Traceability expectations for autoimmune testing

The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)
Harmonisation of Autoantibody Testing Working Group

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Protein Reference Unit
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London

With thanks to
• The patients who generously donated their samples
• IFCC and IRMM
• The members of the WG-HAT
  • Ingrid Zegers (IRMM)
  • Allan Wiik
  • Pier Luigi Meroni
• The companies for their support and participation
  • Dr. Heinz Schimmel, Dr. Evanthia Monogioudi, Dr. Gustavo Martos-Sevilla Dr. Dana Hutu from the IRMM
  • Dr. Emma Tuddenham from St. George’s
Autoantibody testing..... what are we trying to do?

⭐ detect or quantify

⭐ IgG antibodies (or IgA, IgM)
⭐ to cell or tissue components “antigens”

⭐ support or exclude diagnosis
⭐ monitor disease
⭐ suggest prognosis
# Autoantibody testing

<table>
<thead>
<tr>
<th>Time</th>
<th>Development</th>
<th>Driver</th>
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</thead>
<tbody>
<tr>
<td>1970</td>
<td>Indirect immunofluorescence (IIF) for autoantibodies</td>
<td>• Important “minimally” invasive test to support diagnosis of autoimmune disease</td>
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<tr>
<td></td>
<td></td>
<td>• Improving patient diagnosis and management</td>
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<tr>
<td>1980 - 1990s</td>
<td>Manual ELISA based assays for autoantibodies</td>
<td>• Support IIF and add some Specificity to the sensitive but less specific IIF results</td>
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<td>General process of screen by IIF and follow on with ELISA for specific antibodies to relevant antigens</td>
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<tr>
<td>2000-date</td>
<td>• Development of automated immunoassay analyses for autoantibodies</td>
<td>• Increasing workload</td>
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<tr>
<td></td>
<td>• Development of multiplex methods to screen for multiple autoantibodies</td>
<td>• De-skilling</td>
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<tr>
<td></td>
<td></td>
<td>• Enthusiasm for “not missing” anything</td>
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<tr>
<td></td>
<td></td>
<td>• “Because we can”</td>
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<tr>
<td></td>
<td>• Increase in number of “quantitative” tests done</td>
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<tr>
<td></td>
<td>• Labs doing <em>automated</em> quantitative tests alone</td>
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<td></td>
<td>• Increasing use of multiplex tests</td>
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</tbody>
</table>
Detect - sensitive

Various substrates
- ethanol fixed neutrophils
- HEp2 cells
- Monkey kidney

Reported as
- Neg/pos
- Pattern
  - Homogeneous, speckled
  - c-ANCA or p-ANCA
  - Titre or weak, strong, very strong etc.

Follow-up testing
- (more) specific
- ELISA based assays

- Subjective
- Skilled
- Hard to automate

ANCA negative
- cANCA staining pattern
  - Antibodies to proteinase 3
- pANCA staining pattern
  - Antibodies to myeloperoxidase

ANA negative
- ANA homogeneous staining pattern
  - Antibodies to ds or ss DNA
- ANA speckled staining pattern
  - Antibodies to the extractable nuclear antigens

GBM negative
GBM positive
Quantify – (more) specific

Possible advantages
- ELISA based assays
- Multiplex assays
- Numerical result
- Less Subjective
- Easier to automate

Disadvantages
- If you are giving number you need a standard
- Arbitrary values (although units include IU/ml, IU/L, U/ml, U/L)
- Values infer information that is not supportable
  - Patients with the same “concentration” of antibody may have completely different clinical features
  - Higher concentration worse disease is not true for many auto-antibodies
- Various reference ranges and clinical “cut-off” values
- Marked methodological variation
Autoantibodies – when concentration matters

**Pathogenic**
- antigen on cell surface and only on target cells
- concentration related to disease activity
- Only found in patients with the disease
- transfer disease with antibody – either by giving affected serum or across placenta

**Non-pathogenic**
- antigen in cytosol and in cells unrelated to disease
- concentration unrelated to disease activity
- seen in healthy and with other diseases
- no transfer of disease with antibody

**CONCENTRATION**
- Presence/absence
Is there a problem?
Used with permission of UKNEQAS
Antibodies to myeloperoxidase, known positive sample – distribution of method means (n=38)

- Patients and clinicians move from one hospital to another
- A positive results potentially varying by 10x or 100x or 1000x is NOT SAFE
- Clinicians may not know of this variability
- Patients get different results depending on where the samples is analysed

Inappropriate interpretation of results
Inappropriate diagnosis, management or treatment

Range of method means for IgG anti MPO concentrations U/ml or IU/ml
Autoantibody testing…. the challenges

No robust reference materials

Antibody – variations between patients, during disease, affinity and avidity, comparability with assay standard etc.

Antigen variation - purified, synthetic, degraded, lot to lot variation

Method variation - dilution, diluent, manual, automated, conjugate, capture, direct etc.

Detection system - IgG, IgG & IgM, IgA, IgG subclasses, reactivity of detection antibody

We use arbitrary units because then all our assays look the same

We have bigger worries with glucose or TSH or...

Does it really matter…its OK, we understand the results

It will never work

It is too complicated… and we need to use this method/analyser
Challenge 1 – antibody

Binding of antibodies to antigens is variable – affinity and avidity

★ some patients make high affinity antibodies that bind very tightly
  • form stable complexes in vitro and in vivo
  • often are damaging e.g. through complement activation
  • are resilient to changes in temperature, ionic strength, pH etc.

★ some patients make low affinity antibodies that do not bind tightly
  • do not form very stable complexes
  • not so damaging
  • the complex can be separated by minor changes in temperature, ionic strength, pH etc.

★ the behaviour is not consistent through the disease course
★ the antibody used to “standardise” the method is unlikely to be representative of all patients auto-antibodies
★ QC materials are unlikely to be representative of patients samples
Challenge 2 – antigen

★ Purified
  • extracted from mammalian tissue
  • purification with heat, cold, salt, alcohol etc. may alter structure or denature
  • contaminated with other proteins and antigens
  • stability of preparations
  • reproducibility of preparations
  • expression of relevant antigenic epitopes

★ Synthetic
  • not necessarily identical to native (structurally or antigenically)
  • may lack important epitopes

★ Variability
  • Between manufacturers
  • Between lots
Challenge 3 – method variation

Immunoassay

★ ~40 different methods for IgG anti proteinase 3 in UKNEQAS (including “in house”, “others” and “not stated”)
  ★ Manual ELISA
  ★ Automated ELISA
  ★ Automated variants of ELISA
  ★ Multiplex analysis

Various

★ sample dilution
★ Diluent – e.g. variations in ionic strength
★ “capture” – capture antibody bound to “well” to increase sensitivity
★ direct ELISAs
★ Combination of rapid (minutes) and slow (hours) methods
Challenge 4 – detection system

★ What is detected?
★ IgG
★ IgG and IgM
★ IgA

Possible variation in reactivity between
★ Classes of Ig
★ Subclasses of IgG
★ between standards and patient samples reacting to the detection antibody
Robust reference material for the IgG antibody to the antigen

Where to start? Likely to be more than 1 step

Antigen
– may need more detailed characterisation or definition

Detection system

Method – may need more detailed characterisation or definition
IFCC/IRMM
Harmonisation of Autoantibody Testing
Working Group WG-HAT

★ A joint project between the IFCC and IRMM

★ Bring the excellence of the IRMM in preparation, analysis and validation of reference materials to autoimmune serology testing

★ Use similar rigorous protocols as were used on the preparation of ERM DA 470k (protein ref material)
Identified 5 analytes where the concentration was likely to be important – IgG anti:

- Myeloperoxidase
- Proteinase 3
- Glomerular basement membrane
- Cyclic citrullinated peptide
- Cardiolipin/B2 GP1 antibodies
What do we expect of a lab test?
Precise, Accurate, Timely, Clinically useful, CORRECT

Easy analytes e.g. glucose, calcium, where there analyte is well defined and simple

Where we want to be for Autoimmune Serology

Where we are for Autoimmune serology

Difficult analytes e.g. proteins where defining the exact composition is complicated

Adapted from Traceability of Laboratory Test Results, Randox.
Current Autoantibody “reference” materials

No checking homogeneity
No checking of stability

No statement of homogeneity
Not traceable to any higher order material
Mechanism of value assignment is questionable

Used to calibrate our assay
IgG anti MPO The process - briefly

- The raw material: a plasmapheresis material from a patient with antibodies to myeloperoxidase (and relevant clinical findings)
- Plasma converted into serum by the addition of protamine sulphate solution, incubation and centrifugation to remove the fibrin
- Delipidation by incubation with synthetic amorphous silica
- Dialysis against isotonic saline
- pH adjustment
- Preservatives added (sodium azide, benzamidine hydrochloride monohydrate and aprotinin)
- Sterilised through a 0.22µm filter
- 1ml serum transferred into vials under clean room conditions and lyophilised

- Evaluation process
### Characteristics of a Reference Material and ERM DA 476/IFCC

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Explanation</th>
<th>ERM DA 476/IFCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous</td>
<td>Low and stated variability in concentration of the measurand between vials of the material</td>
<td>The uncertainty contribution for potential inhomogeneity is 0.85%</td>
</tr>
<tr>
<td>Stable</td>
<td>The material must be stable over its expected lifespan</td>
<td>The material is stable e.g. during shipment (up to 2 weeks) and the on storage at -20°C and -70°C</td>
</tr>
<tr>
<td>Traceable</td>
<td>Related to a higher order reference material (usually national or international) through an unbroken chain of comparisons, all with stated uncertainty</td>
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<tr>
<td>Commutable</td>
<td>The characteristic of a reference material to behave in a comparable way to the samples (relevant to the intended use of the reference material)</td>
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<tr>
<td>Safe</td>
<td>Chemically and biologically safe (including tested as negative for HIV and Hepatitis B).</td>
<td>The raw material was tested and confirmed as negative for HIV, Hepatitis B and C</td>
</tr>
<tr>
<td>Ethical</td>
<td>Where relevant, samples from patients have been collected ethically and with appropriate agreement from the patients.</td>
<td>Consent given by patients for their material to be used</td>
</tr>
<tr>
<td>Available</td>
<td>There must be sufficient material that is readily available to relevant laboratories or companies over a time period of approx. 5-10 years. Produced with sufficient documentation to reproduce a comparable material when necessary.</td>
<td>Available from the IRMM</td>
</tr>
<tr>
<td>Certified</td>
<td>Ideally, reference material should be certified with stated uncertainties of the various characteristics</td>
<td>Certified in April 2015</td>
</tr>
</tbody>
</table>
Certified

Ideally, reference material should be certified with stated uncertainties of the various characteristics.

ERM-DA476/IFCC
★ IgG anti MPO
★ Certified value 84mg/L
★ Uncertainty 9mg/L
IgG anti MPO
Traceable

The International Unit – only usable with WHO support

★ used to compare the biological activity of different preparations of the same basic substance e.g. vitamins, hormones, vaccines etc.

★ The mass or volume that constitutes one International Unit varies based on which substance is being measured.

★ The WHO Expert Committee on Biological Standardisation provides a reference preparation of the agent, arbitrarily sets the number of IUs contained in that preparation, and specifies a biological procedure to compare other preparations of the same agent to the reference preparation.

★ The number of IUs contained in a new substance is arbitrarily set, there is no equivalence between IU measurements of different biological agents.

★ Vitamin A: 1 IU is the equivalent of 0.3 μg retinol, or 0.6 μg beta-carotene
★ Vitamin C: 1 IU is 50 μg L-ascorbic acid

★ Does the “arbitrary” International Unit meet our need for a TRACEABLE reference material? Is there anything that can?
ERM-DA470k/IFCC

Produced by the IRMM

Collaboration with Dade Behring (Marburg) and 20 laboratories across Europe

ERM-DA470K/IFCC distributed under strict transport guidelines to participating labs

Value transfer protocol detailed and strict

Storage, reconstitution, pipettes, balances, volumes, timing, operators, reagents, QC, assay performance etc.

Closed and open systems used for value transfer

Specific investigations on particular issues

CERTIFICATE OF ANALYSIS

ERM®-DA470k/IFCC

<table>
<thead>
<tr>
<th>Protein (HPT)</th>
<th>Certified value</th>
<th>Uncertainty</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-macroglobulin (A2M)</td>
<td>1.43 ±1</td>
<td>0.06</td>
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<tr>
<td>α2-acid glycoprotein (AAG)</td>
<td>0.617 ±5</td>
<td>0.013</td>
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<tr>
<td>α1-antitrypsin (AAT)</td>
<td>1.12 ±4</td>
<td>0.053</td>
</tr>
<tr>
<td>albumin (ALB)</td>
<td>37.2 ±6</td>
<td>0.2</td>
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<tr>
<td>complement 3c (C3c)</td>
<td>1.00 ±4</td>
<td>0.04</td>
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<tr>
<td>complement 4 (C4)</td>
<td>0.102 ±4</td>
<td>0.007</td>
</tr>
<tr>
<td>haptoglobin (HPGT)</td>
<td>0.850 ±4</td>
<td>0.021</td>
</tr>
<tr>
<td>immunoglobulin A (IgA)</td>
<td>1.80 ±4</td>
<td>0.05</td>
</tr>
<tr>
<td>immunoglobulin G (IgG)</td>
<td>9.17 ±4</td>
<td>0.18</td>
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<tr>
<td>immunoglobulin M (IgM)</td>
<td>0.723 ±4</td>
<td>0.027</td>
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<tr>
<td>transferrin (TRF)</td>
<td>2.36 ±5</td>
<td>0.06</td>
</tr>
<tr>
<td>transferrin receptor (TTR)</td>
<td>0.200 ±5</td>
<td>0.018</td>
</tr>
</tbody>
</table>

1) When the material is reconstituted according to the specified procedure (see page 3).
2) The certified values are the unweighted means of 6-14 accepted mean values, independently obtained by 5-14 laboratories, using ERM-DA470 as calibrator (Blauert et al., EUR 1523 and 15862 European Communities, Luxembourg (1993)).
3) Calculated using the 
4) The certified mass concentration is traceable to the eKg @ the National Measurement Institute of the USA (USNRC 13957/5C (Bodner et al., Am. J. Clin. Pathol. 77 (1992) 12-19)) used as calibrator in the calibration of the mass concentration in ERM-DA470, applying the procedures described in the certificate of ERM-DA470 and in the report for ERM-DA470/IFCC.
5) The certified value in the calibrator ERM-DA470 was obtained by calibration with a pure protein preparation (DMR-2, NIST, Chem. Lab. Med 69 (2001) 1050 - 1067). Consequently, the certified value in ERM-DA470/IFCC is traceable to the International System of Units (SI) via ERM-DA470, applying the procedures described in the certificate report of ERM-DA470 (see point 2) and in the report for ERM-DA470/IFCC.

This certificate is valid for one year after purchase.

Sales date:
The minimum amount of sample to be used is 2 µL. Accepted as an ERM®, Geel, July 2008

Signed:

Prof. Dr. Hendrik Eroms
Unit for Reference Materials
EC-DG JRC-IRMM
Rechainweg 111
2440 Geel, Belgium

Registration No. 205-REST
ISO Guide 34 for the production of reference materials

Page 1 of 4
IgG anti MPO
Traceable

- We are measuring IgG…with specific antibody activity against myeloperoxidase

- The value assignment of IgG anti MPO was done using:
  - with dilutions of the candidate reference materials
  - Purified IgG anti MPO
    - affinity chromatography using a protein A column
    - Hi-trap column using purified human myeloperoxidase
    - Superdex 200 10/300 column
    - Confirmation of purity of material
  - Dilutions of ERM-DA470k/IFCC (CRM for IgG)

- These materials were measured under strict protocols by a variety of methods
IgG anti MPO Value assignment

- The affinity purified Abs or monoclonals can be assigned values that are traceable to the SI (via traceability to ERM-DA470k or UV-absorption measurements) - VITAL

- They can be used to make the values in the matrix material traceable to the SI.

- Certified values 84 mg/L (uncertainty 9mg/L)
Preliminary commutability study for Myeloperoxidase antibodies

Numerical recalibration of values for clinical samples using a conversion factor based on results for a candidate reference material (RM 5)

- good convergence for 6 out of 7 methods
- outliers remain and become more evident
  - this problem can not be solved by recalibration
IgG anti MPO Commutable

The characteristic of a reference material to behave in a comparable way to the samples (relevant to the intended use of the reference material)
The characteristic of a reference material to behave in a comparable way to the samples (relevant to the intended use of the reference material)

IgG anti MPO Commutable

- Different formats of the reference material, all based on the same raw material have been tested and have been shown to be commutable for combinations of SEVEN methods
- It is expected that ERM-DA476/IFCC will be commutable for the majority of IgG anti MPO methods
- If another method is used, then commutability should be verified
IgG anti MPO Commutable

The characteristic of a reference material to behave in a comparable way to the samples (relevant to the intended use of the reference material)

Correlation coefficients 2nd commutability study

<table>
<thead>
<tr>
<th></th>
<th>Wieslab C</th>
<th>Phadia EliA</th>
<th>Euro-immune</th>
<th>Varelisa</th>
<th>Orgentec</th>
<th>Quanta Lite</th>
<th>IMMCO</th>
<th>Biorad EliA</th>
<th>Bioflash</th>
<th>Aesku</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wieslab C</td>
<td>0.29</td>
<td>0.71</td>
<td>0.58</td>
<td>0.60</td>
<td>0.55</td>
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<td>0.95</td>
<td>0.90</td>
<td>0.84</td>
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<td>0.80</td>
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<td>Biorad EliA</td>
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<td>Bioflash</td>
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<td>Patient population (n) vs comparison group (n)</td>
<td>Method</td>
<td>Sensitivity (%)</td>
<td>Specificity (%)</td>
<td>PPV (%)</td>
<td>NPV (%)</td>
<td>AUC/ROC (Range)</td>
<td>Comments</td>
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<td>GPA (86) vs non-vasculitic disease (450)28</td>
<td>IFT</td>
<td>92</td>
<td>99</td>
<td>Nd</td>
<td>Nd</td>
<td>0.96 (0.94–0.98)</td>
<td>Histological diagnosis Retrospective study</td>
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<td>Direct PR3-ANCA ELISA</td>
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<td>60</td>
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<td>0.80 (0.76–0.83)</td>
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<td>Capture ELISA</td>
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<td>0.86 (0.82–0.89)</td>
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<td>96</td>
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<td>GPA (232) vs inflammatory diseases (661)23</td>
<td>IFT</td>
<td>77.9</td>
<td>90.9</td>
<td>73</td>
<td>93</td>
<td>Nd</td>
<td>Histological diagnosis Prospective study</td>
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<td>Anchor ELISA</td>
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<td>97.4</td>
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<td>GPA (59*) vs inflammatory and infectious diseases (585)30</td>
<td>Hn–hr PR3-ANCA ELISA</td>
<td>94</td>
<td>99 (predefined)</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Histological diagnosis Retrospective study</td>
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<tr>
<td>Capture ELISA</td>
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<tr>
<td>Direct (hn) PR3-ANCA</td>
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<td>GPA (34) vs SLE (65)21</td>
<td>Direct PR3-ANCA</td>
<td>97.1</td>
<td>98.4</td>
<td>Nd</td>
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<td>0.999 (0.947–1.00)</td>
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<td>Anchor ELISA</td>
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<td>GPA (40) vs RA or SLE (20)22</td>
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<td>62.5</td>
<td>95–100</td>
<td>Nd</td>
<td>Nd</td>
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<td>Histological diagnosis Retrospective study</td>
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<tr>
<td>Direct PR3-ANCA (n=5 kits)</td>
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<td>45–55</td>
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<td>Capture ELISA</td>
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<td>GPA (55) vs suspected vasculitis (175)23</td>
<td>IFT</td>
<td>69.1</td>
<td>95.4–96.4</td>
<td>Nd</td>
<td>Nd</td>
<td>0.856–0.879</td>
<td>Clinical diagnosis Retrospective study</td>
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<td>Direct PR3-ANCA ELISA (n=2 kits)</td>
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<td>61.8–72.7</td>
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<td>Capture ELISA</td>
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<td>Other assays (n=2)</td>
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<td>72.7–74.5</td>
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*47 of 59 patients in the GPA group had a cytoplasmic ANCA pattern on IFT. Abbreviations: ANCA, antineutrophil cytoplasmic antibody; AUC, area under the curve; GPA, granulomatosis with polyangiitis; hn, human native; hr, human recombinant; IFT, Indirect Immunofluorescence technique; MPA, microscopic polyangiitis; MPO, myeloperoxidase; Nd, not determined; NPV, negative predictive value; PPV, positive predictive value; PR3, proteinase 3; RA, rheumatoid arthritis; ROC, receiver operating characteristics; SLE, systemic lupus erythematosus.
Standardization in autoimmune testing
IFCC/JRC-IRMM WG-HAT

- We have made huge advances
- We are close to well defined processes for producing robust, traceable reference material for autoantibody testing
- Further materials will be prepared to similar protocols reducing lead-time

Future

- Introducing the materials will be a challenge
- Once embedded, we will need to evaluate the impact on results and EQA and consider further harmonisation or better definition of:
  - Antigen type/source
  - Method
  - Detection system
We can improve the numbers....

Introduction and adoption of traceable commutable reference materials should reduce the variability in the values for autoantibody measurements.

It will not solve the inherent variability in the values given by certain patient samples in different methods.

It should help identify methodological outliers and guide improvements.