Traceability expectations for autoimmune testing

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

Dr. Joanna Sheldon
Protein Reference Unit
St. George's Hospital
London

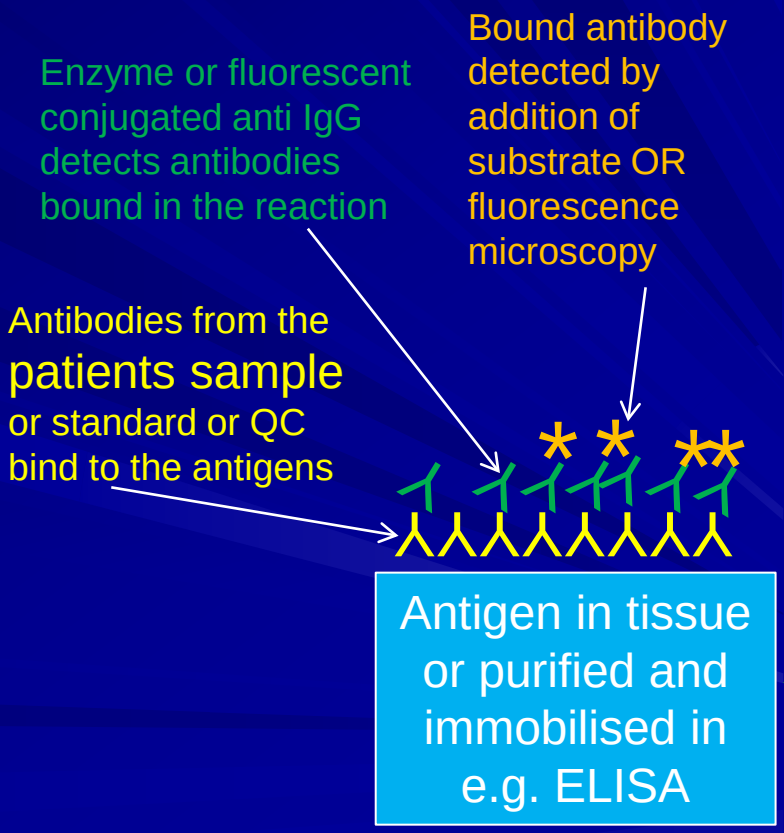
With thanks to
- The patients who generously donated their samples
- IFCC and IRMM
- The members of the WG-HAT
  - Ingrid Zegers (IRMM)
  - Allan Wiik
  - Pier Luigi Meroni
  - The companies for their support and participation

- 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

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

Detect - sensitive

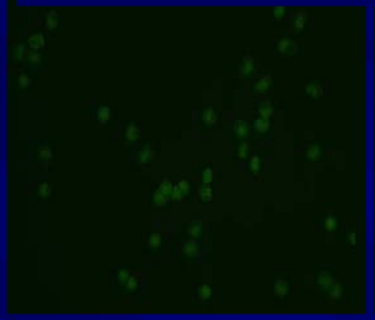
Various substrates
- ethanol fixed neutrophils
- HEp2 cells
- Monkey kidney

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

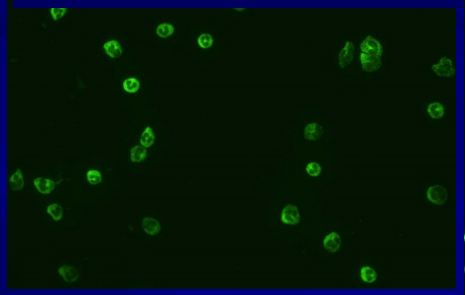
Follow-up testing
- (more) specific
- ELISA based assays

- Subjective
- Skilled
- Hard to automate

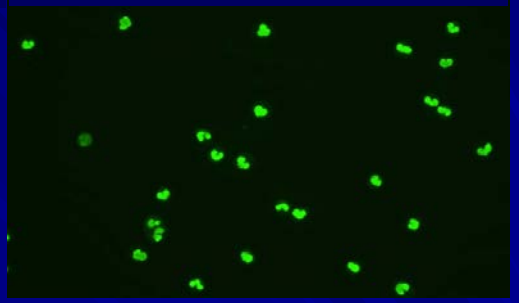
ANCA negative



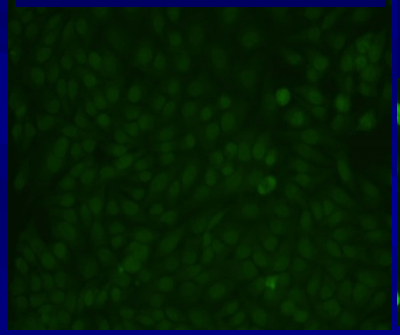
cANCA staining pattern
Antibodies to proteinase 3



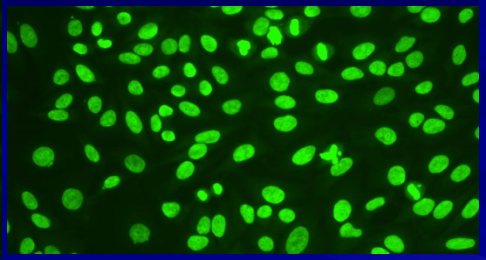
pANCA staining pattern
Antibodies to myeloperoxidase



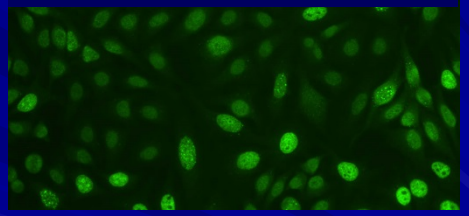
ANA negative

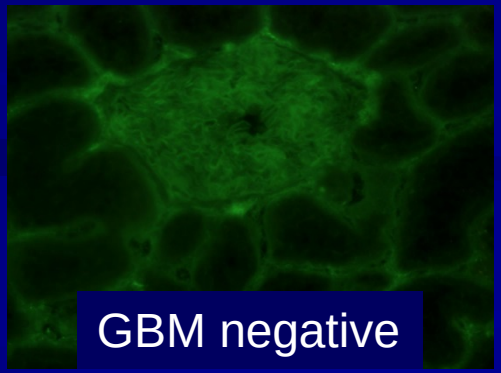


ANA homogeneous staining pattern
Antibodies to ds or ss DNA

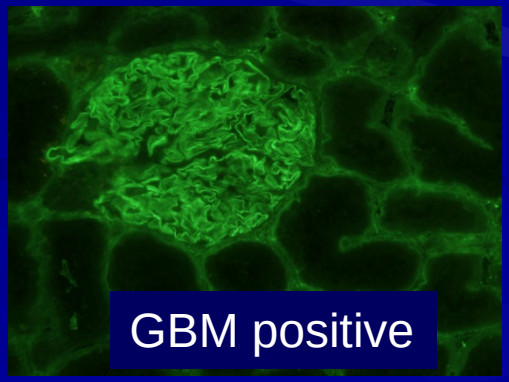


ANA speckled staining pattern
Antibodies to the extractable nuclear antigens





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 may 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

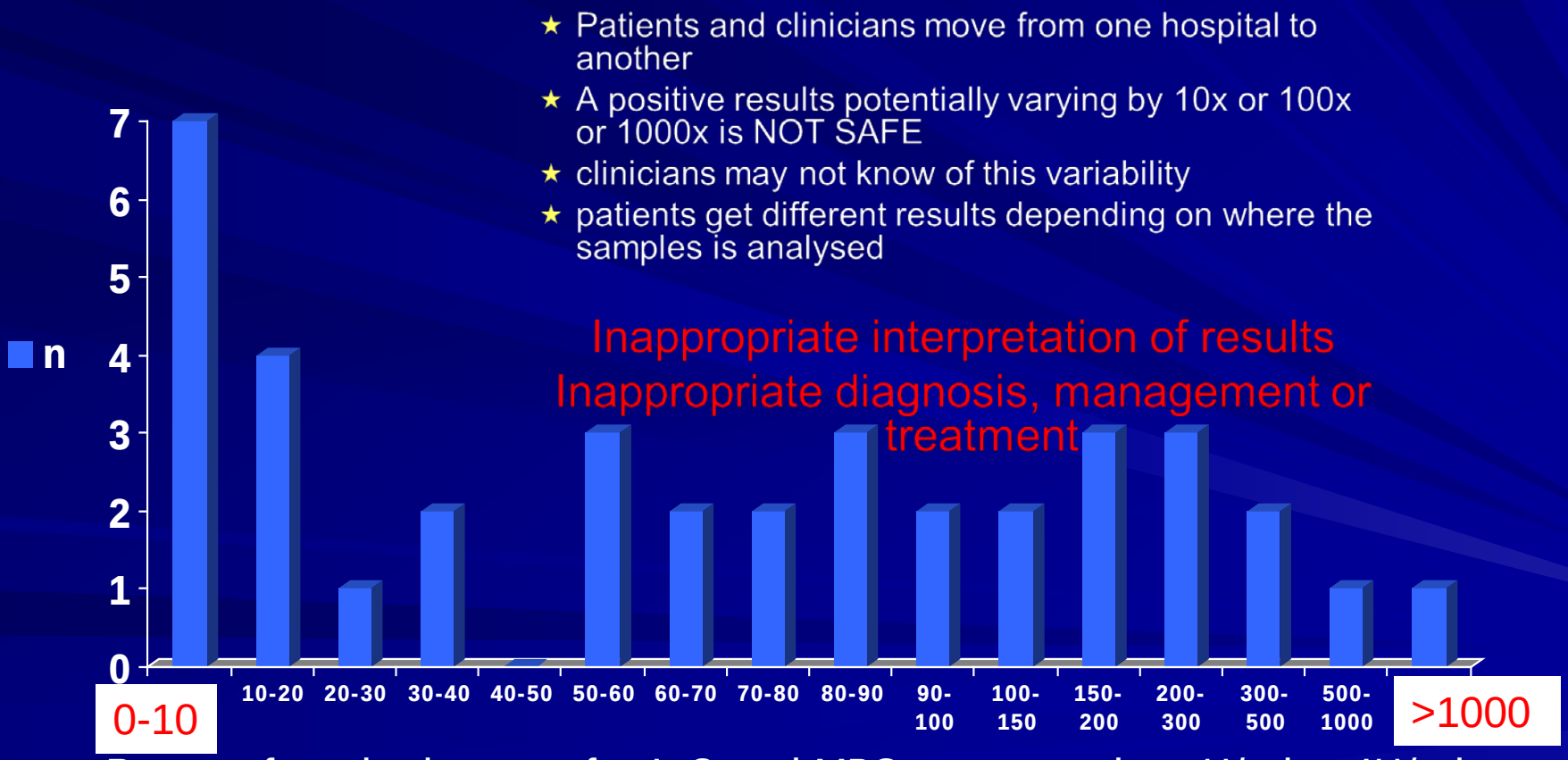
CONCENTRATION

Presence/absence

Is there a problem?

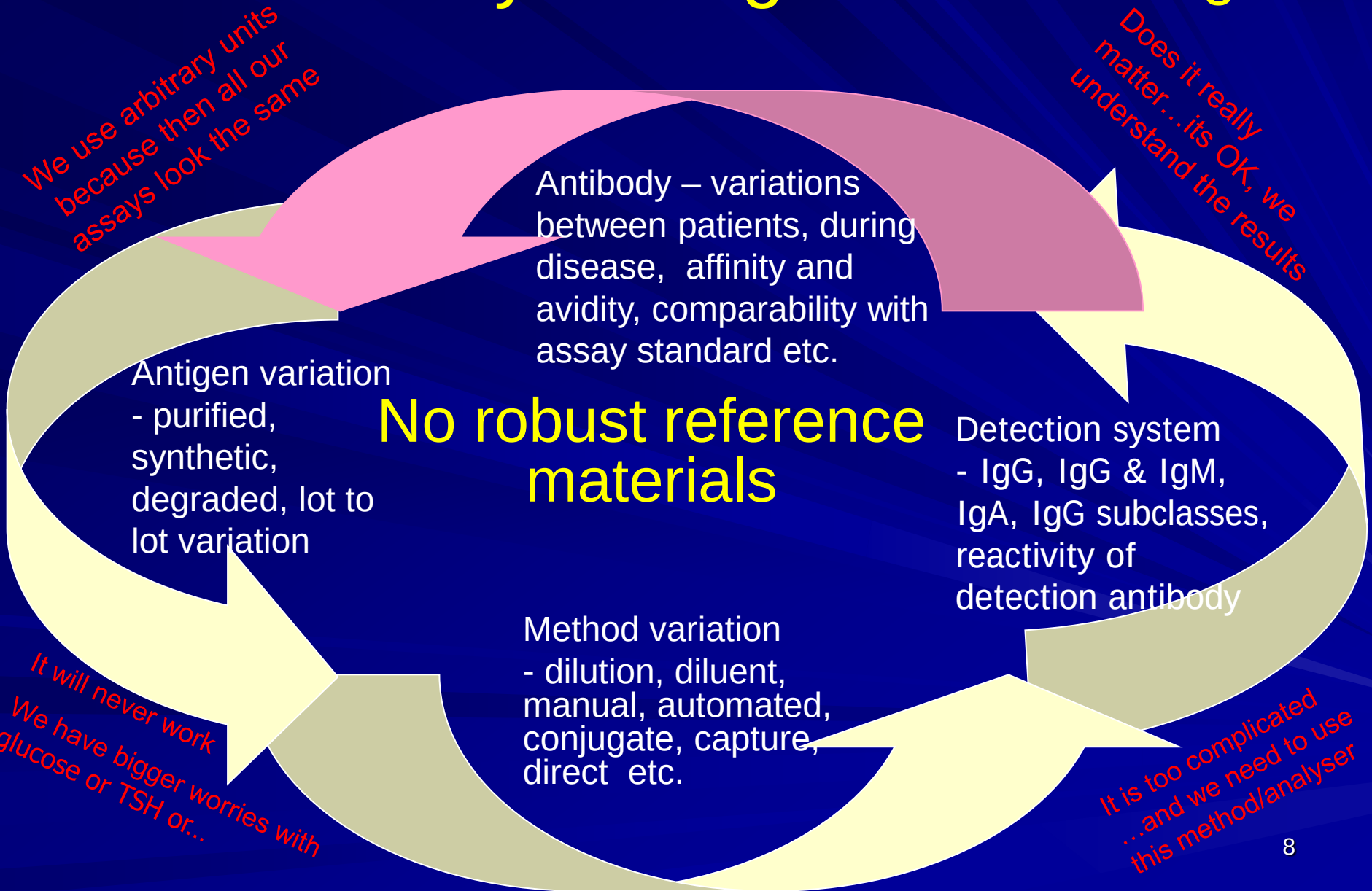
Used with permission of UKNEQAS

Antibodies to myeloperoxidase, known positive sample – distribution of method means (n=38)



Range of method means for IgG anti MPO concentrations U/ml or IU/ml

Autoantibody testing…. the challenges



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)

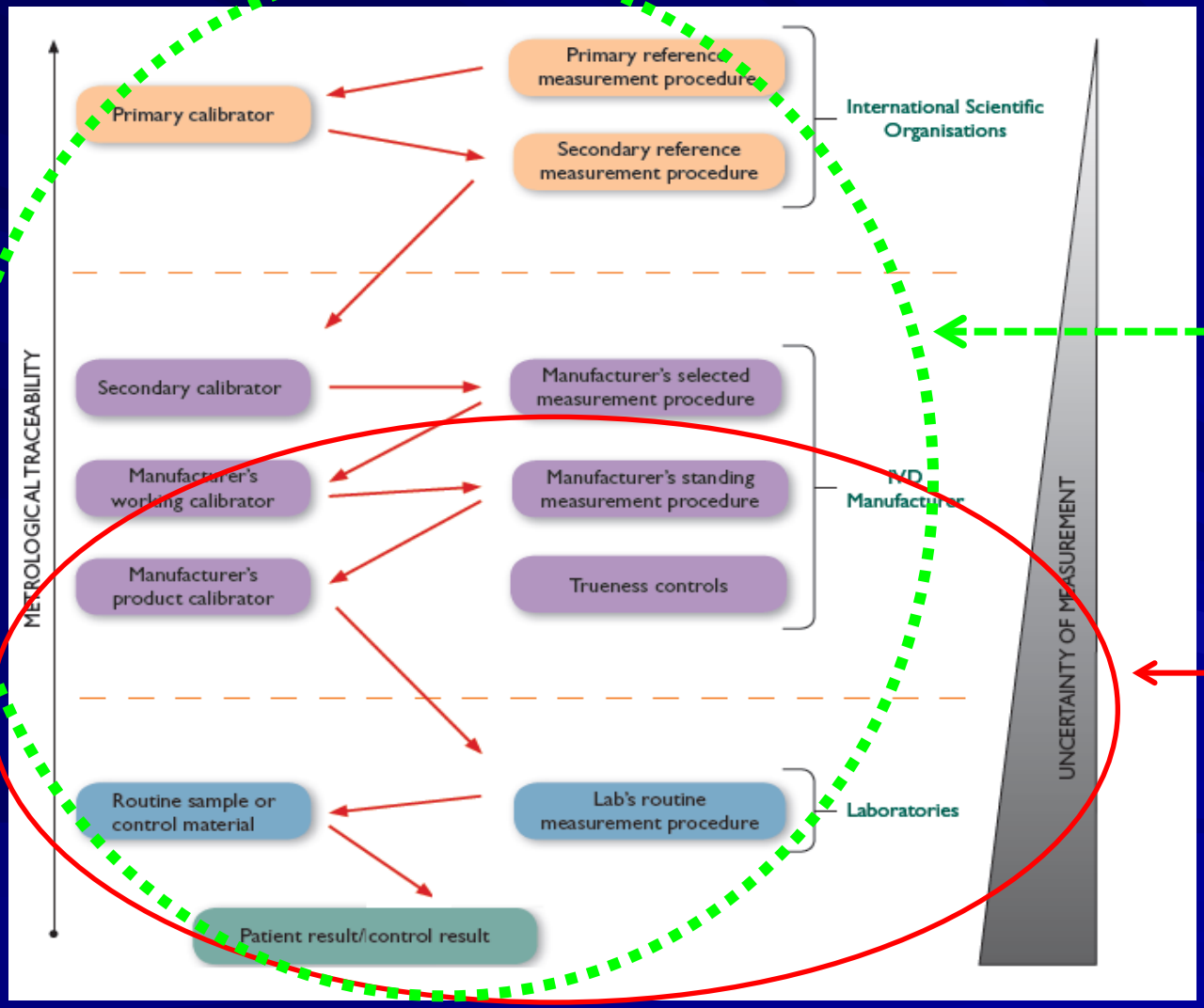
IFCC/IRMM WG-HAT

Identified 5 analytes where the CONCENTRATRION 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 **use**ful, 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.

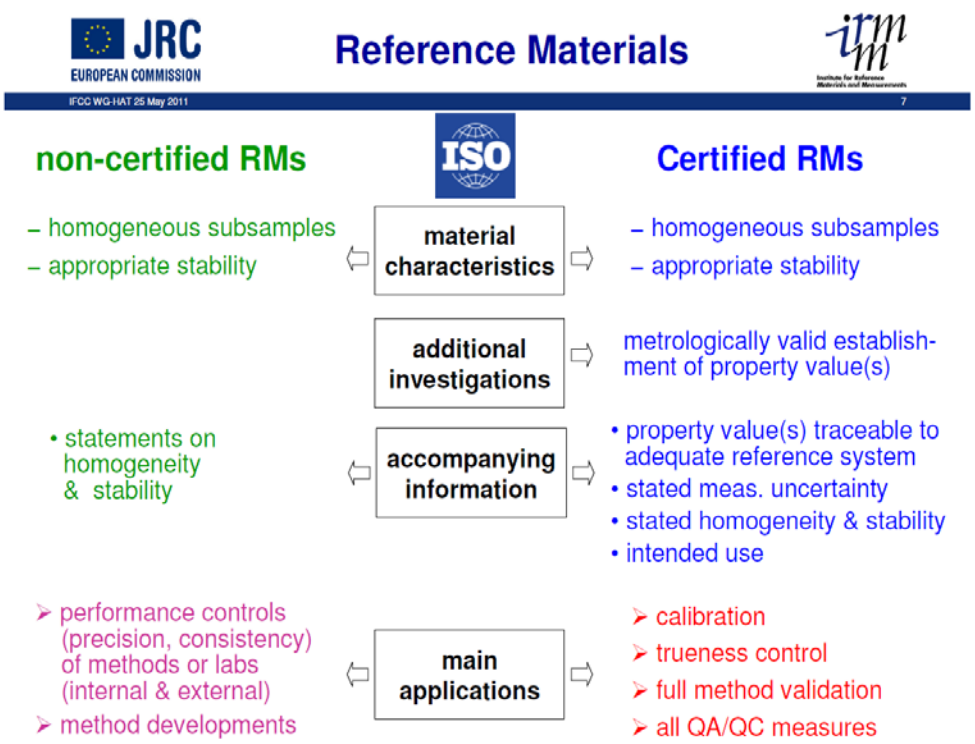
Reference Materials

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

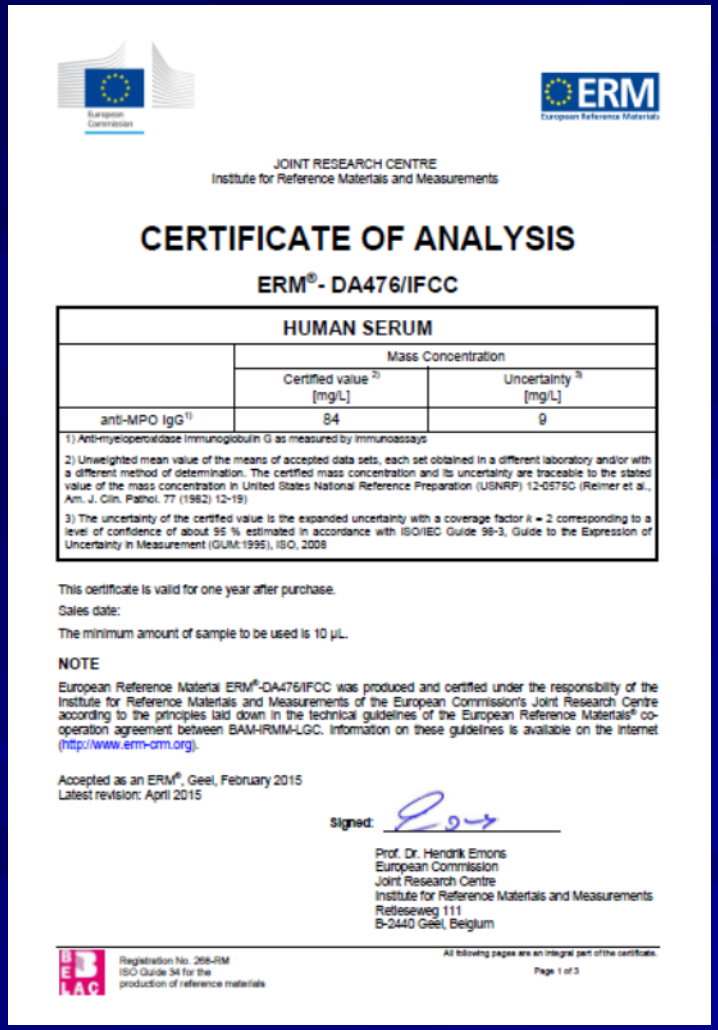
- Evaluation process

Characteristics of a Reference Material and ERM DA 476/IFCC

Characteristic	Explanation	ERM DA 476/IFCC
Homogeneous	Low and stated variability in concentration of the measurand between vials of the material	The uncertainty contribution for potential inhomogeneity is 0.85%
Stable	The material must be stable over its expected life-span	The material is stable e.g. during shipment (up to 2 weeks) and the on storage at -20oC and -70oC
Traceable	Related to a higher order reference material (usually national or international) through an unbroken chain of comparisons, all with stated uncertainty	
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)	
Safe	Chemically and biologically safe (including tested as negative for HIV and Hepatitis B).	The raw material was tested and confirmed as negative for HIV, Hepatitis B and C
Ethical	Where relevant, samples from patients have been collected ethically and with appropriate agreement from the patients.	Consent given by patients for their material to be used
Available	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.	Available from the IRMM
Certified	Ideally, reference material should be certified with stated uncertainties of the various characteristics	Certified in April 2015

Certified

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



JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements

CERTIFICATE OF ANALYSIS

ERM®- DA476/IFCC

HUMAN SERUM		
	Mass Concentration	
	Certified value [2] [mg/L]	Uncertainty [3] [mg/L]
anti-MPO IgG[1]	84	9

1) Anti-myeloperoxidase Immunoglobulin G as measured by immunoassays

2) Unweighted mean value of the means of accepted data sets, each set obtained in a different laboratory and/or with a different method of determination. The certified mass concentration and its uncertainty are traceable to the stated value of the mass concentration in United States National Reference Preparation (USNRP) 12-0575C (Reimer et al., Am. J. Clin. Pathol. 77 (1982) 12-19)

3) The uncertainty of the certified value is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008

This certificate is valid for one year after purchase.

Sales date:

The minimum amount of sample to be used is 10 µL.

NOTE

European Reference Material ERM®-DA476/IFCC was produced and certified under the responsibility of the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre according to the principles laid down in the technical guidelines of the European Reference Materials® co-operation agreement between BAM-IRMM-LGC. Information on these guidelines is available on the internet (http://www.erm-crm.org).

Accepted as an ERM®, Geel, February 2015
Latest revision: April 2015

Signed:

Prof. Dr. Hendrik Emons
European Commission
Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel, Belgium

Registration No. 268-RM
ISO Guide 34 for the production of reference materials

All following pages are an integral part of the certificate.

Page 1 of 3

ERM-DA476/IFCC

- ★ IgG anti MPO
- ★ Certified value 84mg/L
- ★ Uncertainty 9mg/L

IgG anti MPO Traceable

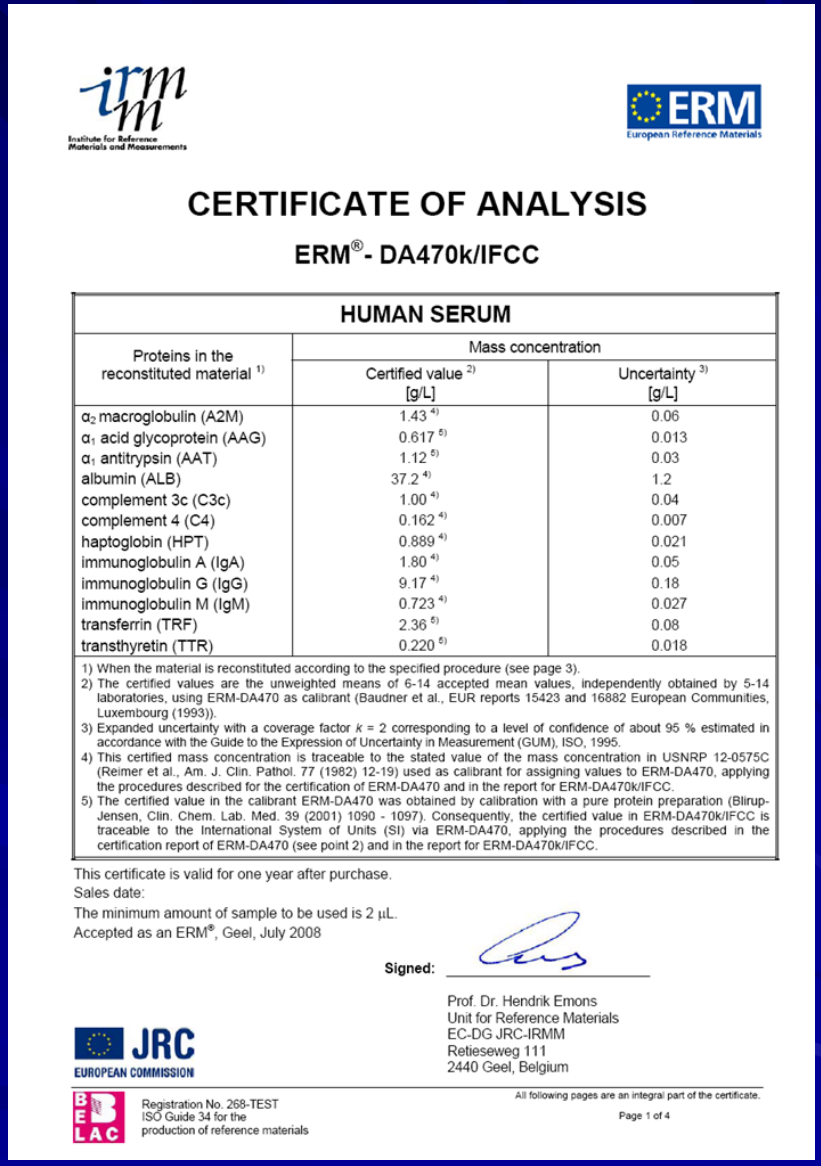
Related to a higher order reference material (usually national or international) through an unbroken chain of comparisons, all with stated uncertainty

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



Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATE OF ANALYSIS

ERM® - DA470k/IFCC

HUMAN SERUM

Proteins in the reconstituted material [1]	Mass concentration	
	Certified value [2] [g/L]	Uncertainty [3] [g/L]
α_2 macroglobulin (A2M)	1.43 [4]	0.06
α_1 acid glycoprotein (AAG)	0.617 [5]	0.013
α_1 antitrypsin (AAT)	1.12 [5]	0.03
albumin (ALB)	37.2 [4]	1.2
complement 3c (C3c)	1.00 [4]	0.04
complement 4 (C4)	0.162 [4]	0.007
haptoglobin (HPT)	0.889 [4]	0.021
immunoglobulin A (IgA)	1.80 [4]	0.05
immunoglobulin G (IgG)	9.17 [4]	0.18
immunoglobulin M (IgM)	0.723 [4]	0.027
transferrin (TRF)	2.36 [5]	0.08
transthyretin (TTR)	0.220 [5]	0.018

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 calibrant (Baudner et al., EUR reports 15423 and 16882 European Communities, Luxembourg (1993)).
3) Expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM), ISO, 1995.
4) This certified mass concentration is traceable to the stated value of the mass concentration in USNRP 12-0575C (Reimer et al., Am. J. Clin. Pathol. 77 (1982) 12-19) used as calibrant for assigning values to ERM-DA470, applying the procedures described for the certification of ERM-DA470 and in the report for ERM-DA470k/IFCC.
5) The certified value in the calibrant ERM-DA470 was obtained by calibration with a pure protein preparation (Blirup-Jensen, Clin. Chem. Lab. Med. 39 (2001) 1090 - 1097). Consequently, the certified value in ERM-DA470k/IFCC is traceable to the International System of Units (SI) via ERM-DA470, applying the procedures described in the certification report of ERM-DA470 (see point 2) and in the report for ERM-DA470k/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 Emons
Unit for Reference Materials
EC-DG JRC-IRMM
Retieseweg 111
2440 Geel, Belgium

JRC EUROPEAN COMMISSION

BELAC Registration No. 268-TEST
ISO Guide 34 for the production of reference materials

All following pages are an integral part of the certificate.

Page 1 of 4

IgG anti MPO Traceable

Related to a higher order reference material (usually national or international) through an unbroken chain of comparisons, all with stated uncertainty

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

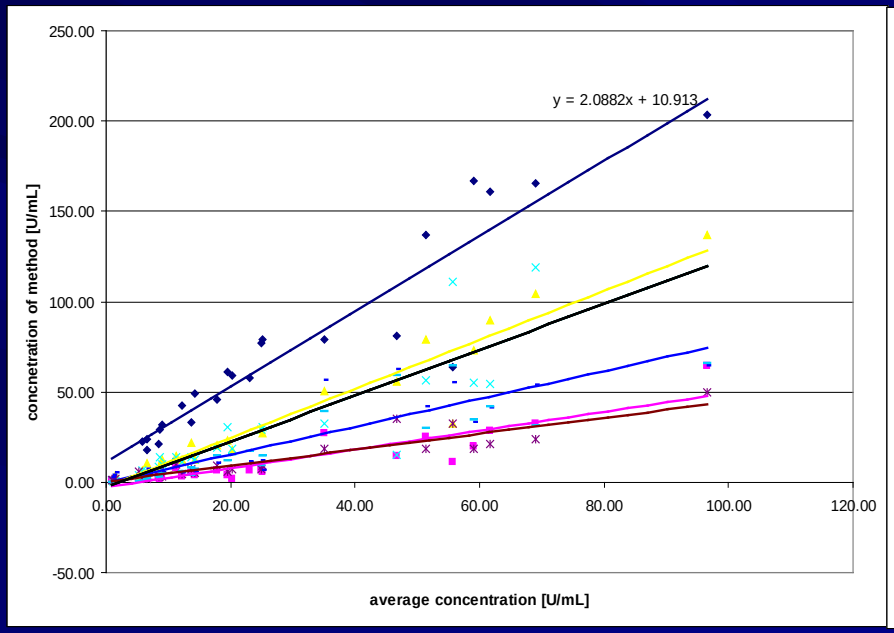
- The value assignment of IgG anti MPO was done using:
  - with dilutions of the candidate reference materials
  - Purified IgG anti MPO
    - affinity chromatography using a protein A column
    - Hi-trap column using purified human myeloperoxidase
    - Superdex 200 10/300 column
    - Confirmation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

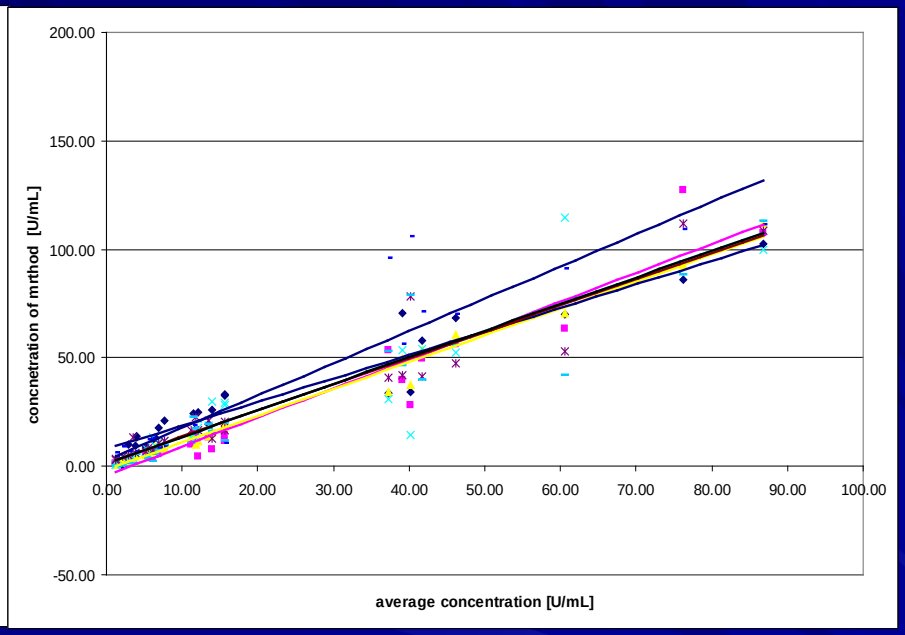
- These materials were measured under strict protocols by a variety of methods

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



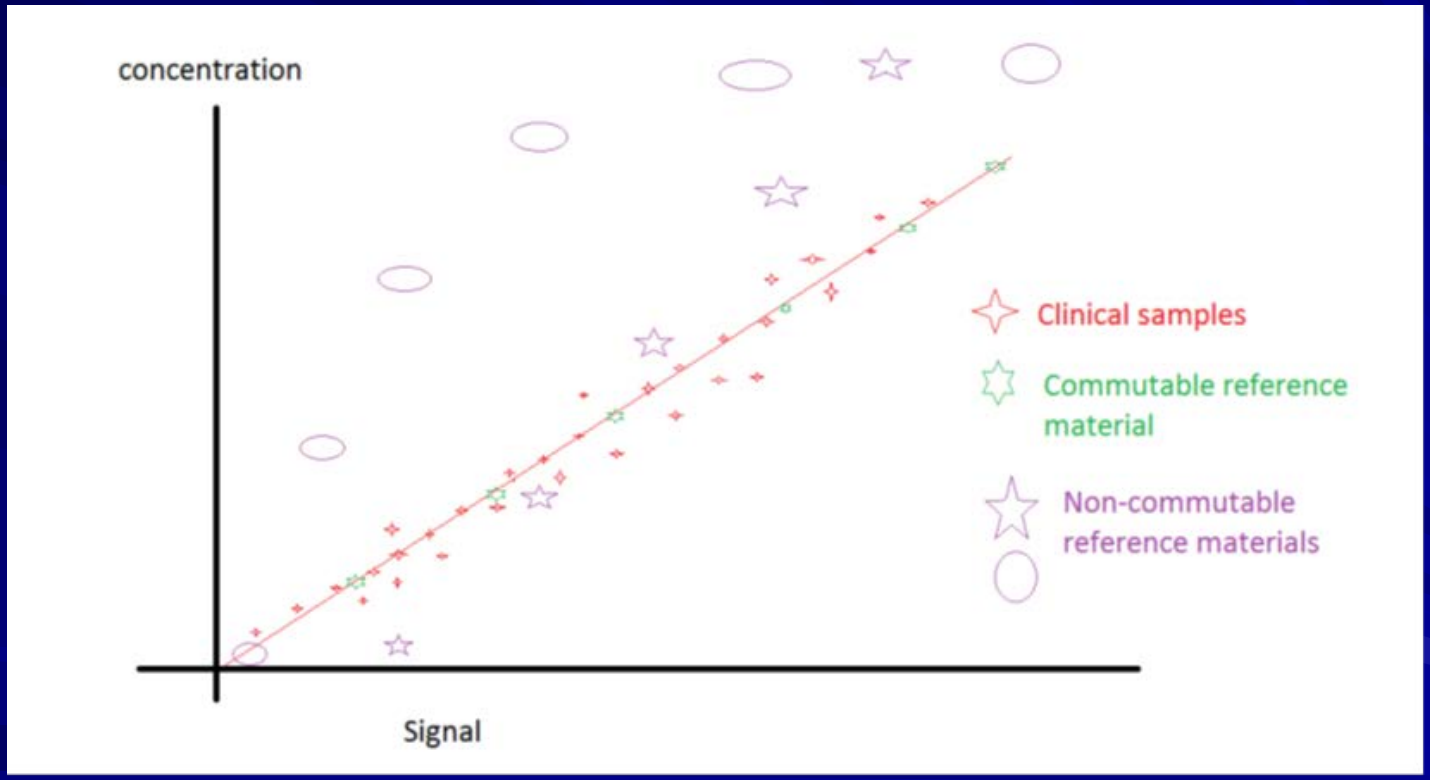


Numerical recalibration of values for clinical samples using a conversion factor based on results for a candidate reference material (RM 5)

- 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)



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)

- ★ 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

	Wieslab C	Phadia EliA	Euro-immune	Varelisa	Orgentec	Quanta Lite	IMMCO	Biorad EIA	Bioflash	Aesku
Bioplex	0.29	0.71	0.58	0.60	0.55	0.79	0.69	0.65	0.71	0.39
Wieslab C		0.69	0.74	0.84	0.74	0.77	0.55	0.65	0.59	0.79
Phadia EliA			0.83	0.88	0.90	0.94	0.79	0.95	0.90	0.84
Euroimmune				0.79	0.90	0.84	0.66	0.79	0.65	0.80
Varelisa					0.83	0.95	0.79	0.90	0.85	0.87
Orgentec						0.88	0.74	0.89	0.79	0.95
Quanta Lite							0.85	0.93	0.87	0.89
IMMCO								0.88	0.81	0.72
Biorad EIA									0.92	0.84
Bioflash										0.74

Table 1 | Comparison of methods for testing for PR3-ANCA and MPO-ANCA in ANCA-associated vasculitis

Patient population (*n*) vs comparison group (*n*)	Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC/ROC	Comments
GPA (86) vs non-vasculitic disease (450)[28]	IFT Direct PR3-ANCA ELISA Capture ELISA Anchor ELISA	92 60 72 96	99 99 99.3 98.5	Nd	Nd	0.96 (0.94–0.98) 0.80 (0.76–0.83) 0.86 (0.82–0.89) 0.96 (0.94–0.98)	Histological diagnosis Retrospective study
GPA (232) vs inflammatory diseases (661)[29]	IFT Anchor ELISA	77.9 80.4	90.9 97.4	73 88	93 93	Nd	Histological diagnosis Prospective study
GPA (59*) vs inflammatory and infectious diseases (585)[30]	Hn–hr PR3-ANCA ELISA Capture ELISA Direct (hn) PR3-ANCA	94 66 64	99 (predefined)	Nd	Nd	Nd	Histological diagnosis Retrospective study
GPA (34) vs SLE (65)[31]	Direct PR3-ANCA Anchor ELISA	97.1	98.4	Nd	Nd	0.999 (0.947–1.00)	Clinical diagnosis Retrospective study
GPA (40) vs RA or SLE (20)[32]	IFT Direct PR3-ANCA (*n* = 5 kits) Capture ELISA (*n* = 2 kits) Anchor ELISA (*n* = 4 kits)	62.5 45–55 60–62.5 60–62.5	95–100	Nd	Nd	Nd	Histological diagnosis Retrospective study
MPA (40) vs RA or SLE (20)[32]	IFT Direct MPO-ANCA ELISA (*n* = 8 kits) Capture ELISA (*n* = 2 kits) Anchor ELISA (*n* = 1 kit)	82.5 62.5–85 80 75	95–100	Nd	Nd	Nd	Histological diagnosis Retrospective study
GPA (55) vs suspected vasculitis (175)[33]	IFT Direct PR3-ANCA ELISA (*n* = 2 kits) Capture ELISA (*n* = 2 kits) Anchor ELISA (*n* = 3 kits) Other assays (*n* = 2)	69.1 61.8–72.7 70.9–72.7 61.8–72.7 72.7–74.5	100 95.4–96.4 95.9–99.5 98.5–99.0 95.9–97.9	Nd	Nd	Nd 0.856–0.879 0.862–0.878 0.833–0.881 0.878–0.902	Clinical diagnosis Retrospective study

*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.

Csernok, E. & Moosig, F. *Nat. Rev. Rheumatol.* 10, 494–501 (2014); published online 3 June 2014; doi:10.1038/nrrheum.2014.78

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