Publishable JRP Summary for Project T2 J11 (CLINBIOTRACE).
TRACEABILITY OF COMPLEX BIOMOLECULES AND BIOMARKERS IN DIAGNOSTICS – EFFECTING MEASUREMENT COMPARABILITY IN CLINICAL MEDICINE

PROJECT RATIONALE

The implementation of reference measurement systems in laboratory medicine, incorporating reference methods and standards has provided a framework for improved clinical laboratory processes and more robust measurements and has been strongly supported by EQALM (European Committee for external Quality Assessment Programmes in Laboratory Medicine). The International Federation of Clinical Chemists (IFCC), the Joint Committee for Traceability in Clinical Measurements (JCTLM) and other stakeholders representing diagnostic industry, academia and healthcare quality assessors, have also highlighted the need for the further development of traceable reference measurement systems for complex biomolecules (e.g. disease state biomarkers and protein markers relevant for therapeutic intervention) to enable IVD and clinical measurement comparability - a value-added exercise that will improve patient care, testing, accuracy and reliability.

The fundamental issue, debated by the clinical, WHO, healthcare and metrology communities lies in the quest to establish traceability for macromolecules of biological origin in the same way as for simpler chemical measurands through assigning values traceable to the SI. Many calibration chains are built on standards which have values assigned in international units, defined arbitrarily for the highest order standard to which secondary and lower order standards are traceable. Complex biomolecules such as proteins have additional chemical and plasma-based heterogeneity so the concept of “a unique, homogeneous chemical entity” does not apply, few reference measurement methods exist, and the measurands are difficult to define. Multiple parameters influence the properties of biomolecular standards and will have to be considered for the establishment of a traceability chain in order to contribute effectively to improving the comparability of biological measurements. The critical measurement is not only the “amount of substance”, which is often appropriate for well characterised measurands, but quantity of “active/functional” component, in an appropriate matrix.

This challenging project takes the first steps towards addressing the SI /IU debate by investigating novel methods for linking structural and functional (“activity”) SI traceable measurements to the amount of protein present. If this can be realised, it will represent a very considerable advance in measurement science, providing a route to traceable complex protein measurements that will impact significantly on healthcare measurement comparability

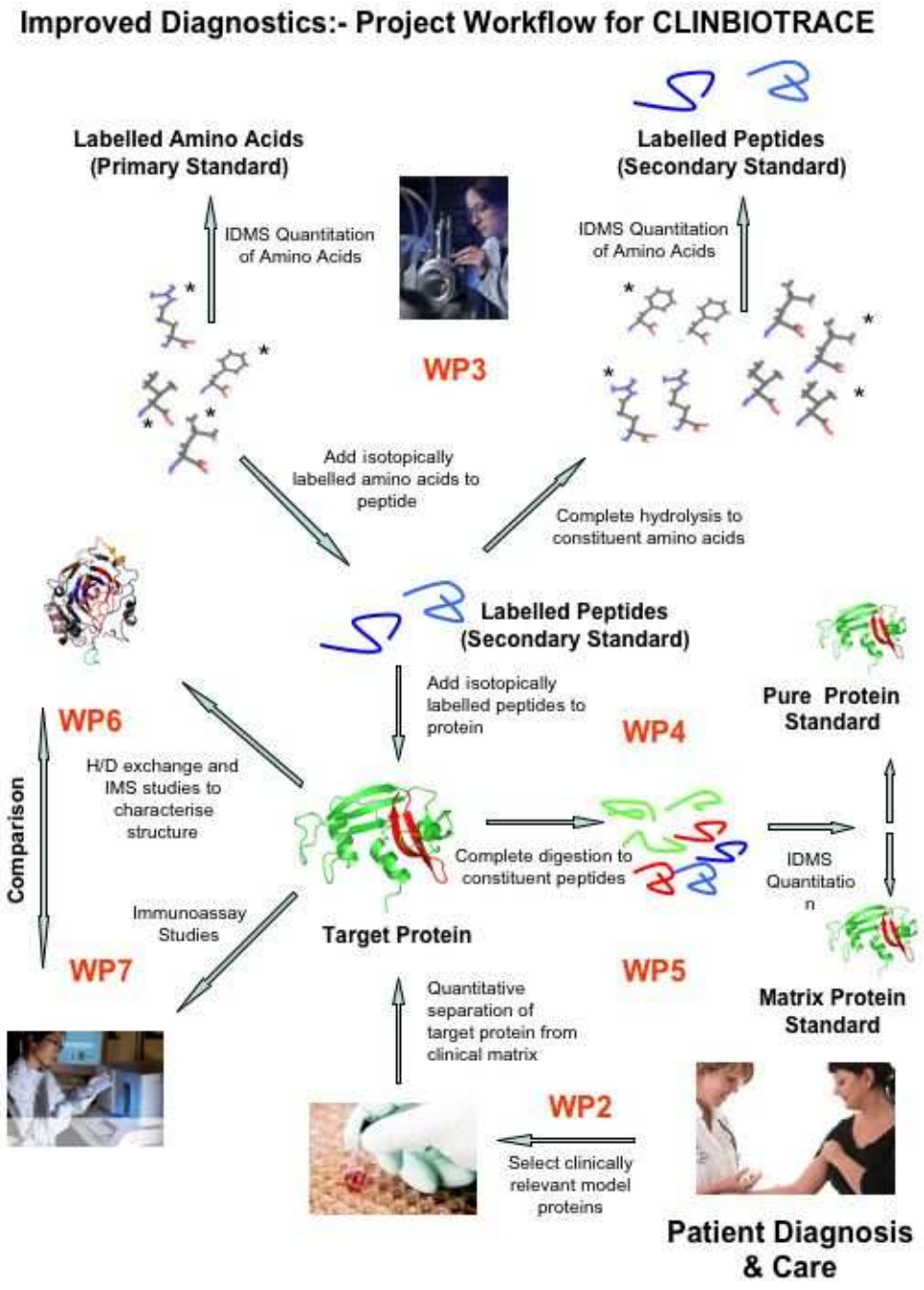
PROJECT OBJECTIVES

To expand the available range of reference measurement procedures and reference materials of a higher order for bio-molecules of clinical significance

Through improving the comparability of measurement of bio-molecules by the (further) development of SI traceable mass-spectrometry based methods, and linking the results of these to measurements results obtained with functional activity-based methods by:

- Refining the current methods for the traceable value assignment of peptides, which are to be used to quantify proteins
- Devising methods for the reproducible and quantitative digestion of target proteins to enable the accurate quantification of the protein using the peptide standards
- Investigating emerging and novel methods for the quantification of different protein structures and evaluate these against diagnostic immunoactivity measurements

WORKPLAN



EURAMET European Association of National Metrology Institutes
EMRP European Metrology Research Programme
Programme of EURAMET

PROGRESS TO DATE

1. Selection, in consultation with key stakeholders, of model diagnostic protein biomarkers for study

A review of appropriate clinical proteins for the project has been undertaken. In consultation with key stakeholders, and taking into consideration the need to demonstrate proof of concept on a spectrum of proteins, 5 model diagnostic protein biomarkers were chosen and prioritised for the initial study:- human growth hormone (hGH), C-reactive protein (CRP), ceruloplasmin, cystatin C and troponin I. Integration of published and our experimental data (peptide mapping, isoform profiling etc) for each of these proteins has enabled informed decision making on study suitability with respect to clinical impact, immunological knowledge and assessment by MS structural analysis. The consortium is now giving highest priority to hGH and CRP, with a preliminary assessment of ceruloplasmin. All are clinically important analytes, for which significant traceability issues exist. The Clinical Chemistry community has been working on standardising hGH for many years, and this project will not be able to solve all the remaining problems. However, the proposed studies should be a significant contribution. CRP is an excellent model system for the study of the impact of structural heterogeneities on the relation between the amount of substance and the functional quantification. Furthermore, the opportunity to integrate the project with a commutability study in collaboration with the IFCC for hGH offers higher impact potential for the project.

2. Traceable Quantification of: a) Amino Acids derived from peptide standard, b) Protein Derived Peptides c) Protein in a Complex Biological Matrix

High accuracy isotope dilution mass spectrometry (MS) has been used to produce amino acid and peptide standards through which the quantification of the primary structure of the protein can be made. To date a standard operation procedure based on LC-MS/MS had been developed and validated for amino acid analysis. Further improvements to the method, employing GC/MS resulted in a significant reduction (3% to 1.5%) in the method related measurement uncertainty.

A 2-step semi- preparative LC method has been developed for the clean-up of peptides for the production of the purified peptide reference solutions which are required as a prerequisite for the accurate quantification of proteins. To date the selected growth hormone tryptic peptides T6 and T13 have been purified. The choice of tryptic peptides has been informed by the requirement to align the project more closely with the form of hGH recommended by the IFCC WG on hGH clinical assays.

The development of IDMS methodologies for traceable peptide quantification has been completed for hGH and used for the accurate quantification of hGH peptides and standard preparations. The approach has been fully validated between two laboratories for hGH. Preliminary work has begun on CRP target peptides.

For the development of digestion/hydrolysis strategies for the production of peptides from the chosen proteins the digestion of four hGH preparations has been assessed using a number of different digestion methods. Complete digestion was assessed using standard additions and proteomic based relative quantification methods (such as iTRAQ). An assessment of the equimolar release of 4 peptides from the target protein has been made.

The feasibility of traceable value assignment of protein mass fraction in a standard solution with a full uncertainty evaluation has been completed for hGH and standard preparations, including the World Health Organisation (WHO) international standard and European pharmacopeia (EurPh) materials have been fully characterised using the methods, and the work has now been published in Clinical Chemistry.

A protocol for digestion of serum samples followed by extraction of the released hGH-typical peptides and quantification by isotope dilution mass spectrometry has also been developed and validated and a demonstration of its applicability to serum samples, used in external quality assessment exercises, has been published in Analytical Biochemistry. Further approaches to generation and extraction of the proteotypic peptides from a complex matrix are currently being tested, focusing on removal/ depletion of highly abundant proteins prior to digestion.

3. Traceable Quantification of a Proteins Tertiary Structure

We aim to develop methods capable of determining the relative concentrations of different protein isoforms and structures. Work has commenced on the evaluation of advanced mass spectrometry-based techniques for the quantitative determination of protein structure.

- hydrogen deuterium (H/D) exchange. This enables interrogation of protein structure under physiological conditions
- Ion mobility MS enables proteins of the same mass but different folding states to be separated.

It is envisaged that by combining the above with high resolution chromatography, capable of separating protein isoforms, the quantity of the functional form can be determined

An H/D exchange method has been developed for the comparison of protein structures and preliminary work undertaken on hGH to assess differences in the currently available reference standards as well as investigating the structural stability of different parts of the intact molecule. The potential of the HDX methods for distinguishing and quantifying known amounts of different molecular structures and measuring protein binding constants is being assessed with a “model” peptide in the first instance.

Ion mobility mass spectrometry methods have been assessed for distinguishing differences in standard preparations of the target proteins. Preliminary investigations using hGH have identified a number of molecular species with different gas phase collision cross sections that may be indicative of different folding states and aggregates of the proteins present.

4. **Linking immunoassay measurements with SI traceable approaches**

The feasibility of these physico-chem approaches for traceable structural analysis will be demonstrated by evaluation against immunoassay studies (the routinely applied clinical laboratory diagnostic measurement for protein) to determine the link between functional activity and protein quantification/structure,

An initial evaluation of the oligomerisation and aggregation state was undertaken for pure protein preparations of hGH, CRP, CysC, TnI and the results used to inform prioritisation of hGH and CRP proteins for more detailed study.

CRP isoform profiling in matrix preparations has been performed by a combination of size exclusion chromatography, different gel electrophoresis techniques, denaturating, native and semi-native gel electrophoresis, and Western Blotting using specific antibodies. This has enabled correlation of the isoforms pattern of CRP with the functional activity in

immunoassays in a semi-quantitative manner. The results may also provide an explanation of biases observed in the values of CRP in (candidate) reference materials. Different isoforms of CRP have been prepared both artificially, and purified from matrix materials. These have been measured by a range of immunoassays, making it possible to link the isoforms composition with the functional activity of this protein

POTENTIAL IMPACT OF PROJECT

Successful development and validation of this functional traceability concept will impact on key stakeholders in biological standardisation including the JCTLM, IFCC, WHO, reference material producers, competent authorities, producers of commercial measuring systems and practitioners of Laboratory Medicine by:

1. Systematically studying and raising awareness of factors influencing existing immunoassay measurement comparability, and developing multiparametric traceable potential reference measurement methods for key diagnostic proteins of clinical relevance
2. Informing the stakeholders involved in the SI /IU debate on the need and feasibility of developing traceable clinical measurements for protein structure related “activity” in *in vitro* diagnostics
3. Enabling reference material producers to prepare Certified Reference Materials better suited for the calibration of *in vitro* diagnostic devices and in line with ISO 17511 in order to achieve acceptable comparability of IVDs on individual patient samples which could then be considered for recommendation by JCTLM

CONSORTIUM PUBLICATIONS / PRESENTATIONS:

1. Pritchard C*, Quaglia M, Mussell C, Burkitt WI, Parkes H, O’Connor G, Fully Traceable Absolute Protein Quantification of Somatropin that Allows Independent Comparison of Somatropin Standards, Clin. Chem 55 (2009) 1984-1990 (SI-traceable quantification of recombinant hGH)
2. Arsene CG, Ohlendorf R, Burkitt W, Pritchard C, Henrion A, O’Connor G, Bunk DM, Guttler B, Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy, Anal. Chem. 80 (2008) 4154-4160
3. Arsene CG, Henrion A, Diekmann N, Manolopoulou J, Bidlingmaier M, Quantification of growth hormone in serum by isotope dilution mass spectrometry, Anal. Biochem. 401 (2010) 228 – 235
4. “The NMI Metrology Perspective: Towards Traceable Biologic Structure and Function” Dec 2008 CCQM /USP Workshop “Measurement Traceability for Pharmaceutical and Bio-pharmaceutical Measurements.”
5. “Mass Spectrometry in Biomeasurand Design: Primary and equivalent methods to address present and coming challenges in life science” April 2009 CCQM International Workshop on the “Frontiers of Traceability in Chem / Bio Measurement” Andre Henrion PTB & Gavin O’Connor LGC
6. “CLINBIOTRACE” project presentation at the health care session of, Metrologie, Paris June 2009 Helen Parkes, LGC
7. “Investigating proteomic methods for the traceable value assignment of protein biomarkers”. The 12th Biological and Environmental Reference Material Symposium BERM12, Oxford, UK, July 2009 O'Connor, Gavin; Pritchard, Caroline; Burkitt, William; Quaglia, Milena; Parkes, Helen; Henrion, A.; Arsene, C.; Schimmel, Heinz; Zegers, Ingrid; Hills, Anna:
8. Isotope dilution mass spectrometry (IDMS) in protein quantification: A reference method for determination of human growth hormone from serum. The 12th Biological and Environmental Reference Material Symposium BERM12, Oxford, UK, July 2009. Arsene, C.; Henrion, A.
9. “MiS-MALDI: Microgel-Selected on-probe detection of protein biomarkers by MALDI-ToF mass spectrometry." E.Cerasoli, P.D.Rakowska, A.Horgan, J.Ravi, M.Bradley, B.Vincent, M.G.Ryadnov (submitted).

EMRP
European Metrology Research Programme
Programme of EURAMET

JRP Contract Number:	T2 J11
JRP Title - JRP Acronym:	Traceability of Complex Biomolecules and Biomarkers in Diagnostics – Effecting Measurement Comparability in Clinical Medicine - CLINBIOTRACE
JRP start date and duration:	1st June 2008 for 36 months
Date of this Publishable JRP Summary:	June 2010

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IRMM, EU

NPL, UK

The research within this EURAMET joint research project receives funding from the European Union Seventh Framework Programme, ERA-NET Plus, under the iMERA-Plus Project - Grant Agreement No. 217257.