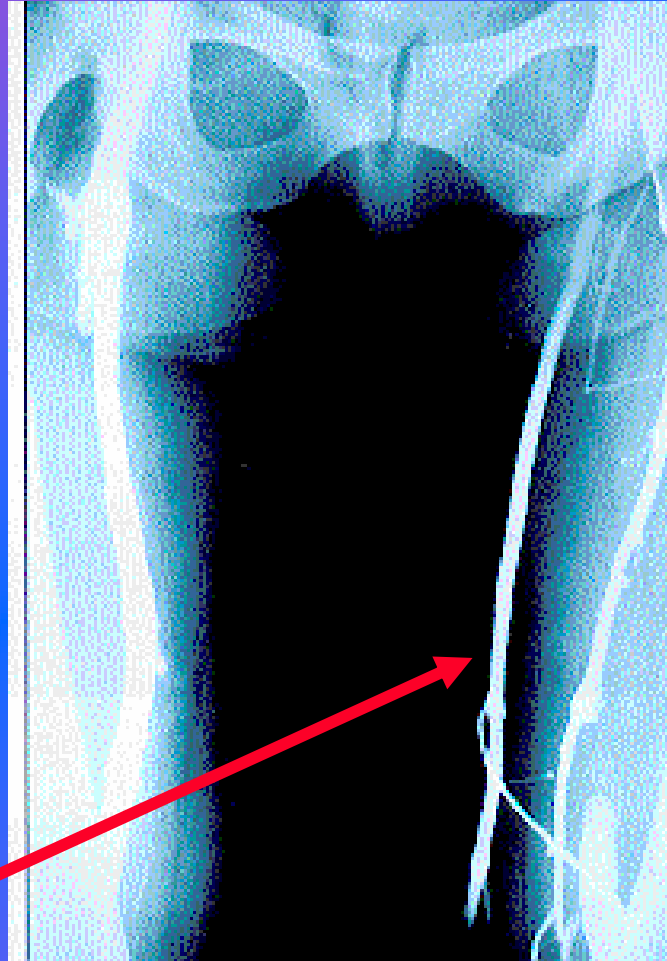
When a thrombus stops blood flow the system supply by generating a new network of blood vessels

Normal blood flow

Occlusion by a thrombus stops blood flow



Usually blood find new ways to
circumvent the thrombus



The Factor V protein...

- is a 330 Kda protein
- is activated by factor Xa to form an heterodimer of 105/220 Kda
- is activated by thrombin to form an heterodimer of 105/74 Kda
- APC (activated protein C) binds to activated factor V (Va) and cut the Va at position Arg506
- in APC-resistant patients Arg506 is replaced by Gln (Leiden mutation)

The sequence of Factor V.

P D I T V C A H D H I S W H L L G M S S G P E L F S I H F N G Q V L E Q
OCA GAT ADA ACA GTT TGT GGC CAT GAC CAC ATC AGC TGG CAT CTG CCA ATG AGC TGG GGG CCA GAA TTA TTC TOC ATT CAT TTC AAC GGC CAG GTC CTG GAG CAG

V S A A I T C L V S A T S T T A N N T V G G C P A E G G A K W I I S S L T P K A H L L O A A C T
GTC TCA GGC ATC ACC CTT GTC AGT GCT ACA TCC ACT ACC GCA AAT ATG ACT GTG GGC CCA EGG GGA ANG TGG ATC ADA TCT TCT CTC ACC CCA AAA CAT TTG CCA OCT

Y I D I K N C P K K T R R N L K A I T R E O R R R H M K R W E V F I A A E E
TAC ATT GAC ATT AAA AAC TGC CCA ANG AAA ACC AGG AAT CTT MAG AAA ADA ACT GGT GAG CAG AGG GGG CAC ATG ANG AGG TGG GAA TAC TTC ATT GCT GCA GAG GAA

Y A P V I A P A G A N M D K K A Y R G T C O G H L D N P F S N O I T G A K H Y T K K A V M Y T A C O G
TAT GCA OCT GTA ATA CCA GGG AAT ATG GAC AAA AAA TAC ANG TCT CAG CAA ATG TCA AAC CAA ATT GGA AAA CAT TAT ANG AAA GTT ATG TAC TCA OCT

T C E T K H T V N P N M K E D G I L G T P I I R A O V R A D T L K I V F K N M
TCC TAT ACC AAA CAT ACA GTG AAT OCC AAT ATG AAA GAA GAT GGG ATT TTG GGT OCT ATT ATC AGA GGC CAG GTC AGA GAC ACA CTC AAA ATC GTG TTC AAA AAT ATG

V S I V Y C H G V A C F S P T Y T E D A O C L T R P V V S D V T D I M R A D I A S G
TAT AGC ATT YAC OCT TGA GTG ACC TTC TGS PCT YAT GAA GAT GAA GTC NAC TCT TCT ACC TCA GGC AGG AAC AAC ACC ATG ATC AGA GCA GTT OCA P G G

Y K W N I L E F P C A E A M D A O C L T R P V V S D V T D I M R A D I A S G
TAT ANG TGG AAC ATC TTA EFG TTT GAT ACC ACA GAA AAT GAT GGC CAG TCC TTA ACA AGA CCA TAC TAC AGT GAC GTG GAC ATC AGA GAC ACA GGC TCT GGG

L L I C K S R S R S L D R S I O R A A D I E O O A V F A V F D E N K S W Y
CTT CTA ATC TGT ANG AGC AGA TOC CTG GAC AGC CCA GCA ADA CAG GCA CCA GCA GCG GCT GTG TTT GCT GTG TTT GAT GAG AAC AAA AGC TGG TAC

I N K F F C E A N P D E V K R D D D P F F V E S N I M S T I N G T V P E S I T
ATC AAC KAG TTT TGT GAA AAT OCT GAT GAG GTG AAA OCT GAT GAC OCC ANG TTT TAT TAT GAA TCA AAC ATC M S T I N G T V P E S I T

C F D D D T V Q H H F C S V G T M D N V G T W H L T I H F T G H S H I V G R H F
TGC TTT GAT GAC ACT GTC CAG TGG CAC TTC TGT AGT GTG GGG ACC CAG AAT GAA ATT TTG ACC ATC CAC TTC ACT GGG CAC TCA TTC ATC TAT GGA ANG AGG CAT GAG

L F P C M R G G A S V T V T M D N V G T W H L T I H F T G H S H I V G R H F
CTC TTC OCC ATG GGT GGA GAA TCT GTG ACG GTC ACA ATG GAT AAT GTT GGA ACT TGG ATG TTA ACT TOC M AAT TCT TCT S S P R S K K L R L F K

K C I P D D D E D S Y E I P E P E S T V M A T R K M H D R L E P E D E
AAA TGT ATC CCA GAT GAT GAT GAA GAC TCA TAT GAG AAT TTT GAA OCT CCA GAA TCT ACA GTC ATG GCT ACA CCG AAA ATG CAT GAT OCT TTA GAA OCT GAA GAT GAA

D Y D Y Q N R L A A A L G I R S F R N S S L N Q E E E F N L A L A L
TAC TAT GAT TAC CAG AAC AGA CTG GCT GCA GCA TTA GGA AAT AGG TCA TTC CCA TCA TTA TGG AAC CAG GAA GAA GAG TTC AAT CTT ACT GGC CTA GCT CTG

E F V S S N T D I I V G S N Y S S P S N I S K F T V N N L A E P Q K A P
GAA TTC GTT TCT TGS AAC ACA GAT ATA ATT GTT GGT TCA AAT TAT TCT TOC CCA AGT AAT ATT AGT ANG TTC ACT GTC AAT AAC CTT GCA GAA OCT CAG AAA GGC OCT

A T T A G G S P L R H L I G K N S V L N S S T A E H S S P Y S E D P I E G D
OCC ACC ACA OCT GGT TOC CCA CTG AGA CAC CTC ATT GGC AAG AAC TCA GTT CTT AAC TET TOC ACA GCA GAG CAT TOC ACC CCA TAT TCT TCT GAA GAC OCT ATA GAT

D V T G I R L L S L G A G E F R S Q E H A K R K G P K V E R D O A A K H
TAT GTC ACA GGG ATA OCT CTA CTT CTA CTT GGT GCT GGA GAA TTC AGA AGT CAA GAA CAT GCT MAG GGT MAG GGA CCC ANG GTA GAA AGA GAT CAA GCA GCA AAG CAC

M K A L L A H K V G R H L S Q D T G S P S G M R P W E D L P S O D T G S P
ATG AAA TTA CCA CAT AAA GTT GGG AGA CAC CTA AGC CAA GAC ACT GCT TCT OCT TOC GCA ATG AGG OCC TGG GAG GAC CTT OCT AGC CAA GAC ACT GCT TCT OCT

P W E D P P S D L L L L K A Q S N S S K I L V G R W H L A S E K G S Y E I
OCC TGG GAG GAC OCT OCT AGT GAT CTG TTA CTC TTA AAA CAA AAT AAC TCA TCT AAG ATT TTG GTT GGG AGA TGG CAT TTG GCT TCT GAG AAA GGT AGC TAT GAA ATA

D E D T A V N N W L I S P Q N A S R A W G E S T P L A N K P G K O S G C H
TAT GAA GAC ACA GAT TGG CTG ATC AGC OCC CAG AAT GAT GAC TCA GCT GCT TGG GCA GAA ACC OCT CTT GGC AAC ANG OCT GGA ANG CAG AGT GGC HAC

R V R H K S L O V R Q D G G A K S R L K K S O F L I K T R K K K K E K H T
GAT GTT AGA CAT AAA TCT CTA CAA GTA AGA CAG GAT GAT GGA GAG AGT AGA CTC AAG AAA ACC CAG TTT CTC ATT AAG ACA CCA AAA AAG AAA AAA GAG AAG CAC ACA

L S C P R A T F H P L R S E A Y N T F S E A R R L L K H S L V L L H K S N E T S C L T
TAT TCT OCT AGG ACC TTT CAC OCT CTA AGA AGT GAA GGC TAC AAC ACA TTT TCA GAA ERA RGA CTT AAG CAT TCG TTG GTG CTT CAT AAA TOC AAT GAA TCA TCT CTT

N O T L P S M D F G W I A S L P D H N O N S S N D T G Q A S C P P G L Y
TAT CAG ACA TTG OCC TCT AIG GAT TTT GGC TGG ADA GGC TCA CTT OCT GAC CAT AAT CAG AAT TOC TCA AAT GAC ACT GGT CAG GCA AGC TGT OCT CCA GGT CTT TAT

"Leiden"

Epidemiology:

- Frequency in the general population: 2-5%
- The thrombotic risk in healthy women (wt) taking the pill is 2-8 fold, in women w/m (heterozygous) is 35 higher, and in homozygous women is 150-500 fold
- The 60% of cases of thrombosis is found in pregnant women (APC resistance), of them 90% bear the "Leiden" mutation
- In presence of the "Leiden" mutation, a post-operative profilaxis reduces of 50% the risk of thrombosis

“Leiden”

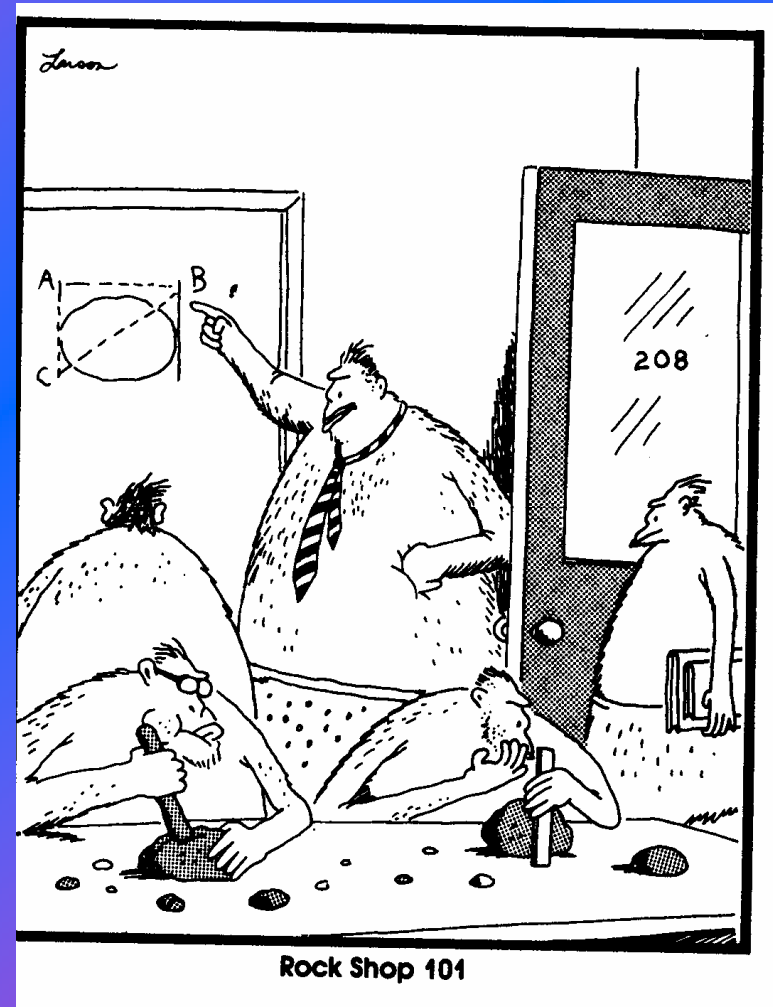
Indications

- Familial thrombotic events
- Anti-conceptionals
- Immobilization causing venous stasis
- Previous thrombotic events

"Leiden"

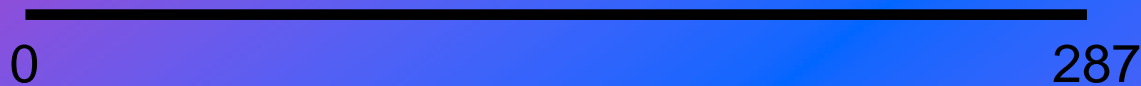
Technical aspects

- DNA extraction from peripheral blood (EDTA/ACD)
- PCR amplification
- Restriction analysis
- Results: wild-type, heterozygous, homozygous

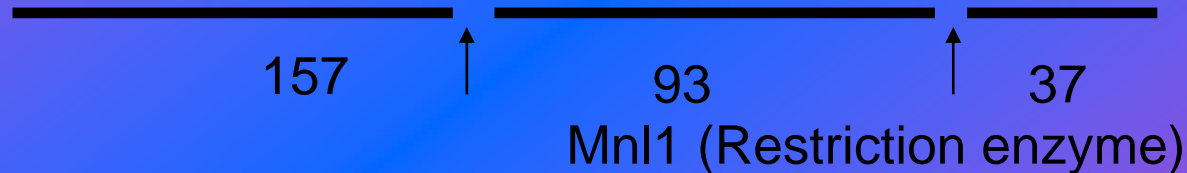


"Leiden" with Mnl1 restriction

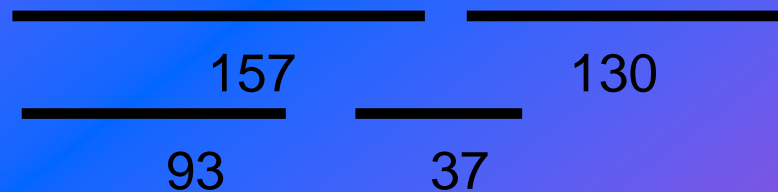
Amplified fragment :



Wild-type: restriction produces three fragments:



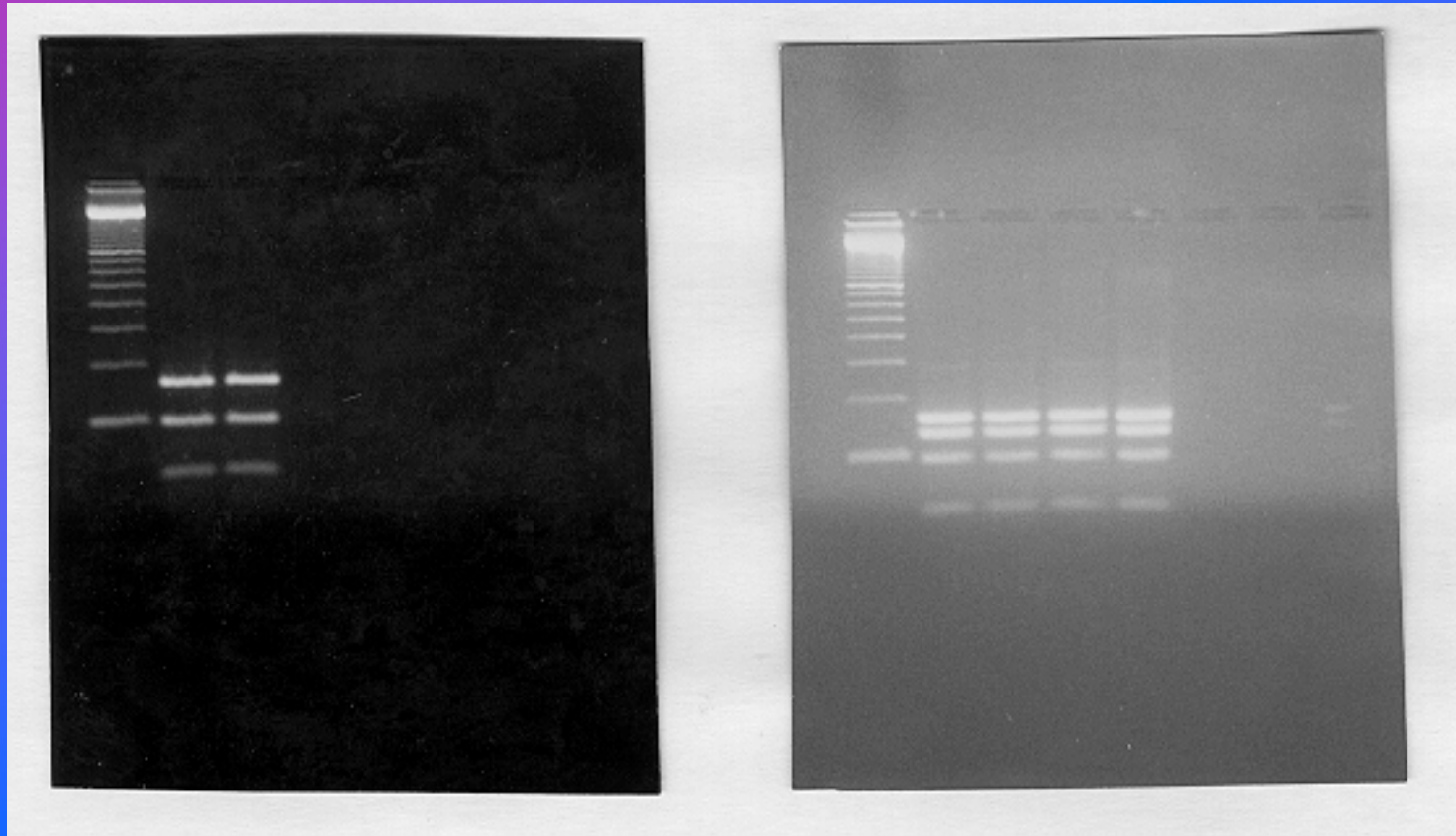
Heterozygous: restriction produces four fragments:



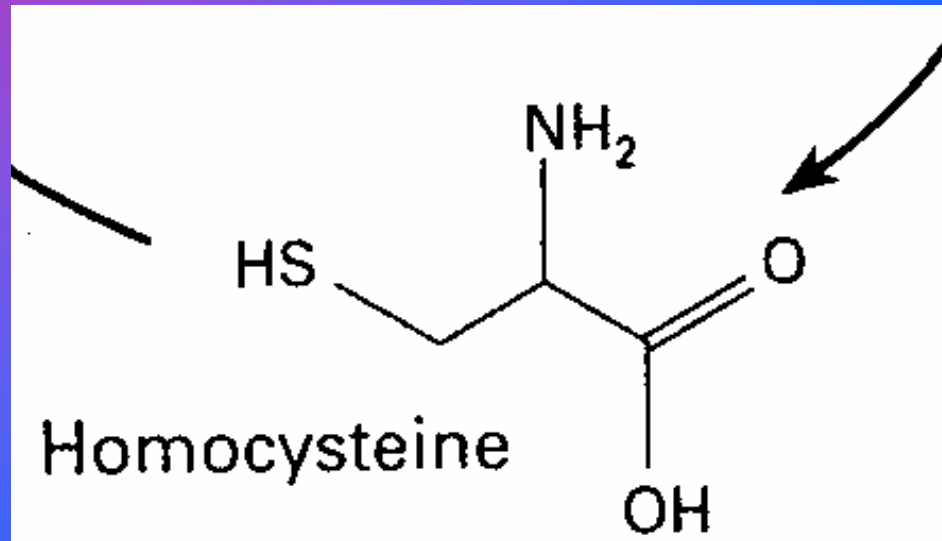
Homozygous: restriction produces two fragments:



Factor V (Leiden) results.



Homocysteine

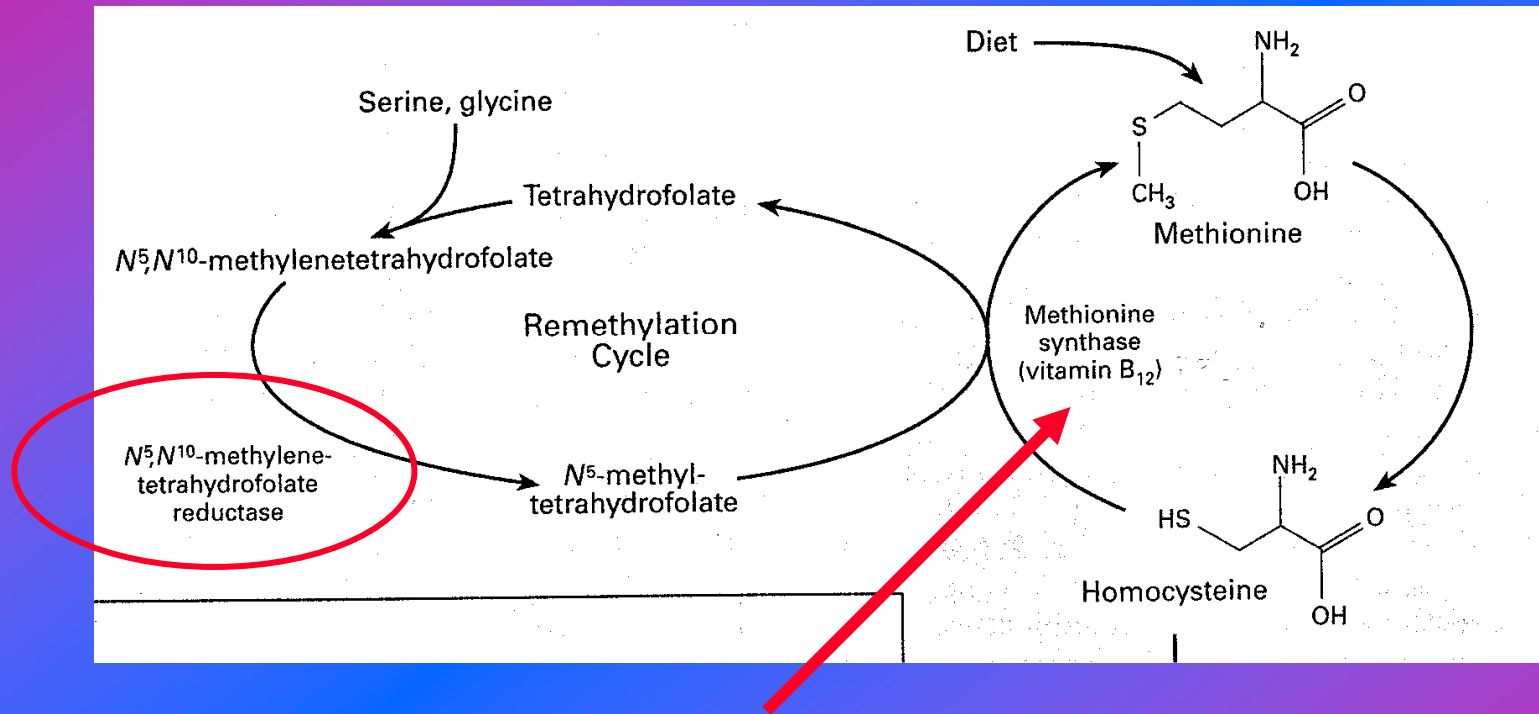


Intermediate aminoacid formed during the metabolism of methionine

It is metabolized by one of two pathways:

- remethylation
- transsulfuration

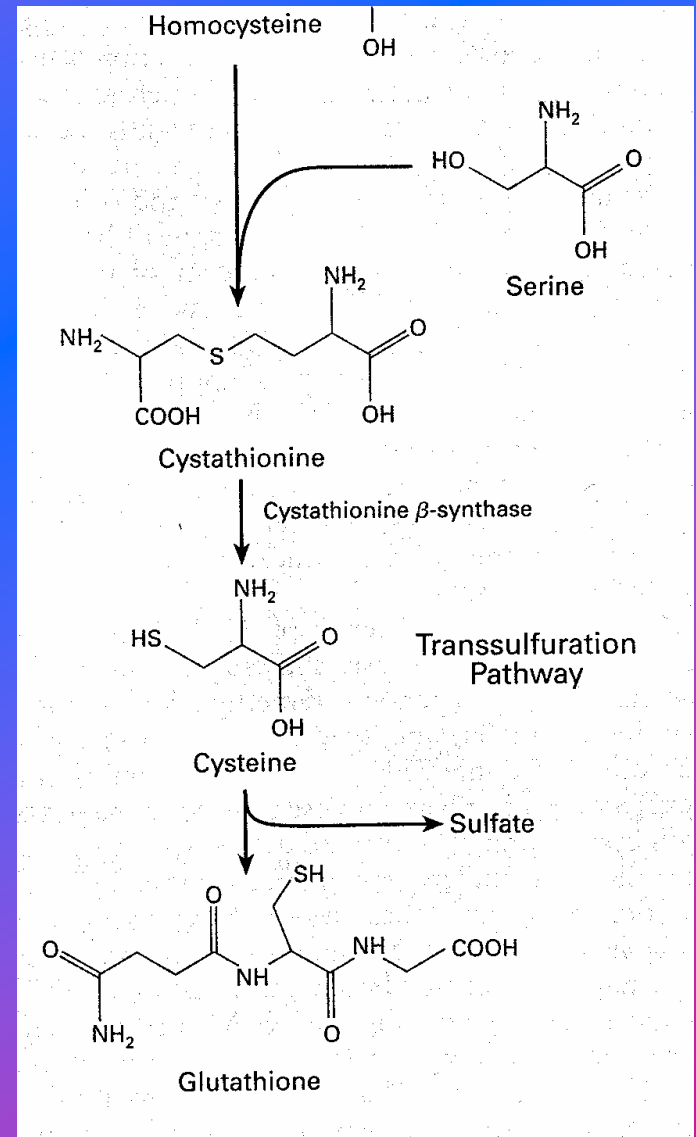
Homocysteine metabolism



In the remethylation cycle homocysteine is salvaged by the acquisition of a methyl group in a reaction catalyzed by the enzyme methionine synthase.

Homocysteine metabolism

In conditions of methionine excess or cysteine requirement, homocysteine enters the transsulfuration pathway to end with the formation of glutathione or further secreted metabolized to sulfate and excreted in the urine.



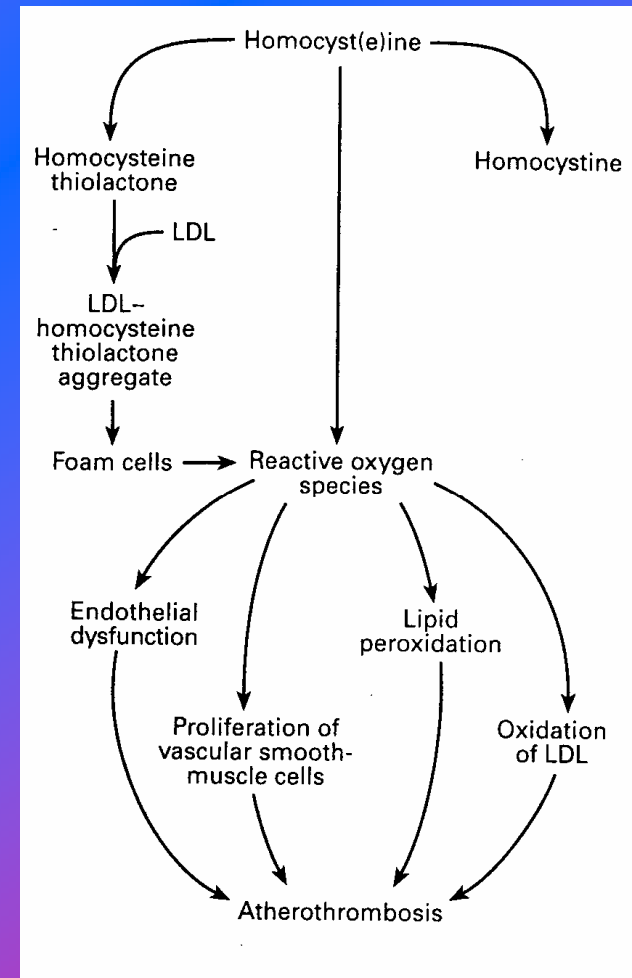
Homocysteine physiopathology

1. Homocysteine is rapidly auto-oxidized in plasma, forming homocystine and homocysteine thiolactone

2. Potent reactive oxygen species including superoxide and hydrogen peroxide are produced during the auto-oxidation process and hydrogen peroxide has been implicated in the vascular toxicity of hyperhomocysteinemia

3. Oxidative damage to vascular endothelial cells induces platelet accumulation, platelet-rich thrombus formation and smooth-muscle cell proliferation in areas of endothelial injury

4. Consequent platelet and leukocyte activation



Homocysteine physiopathology (2)

Cytotoxic reactive oxygen species, including superoxide anion radicals initiate lipid peroxidation, occurring at the endothelial plasma membrane level and within lipoprotein particles resulting with:

- enhancement of the activity of factor XII and factor V and
- depression of the activity of protein C
- induction of the expression of the tissue factor

facilitating ultimately to the formation of **thrombin** and creating a **prothrombotic environment**

Methylenetetrahydrofolate reductase (MTHFR)

Epidemiology

- the C677T mutation is independent of other classical risk factors
- the homozygous form of the mutation is found 5-18% of the general population (16.2% in Switzerland) and in 19% of patients with CAD

Methylenetetrahydrofolate reductase (MTHFR)

Indications

- Homocysteine is harmful to epithelial cells which lay in internal blood vessels (cytotoxic)
- elevated levels of homocysteine is found in patients with angina pectoris, MI and ictus
- if homocysteine is $>15 \mu\text{M}$ in patients with coronaric disease the mortality risk increases 5 times compared to a patient with normal homocysteine
- the presence of an elevated amount of homocysteine correlates with a mutation on the MTHFR gene (C677T)

The sequence

	550	560	570	580	590	600
	* GGC CTG GTG AAG CAC ATC CGA AGT GAG TTT GGT GAC TAC TTT GAC ATC TGT GTG GCA GGT	* Gly Leu Val Lys His Ile Arg Ser Glu Phe Gly Asp Tyr Phe Asp Ile Cys Val Ala Gly				
	610	620	630	640	650	660
	* TAC CCC AAA GGC CAC CCC GAA GCA GGG AGC TTT GAG GCT GAC CTG AAG CAC TTG AAG GAG	* Tyr Pro Lys Gly His Pro Glu Ala Gly Ser Phe Glu Ala Asp Leu Lys His Leu Lys Glu				
	670	680	690	700	710	720
	* AAG GTG TCT GCG GGA GGC GAT TTC ATC ATC ACG CAG CTT TTC TTT GAG GCT GAC ACA TTC	* Lys Val Ser Ala Gly Ala Asp Phe Ile Ile Thr Gln Leu Phe Phe Glu Ala Asp Thr Phe				
	730	740	750	760	770	780
	* TTC CGC TTT GTG AAG GCA TGC ACC GAC ATG GGC ATC ACT TGC CCC ATC GTC CCC GGG ATC	* Phe Arg Phe Val Lys Ala Cys Thr Asp Met Gly Ile Thr Cys Pro Ile Val Pro Gly Ile				
	790	800	810	820	830	840
	* TTT OCC ATC CAG GGC TAC CAC TCC CTT CGG CAG CTT GTG AAG CTG TCC AAG CTG GAG GTG	* Phe Pro Ile Gln Gly Tyr His Ser Leu Arg Gln Leu Val Lys Leu Ser Lys Leu Glu Val				
	850	860	870	880	890	900
	* CCA CAG GAG ATC AAG GAC GTG ATT GAG CCA ATC AAA GAC AAC GAT GCT GCC ATC CGC AAC	* Pro Gln Glu Ile Lys Asp Val Ile Glu Pro Ile Lys Asp Asn Asp Ala Ala Ile Arg Asn				
	910	920	930	940	950	960
	* TAT GGC ATC GAG CTG GCC GTG AGC CTG TGC CAG GAG CTT CTG GCC AGT GGC TTG GTG CCA	* Tyr Gly Ile Glu Leu Ala Val Ser Leu Cys Gln Glu Leu Leu Ala Ser Gly Leu Val Pro				
	970	980	990	1000	1010	1020
	* GGC CTC CAC TTC TAC ACC CTC AAC CGC GAG ATG GCT ACC ACA GAG GTG CTG AAG CGC CTG	* Gly Leu His Phe Tyr Thr Leu Asn Arg Glu Met Ala Thr Thr Glu Val Leu Lys Arg Leu				
	1030	1040	1050	1060	1070	1080
	* GGG ATG TGG ACC GAG GAC CCC AGG CGT CCC CTA CCC TGG GCT CTC AGT GCC CAC CCC AAG	* Gly Met Trp Thr Glu Asp Pro Arg Arg Pro Leu Pro Trp Ala Leu Ser Ala His Pro Lys				
	1090	1100	1110	1120	1130	1140

CGATT

Ala - Val

Pro
GGASTC
CCACAG

HofE GANTC

Methylenetetrahydrofolate reductase (MTHFR)

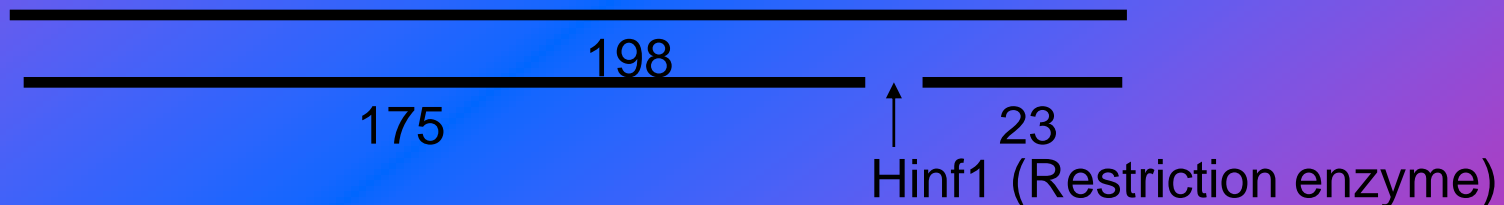
Amplified fragment :



Wild-type: the fragment is not cut :



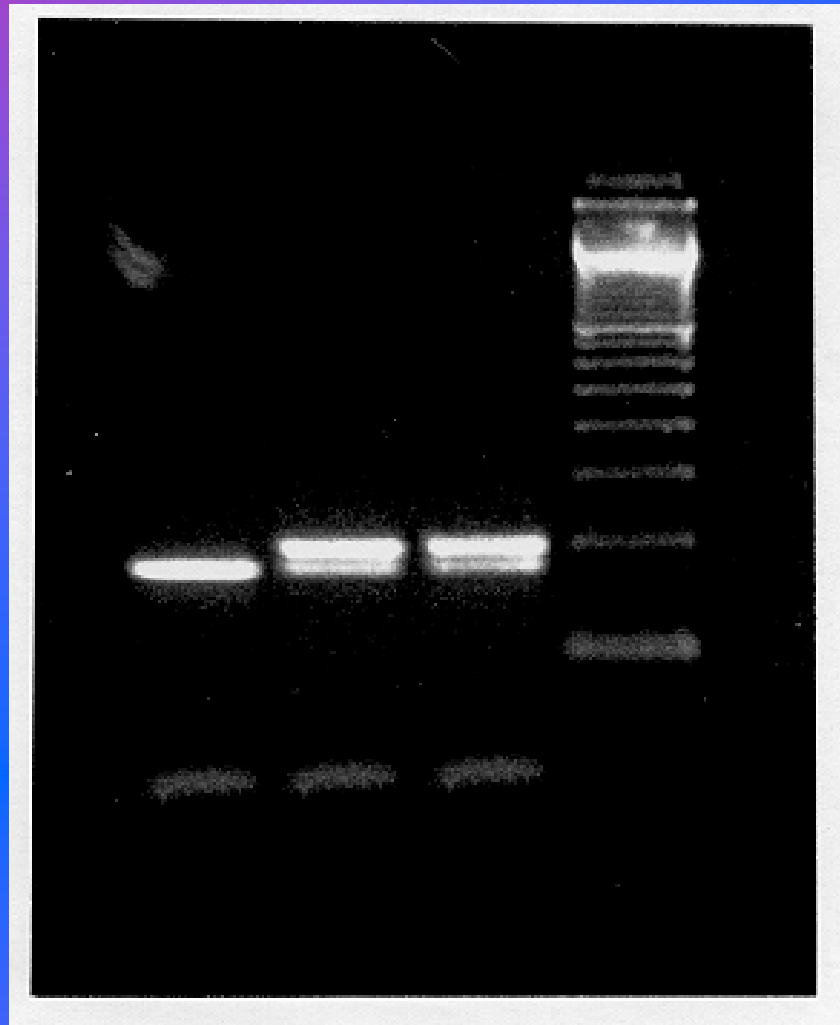
Heterozygous: restriction produces three fragments:



Homozygous: restriction produces two fragments:



The results are here.

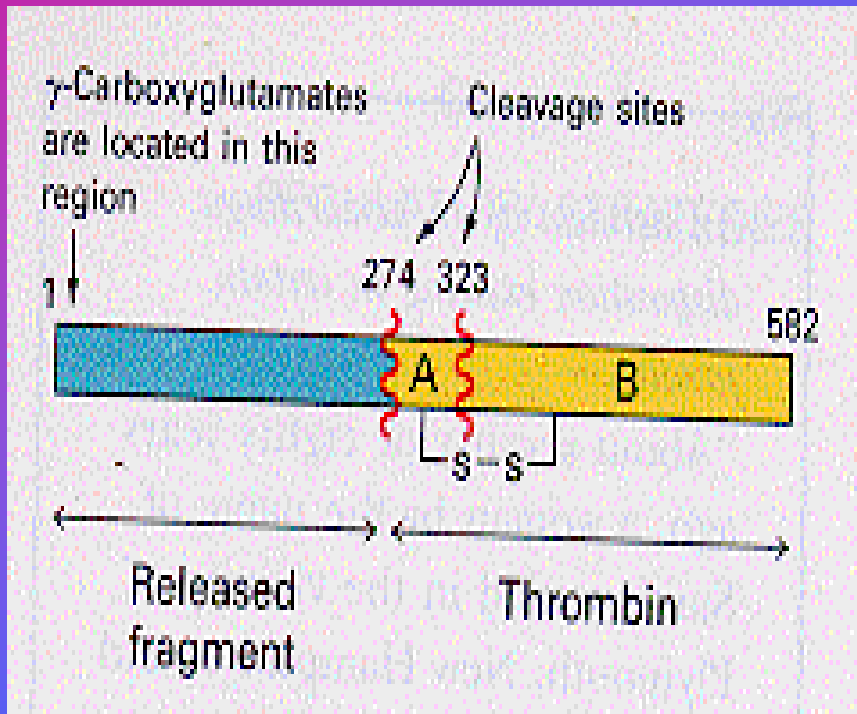


Prothrombin, another same old story.



Horse hospitals

What is Prothrombin?



e codifica la protrombina (estratto da GeneBank, Internet)

```

ctgttgggtg gagaatagac tgtaggtggg caaagaatga aggaaactag
agctcgagct agaagtgggtg agaaggggtt ggatttgggg tctatgctga
gacaagattt gctaggattg gatgtgtagg gtgaggaagt ggggacagca
gaggggtaag tggactctca ccagctgtgt ctctggaagg ggcgtggctg
tatgctctcg agcacagacg gctgttctct ttcaaggta caagcctgat
gaggggatgc ctgtgaaggt gacagtgggg gaccctttgt catgaaggta
aagcccaggg cctggtgaac acatcttctg ggggtgggga gaaactctag

```

```

26461 tatctagaaa cagttgctg gcagaggaat actgatgtga ccttgaactt gactctattg
26521 gaaacctcat ctttcttctt cagagcccct ttaacaaccg ctggtatcaa atgggcatcg
26581 tctcatgggg tgaaggctgt gaccgggatg ggaaatatgg cttctacaca catgtgttcc
26641 gctgaagaa gtggatacag aaggtcattg atcagtttgg agagtagggg gccactcata
26701 ttctgggctc ctggaaccaa tcccgtgaaa gaattatfff tgtgtttcta aaactatggt

26761 tccaataaa agtgactctc agcGagcctc aatgctccca gtgctattca tgggcagctc
26821 tctgggctca ggaagagcca gtaactactac tggataaaga agacttaaga atccaccacc
26881 tgggtgcacgc tggtagtccg agcaactcggg aggctgaggt gggaggat

```

“a” nelle persone con rischio trombotico

Prothrombin

- 34 KDal mass and contains two chains
- cleaves only certain arginine-glycine bonds
- synthesized as a zymogen called prothrombin
- Proteolytic cleavage of the Arg274-Thr275 bond releases a 32 Kdal fragment from the 66 Kdal zymogen
- Cleavage at the Arg323-Ile324 bond yields active thrombin
- The **vitamin K**-dependent carboxylation reaction converts glutamate, a weak chelator of Ca^{++} , into gamma-carboxyglutamate a much stronger chelator.
- The binding of Ca^{++} by prothrombin anchors it to **phospholipid membranes** derived from blood platelets bringing prothrombin in close proximity to **factor Xa and Factor V**, two proteins that mediate its conversion to thrombin

The sequence of the prothrombin gene

Sequenza del gene che codifica la protrombina (estratto da GeneBank, Internet)

```
....26041 attattctgc ctggtgggtg gagaatagac tgtaggtggg caaagaatga aggaaactag
26101 tgggttcagg agctcgagct agaagtgggtg agaagggttt ggatttgggg tctatgctga
26161 aggtagagcc gacaagattt gctaggattg gatgtgtagg gtgaggaagt ggggacagca
26221 agaatgactg gaggggtaag tggactctca ccagctgtgt ctcgtgaagg ggcgtggctg
26281 ggctatgagc tatgctcctg agcacagacg gctgttctct ttcaaggtta caagcctgat
26341 gaagggaaac gaggggatgc ctgtgaaggt gacagtgggg gaccctttgt catgaaggta
26401 agcttctcta aagcccaggg cctggtgaac acatcttctg ggggtgggga gaaactctag
26461 tatctagaaa cagttgcctg gcagaggaat actgatgtga cttgaactt gactctattg
26521 gaaacctcat ctttcttctt cagagcccct ttaacaaccg ctggtatcaa atgggcatcg
26581 tctcatgggg tgaaggctgt gaccgggatg ggaaatatgg cttctacaca catgtgttcc
26641 gcctgaagaa gtggatacag aaggtcattg atcagtttgg agagtagggg gccactcata
26701 ttctgggctc ctggaacca tcccgtgaaa gaattathtt tgtgtttcta aaactatggt

26761 tcccaataaa agtgactctc agcGagcctc aatgctccca gtgctattca tgggcagctc
26821 tctgggctca ggaagagcca gtaactactac tggataaaga agacttaaga atccaccacc
26881 tggtgcacgc tggtagtccg agcactcggg aggctgaggt gggaggat
```

“a” nelle persone con rischio trombotico

The amplification produces a 345 bp fragment and the transition G to A creates a restriction site for Hind III.

Mutation on the prothrombin gene (G20210A)

Epidemiology

- The prevalence in the general population is about 1-2%
- The incidence of deep venous thrombosis (DVT) in the general population is 1/1000
- The mutation on the prothrombin gene gives a 8-fold greater risk to develop a DVT and a 4-fold risk to develop a MI
- The mutation is found in 18% of patients with a personal history or familial of DVT (2.3% in healthy)
- 87% of patients with the polymorphism are in the higher quartile of prothrombin levels

Mutation on the prothrombin gene (G20210A) and more...

- The G20210A transition is more frequent in women which had a first MI (5.1%) than controls (1.6%)
- The relative risk is much higher if another risk factor like smoke is present. The odds ratio goes up from 4.0 to 43.3.

Mutation on the prothrombin gene (G20210A)

Indications

- Familial thrombotic events
- Anti-conceptionals
- Immobilization causing venous stasis
- Previous thrombotic events

Mutation on the prothrombin gene (G20210A)

Amplified fragment :

0 345

Wild-type: the fragment is not cut:

345

Heterozygous: restriction produces three fragments:

345

323

22

Homozygous: restriction produces two fragments :

322

22

Which looks like this.

