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 health care in the EU. Cardiac biomarkers help to confirm the diagnosis, provide prognostic information and, thus, enable successful treatment. The aim of the project was 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 was investigated to improve the respective reference methods. Potential reference measurement procedures were developed within this project. The requirement calibration materials were either purchased or produced and thoroughly characterised so that they are now available. A biosensor for the continuous measurement of cardiac troponin was developed.

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 determination 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 *N*-terminal proBNP (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 was 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 were:

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. Results from this project show that 14.9 % of patients with MCI had no standard modifiable cardiovascular risk factors 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 has been determined and will be 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 (RMP). 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 built upon their efforts and provided a more sensitive method using IDMS as well as a complementary method using isotope dilutions

inductively coupled plasma mass spectrometry (ID-ICP-MS) 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 included 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 has developed 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. Based on this it can be examined in the future 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. However, little has been done towards measurement standardisation that could lead 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 support to 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

It has been suspected that a considerable number of patients at risk for MCI are not picked up by the conventional lipid biomarkers, Therefore, an extensive data analysis has been conducted by this project using the Swedish cardiac registry SWEDEHEART to identify the subgroups that will most benefit 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. Long-term prognosis in these patients with up to 11 years of follow-up is being assessed. Results show that 14.9 % of patients with MCI had no standard modifiable cardiovascular risk factors such as hypertension, diabetes, hypercholesterolaemia, and smoking, thus being less likely to receive preventive treatment. Currently, low density lipoprotein cholesterin (LDLc) levels are also used as clinical markers during MCI and for prognosis. To assess the suitability of this marker, UPP has conducted two large-scale studies: One analysis investigated the level of LDLc at the time of an MCI in 60 000 patients, the other looked at the trend of the LDLc concentration during cardiac rehabilitation and its association to long-term prognosis. In both analyses, prior and subsequent lipid modifying therapy was taken into account. Results show that the residual risk is impossible to estimate beyond rates or hazards by LDLc change or statin intensity.

Routine methods for the quantification of concentration of conventional biomarkers (e.g. LDLc, non-HDLc, triglycerides (TG) are used in day-to-day clinical practice. In order to document the state of the art to predict the CVD risk based on the results of these diagnostic tests and determine what is the measurement uncertainty needed for routine methods to accurately stratify patients, an EQA scheme has been executed. To ensure that the samples used in the EQA scheme will behave identically to fresh patient samples in the clinics, an initial commutability study has been organised. In this study the results of fresh single patient samples, pooled native serum pools and conventional EQA material has been compared using the major measurement techniques. The laboratories performing the measurements have reported their data and new analytical performance specifications have been proposed based on the results. In addition, LNE sourced samples from the Cholesterol Reference Method Laboratory Network (CRMLN) that were already value assigned for LDLc 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 has been distributed to clinical laboratories running the most popular assays for LDLc. To ensure that the results from this project will be included in future recommendations for analytical performance criteria that assays for conventional biomarkers to estimate long-term CVD risk have to fulfil, LNE joined CDC's working group on lipids analytical performance criteria. That brings together world class experts of this field. Recommendations which are currently discussed between these world class experts and assay manufacturers will be published.

A candidate reference measurement procedure 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 % has been developed and published. The procedure for sample

preparation is in place: A list of 31 target peptides was established. After producing pilot batches of these peptides, a preliminary purity assessment has been conducted which demonstrated satisfactory purity for the peptides to be used as calibrators. After upscaling the production, the purity has been assessed thoroughly. After the experiments to verify equimolarity have been finished, 7 peptides have been selected as quantifiers while the others remained as qualifiers. Equimolarity of digestion is a key step to demonstrate that the specific peptides are suitable to be used to quantify the whole protein and thus to establish the traceability of the results to the International System of Units (SI). Digestion conditions were optimised, a protocol for isolation of proteotypic peptides was established and liquid chromatography coupled to mass spectrometry (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 were identified and implemented. Further comparisons are planned when the method is fully validated. Three candidate secondary reference materials were sourced and evaluated by liquid chromatography isotope dilution mass spectrometry (LC-IDMS) to be used as trueness verifiers and/or EQA materials for absolute quantification of a panel of apolipoproteins. Materials have been value assigned by the proposed RMP. The commutability of the materials has been assessed in collaboration with the IFCC WG-ApoMS to estimate the accuracy of routine assays that are available for ApoB, ApoA-I and Apo(a). It turned out that unspiked serum samples are best suited for the purpose.

Theoretical subgroups of patients with acute coronary syndrome and specific lipid disorders have been identified, which are expected to have the highest value added for CVD risk assessment and patient stratification using apolipoprotein profiling and other advanced lipoprotein testing methods compared with conventional makers. A clinical study was conducted with the objective to compare the apolipoprotein profile of patients with familial hypercholesterolemia (FH) with and without hypertriglyceridemia (HTG). The results from this small-scale study suggest that some apolipoproteins may have a specific role in anticipating the atherosclerotic process in FH, on top of classical lipid biomarkers. This will need to be confirmed in larger cohorts, but this was not planned to be part of this project. This objective has been achieved as planned.

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

CTn is a protein consisting of three subunits that is released into the bloodstream on damage to the heart muscle. It is analysed routinely in clinical laboratories to exclude MCI and RMPs for cTnI has been developed in this project. All subunits of cTn and their complex were expressed successfully in *E. coli* and were isolated using affinity and size exclusion chromatography. Furthermore, production and purification of the binary complex cTnI-cTnC, and the ternary complex cTnI-cTnC-cTnT were accomplished. A comparison of different materials showed that the recombinantly produced cTnI and the cTnI purchased from Hytest, which is isolated from human serum, behave very similarly in the ELISA (enzyme-linked immunosorbent assay) developed in this project. Besides the cTn subunits with a natural isotopic composition, all subunits could also be expressed containing ^{15}N-isotope labels. Selenomethionine-labelled cTnC was successfully prepared and is available for shipment to the partners. Besides the production of recombinant human troponin subunits, experiments characterising the phosphorylation status of the troponins were performed as this might affect the results of antibody-based tests. Recombinantly expressed cTnI has been successfully phosphorylated and the phosphorylation sites were identified using MS.

A method for the separation and quantification of cTn via its sulphur content using LC coupled to ICP-MS was developed. However, the sensitivity was not sufficient for clinical samples. A new strategy using lanthanide labels of the cysteines in the cTnI was tested, followed by either detecting the whole protein or the labelled specific peptides after enzymatic digestion. To improve the sensitivity, cTnI is captured on magnetic beads modified with protein G and monoclonal antibodies (mAb) against cTnI. However, optimisation regarding the sensitivity and specificity is still required for both approaches to be fit for purpose in clinical samples. Additionally, the quantification method via specific peptides using the LC-MS method published by Schneck et al. (Anal Bioanal Chem 2018, 410:2805–2813) could be improved using nano-LC-MS, so that complementary approaches are now available for mutual validation. The currently most sensitive method developed in this project is the isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) with a limit of detection (LOD) in serum of currently 600 ng/L. To achieve a LOD of 5 ng/L reached by hs-cTNI assays further improvement is necessary.

A conventional sandwich enzyme-linked immunosorbent assay (ELISA) was established which was used for the comparison of RMPs 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. Having these results, the project achieved only partly the objective and the partners will continue to work on the improvement of the methods after the end of the project.

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

For the diagnosis of MCI the change in the concentration of cTn is even more important than the concentration. Therefore, 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 and the best ones were produced in higher quantity and used to prepare the affinity column used in the sensor. Interferences issues were solved switching from fluorescence to chemiluminescence for detection and quantification. No interferences from other troponin variants could be excluded. For emergency settings, the sensitivity still has to be improved further as the current LOD is 23 µg/L in plasma and 87 µg/L in serum. Having these results, the project achieved only partly the objective and the partners will continue to work on the improvement of the biosensor after the end of the project.

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 to preliminarily 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. Different O-glycosylated forms of NT-proBNP were purchased, their purity assessed, and value assigned using amino acid analysis. The samples were then distributed within EQA schemes organised by UK NEQAS CM. The preliminary results for S44 indicate that O glycosylation may have a significant influence on the performance of routine assays and, thus, arises the question of the definition of the measurand. A targeted LC-MS method for the quantification of NT-proBNP in plasma was developed. However, the first attempt using tryptic digestion was not successful due to issues with degradation of the selected tryptic peptides. Therefore, a new digestion method was tested using a different enzyme. Glu-C provides two stable signature peptides of NT-proBNP which were used for the quantification of NT-proBNP. To achieve the required sensitivity in biological matrices, different clean-up methods were assessed.

Preliminary experiments were carried out to increase the sensitivity of the published method on quantification of 1-32 BNP in plasma. Additionally, a method for quantification of total BNP (1-32 BNP + main truncated forms) has been developed using a peptide in common between the 1-32 BNP and its proteoforms. The peptide and its isotopically labelled form were sourced and characterised. Having these results, the project achieved only partly the objective and the partners will continue to work on the improvement of the method after the end of the project.

Impact

The consortium presented results as part of 23 national and international conferences (such as LCME / KSLM Congress 2019 International congress of metrology 2019, EQALM Symposium 2019, International congress of metrology 2021, Congress of the European Society of Atherosclorosis 2021 and others). Updates on the project's progress and regular input were provided to standardisation bodies such as JCTLM and industry working groups such as IFCC. Three papers have been published and another one has been submitted. In addition, a project website was established (https://www.ptb.de/empir2019/cardiomet/home/) and the initial stakeholder committee was 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 have organised special sessions at relevant conferences and the developed

RMP 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. Aliquots of NT-proBNP primary calibrator and selected peptides were sent to SPMD-RfB in preparation to transfer the RMP developed at LGC to SPMD-RfB. After successful transfer, SPMD-RfB will use the measurement procedure to provide reference values for their EQA scheme. Furthermore, all developed reference methods as well as the reference values will be provided through 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 have been 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 and 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. The AACC podcast “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” given by Christa Cobbaert (LUMC) was aired in Nov 21 (https://www.aacc.org/science-and-research/clinical-chemistry/clinical-chemistry-podcasts/2021/si-traceable-reference-measurement-system-for-seven-serum-apolipoproteins).

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.. Another example is the establishment of guidelines and certified reference materials prepared under the umbrella of IFCC working groups focused on cardiac markers standardisation. The partners are collaborating with all relevant IFCC working groups. 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 MCI 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. The recommendations are currently discussed and are expected to be published soon.

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

- L Renee Ruhaak et al., Development of an LC-MRM-MS-Based Candidate Reference Measurement Procedure for Standardization of Serum Apolipoprotein (a) Tests, Clinical Chemistry, 2023; hvac204, https://doi.org/10.1093/clinchem/hvac204
- Ioannis Dikaios et al., Commutability Assessment of Candidate Reference Materials for Lipoprotein(a) by Comparison of a MS-based Candidate Reference Measurement Procedure with Immunoassays, Clinical Chemistry, 2023;, hvac203, https://doi.org/10.1093/clinchem/hvac203
- Robert Tannenberg et al., Chemiluminescence Biosensor for the Determination of Cardiac Troponin I (cTnI), https://doi.org/10.3390/bios13040455

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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Internal Funded Partners: 1. PTB, Germany 2. BAM, Germany 3. LGC, United Kingdom 4. LNE, France 5. TUBITAK, Turkey	External Funded Partners: 6. APHP, France 7. GGHB, United Kingdom 8. GUF, Germany 9. ICAN, France 10. LUMC, Netherlands 11. SPMD-RfB, Germany 12. UPP, Sweden	Unfunded Partners:
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