Plasma proteins standardization: an accomplished fact?

Dr. Joanna Sheldon
PRU St. George’s London
(IFCC Committee on Plasma Proteins)

Protein standardisation

We think we are so clever!

★ we have labs full of fancy equipment
★ we run a huge selection of tests 24/7
★ we use primary tube samples, bar codes, pre-prepared reagents
★ we have standards, QC, QA, accreditation, GLP, delta checking, reflex testing etc.
A question
What is acceptable variation from the “true” value of a test?
±10%
± 7%
± 5%
± 2%
± 1%
0%?

Now think of pay day
What is acceptable variation in your pay?
± 10%, ± 7%, ± 5%, ± 2%, ± 1%, 0%?
…..does it matter – it should even out in the long run
…..what happens if it is consistent bias?
Standardisation – why bother?

- Result today will be the same as tomorrow
- Result in Milan will be the same as the result in London
- We can set reference ranges and decision points
- We all measure to the same set of rules

.........so we can diagnose, monitor and treat patients appropriately

Protein standardisation

Who is from a lab where they measure plasma protein concentrations?

Do you know....
...what your assays are calibrated against?
...when your reference ranges were set?
...how your reference ranges were set?
...where your standards are from?
...where your antiserum is from?
Protein standardisation

**Wisdom**... Taking the data, knowledge and understanding, putting it into a context and making a judgement.

**Understanding**... the process of taking knowledge and synthesize new knowledge from the previously held knowledge.

**Knowledge**... a collection of information so it can be useful.

**Data and information**

**Data**... it simply exists and has no significance beyond its existence.

**Information**... data that has been given meaning.

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Protein standardisation

**Wisdom**... an IgA of 0.06 g/L could be due to an analytical error, to the patient being IgA deficient or having secondary immune suppression. IgA deficiency is fairly common but other causes should be considered and the results interpreted with other immunoglobulin results and EP.

**Understanding**... The reference range means that 95% of people who are normal will have an IgA concentration of between 0.8 and 4.0 g/L so an IgA of e.g. 0.06g/L is low.

**Knowledge**... The adult reference range for IgA is 0.8 – 4.0 g/L.

**Data**... 0.8, 4.0
Protein standardisation

With all the fancy analyses and analysers we still have issues with standardisation.

A recent UKNEQAS distribution the method means for IgA varied from:

0.012 g/L (low) to 5.89 g/L (high)

The lower limit of detection was between 0.02 g/L and 0.7 g/L

Some labs reported the IgA concentration to 4 decimal places!

So what is the problem?

Protein standardisation

Not easy!
★ there is molecular heterogeneity and genetic variability
★ presence of binding proteins
★ the protein needs to be purified from human serum
★ this needs precipitation, heating, alcohol, solvents etc. all can denature the protein.
★ the pure preparation needs to be dried – further degradation and variable water of hydration
★ the pure preparation needs to be weighed and made up accurately – accurate balances and volumetric flasks

We can’t just buy pure IgG and make a 10g/L solution
Protein standardisation

★Whole process is likely to change the protein from its native form
★minimal processing is desirable.

★Now we have recombinant proteins…
★best for proteins with minimal molecular variation
★but these can vary from native proteins
...... so are of limited use.

Protein standardisation

History
WHO 67/86
★used in the early days of protein quantification
★Freeze dried preparation
★Turbid on reconstitution - o.k. for RID or rockets but not good for nephelometry
Protein standardisation

In 1979 the IFCC expert panel on proteins published details of the new protein standard IFCC 74/1.

Protein standardisation

History
IFCC 74/1
★ Prepared to replace WHO 67/99
★ Young male donors 20-30 years
★ Screened for known viruses, hyperlipidaemia and genetic variation e.g. AAT, AAG, C3, TRF
★ Fasted for 18 hours before donation
★ Discard first 20 mls of collection
★ Separate sample from cells within 40 mins
★ Handled aseptically
★ Sodium azide added
★ Liquid frozen in glass ampoules at -70°C
IFCC 74/1

Calibrated for
★ Albumin
★ IgG, IgA and IgM
★ Other proteins gradually added
★ Local material cross calibrated to IFCC 74/1 in 18 countries

IFCC 74/1

Introduction of IFCC 74/1 showed that:
★ a major cause of variation in protein analysed had been the methodologies used
★ using recommended methods with IFCC 74/1 gave between lab variation never before achieved

★ Proposal to designate reference antiserum
Other factors

★there were other reference preps
  - WHO 6HSP and USNRP
★there were big changes in method
  - Automated rate nephelometers
★BUT by the late 80s we should have seen excellent comparability between methods

Unfortunately…..

★EQA showed values for some proteins varied by 100%
★This was often due to variations in the calibrants
★A single international reference preparation was needed
IFCC working group on plasma protein standardisation

★ Project to make a new reference material
★ Produced by the Community Bureau of Reference of the Commission of the European Communities
★ Managed by the IFCC Committee for Plasma Protein Standardisation and the College of American Pathologists
★ Certified Reference Material (CRM) 470

CRM 470 Released in 1993
CRM 470

★ Fresh serum, naturally clotted
★ Several hundred donors across Europe
★ Patient demographics noted
★ Tested for known viruses and RhF, monoclonal proteins
★ Alpha-1 antitrypsin and haptoglobin phenotypes
★ Haemolysis, bilirubin and turbidity
★ Sodium azide added and then frozen to be sent for processing

CRM 470

★ Collections thawed, pooled, delipidated and stabilised
★ Pure CRP added
★ Buffered
★ Sterile filtered
★ Bottled and freeze dried (40,000 ampoules)
★ It was expected that this batch of CRM 470 would last for some years
CRM 470

★ Values assigned against WHO 6HSP and USNRP and WHO CRP
★ 27 labs participated
★ Albumin, ceruloplasmin, C3, C4, IgG, IgA IgM, α2 macroglobulin, α antitrypsin, haptoglobin, CRP
★ Where possible, checked against pure preparations
★ There were some marked differences from existing standards and consequent changes in reference ranges
★ CRM470 was adopted by reagent manufacturers across the world

Effect of CRM 470 on Protein Assay Quality Control

★ Two serum samples with ~35% difference in concentrations were sent in 1992 to national QC programs in Europe and the South Pacific
★ Two new but similar samples were distributed in 2002-3
★ Results are shown as Youdin plots, with the axes the same for both graphs in each case

with thanks to Myron Johnson
Effect of CRM 470 on Protein Assay Quality Control

★ With general usage of CRM 470, uncertainty for the plasma proteins

– Decreased markedly: $\alpha_1$-antitrypsin, haptoglobin, transferrin, C3, C4, IgA, IgG, IgM
– Remained essentially unchanged: orosomucoid ($\alpha_1$-acid glycoprotein), $\alpha_2$-macroglobulin, ceruloplasmin
– Increased: C-reactive protein

$\alpha_1$-Acid Glycoprotein
$\alpha_1$-Antitrypsin

![Graph showing $\alpha_1$-Antitrypsin levels from 1993 and 2002.]

Ceruloplasmin

![Graph showing Ceruloplasmin levels from 1993 and 2002.]
Haptoglobin

\[ \text{Hp} \]

1993

1999

Beckman

DadeBehring

\[ \text{A2M} \]

2002

2002

Beckman

DadeBehring

\[ \alpha_2 \text{-Macroglobulin} \]
**IgG**

**IgM**
### C-Reactive Protein

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<td>0.04</td>
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**CRP 1993**
- Beckman
- Roche
- Abbott

**CRP 2002**
- Ortho, Ilab
- Dako, Bayer
- Olympus, Kone
- Beckman, Orion

### Some Reasons High Uncertainty

- Inadequate accuracy and precision of transfer of values by manufacturers from CRM 470 to controls and calibrators
- Matrix differences among reference materials (CRM 460 is a delipidated pool of normal serum)
- Epitopic differences in proteins (genetic variants, partial catabolism, etc.)
Some recent examples of poor standardisation

★ Ceruloplasmin – marked difference between various methods – probably related to how the antisera behave with fresh and aged sera
★ C4 – a manufacturer incorrectly transferred C4 values to its calibrants giving their C4s significant bias
★ IgG – a manufacturer NEVER checked its value transfers back to CRM 470 and gradually generated a 10% positive bias
★ AAT – a manufacturer consistently shows 10% negative bias

A huge step forward…since 1993

★ 2006 we knew that CRM 470 was running out
  ★ It remained stable and valid but we needed a new preparation
★ IFCC committee for plasma proteins met to collaborate with the IRMM to produce a new reference material
★ Discussions on
  ★ which proteins to include
  ★ whether to use recombinant proteins where necessary
  ★ sample collection protocols
2008 ERM-DA470k

★ Produced by the IRMM
★ Similar protocols to CRM470
★ Normal human serum with added
  ★ CRP
  ★ Recombinant β2 microglobulin
★ Processed, virus tested, pre-tested to proteins, bottled, lyophilised.
★ Value assigned against CRM470 and where possible the pure proteins
★ Value transfer protocols developed
★ Tested as calibrant with samples
★ Stability tested - ongoing

2008 ERM-DA470k

★ Produced by the IRMM
★ Collaboration with Dade Behring (Marburg) and 20 laboratories across Europe
★ Vials of ERM-DA470K 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
Some issues with ERM-DA470k

★ We are doing some final investigations for some proteins
★ The ceruloplasmin problem seen with CRM470 is still seen – likely related to a difference in how the assays recognise fresh or aged serum
★ $\beta2$ microglobulin will be value assigned soon
**Wisdom**... Considering all the components of an analysis to make a judgement about the validity – for the patient today, and in the future. To continually assess our standardisation so we can improve rather than always aiming for the mean.

**Understanding**... having a standards does not mean our assays are standardised – we need to understand what standardisation means and how to maintain it.

**Knowledge**... We know the values for ERMDA470K.

**Data and information**

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**Data**... We know it exists

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**ERMDA470K**

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ERMDA470K

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Summary

★ Standardisation is vital
★ We have to keep it as a high priority in our labs
★ It is the first step in generating quality results
★ It should be the main reason we select an analyser – not speed or throughput
★ It should mean that we generate high quality results for our patients....and we can all be the patient!