Publishable Summary for 18HLT10 CardioMet

Providing the measurement infrastructure to allow quantitative diagnostic methods for biomarkers of coronary heart diseases

Overview

With 11.3 million new cases of cardiovascular disease and 1.8 million deaths per year, heart diseases remain one of the main challenges for healthcare in the EU. Cardiac biomarkers help to confirm the diagnosis, provide prognostic information and, thus, enable successful treatment. The aim of the project is to standardise and improve commercially available quantification methods by establishing reference methods for biomarkers such as cardiac troponin and apolipoproteins for cardiovascular disease and B-type natriuretic peptides for heart failure. Furthermore, the structural heterogeneity of these cardiac biomarkers will be investigated to improve the respective reference methods.

Need

Cardiac troponin (cTn) levels in the blood are routinely used in the diagnosis of heart attacks (myocardial infarctions, MCI). Blood samples are taken from a patient at appropriate intervals to assess whether their cTn level is elevated or not, and whether its level is increasing, stable or decreasing. These levels indicate whether or not, an MCI is in progress, or has recently occurred. cTn is, therefore, known as a ‘cardiac biomarker’. Similarly, apolipoproteins, which can carry cholesterol, are used as biomarkers for the risk of future cardiovascular diseases (CVD), and brain natriuretic peptides (BNP) are used as biomarkers to assess the risk of future heart failure (HF).

Analysis of patient blood samples must be undertaken by accredited laboratories, each of whom uses a variety of measurement devices which must, in turn, be calibrated to ensure accuracy, reliability and comparability between laboratories. In directives such as the Directive of the German Medical Association (RiliBÄK), important health relevant parameters such as cTn and its derivatives and their respective concentrations and permissible deviations are defined. However, there are no reference values to properly calibrate the diagnostic equipment and variations of up to 60 % can be found when comparing the results of different laboratories using the same nominal equipment. These variances can lead to incorrect diagnoses resulting in poor patient outcomes.

The measurement of biomarker kinetics is an innovative way to distinguish real heart attacks from other, less acute diseases. This is important because nearly all diagnostic determinations of biomarkers of CVD are based on static measurements. Since the decay of coronary tissue is a dynamic process, the change of the respective parameters should be more relevant than the absolute values. Therefore, a mobile, quick and highly sensitive biosensor system will improve the diagnostics for CVD.

The European Society of Cardiology (ESC) guidelines on the prevention, diagnosis and treatment of heart diseases name the natriuretic peptides (NT-proBNP and 1-32 BNP) as especially important biomarkers for the assessment of the status for HF. However, the high measurement variability limits the ability of those markers to be used to their full potential.

Besides the treatment of CVDs, their prevention is a major focus of EU initiatives. The crucial role of dyslipidaemia (alterations in lipid metabolism), especially hypercholesterolaemia, in the development of CVD is particularly documented. Lipids such as cholesterol and triglycerides circulate in blood plasma bound to apolipoproteins. Measurements of apolipoprotein panels have increasingly come into focus as possible biomarkers for CVD risk and to enable a more personalised treatment of patients. Such biomarkers need to...
be assessed for efficacy in a clinical setting, and if they are found to offer added value for medical diagnostics, establishing a higher order reference system will be required.

Regulation (EU)2017/746 of the European Parliament and the Council ("IVDR") requires the metrological traceability of values assigned to calibrators and/or control materials to be assured through suitable reference measurement procedures and/or suitable reference materials of a higher metrological order. This new regulation is proving challenging to meet for the assay manufacturers.

Objectives

The overall aim of this project is the development of reference measurement procedures for the traceable quantification of selected cardiac biomarkers for the diagnosis and risk management of CVD and HF, and to characterise the reference materials needed for these procedures, to ensure accuracy and comparability of medical diagnostic equipment. The specific objectives are:

1. To develop reference measurement procedures for the traceable quantification of apolipoproteins with an expanded uncertainty < 15 % and a target limit of quantification (LOQ) of 1 μmol/L. Further, to assess the clinical utility, performance criteria and suitable routes for standardisation of advanced lipoprotein testing methods that could be used to reduce undiagnosed CVD risk.

2. To develop reference measurement procedures for the traceable quantification of cTn which acts as a biomarker for coronary heart diseases. Further, to develop selective and highly efficient enrichment methods such as immunoaffinity to achieve the target LOQ of 3-4 ng/L and uncertainty < 15 %. In addition, to use the new procedures to measure cTn in calibration material and clinical samples, and to compare the procedures in terms of LOQ, uncertainty and specificity.

3. To develop a biosensor capable of fast (one measurement per 10 minutes) and quasi-continuous monitoring of cardiac biomarkers to enable a very early diagnosis of heart attacks.

4. To develop a reference measurement procedure for the quantification of HF biomarkers such as brain natriuretic peptides (BNP), including the development of isotope dilution mass spectrometry (IDMS) approaches targeting the biomarkers NT-proBNP, 1-32 BNP and its metabolites and the quantification of appropriate primary calibrators to ensure SI traceability (target uncertainty ≤ 15 %). Further, to evaluate the potential of the methods developed to be used for standardisation of BNP measurements and to define commutability requirements of external quality assessment (EQA) scheme samples.

5. To facilitate the uptake of the methods and results developed in the project by clinical reference laboratories, in vitro diagnostic medical device (IVD) producers, relevant national clinical associations and standards developing organisations, including the Joint Committee of Traceability in Laboratory Medicine (JCTLM), International Organization for Standardization (ISO) and European Committee for Standardization (CEN).

Progress beyond the state of the art

Current biomarkers and diagnostic tools only modestly predict CVD events and important progress remains to be done before achieving proper CVD risk assessment and accurate patient stratification. Apolipoproteins and other advanced lipoprotein testing methods offer another route to evaluate CVD risk and to enable a more personalised treatment of patients. Furthermore, the development of a new traceability chain based on IDMS, including the preparation and characterisation of the necessary isotopically enriched spike materials, will enable an evaluation of the additional value of these biomarkers. The performance specification of advanced lipoprotein testing methods for accurate CVD risk assessment and patient stratification will be determined and provided to routine laboratories and clinics. A special focus is on patients lacking the classical risk factors.

In the case of CVD diagnosis, the subunits cTnI and cTnT are the most commonly used biomarkers. To date, the decision limits (the levels at which treatment is recommended or not) for both men and women are the same. A previous study has shown that if cohorts were differentiated between men and women, the upper reference limit for men should be 0.034 μg/L whereas the upper reference limit for women should be as low as 0.016 μg/L. Hardly any routine method provides the necessary sensitivity let alone any reference measurement procedure. The currently published state of the art for a potential RMP achieves a LOQ of 2 μg/L. The International Federation of Clinical Chemistry and Laboratory Medicine working group on troponin I (IFCC WG-TNI) have already made a significant effort in this field. This project will build upon their efforts by providing a complementary and more sensitive method using IDMS to enable a traceable quantification of cTn at the low
concentrations in human samples, with a target LOQ of 0.003-0.004 μg/L. This also includes the preparation and characterisation of the necessary isotopically enriched spike materials.

Nearly all diagnostic means for the quantitative determination of biomarkers of coronary heart disease are based on static measurements, sometimes repeated after a time. Since the decay of coronary tissue is a dynamic process, the change of the respective parameters should be more relevant than the absolute values, which are influenced by many boundary conditions, which are often not under control of the medical staff. The measurement of biomarker kinetics is an innovative way to distinguish real heart attacks from other, less acute diseases. The project will develop a prototype for a biosensor system that can deliver a diagnostic value every 10 minutes making it possible to calculate a kinetic value (increase) of the parameter after 20 minutes and beyond. It will be examined whether a decision limit of the slope can be defined, instead of an absolute value.

Moreover, various BNP forms (such as NT-proBNP and 1-32 BNP) are routinely measured using immunoassays, but little has been done towards measurement standardisation leading to improved thresholds and measurement accuracy. Furthermore, these methods often cannot distinguish between glycosylated and non-glycosylated forms (cross-reactivity) or cannot detect glycosylated forms. The results of this project will define standardised routes to report measurement uncertainty from EQA schemes and identify the need for EQA samples to behave like patient samples in routine measurements (commutability).

Results
Reference measurement procedures for the quantification of apolipoprotein biomarkers for CVD risk assessment

An extensive data analysis has been conducted using the Swedish cardiac registry SWEDEHEART to identify the subgroups that will most profit from determining lipoproteins as additional biomarkers for CVD risk. Based on the outcome of the analysis a clinical study has been devised and submitted to ethical approval. Currently, 63,000 patients are included in the analyses. Baseline data and definitions have been performed. Long-term prognosis in these patients with up to 11 years of follow-up is being assessed. Further, UPP is conducting two large-scale studies aiming at assessing low density lipoprotein cholesterol (LDL-C) levels during MI and prognosis: One analysis is investigating the level of LDL-C at the time of an MI in 60,000 patients and its association to long-term prognosis. In order to document the state of the art regarding CVD risk prediction on the basis of diagnostic tests currently used in day to day clinical practice and determine what is the measurement uncertainty needed for routine methods to accurately stratify patients based on concentration of conventional biomarkers (e.g. LDL-C, non-HDL-C, TG, ...), preparation of an EQA scheme has been initiated. Clinical specimens that are intended to be used in an EQA scheme later in 2021 have been sourced. An initial commutability study has been organised, comparing the results of fresh single patient samples, pooled native serum pools and conventional EQA material using the major measurement techniques. In addition, LNE sourced additional samples from the Cholesterol Reference Method Laboratory Network (CRMLN) that were already value assigned for LDL-C by the Center for Disease Control and Prevention’s (CDC) reference method by beta quantification. 25 pools of human frozen serum were received by LNE and will be distributed to clinical laboratories running the most popular assays for LDL-C. In order to establish recommendations for analytical performance criteria for assays relying on conventional biomarkers currently used to estimate long-term CVD risk, LNE joined CDC’s working group on lipids analytical performance criteria that brings together world class expert of this field. Recommendations which are currently discussed with assay manufacturers will be published soon.

Substantial progress was made in the development of a candidate reference measurement procedures for a panel of apolipoproteins (ApoA-I, B, C-I, C-II, C-III, E and Apo(a)) with a target uncertainty of below 15 %. The procedure for sample preparation is almost in place: a list of target peptides was established, digestion conditions were optimised, a protocol for isolation of proteotypic peptides was established and LC-MS conditions were optimised. The developed preliminary standard operating procedure (SOP) was tested among three clinical reference laboratories using serum-based calibrators and the needs for further improvement have been identified. Sourcing and evaluation of calibration materials for absolute quantification of a panel of apolipoproteins by LC-IDMS has been started. Preliminary purity assessment for four peptides showed sufficient purity to proceed. Equimolarity of digestion is a key step to demonstrate the suitability of the bottom-up approach and specially to establish the traceability of the results to the International System of Units (SI). Ongoing experiments will result in validating the final list of peptides that will be used to quantify the whole protein(s) and, therefore, to decide which primary calibrators are needed. Meanwhile, a preliminary purity assessment of 5 candidate peptides for apo(a) was conducted to ensure that preliminary concentrations
determined by amino acid analysis are sufficiently accurate to conduct the equimolarity experiments. After this work has been completed, additional purity evaluation and amino acid analysis will be conducted with the objective to perform formal certification of the primary calibrators that will be used to calibrate the candidate reference method.

**Reference measurement procedures for the quantification of biomarkers for coronary heart disease**

As a first step in producing the isotopically enriched spike material, all subunits of cTn and the complex could be expressed successfully in *E. coli* and were isolated using affinity and size exclusion chromatography. Selenomethionine-labelled cTnC was successfully prepared and is available for shipment to the partners. A method for the separation and quantification of cTn via its sulphur content using LC coupled to ICP-MS has been developed. However, the sensitivity is not sufficient for clinical samples; so a new strategy using lanthanide labels is currently under investigation, either detecting the whole protein or specific peptides after enzymatic digestion. Additionally, the quantification method via specific peptides using LC-MS published by Schnick et al. (Anal Bioanal Chem 2018, 410:2805–2813) could be improved, so that complementary approaches will be available for mutual validation.

A conventional sandwich enzyme-linked immunosorbent assay (ELISA) has been established which will be used for the comparison of reference measurement procedure and routine methods. However, the sensitivity of the assay has still to be improved to be comparable to the high sensitivity assays in use at clinical laboratories nowadays. Furthermore, experience with different antibodies during the development of the ELISA could be used in the development of the biosensor for cTn.

**Biosensor system for fast (10 minutes) and quasi-continuous monitoring of cardiac biomarkers**

A flow injection assay (FIA) – based biosensor setup (hardware and initial software) has been established and the assay has been tested on blood plasma. Different combination of antibodies has been tested to give the best sensitivity and selectivity. Still some interferences as well as sensitivity issues need to be resolved before the biosensor is fit-for-purpose to be used as point-of-care test.

**Reference measurement procedures for the quantification of biomarkers for heart failure**

Different BNP materials (recombinant, synthetic and glycosylated) were sourced and intact protein analysis was carried out to confirm their identity and preliminary assess their purity. Recombinant NT-proBNP was selected as primary calibrator of choice and quantified traceably to the SI. Hydrogen-deuterium-exchange experiments were carried out to define higher order structural differences between recombinant and glycosylated NT-proBNP that may cause discrepancies in immunoassay binding. No structural differences were observed and immunoassay measurements are currently on-going on the samples analysed. A targeted LC-MS method for the quantification of NT-proBNP in plasma was preliminary developed. The NT-proBNP signature peptides were selected and the optimisation of a tryptic digestion and sample clean-up protocol is on-going. Preliminary experiments were carried out to increase the sensitivity of the published method on quantification of 1-32 BNP in plasma.

**Impact**

Presentations have been given at 5 international conferences (such as LCME / KSLM Congress 2019 International congress of metrology 2019 and EQALM Symposium 2019). Updates on the project’s progress and regular input have been provided to standardisation bodies such as JCTLM and industry working groups such as International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). Further conference participation and input to standardisation bodies has been quite limited in 2020 due to the Covid-19 pandemics. In addition, a project website has been established and the initial stakeholder committee has been formed consisting mainly of the stakeholders involved in the project who are greatly linked to stakeholder organisations such as IFCC, EQALM and others and, thus, have a good understanding of their most pressing needs.

**Impact on industrial and other user communities**

As the IVDR requires metrological traceability, the results of this project will help the IVD industry to comply with this regulation. Also, patients and the healthcare sector will benefit from the metrological underpinning of medical test results with proven clinical utility for CVD and HF management due to comparable and traceable measurement results provided by the long-term stability of the reference system. This has several advantages: reference values and decision limits for healthy control groups can be established which are valid for all test kits. This renders it unnecessary for each manufacturer to determine reference values or decision limits themselves for each kit and enables the extrapolation of clinical trial results and, thus, prevents under- and overdiagnosis. To ensure a timely dissemination of the results to the relevant stakeholders in industry and clinical laboratories, the partners will organise a stakeholder workshop and the developed reference measurement procedures will be disseminated through the reference laboratories involved in this project, who
will use the results as references in the EQA schemes for clinical laboratories of Germany, UK, France, Sweden and the Netherlands. A first interlaboratory comparison for seven apolipoproteins including three clinical laboratories from three different countries has been organised successfully. This demonstrated the robustness of the method and the feasibility to establish a network of reference laboratories. Furthermore, the methods as well as the reference values will be provided to the European Metrology Network for Traceability in Laboratory Medicine (EMN-TLM) where most of the relevant stakeholders are involved.

*Impact on the metrology and scientific communities*

To facilitate the European and international metrological community to measure cardiac biomarkers and offer services in their countries, the achievements and results of the project will be presented to the other National Metrology Institutes (NMIs) and Designated Institutes (Dis), at the annual EURAMET meetings as well as at the protein analysis working group (PAWG) meetings of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM).

The results of this project will help scientific communities to better understand the behaviour of the biomarkers in routine test kits and, thus, make them aware of some pitfalls in using them on patient samples, preventing incorrect diagnosis. On an international level, the partners have and will continue to present the project progress to the relevant working groups of the IFCC and the American Association for Clinical Chemistry (AACC), as well as at the biannual stakeholder workshop organised by JCTLM in Paris.

*Impact on relevant standards*

There are currently no relevant standards for cardiac biomarkers or standardisation bodies working in this area. The partners will be in contact with the national committees concerned with the implementation of the IVDR to provide input on establishing traceability to the SI where possible, using the example of cardiac markers. The partners who are members of technical committees relevant to this project will inform them about the project results and will endeavour to ensure they are incorporated in any updates to standards or guidelines. One example is the standard EN ISO 17511:2003 which is currently under revision to fit the requirements of the IVDR and demands a reference measurement system for the determination of analytes in samples of human origin. Another example is the establishment of guidelines and certified reference materials prepared under the umbrella of IFCC working groups focused on cardiac markers standardisation. Additionally, a project partner is on the advisory board of National Institute for Health and Care Excellence Diagnostic Assessment Programme: High-sensitivity troponin tests for the early rule out of acute myocardial infarction and can provide the results of the project for cTn as input to improve patient care.

Furthermore, a partner of the consortium has joined the working group on lipid analysis performance criteria of the US Centers for Disease Control and Prevention (CDC) to help establishing recommendations for analytical performance criteria for assays relying on conventional biomarkers currently used to estimate long-term CVD risk.

*Longer-term economic, social and environmental impacts*

Earlier and more accurate diagnoses of CVD and HF result in decreasing mortality and, thus, result in lower health-care costs which are currently estimated by the European Cardiovascular Disease Statistics (2017 edition) to burden the EU economy with € 210 billion a year. By earlier and more accurately identifying risk patients, timely treatment can also prevent acute events.

In the case of MCI, a timely clinical treatment such as catheter intervention or bypass is lifesaving. A prerequisite is a fast, sensitive and reliable diagnosis which is often based on cTn concentrations. New and reliable measurement procedures can lead to improvements and decrease in mortality especially for women.

Furthermore, the sensors for cTn developed within this project will enable the emergency physician to conduct first measurements at first contact with the patient, decreasing the overall measurement time in the hospital.

*List of publications*

*(Only include peer-reviewed publications that are open access and published in the public domain and include a clickable link (persistent identifier eg DOI) that links directly to each publication)*

This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/
Project start date and duration: 01 July 2019, 42 months

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