Laboratory assessment of the Antiphospholipid Syndrome

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The antiphospholipid syndrome (APS)

- autoimmune disease
- antiphospholipid antibodies (aPL)
- thrombosis
- pregnancy morbidity

- How to measure the aPL
- How to use these tests in risk stratification
The antiphospholipid syndrome (APS)

Introduction

One clinical and one laboratory criterion
# Laboratory criteria

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>LAC</strong></td>
<td>Screening-, mixing and confirmation test</td>
<td>Screening-, mixing- and confirmation test</td>
</tr>
<tr>
<td></td>
<td>Interval 6 weeks</td>
<td>Interval 12 weeks</td>
</tr>
<tr>
<td><strong>aCL</strong></td>
<td>ELISA (β2GPI), IgG and IgM</td>
<td>ELISA, IgG and IgM</td>
</tr>
<tr>
<td></td>
<td>high or medium titer</td>
<td>&gt; 40 GPL/MPL of &gt; 99th perc.</td>
</tr>
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<td></td>
<td>Interval 6 weeks</td>
<td>Interval 12 weeks</td>
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Antiphospholipid antibodies

β2 glycoproteïne I (β2GPI)

Antiphospholipid antibodies

Lupus anticoagulants (LAC)

Anticardiolipin antibodies (aCL)

Beta-2-glycoprotein I antibodies (β2-GPI)

Introduction
Antiphospholipid antibodies

- Lupus anticoagulants (LAC)
- Anticardiolipin antibodies (aCL)
- Beta-2-glycoprotein I antibodies (ß2-GPI)

Solid phase assays (ELISA) vs. Coagulation assays

Introduction
Diagnosis of the APS

Incidence of clinical features is high and often determined by other underlying factors.

Diagnosis of the APS relies predominantly on the laboratory results.

Assays with optimal diagnostic power: Sensitivity and specificity
Importance of sensitivity:
- Prevent false negatives
- Patients with APS need long-term anticoagulation to prevent recurrence

Importance of specificity
- Prevent false positives
- Patients without APS getting anticoagulation (the bleeding risk) without the benefit
Laboratory diagnosis of the APS

• Lack of **standardization** in the assays
  – Overdiagnosis/misdiagnosis
  – Inter-laboratory variation

• Diagnosis of APS: lack of a “golden standard”
  – LAC (*ISTH SCC Pengo et al, 2009*)
  – Solid phase assays
    • aCL antibodies
    • aβ₂GPI antibodies
## Antiphospholipid antibodies

<table>
<thead>
<tr>
<th>Assays</th>
<th>Dependent on antibodies against</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC</td>
<td>“all” aPL: β2GPI antibodies, prothrombin antibodies, other?</td>
</tr>
<tr>
<td>aCL ELISA</td>
<td>aCL (β2GPI-dependent) antibodies</td>
</tr>
<tr>
<td>aβ2GPI ELISA</td>
<td>β2GPI antibodies</td>
</tr>
</tbody>
</table>

**Laboratory diagnosis**

**Strengths and weaknesses**
Laboratory diagnosis of the APS

- Laboratory criteria
  - LAC
  - aCL
  - aβ2GPI
Laboratory diagnosis of the APS

LAC

- Phospholipid dependent coagulation tests
  - functional activity, qualitative test
- Multiple steps (Brandt et al 1995, Pengo et al 2009)
LAC: methodology

- **Preanalytical conditions**
  - Screening assays: low concentration of PL
  - Blood collection
    - Platelet poor plasma (<$10^7$/mL)
      - residual platelets: give false negative results
    - double centrifugation:
      1) 2000g 15 min RT; 2) 2500g 10 min
  - avoid repeated freeze/thawing cycles
LAC: methodology

Choice of assays
Any PL-dependent assay?

Pengo et al, J Thromb Haemost, 2009

two phospholipid-dependent coagulation tests
different assay principle

assay of choice?
-reduce the inter-laboratory variation
Commercially available and quality controlled
-robust and highly reproducible
dRVVT and aPTT (low conc. PL and silica activator)

One of the two assays positive
### LAC: methodology

**• Calculation cut-off values**

<table>
<thead>
<tr>
<th>Screening</th>
<th>Mixing</th>
<th>Confirmation</th>
</tr>
</thead>
</table>
| - Express results as normalized ratios  
- Use local cut-off values  
- > **99th percentile** of a normal population | > Rosner index  
((APTT 1:1mix – aPTT NPP)/ aPTT PP)) x 100  
> clotting time of a normal distribution in sec | > % correction:  
(screen-confirmation) / screen x100 |
LAC: methodology

Integrated test systems

- Screening+confirmation in one assay:
  dRVVT or aPTT:
  low (screen) PL
  high (confirmation) PL

  on mixture of PP and NPP (Staclot-LA)
  neat plasma (dRVVT)

- Interpretation:
  - Lupus ratio: screen/confirm
  - % correction: (screen-cfr)/screen x100
LAC: methodology

**traditional test**

- **screening test**
  - (+)
  - screening test on mix
    - (-)
    - (+)
      - confirmation test
        - (-)
        - (+)
          - LAC negative
          - LAC positive

**Three step method**

- Yes Mixing test
- No
LAC: methodology

• To mix or not to mix?
  – False positive results by no mixing
    • Screen/Confirm positive on neat plasma with mixing test on screen negative (*Devreese JTH 2010*)
      majority aCL and/or aβ2GPI negative
  – False negative results by no mixing
    • Strong LAC (*Favaloro et al, JTH 2010*)
    • “lupus cofactor” (*Tripodi and Pengo, JTH 2011*)
  – False negative results by mixing
Interference with anticoagulant therapy

<table>
<thead>
<tr>
<th>LAC: pitfalls</th>
<th>AVK</th>
</tr>
</thead>
<tbody>
<tr>
<td>check</td>
<td>INR</td>
</tr>
<tr>
<td>Analysis of LAC</td>
<td>After AVK, INR&lt;1.5</td>
</tr>
<tr>
<td></td>
<td>Mix 1:1 PPP/NPP</td>
</tr>
<tr>
<td></td>
<td>No analysis INR &gt;3</td>
</tr>
<tr>
<td>Interference</td>
<td>aPTT and dRVVT</td>
</tr>
<tr>
<td></td>
<td>FP and FN</td>
</tr>
</tbody>
</table>
LAC: pitfalls

Samples spiked with CRP:
PTT-LA & LA-Screen normalised ratio

ECAT exercise in 2010: NPP enriched with CRP
“positive for LAC”
94% screening (PTT-LA)
72% positive mixing
86% positive confirm (Staclot)

(Schouwers and Devreese, Thromb Res 2010)
LAC: pitfalls

- Analytical interference of CRP: imitates aPL
  Binding of CRP to negatively charged PL can influence test results for LAC, causing false positive results

- Type of PL in the reagent is important
  - PTT-LA and Staclot-LA (aPTT) are sensitive
  - No interference observed for dRVVT reagents used

- Interference increases with increasing CRP
LAC: pitfalls

• **False positive results**
  – Cut-off 99th percentile
  – Two PL-dependent assays
  – Three steps: screen, mix and confirm
  – Anticoagulation: heparin contamination, new anticoagulants, AVK INR >3
  – Specific coagulation factor inhibitors
  – CRP
  – Repeat testing after 12 weeks

• **False negative results**
  – Improper plasma preparation
  – Diluting effect of mixing studies
LAC: patient selection

• **Clinical characteristics**  *(Pengo et al, 2009)*
  
  – **Low:**
    • VTE or AT in elderly patients
  
  – **Moderate:**
    • accidentally prolonged aPTT without symptoms
    • Recurrent spontaneous early abortion
    • Provoked VTE in young patients
  
  – **High:**
    • Unprovoked VTE and AT in young patients (<50y)
    • Thrombosis at unusual sites
    • Late pregnancy loss
    • TEC or pregnancy complications in AID

No generalized searches in asymptomatic patients (increase of FP)
Laboratory diagnosis of the APS

- Laboratory criteria
  - LAC
  - aCL
  - aß2GPI
aCL and β2GPI antibodies

Adapted from Giannakopoulos B et al. Blood 2009;113:985-994
aCL and β2GPI antibodies

• Guidelines  (Miyakis et al, 2006)
  ELISA, IgG and IgM
  > 40 GPL/MPL (aCL) of > 99th perc.
  Interval 12 weeks

• Minimum requirements
  -Calculate cut-off values in percentiles (99th)
  -aCL in GPL/MPL units and range and aβ2GPI in universal units
  -Inter-assay variation should be less than 20% (CV) (10%)
  -Samples should be run in duplicate (automated systems?)
  -reliable standard with traceability for calibrators

Methodological problems
  Interassay and interlaboratory variation

(Tincani et al, 2004; Wong et al, 2004; Reber et al 2005; Pierangeli et al 2008; Lakos et al 2012)
aCL and β2GPI antibodies

Comparison of titers between systems

• IgG Sapporo standard (HCAL) 10.7 µg/ml

ELISA vs new automated systems

Laboratory diagnosis

(Van Hoecke and Devreese, Int J Lab Hematol, 2012)
aCL and β2GPI antibodies

- Commercial ELISAs
- Same samples
- Tested in different labs

(Pengo et al, 2007)

Laboratory diagnosis
Interassay variation

- Lack of uniformity in calibration resulting in large titer variation between assays
  - Harris standards (polyclonal IgG and IgM)
  - Secondary standards (heterogenous)
  - Monoclonal antibodies HCAL (IgG) and EY2C9 (IgM)
  - Internal standard

- No universal units
  - aCL expressed as GPL/MPL, U/ml
  - β2GPI expressed as AU, IU/ml, U/ml, µg/ml,…

- Differences in production of the kits (type of microtiter plate, PL, blocking agents, source of β2GPI, …)

Interlaboratory variation

- In numeric results
- Cut-off values

Laboratory diagnosis

Laboratory diagnosis of the APS

- Laboratory criteria
  - LAC
  - aCL
  - aß2GPI

- Clinical relevance?
LAC and thrombosis

The lupus anticoagulant (LAC) correlates better with thrombosis than aCL.

<table>
<thead>
<tr>
<th>Type of event</th>
<th>Anticardiolipin isotype</th>
</tr>
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<tbody>
<tr>
<td>Cerebral Stroke</td>
<td></td>
</tr>
<tr>
<td>Any</td>
<td>G/M</td>
</tr>
<tr>
<td>Deep Vein Thrombosis</td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>G</td>
</tr>
<tr>
<td>Recurrence</td>
<td>G</td>
</tr>
<tr>
<td>Any</td>
<td>G</td>
</tr>
<tr>
<td>Any Thrombosis</td>
<td></td>
</tr>
<tr>
<td>Any</td>
<td>G</td>
</tr>
</tbody>
</table>

Galli et al, Blood 2003; 101: 1827-1832
LAC and thrombosis

• Discrepancies in reported risk:
  
  Odds Ratio
  
  

Causes:

- Study population (age, sex, control population)
- Methodology for LAC (choice of assays, cut-off values, two separate analyses...)

APS diagnosis
aCL and aβ2GPI: clinical relevance

aCL

- Isotype:
  - IgG (Pengo et al, JTH 2010) (Galli, JTH 2010)
  - Only IgM: no firm association in clinical studies

- High titer aCL (99th percentile)

- Odds Ratio aCL
  - VTE: 4.7-5.5 (Sanmarco et al, 2007; Ginsburg et al, 1992)
  - Arterial thrombosis: 1.4-15 (Wu et al, 1992; Saidi et al, 2009)

- Discrepancies: study design (small studies, retrospective studies, control population), different assays, single sample measurement, …
β2GPI antibodies

- No or modest risk:
  VTE: 1.6-2.4  \((Petri\ et\ al\ 2010;\ de\ Groot\ et\ al,\ 2005)\)
  MI: 2.5  \((Meroni\ et\ al,\ 2007)\)
  stroke: 2.3  \((Urbanus\ et\ al,\ 2009)\)

- Current available ELISA for IgG β2GPI antibodies
  \(-\ 4\ -15.4\ \(Devreese\ et\ al,\ Blood\ 2010;\ Van\ Hoecke\ and\ Devreese,\ Int\ J\ Lab\ Hematol,\ 2012)\)
  \(-\ 7.6-11.7\ \(de\ Moerloose\ et\ al,\ JTH\ 2010\)\)

Study population and type of assay

APS diagnosis
aCL and aß2GPI: clinical relevance

ß2GPI antibodies

Diagnostic weakness:

- more specific, easy to standardise?
- Heterogeneous group of antibodies

- subpopulation of ß2GPI antibodies (domain I)
  OR - 18.9  \( (de\ Laat\ et\ al,\ Blood\ 2005) \)
  - 3.5  \( (de\ Laat\ et\ al,\ JTH\ 2009) \ (n=442) \)

Giannakopoulos B et al 2009
Laboratory diagnosis of the APS

- Laboratory criteria (Miyakis et al, 2006)

- Relation with thrombosis:
  - LAC: +
  - aCL: ?
  - aβ2GPI: ±
# Diagnostic value of LAC, aCL, β2GPI antibodies

<table>
<thead>
<tr>
<th>Isolated LAC</th>
<th>Isolated aCL</th>
<th>Isolated aβ2GPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No association with thrombosis</td>
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<td>No association with thrombosis</td>
</tr>
<tr>
<td></td>
<td>Except in SLE</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(Les et al, Semin Thromb Hemost 2009)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>not β2GPI-dependent</td>
<td>not β2GPI-dependent</td>
<td>Non pathogenic antibodies</td>
</tr>
</tbody>
</table>
aCL and β2GPI antibodies

ELISA A

ELISA B

54 LAC positive patients

(Devreese et al, Thromb Haemost 2011)
Diagnostic value of LAC, aCL, β2GPI antibodies

- Antibody profiles (Pengo et al, 2005)
  - LAC, aCL, β2GPI antibodies
  - LAC+ aCL and β2GPI antibodies same isotype (IgG) = high risk for thrombosis

Positivity on multiple assays (LAC/CL-ELISA/direct β2GPI-ELISA) is associated with an increased risk of thrombosis and pregnancy complications.

Diagnostic value of LAC, aCL, β2GPI antibodies

Miyakis et al 2006: Classification into subcategories:

I: more than one laboratory criterion present
IIa: LAC present alone
IIb: aCL present alone
IIc: aβ2GPI present alone

Pengo et al 2010: type and number of positive assays

I: triple positivity (LAC, aCL and aβ2GPI)
II: double positivity (aCL and aβ2GPI)
III: single positivity

(Pengo et al, Lupus 2010)
## Diagnostic value of LAC, aCL, ß2GPI antibodies

### Triple positivity

- Cumulative increasing incidence of thrombosis (recurrence)
- Carriers are at risk for a first event

<table>
<thead>
<tr>
<th>Triple positivity</th>
<th>Marked association with thrombosis</th>
</tr>
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<tbody>
<tr>
<td>(LAC, aCL, ß2GPI)</td>
<td>Clear definite APS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Double positivity</th>
<th>Lower risk?</th>
</tr>
</thead>
<tbody>
<tr>
<td>(aCL, ß2GPI)</td>
<td>Classification as APS?</td>
</tr>
</tbody>
</table>

| Single positivity | |
|-------------------| |

**APS diagnosis**
Diagnostic value of LAC, aCL, β2GPI antibodies

• Results depend on quality and standardization of assays!
• Interassay variability
  • Methodological shortcomings
    – LAC: progress++: ISTH SSC updated guidelines
    – aCL and αβ2GPI: more guidelines and standardization needed

• Relate lab results to clinical symptoms
  – Integration and interaction of laboratory and clinician
  – Clinical probability for APS/ laboratory probability for APS
Laboratory diagnosis of the APS

- Perform all three assays: LAC, aCL, β2GPI AB

- Antibody profiles
  - LAC, aCL, β2GPI antibodies

- Medium/ high titers
- IgG > IgM > IgA

- Persistent antibodies (> 12 weeks)
“What is the opposite of ‘Eureka!’ ?”

Thank you for your attention