Clinical Chemistry

Towards an SI-Traceable Reference Measurement System for Seven Serum Apolipoproteins Using Bottom-Up Quantitative Proteomics: Conceptual Approach Enabled by Cross-Disciplinary/Cross-Sector Collaboration

Article:
Christa M. Cobbaert, Harald Althaus, Ilijana Begcevic Brkovic, Uta Ceglarek, Stefan Coassin, Vincent Delatour, Liesbet Deprez, Ioannis Dikaios, Julia Dittrich, Andrew N. Hoofnagle, Gerhard M. Kostner, Florian Kronenberg, Zsusanna Kuklenyik, Urban Prinzing, Hubert W. Vesper, Ingrid Zegers, and L. Renee Ruhaak, for the IFCC Working Group for Standardization of Apolipoproteins by Mass Spectrometry
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Clin Chem 2021; 67:3 478-489 https://doi.org/10.1093/clinchem/hvaa239

Guest: Dr. Christa Cobbaert, head of the Clinical Chemistry and Laboratory Medicine department at the Leiden University Medical Center in the Netherlands

Bob Barrett: This is a podcast from *Clinical Chemistry* sponsored by the Department of Laboratory Medicine at Boston Children's Hospital. I'm Bob Barrett.

In evaluating the diagnosis of cardiovascular disease, first came measuring lipids. Then came measuring the lipoproteins and following that, measuring the individual apolipoproteins. Well, this is a well-accepted heritage and evolution of the field. This situation presents problems and comparisons of clinical studies and comparing laboratory methods. The lack of standardized analytical measurements prevents the compilation of data from different laboratories into large-scale epidemiologic studies which will be necessary for the newer apolipoproteins biomarkers to be accepted as valid risk markers.

A special report appearing in the March 2021 issue of *Clinical Chemistry* presents progress towards development of a reference measurement system for serum apolipoproteins to help address this issue. The report was prepared by the IFCC Working Group for Standardization of Apolipoproteins by Mass Spectrometry. And the chair of the working group is Dr. Christa Cobbaert, head of the Clinical Chemistry and Laboratory Medicine department at the Leiden University Medical Center in the Netherlands. Her scientific research is on medical test development and evaluation for the sake of better patient outcomes and she is our guest in this podcast.

So, Dr. Cobbaert, why is the development of a reference measurement system for serum apolipoproteins ranked so highly as a goal by the IFCC?

Christa Cobbaert: The development of reference measurement system is ranked highly by the Scientific Division of the International Federation of Clinical Chemistry because medical tests often have critical test roles and clinical indications in clinical pathways such as detection of disease, risk classification of patients, and guidance of treatment. In case of

unstandardized medical tests and non-equivalence of test results among medical laboratories, patients may go undetected. They may be misclassified or may be withheld their treatment. And also, as we live in a global world, clinical decision limits determined, for instance, in randomized clinical controlled trials, should preferentially be comparable and exchangeable in time and space in order to allow universal use of these limits and to prevent patient harm.

So, IFCC's scientific division strives in general for medical test standardization of prioritized analytes to guarantee safe and effective use of medical tests at a global scale. And then, specifically, why do we want to develop reference measurement system for apolipoproteins? We are faced with recent discoveries in basic science and translational medicine. And these have set a stage for the establishment of the IFCC working group on apolipoproteins by mass spec. The main drivers for doing that were the convergence of unmet clinical needs and cardiovascular disease patients and evolution in metrology designs of measurement. Let me explain this before major arguments.

First, we have residual cardiovascular risk. Quite huge after accounting for established risk factors and demonstrating that the current way of screening for this lipidemia with a lipid panel is too limited to capture the full complexity of the pigment metabolism in patients. So, atherogenic phenotypes go unnoticed such as remnant disease and also an excess of lipoprotein(a) called apolipoprotein(a) which cause patient harm.

Second, there is a need for accurate test results and highly polymorphic and atherogenic apolipoproteins such as the apo(a) and lipoprotein(a).

Third argument, there is now sufficient robustness of mass spectrometry technology and it allows reproduceable protein quantitation at the molecular protein level and that opens a world of defining health and disease in a molecular way and going to a more granular definition of health and disease.

The fourth argument for doing this is we recently had the revision of ISO 17511. That's a guideline which describes several calibration hierarchies for test results. And the highest achievable standard is creating test results that are traceable to SI (that means the international system of units). And we do that specifically with the IFCC working group for a panel of apolipoproteins.

We choose for a panel because a panel of seven apolipoproteins, serum apoA1 and B measurements, these allow for much more direct quantification of either the protective or atherogenic lipoprotein particles. There is also

heightened interest nowadays and apo(a) and its clinical implication as it is now really considered a strong genetically determined risk factor for cardiovascular disease. So, it's also added to the panel and also because there is now new apo(a) lowering medication under investigation.

And finally, to unravel and treat also the atherogenic remnant diseases, there is also interest in the clinical utility of the serum apolipoproteins C-I, C-II, C-III and E and the e-phenotype. With the selection of this apo panel 4 putting it into the reference measurement system for apolipoproteins, a more comprehensive picture of the dyslipidemia underlying cardiovascular disease can be revealed.

Bob Barrett: So, what is the rationale for developing an SI based multiplex reference measurement system for serum apolipoproteins?

Christa Cobbaert: Thanks for this question. It is well-known that internationally endorsed reference measurements systems are the key to ensure metrological traceability of medical test results. And that happens through an unbroken sequence of calibrators and measurement procedures to internationally recognize higher order materials and methods in line with the ISO 17511 calibration hierarchies. Six calibration hierarchies are currently defined; the most complete one traces and test results back to SI Units.

The second best calibration hierarchy makes test results traceable to endorsed reference materials such as WHO, IFCC reference materials. And in the 90s, so in the past century, IFCC, WHO standardization efforts led to the development of reference measurement systems for free of the apolipoproteins which I just named. In this case, for apoA1, for apoB and Lp(a). And the contemporary metrological traceability change for these free analytes demonstrate traceability of test results to WHO secondary reference materials. So, not to SI. Not to the primary reference material as that was not existing.

And currently, this WHO, IFCC reference materials are running out of stock. And in case of Lp(a), it's even almost depleted. On top, the immunoassay-based reference methods which were developed at that time are also no longer available nowadays. So, replacement of the higher or the reference materials and procedures for apoA1, B and Lp(a) are needed. And now being 40 years beyond the first apolipoprotein standardization efforts for these free analytes, and considering the evolution in metrology and technology, it was decided by the IFCC working group to establish indeed a SI based multiplex apolipoprotein reference measurement system both for the conventional apolipoproteins A1, B and the Lp(a) but also for emerging apolipoproteins C-I, C-II, C-III and apoE.

And by striving for SI traceability of test results, the most complete calibration hierarchy will be established and will allow the community to trace results back to a well-defined system of units based on the international system of quantities, that's the SI system, and it's adopted by the general conference ways and measures. SI traceability of apolipoproteins should lead to improve accuracy and global equivalence of apolipoprotein test results.

Bob Barrett: The IFCC report recommends bottom-up proteomics for SI traceability of apolipoprotein test results. What exactly is bottom-up proteomics and what advantage does it have compared to immunoassay-based reference measurement procedures?

Christa Cobbaert: Bottom-up proteomics is a common approach used to identify proteins to characterize their amino acid sequences and polymorphisms and also post-translational modifications. It is done by proteolytic digestion of the proteins in the serum, for instance, and that's done prior to mass spectrometry analysis. And in addition, with that technology, accurate and reproduceable quantitation of serum proteins has been demonstrated with triple-quad mass spec technology.

And this paved the way for selecting quantitative proteomics on triple-quad mass spectrometry for developing the apolipoprotein reference measurement system.

In the proposed bottom-up proteomics system for apolipoproteins, the apolipoproteins in serum are unsymmetrically digested into proteolytic peptides which are then quantified by liquid chromatography of triple reaction monitoring mass spectrometry. And by comparing the masses of the proteolytic peptides or from the mass spectra with those predicted from the sequence database are annotated in a peptide spectro-library. Proteotypic peptides can be identified, and proteotypic peptides concentrations assembled into an apolipoprotein identification.

Major advantages of a bottom-up proteomics space reference measurement system are therefore related to the analytical specificity which can be achieved through liquid chromatography separation in combination with the selection of specific peptide fragments in the third quadruple and its capability of measurements at the molecular level. Another metrological advantage of bottom-up proteomics is related to the fact that common immunoassay independent reference measurement system is established for quantitation and standardization of those seven clinically relevant apolipoproteins in one test, whereas the former immunoassay-based reference measurement systems are uniplex and depend on the specificity of the antibodies used.

So, immunoassay-based reference measurement systems, the former ones, are generally blind also from molecular variation of proteins and apolipoproteins. These are the reasons.

Bob Barrett: So, which tools are being developed to enable manufactures and end users to standardize or re-standardize their lab developed tests or even commercial IVDs?

Christa Cobbaert: Once the SI traceable multiplex reference measurement system for apolipoprotein is in place, secondary human matrix based commutable and value assigned reference materials will be available at the European Union Joint Research Center in Geel, Belgium for all seven apolipoproteins at clinically relevant concentrations. Manufacturers and clinical trial labs that aim to standardize or re-standardize their commercial or lab developed tests can purchase these commutable reference materials.

Secondly, organizations like the Center for Disease Control in Atlanta, Georgia USA will foresee also in certification programs that are offered to IVD manufacturers through a global network of calibration labs running the apolipoprotein reference measurement system.

And thirdly, also external quality assessment organizations and metrology institutes like LNE in Paris, France will develop what we named external quality assessment materials but of a very high quality the so-called trueness verifiers that are also commutable and value assigned by one of the calibration labs running the endorsed apolipoprotein reference measurement system. And medical labs can participate in this phase and will receive these trueness verifiers in a blinded manner to investigate trueness of their test results.

Bob Barrett: Well finally, doctor, let's talk about resources. What is needed for organizing standardization programs of apolipoprotein tests in a global and sustainable way?

Christa Cobbaert: The effort to establish and SI traceable reference measurement system in a network of calibration labs and the collaboration with all stakeholders and the IFCC working group is indeed a big elephant. Yet, the subsequent global implementation which you query about of the revised SI traceable apolipoprotein reference measurement system will even be more challenging. There's understandable resistance to changes due to conservatism, sticking to traditional lipid screening guidelines and different regulatory requirements across the globe.

Yet, both IVD manufacturers and lab professionals should take responsibility in implementing (respectively, advocating)

test standardization and they should strive to make standardization inclusive. That means tests should be standardized before they are put on the market for availability and use in medical labs.

Also, new regulations such as for instance in Europe, the IVD regulation 217/746, these new regulations demand safety and effectiveness of medical tests to protect their citizens.

I personally believe that standardization of medical tests to the highest order of reference materials and methods available is a key requirement to guarantee the safety and effectiveness of medical testing. Only when medical labs produce equivalent results in relation to universal decision limits, the downstream consequences for patient management can be handled by doctors in an appropriate way.

Whether upfront medical test standardization is cost-effective and worth the investment early on should be balanced against the patient benefit harm risks and ratios of using standardized versus non-standardized medical test and clinical care pathways. During the entire life cycle of tests, a paradigm shift of the inclusiveness of medical test standardization before commercializing tests is in my perception highly needed.

Bob Barrett: That was Dr. Christa Cobbaert, professor and head of the Clinical Chemistry and Laboratory Medicine department at the Leiden University Medical Center in the Netherlands. She is chair of the IFCC Working Group for Standardization of Apolipoproteins by Mass Spectrometry which published the special report in the March 2021 issue of *Clinical Chemistry*.

I'm Bob Barrett. Thanks for listening.