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 azathioprin
  - Example 2: Clozapine in the treatment of psychiatric diseases
  - CXP3A4 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.
Visualizing blood easily flowing through a vessel.

Doppler analysis of two parallel normal blood vessels
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.

Blood (liquid)  

hemostasis  

Blood (solid)
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 clotting pathway.

Blood trauma

XII to XIIa

Xi to Xia

IX to Ixa

platelets phospholipids

X to Xa = prothrombin activator

1 prothrombin to thrombin

fibrinogen to fibrin clot

Vit K

activated protein C (APC)

degradation of factor Va

degradation of factor VIII

Protein C (serine protease)
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-resistent patients Arg506 is replaced by Gln (Leiden mutation)
The sequence of Factor V.
“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:

0 287

Wild-type: restriction produces three fragments:

157 93 37

Mnl1 (Restriction enzyme)

Heterozygous: restriction produces four fragments:

157 130

93 37

Homozygous: restriction produces two fragments:

157 130
Factor V (Leiden) results.
Homocysteine

Intermediate aminoacid formed during the metabolism of methionine
It is metabolized by one of two pathways:
- remethylation
- transsulfuration
In the remethylation cycle homocysteine is salvaged by the acquisition of a methyl group in a reaction catalyzed by the enzyme methionine synthase.
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
Methylenetetrahydrofolatoe reductase (MTHFR)

Epidemiology

- the C677T mutation is independent of other classical risk factors
- the homozigous 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 armful 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 µM in patients with coronary 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
Methylenetetrahydrofolate reductase (MTHFR)

Amplified fragment:

| 0 | 198 |

**Wild-type:** the fragment is not cut:

| 0 | 198 |

**Heterozygous:** restriction produces three fragments:

| 175 | 198 | 23 |

**Hinf1** (Restriction enzyme)

**Homozigous:** restriction produces two fragments:

| 175 | 23 |
The results are here.
Prothrombin, another same old story.
What is Prothrombin?
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 ctgttggttg gagaatagac tgtaggtggg caaagaatga aggaaactag
  26101 tgggttcagg agctcgagct agaagtggtg agaaggttta ggatttgggg tctatgctga
  26161 aggtagagcc gacaagattt gcctaggatt gatggtttag gtagggaagt gggacagca
  26221 agaatgactg gagggaataag tggactctca ccagctgtgt ctcgtgaagg ggctggtgctg
  26281 ggtcatagac tattgcctctg agccacagacg cgtgttctct ttcaggtta caagctgtat
  26341 gaagggaaac gagggaatgc cttgtaaggt gaaagttggg gacccctttgt cttgaaggta
  26401 aggcttctta aagcccaagg ccctggtagac acctcttctg ggggtggaag gaaacctctag
  26461 tatctagaaa caagttgctg gcagaggtat acctgatgtg ctttgatacct gcctctattg
  26521 gaaactcact cttctctctt cagagcccttt ttaacaaaccg cttgtatcga atggccatcg
  26581 tctctctggt ggaggtgtgt gaccgggtat gggaatatgg cttctacaca cattgtttcc
  26641 gcctgaagaa gttgataaga aaggtcattg atcagtttg gaggtaggg gcccactcata
  26701 tctttgcttc cttgaaacca tccccgtgaaa gatatttttt tttgttctct cttgccattg
  26761 tcctaaaag aagtgaacttc acgcggtcc aatgtcctcct gttgtaattt tgggtcagttc
  26821 tctgttctca ggaagagcca gtaatacata tggataaaga agaccttta aatcaccacc
  26881 tggtcagcgc tttgtagctcc agcactctggg aggctgaggt gggaggt

"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)

<table>
<thead>
<tr>
<th>Amplified fragment:</th>
<th>0</th>
<th>345</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild-type:</strong> the fragment is not cut:</td>
<td></td>
<td>345</td>
</tr>
<tr>
<td><strong>Heterozygous:</strong> restriction produces three fragments:</td>
<td></td>
<td>345</td>
</tr>
<tr>
<td></td>
<td>323</td>
<td>22</td>
</tr>
<tr>
<td><strong>Homozygous:</strong> restriction produces two fragments:</td>
<td></td>
<td>322</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>
Which looks like this.