OBJECTIVES

After reading this article, the reader will be able to:
- List the mutations that cause Factor V Leiden and Prothrombin Mutation.
- Outline the incidence of Factor V Leiden and Prothrombin Mutation.
- Diagram the normal negative feedback loop with the coagulation pathway, specifically the role of Thrombin, Protein C, and Factor V.
- Compare how Factor V Leiden mutation differs in the coagulation pathway to wild-type Factor V.

OBJECTIVES

- Predict thrombotic risk status of individuals if given environmental and genetic factors.
- Explain the various molecular methods used to test for inherited thrombophilia.
- Differentiate between Light Cycler and TaqMan assays, including the advantages and disadvantages of each method.
- Justify the importance of quality control in molecular testing.
- Defend the importance of inherited thrombophilia testing.

Venous Thromboembolism

- Natural balance between bleeding and clotting
  - Number of conditions that can sway that balance
    - Surgery, hormone therapy, pregnancy, oral contraception, and immobility due to travel or bed rest
    - Genetic predisposition
  - Venous Thromboembolism (VTE)
    - Deep Venous Thrombosis (DVT)
      - Occurs in the legs resulting in unilateral leg
    - Pulmonary Embolism (PE)
      - Clot travels to the lungs and blocks either pulmonary arteries or lung stems

VTE Detection

- Diagnostics:
  - D-Dimer and ultrasound
- Guidelines for genetic testing:
  - a VTE younger than 50 years old
  - recurrent/unprovoked VTEs
  - clots that occur in uncommon locations
  - a family history of homozygous inherited mutations
  - family history of VTE and has had VTE in self
  - unknown loss of pregnancy in 2nd or 3rd trimesters

Inherited Disorders

- Disorders:
  - Protein C Deficiency
  - Protein S Deficiency
  - Antithrombin Deficiency
  - Factor V Leiden Mutation
  - Prothrombin G20210A Mutation
  - Methylenetetrahydrofolate reductase (MTHFR)
- Functional tests available:
  - Protein C deficiency, Protein S deficiency, Antithrombin deficiency, Factor V Leiden (FVL)
  - APCR-APTT is a phenotypic test for Factor V Leiden
Coagulation Pathway

Factor V
- OMIM 612309
- 1q23
  - 25 exons
- In wild-type individuals
  - FV → (FVa)
  - Cofactor for Factor X
- Excess thrombin initiates a negative feedback loop
  - Thrombomodulin (TM), Endothelial Protein C Receptor (EPCR), Thrombin (T), Protein C (PC)
  - Activated Protein C (APC)
  - Cleaves FVa at 3 sites, Arg 306, Arg 506, and Arg 679

Factor V Leiden
- Inherited autosomal dominant
- G1691A
  - Missense substitution in exon 10
  - Removes the Arg 506 cleavage site
- Heterozygotes: 5-fold increase risk in VTE
- Homozygotes: 80-fold increase
  - Incidence of 2 in 10000 individuals
- Incidence:
  - 5% in Caucasian population
  - Much lower incidence in Asians and African Americans

Additional FV Mutations
- FV Cambridge (R306T) & FV Hong Kong (R306G)
  - Very rare
  - Evidence of increasing risk of thrombosis
- FV R2 haplotypes (A4070G)
  - exon 13
  - Decreases FV levels
  - Increases risk of thrombosis 3-fold
  - Numerous polymorphisms
  - Can be discriminated from FVL by some molecular methods

Prothrombin (Factor II)
- OMIM 176930
- 11p11-q12
  - contains 14 exons
- Synthesized in the liver
  - vitamin K dependent
- Cleaved by FXa
- Forms fibrin which is the foundation of clot formation

Prothrombin Mutation
- Second most common inherited cause of thrombophilia
- G20210A
  - 3' untranslated area from q11-12
  - Increased efficiency of the 3' cleavage signal
  - Increases production of prothrombin
- Heterozygotes: 30% increase
- 2-3 fold increase risk of VTE
- Homozygotes: 70% increase
- Incidence:
  - Caucasian population
  - Southern European descent
  - Heterozygotes: 2%
  - Homozygosity: very rare
  - Rarely seen in Asians and African Americans
Methods of Detection

- The United Kingdom National External Quality Assessment Service (UK NEQAS)
  - surveyed 97 laboratories in Oct 2005
    - 26 laboratories used PCR-RFLP
    - 26 laboratories used LightCycler
    - 14 laboratories used Fluorescence with Allele Specific Discrimination
    - 13 laboratories used ABI TaqMan
    - and less than 5 laboratories used Invader, ARMS, ELISA
- "Gold standard" method
  - Bidirectional sequencing of the SNP region
  - PCR-RFLP or allele-specific PCR may be used as alternatives
- Considerations:
  - PCR
  - QC
  - Instrumentation

Restriction fragment length polymorphism (RFLP)

- The loss of the Arg 506 cleavage site → loss of a MnlI endonuclease site
  - MnlI endonuclease: unstable and expensive
  - Alternatives: TaqI, SacI, and HindIII
- RFLP Steps:
  - DNA extraction
  - PCR amplification
  - Restriction enzyme digestion
  - Visualization on 3% agarose gel
- QC
  - Second internal restriction site
  - PCR controls

RFLP Interpretation

- Digested amplicon
  - Fragments along the length of the gel
  - Loss or gain of a particular fragment
  - For example
    - wild-type: 123 bps
    - Mutation: 148 bps

RFLP: Pros & Cons

- Advantages:
  - Accurate
  - Relatively inexpensive
  - FVL and Prothrombin can be multiplexed
  - Little dedicated molecular equipment
- Disadvantages:
  - Significant hands-on time
  - Post-PCR manipulation
  - Less effective with large numbers
  - Ethidium bromide and UV light
  - Polymorphisms near to SNP affect results

Real-Time PCR: TaqMan

- Two unlabeled primers and probe
  - forward and reverse
  - sequence-specific probe
  - 5' fluorophore
  - 3' quencher
- During the annealing phase of amplification
  - Hybridization to patient DNA
- During the extension phase of amplification
  - 5' to 3' exonuclease activity of DNA polymerase
  - Breakdown of probe
  - Measureable fluorescent signal

TaqMan: Pros & Cons

- Advantages:
  - Sophisticated instrumentation
  - Multiplexing
  - High throughput
  - No post-PCR manipulation
  - Closed system
- Disadvantages:
  - Success depends on proper primer/probe design
  - SNPs
  - Expense
Real-Time PCR: LightCycler

- **Master mix**: Diluent, 2 primers (forward and reverse), 2 probes (fluorescence donor and acceptor), Taq DNA polymerase, dNTPs, Brij 35 (a non-ionic detergent), and MgCl₂.
- **Mutation Probe**: Fluorescein attached to the 3’ end
- **Anchor Probe**: LightCycler Red 640-NH₂ hydroxy succinimide ester at the 5’ end
- **Fluorescence resonance energy transfer (FRET)**: Light energy (hv) excites the fluorescein. Energy will then transfer to the Red 640-NHS ester, producing a fluorescent signal.

LightCycler: Melting Curve

- **End of the amplification**: Cooling the samples to 35°C
- **Increasing the temperature**: 0.1°C/s to 75°C.
- **If the mutation is present**: Mismatch will have lower Tm.
  - Wild-type: 65°C ± 2.5°C
  - Mutant: 57°C ± 2.5°C
- **Allow to distinguish heterozygotes and homozygotes**

LightCycler: Pros & Cons

- **Advantages**:
  - Multiplex
  - No post-PCR manipulation
  - Little hands on time
  - Closed system
  - IVD FDA approved for FVL and FII G20210A
- **Disadvantages**:
  - Sample identification
  - Expensive
  - Affected by polymorphisms

Future Work

- **Algorithms**
  - Incorporate all risk factors
  - Provide better risk assessment to patients
  - Better management of pregnancies
- **Microarrays**
  - Assess several factors at the same time
  - Drug personalization
  - Cytochrome p450 and vitamin K epoxide reductase (VKORC1)
- **Additional mutations**
  - African Americans populations
  - Coagulation pathway

References