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Coordinator: Claudia Swart, PTB Tel: +49-(0)531-592-3220 E-mail: Claudia.swart@ptb.de Project website address: https://www.ptb.de/empir2019/cardiomet/home/		
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TABLE OF CONTENTS

1 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.

2 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.

3 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).

4 Results

4.1 Development of reference measurement procedures for the traceable quantification of apolipoproteins

Metrology for conventional biomarkers for CVD risk assessment

As current dyslipidemia management in patients with atherosclerotic cardiovascular disease (ASCVD) is based on traditional serum lipids, laboratory measurements need to have consistent accuracy and reliability to ensure correct diagnosis and treatment. Therefore, accurate and reliable blood lipid measurements are critical for the correct assessment of CVD risk and the appropriate treatment of patients.

In this project, accuracy of conventional biomarkers for CVD risk assessment was evaluated through the organisation of an external quality assessment (EQA) scheme. Results showed that the most popular assays used to measure TC, triglycerides (TG) and HDLc in serum have appropriate accuracy, but that comparability for results obtained with assays for LDLc have to be improved and that accuracy of some assays do not have the required analytical performance to meet clinical needs. According to the Swedish Diabetes registry, there is a regional increase in the prescription of statins in Sweden, which raises the question of whether this could be due to inaccuracy of LDLc measurements. The present study confirmed that laboratories from regions preferably using some of the direct LDLc assays on the Swedish market have the largest calibration bias. The study, therefore, made it possible to identify assays with inappropriate accuracy and demonstrate that this has an important impact on patient management: some patients will unduly be prescribed medications that were not needed and cause unjustified costs to the healthcare system. The concerned assay manufacturers are approached to understand the causes for the calibration bias that was observed and correct it.

In addition to evaluating the causes for assays inaccuracy, assessing whether analytical performance goals are appropriate is also of major importance. The project contributed to assessing the suitability of current **performance criteria for conventional testing methods** and proposed new approaches to improve measurement accuracy and reliability that support the latest clinical practice guidelines. Different approaches were assessed to modify current criteria and develop approaches that would allow i) maintaining analytical performance at high lipid levels, ii) improving accuracy and reliability at low lipid levels and iii) addressing variability related to sample-specific factors. The selected approach is to establish bias and imprecision criteria applied to individual samples using a probabilistic approach and binomial distribution procedures. The advantages of this approach are that i) it addresses individual sample bias and takes imprecision into consideration, ii) fail rates are based on probability statistics and iii) it better addresses bias at low lipid concentrations.

Traceability chain for apolipoprotein measurements

Although current dyslipidemia management in patients with ASCVD is based on traditional serum lipids, a considerable number of patients with low-risk profiles still experience cardiovascular events and intervention studies on cholesterol-lowering therapies show that residual risk remains, even after achieving optimum cholesterol concentrations. The residual cardiovascular risk after accounting for established risk factors demonstrates that the current lipid panel is too limited to capture the full complexity of lipid metabolism in patients. In an era of precision medicine, this observation has stimulated further research on lipoprotein-related factors to provide physicians with more predictive and comprehensive biomarkers for CVD risk assessment. A**dvanced lipoprotein testing** methods were developed to measure various properties of lipoproteins rather than their lipid content. There is some indication from basic research that serum apolipoproteins A-I, (a), B, C-I, C-II, C-III, and E may give better pathophysiological insight into the root causes of dyslipidemia. To facilitate the future adoption of clinical serum apolipoprotein profiling for precision medicine, strategies for accurate testing are needed. In the revised ISO 17511:2020 guideline, several calibration hierarchies are described for establishing metrological traceability of test results, the highest achievable standard being traceability to the International system of units (SI). In cooperation with the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) working group on quantitating apolipoproteins by mass spectrometry (WG APO MA), a next-generation reference measurement system (RMS) was developed, for seven apolipoproteins. It consists of i) SI-traceable peptide-based primary calibrators, ii) a robust, antibody-independent, multiplexed reference measurement procedure (RMP) based on isotope dilution mass spectrometry (IDMS) and ii) secondary serum-based reference materials (RMs) certified for their apolipoprotein content. The MS-based candidate RMP for serum apolipoproteins was developed using quantitative bottom-up proteomics targeting 32 proteotypic peptides. The candidate RMP fulfils predefined analytical performance specifications, making it a promising RMP candidate in an SI-traceable MS-based RMS. The RMS will enable standardisation of existing and emerging apolipoprotein assays to the SI, within allowable limits of measurement uncertainty, through a sustainable network of reference laboratories.

A major achievement of the project was the development of a candidate RMP for the genetically determined risk factor lipoprotein(a) (Lp(a)). Indeed, elevated concentrations of Lp(a) are directly related to an increased risk of CVDs, making it a relevant biomarker for clinical risk assessment. However, the lack of global standardisation of current Lp(a) measurement procedures (MPs) leads to inconsistent patient care. There is, therefore, a need for accurate test results for this highly polymorphic and atherogenic apolipoprotein(a) (apo(a)) in Lp(a). Lp(a) measurements are challenging because of the structural heterogeneity of this large and complex protein: Apo(a) comprises a size polymorphism in its KIV- 2 region. Therefore, definition of the measurand at the molecular level is of high importance. The strategy used in the present project allowed for the identification of the measurand at the molecular level through the selection of specific peptides. The quantification of serum apo(a) is possible from 3.8 to 456 nmol/L using the peptide LFLEPTQADIALLK. Results obtained using the peptides GISSTVTGR and TPENYPNAGLTR proved to be in good agreement. The developed candidate RMP will be an essential part of the apolipoprotein traceability chain and future RMS. Especially, it will be used to certify the concentration of apo(a) in secondary certified reference materials (CRMs) to calibrate commercial immunoassays and verify their accuracy before bringing them to the market.

To reach measurement standardisation through this new measurement system, two essential requirements had to be fulfilled: a sufficient correlation among the MPs and appropriate commutability of serum-based reference materials. The correlation among the candidate RMP and immunoassay-based MPs was assessed by measuring a panel of 39 clinical samples. Results of the immunoassay-based MPs and the candidate RMPs demonstrated good linear correlations for the clinical samples, but some significant sample-specific differences were also observed. In addition, the commutability of 14 different candidate reference materials was investigated. The results of the commutability study showed that RMs based on unspiked human serum pools can be commutable with clinical samples, whereas human pools spiked with recombinant apo(a) show different behaviour compared to clinical samples. This led to the conclusion that unspiked human serum pools are the preferred candidate secondary RMs in the future SI-traceable Lp(a) RMS (figures 1-3).

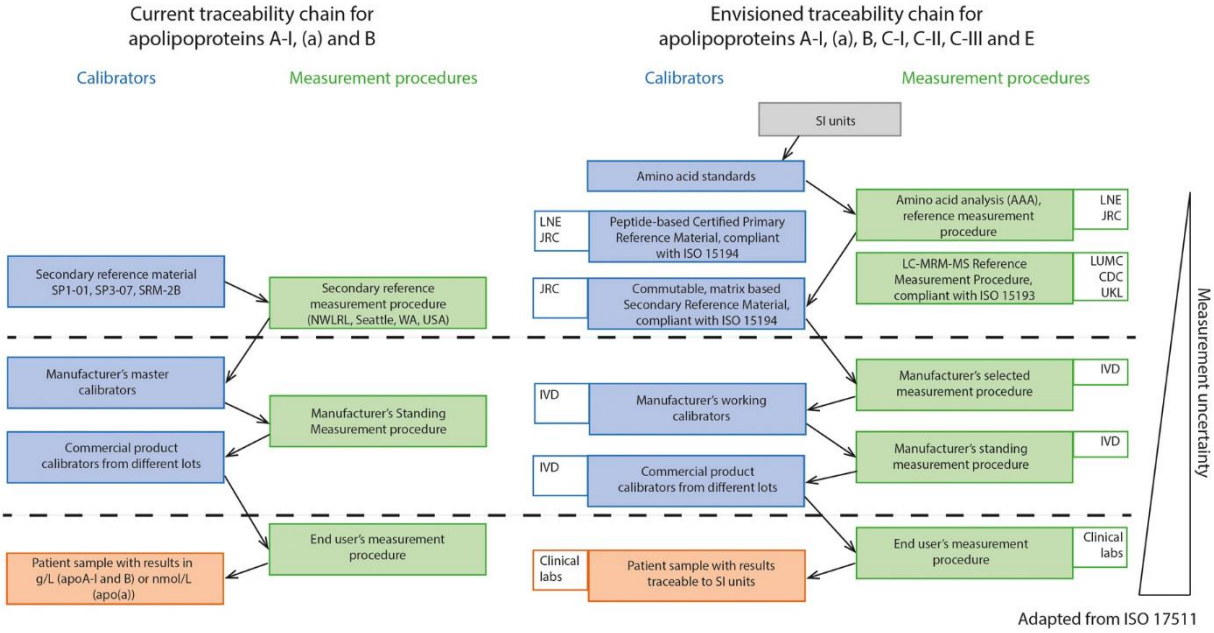


Fig 1. Metrological traceability chains are presented for contemporary and emerging serum apolipoproteins consisting of an unbroken sequence of calibrators and measurement procedures that are used to relate a measurement result to a reference of higher order. Serum apos A-I, B, and (a)/Lp(a) test results are currently traceable to WHO-IFCC secondary reference materials (left), whereas envisioned traceability to SI for serum apos A-I, B, Cs, E, and (a) is presented on the right. JRC: Joint Research Centre, Geel, Belgium; LNE: Laboratoire National de Métrologie et d'Essais, Paris, France; IVD: *In-Vitro* Diagnostic; SI: Système Internationale d'Unités (from C. Cobbaert et al., Clin. Chem. 2021, 67(3), 478).

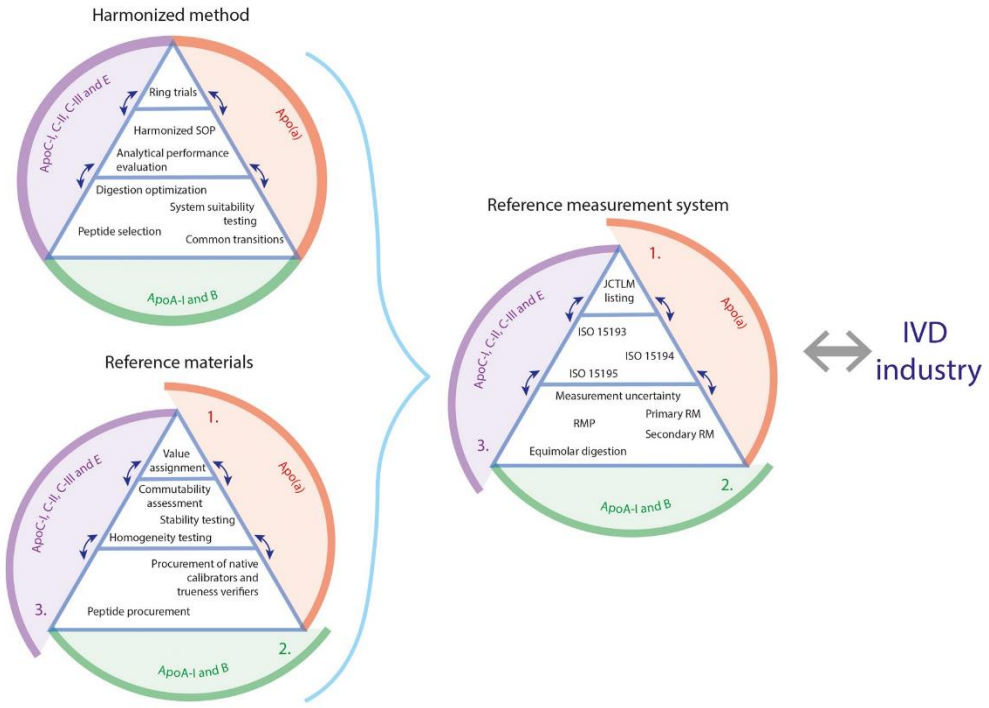


Fig 2. Establishment of a multiplex RMS for seven serum apolipoproteins, including timelines for its deliverables. The apolipoprotein RMS is being developed along two lines: the establishment of a harmonised multiplex RMP and the development of primary respectively secondary, serum-based reference materials (from C. Cobbaert et al., Clin. Chem. 2021, 67(3), 478).

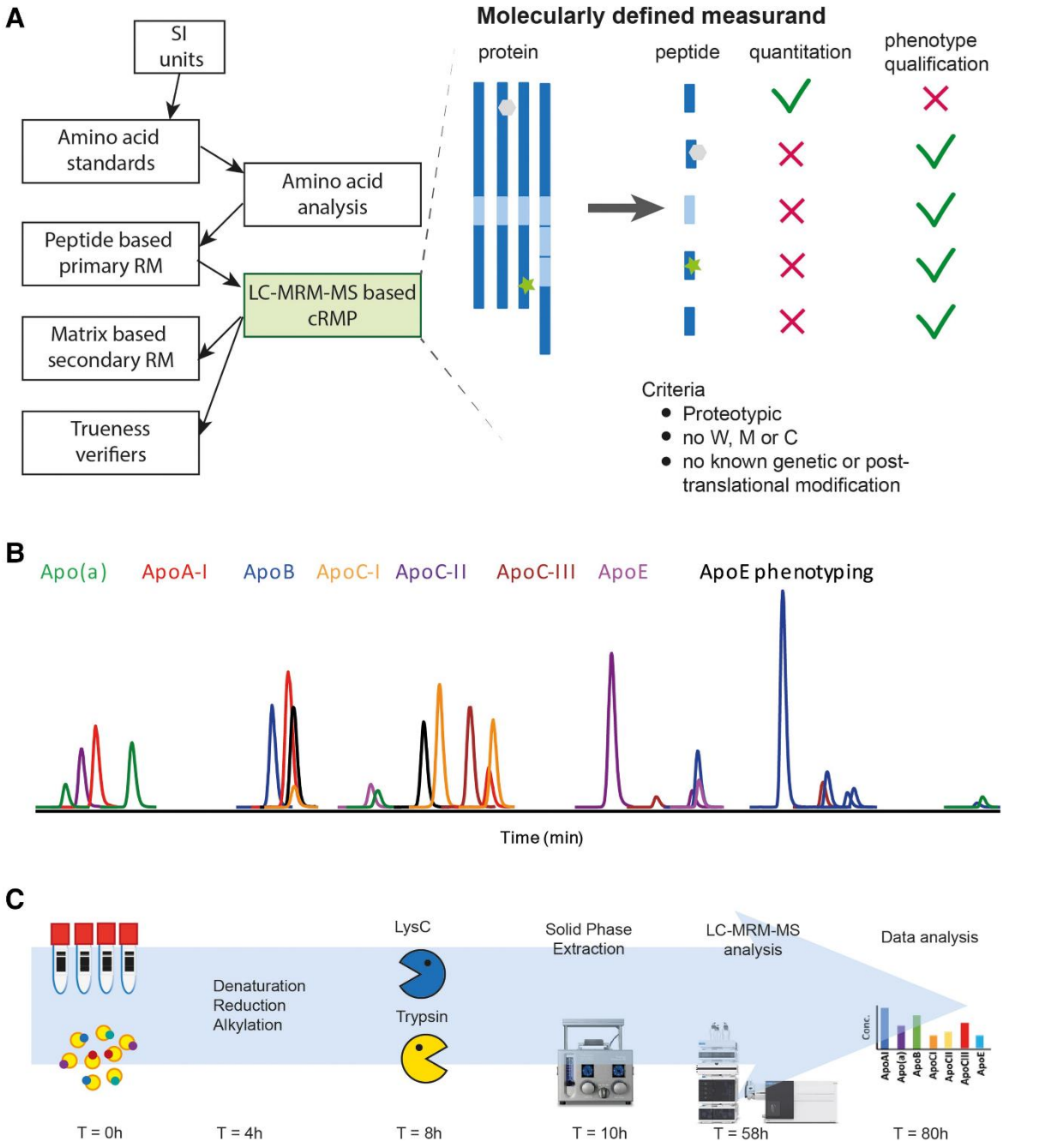


Fig 3. Overview of the liquid chromatography mass spectrometry with multiple reaction monitoring (LC-MRM-MS) candidate RMP for absolute and simultaneous quantification of a panel of apolipoproteins. LC-MRM-MS allows definition of the measurand at the molecular level through the quantification of proteotypic peptides and qualitative measurement of specific peptides representing proteoforms (A). The candidate RMP provides a chromatogram comprising peptide signals from 32 endogenous peptides as well as their synthetic stable isotopic labelled analogues (B) and uses a bottom-up proteomics strategy, with a combined LysC—trypsin digestion. The total analysis time of the procedure, including data evaluation, takes approximately 80 hours (C). W: tryptophan; M: methionine; C: cysteine (from L.R. Ruhaak et al., Clin. Chem. 2023, https://doi.org/10.1093/clinchem/hvac204).

Role and performance specification of advanced lipoprotein testing methods

Additionally, the project contributed to documenting the clinical utility of apolipoprotein profiling. There is increasing evidence that advanced lipoprotein testing methods such as multiplexed measurements of apolipoprotein panels (ApoA-I, A-II, A-IV, B-100, C-I, C-II, C-III, E), provide more detailed information on patient dyslipidaemic profiles compared to classical lipid tests, leading to better understanding of patient conditions and better stratification of patients. A clinical study was conducted with the objective to compare the apolipoprotein profile of patients with familial hypercholesterolemia (FH), a common genetic condition associated with increased LDLc levels and increased cardiovascular risk, by comparing those with associated hypertriglyceridemia (hyperTG) to those with isolated hypercholesterolaemia. The study also aimed at comparing the subclinical impairment of FH patients with and without hyperTG as a measure of coronary atherosclerotic burden (coronary artery calcium - CAC), carotid atherosclerotic burden (ultrasound) and femoral atherosclerotic burden (ultrasound). 98 subjects with a molecular diagnosis of FH, on stable statin treatment, in primary prevention for ASCVD, asymptomatic, were recruited and stratified according to the presence/absence of hyperTG, defined as TG 135-500 mg/dL in a case-control prospective observational study design. Subjects with severe hyperTG (> 500 mg/dL), statin-intolerant, pregnant, uncontrolled diabetes (HbA1c > 85 mmol/mol), receiving treatment for Human Immunodeficiency Virus (HIV) infection or corticosteroids, were excluded from the study. A preliminary analysis was performed on 46 samples but no significant difference in apolipoprotein concentrations was observed between the two study groups after adjusting for age. Then, the correlations of classical lipid biomarkers and apolipoproteins in the two groups were explored. It was found that ApoB and E were correlated with TG levels only in the CAC+ group, while Apo A-IV, Apo C-II and Apo C-III were more strongly associated with the presence of CAC although a significant weaker correlation was found in the CAC- group. These preliminary results 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. The study seems to indicate that serum apolipoprotein test results measured by MS can be used in the context of a modestly large cohort (<100 patients). While the findings should certainly be confirmed in large cohorts, using fully automated immunoassays would obviously be way faster and cheaper, provided that results are accurate. Therefore, the IDMS-based reference method will play a very important role to help end-users and laboratory specialists to select adequately standardised immunoassays and/or measurement procedures that are *fit-for-clinical-purpose*, i.e. produce tests results that are SI-Traceable and are within allowable measurement uncertainty. Accurate apolipoprotein tests will also make it possible to perform clinical studies on large cohorts and to combine data from different studies, even if immunoassays from different manufacturers were used.

Collaboration

The consortium brings together experts in laboratory medicine, metrologist, clinicians and proficiency testing (PT) providers. The clinical partners APHP and UPP provided the clinical data and samples which were then analysed by ICAN, LNE and LUMC. The experts in laboratory medicine at LUMC and metrologist from LNE developed the RMP for advanced lipoprotein measurements and provided and characterised the necessary calibration and candidate reference materials to implement a future RMS for apolipoproteins. UPP which is also a PT provider organised and evaluated an EQA scheme for established lipid biomarkers to understand their limitations and recommend appropriate analytical performance specifications for routine methods.

Key outputs and conclusions

A candidate RMP based on serum-based calibration materials for a panel of apolipoproteins (A-I, (a), B, C-I, C-II, C-III, and E) has been developed. Its suitability has been confirmed in an interlaboratory comparison between calibration labs who will implement this method now. It is the intention to change this method to peptide-based calibration as soon as the last issues with the repeatability are sorted out. Candidate secondary reference materials have also been chosen and characterised to be used in a future new RMS for the panel of apolipoproteins. It turned out that unspiked human serum pools are the preferred candidate secondary RMs in the future SI-traceable Lp(a) RMS as they are commutable for nearly all commonly used measurement kits. Based on data for a large cohort available at LUMC reference intervals for apolipoproteins could be recommended.

The accuracy of conventional biomarkers for CVD risk assessment was evaluated through the organisation of an EQA scheme. The results from a study organised within the present project confirmed previous observations in the Swedish Diabetes Registry that laboratories from regions with higher prescription of statins preferably use direct LDLc assays with a larger calibration bias compared to regions with a lower prescription which preferably use LDLc assays with no or modest calibration bias. The project contributed to assessing the

suitability of current performance criteria for conventional testing methods and propose new approaches to improve measurement accuracy and reliability that support the latest clinical practice guidelines.

A clinical study was conducted with the objective to compare the apolipoprotein profile of patients with FH, a common genetic condition associated with increased LDLc levels and increased cardiovascular risk. The preliminary results 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.

4.2 Development of reference measurement procedures for the traceable quantification of cTn which acts as a biomarker for coronary heart diseases

In the case of acute myocardial infarction (MI), cardiac troponin (cTn) is the most common biomarker for the diagnosis. cTn is a protein located in the heart muscle and is released into the bloodstream when muscle cells are damaged. The protein consists of three different subunits cTnI, cTnT (which are both specific for heart the heart muscle) and cTnC. The release of cTn into the blood is not exclusive to MI but can occur in various acute and chronic conditions. However, specific to MI is the rise and fall of cTn levels over time. A diagnosis is based on the cTnI or cTnT concentration at baseline level and changes in its concentration usually over 2-6 hours, depending on the sensitivity of the assay used, as well as considering other risk factors to distinguish between chronic conditions and MI. As EQA schemes reveal a huge spread in the results achieved by different measurement kits for cTnI in the same sample, standardisation by introducing a RMS based on RMP and calibration materials is urgently required.
Therefore, pure standard material was produced and characterised regarding the identity and modification of cTn using elemental and molecular MS, nuclear magnetic resonance (NMR) and gel electrophoresis (GE). These materials were then used in the development of the RMPs based on IDMS, including the investigation of separation methods of cTn and enrichment strategies. The methods were validated and a method comparison between the different methods was performed.

Preparation and characterisation of reference and spike for cTn

Preparation and characterisation of troponins as reference materials is the key step towards developing potential RMPs. All three individual subunits of the human cTn complex were recombinantly expressed in Escherichia coli (*E.coli*) host cells (figure 4A). cTnI, cTnT and cTnC were purified near to homogeneity. Purification of the binary complex of the cTnI-cTnC, and the ternary complex of the cTnI-cTnC-cTnT were accomplished as shown by the size exclusion chromatography (SEC) (figure 4B). Further, all proteins were characterised by 2D-NMR spectroscopy using ^{15}N-isotopically enriched proteins. Successful formation of the cTnI-cTnC complex was re-confirmed by NMR experiments (figure 4C). Experiments characterising the phosphorylation status of the troponins were performed. Recombinantly expressed cTnI has been successfully phosphorylated, which could be confirmed by ^{31}P-NMR (figure 4D). Subsequent MS analysis revealed that cTnI was seven-fold phosphorylated. Further, the recombinantly produced cTnI and the cTnI purchased from Hytest, which is isolated from human serum, behave very similarly in the ELISA confirming that the proteins are folded and active (figure 4D).

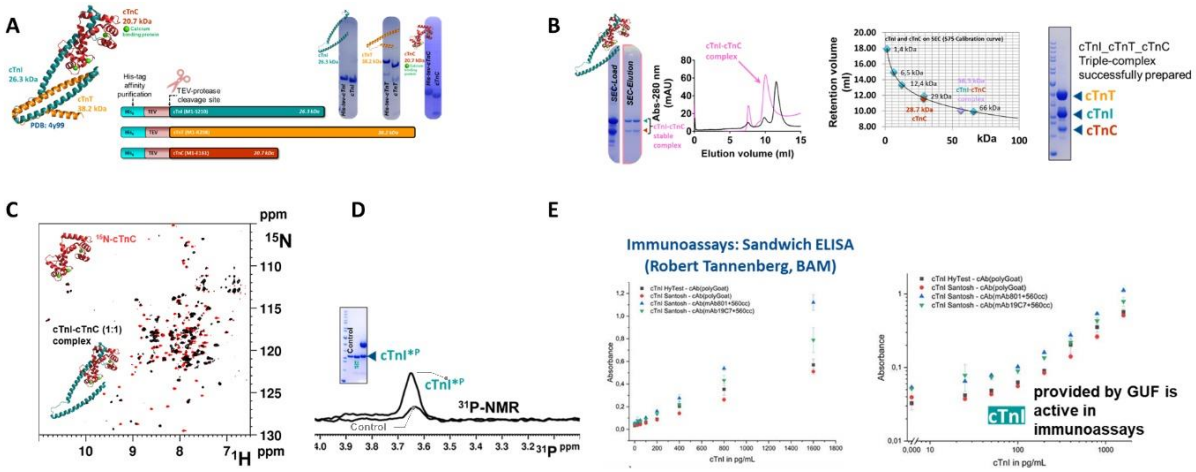


Fig 4. A) Human cTn constructs and expression. PDB deposited crystal structure of the core domain of the human cardiac protein (4Y99) (left), shown as a reference to ease perception and correspond to the colour code of the different constructs designed (middle), intended to be expressed in *E.coli*. All three cTn subunits were recombinantly expressed in *E.coli* and purified with His fusion tag, which could be removed with the TEV protease enzyme (right). B) Human cTn binary and ternary complexes. Purified cTnI and cTnC form a stable binary complex, which is purified with SEC. Fractions containing the binary complex were verified on a sodium dodecylsulfate (SDS)-PAGE (left). The chromatograms of the binary complex in the middle show 1:1 ratio of cTnI and cTnC (in black) and with an excess of cTnI (in pink), and the graphical picture shows the arbitrary molecular weight of the binary complex with reference to the standard molecular weight markers of the SEC 75 tricon (middle). Purified cTn ternary complex on the SDS-PAGE (right). C) $^{1}H,^{15}N$ TROSY HSQC spectra (950 MHz) of the ^{15}N-cTnC (apo, black spectrum) and cTnC-cTnI binary complex (holo, red spectrum). Inset: crystal structure of cTnC or cTnI-cTnC complex. The spectra were acquired at 298 K in 50 mM Tris pH 8.2, 200 mM NaCl, 5 mM CaCl2, 2 mM TCEP. D) ^{31}P-NMR spectrum of phosphorylated cTnI by PKA. Compared to control a clear signal is observed for phosphorylated cTnI. E) Immunoassays (Sandwich ELISA) comparing the recombinantly produced cTn against the commercial protein from Hytest.

Development and validation of a suitable quantification method

ELISA Development for the Detection and Quantification of cTnI

Different ELISAs were developed for quantifying cTnI at low concentrations. Various designs were tested and optimised. A schematic diagram of the assay design is depicted for illustration purposes in figure 5.

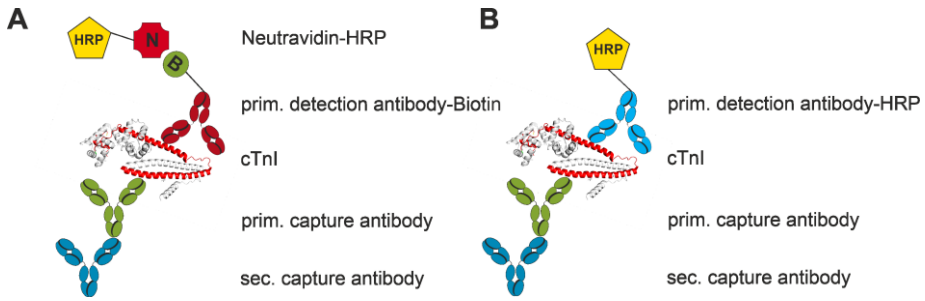


Fig 5. Schematic illustration of the sandwich ELISA formats 1.1 and 1.2. A microtiter plate (MTP) was incubated for both assays with polyclonal goat anti-rabbit antibodies as secondary capture antibodies, followed by a primary monoclonal rabbit anti-cTnI capture antibody (RecR33 from HyTest Ltd.). After blocking and incubation with the analyte (cTnI, HyTest Ltd.), two different approaches have been tested. A) Design 1.1: Incubation with biotinylated polyclonal goat anti-cTnI antibodies (HyTest Ltd.), as primary detection antibodies, followed by an incubation step with Neutravidin-horseradish peroxidase(HRP), before the final reaction with 3,3',5,5'-Tetramethylbenzidine (TMB) was performed. B) Design 1.2: Incubation with HRP-labeled monoclonal anti-cTnI antibody (Mab 9707, Medix Biochemica) as primary detection antibody followed by adding TMB and absorbance measurement after stopping with sulphuric acid.

The corresponding results of both assays (1.1. and 1.2) are presented below (figure 6).

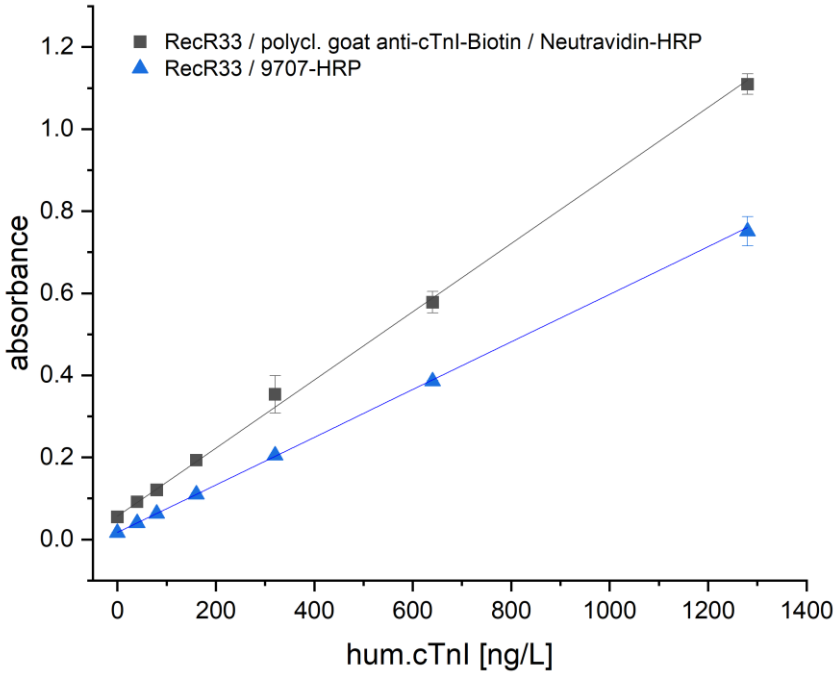


Fig 6. Results of the sandwich ELISA Designs 1.1 (black) and 1.2 (blue). For design 1.1, a LOD of 9 ng/L was achieved. By applying design 1.2, a LOD of 4 ng/L was determined.

Development of a quantification method of cTn with LC-ICP-MS

In its biological function, troponin can bind calcium (Ca) ions, which are present in so-called binding pockets. Therefore, the original idea was to quantify cTn via its Ca content with ICP-MS. Another potential approach is to quantify it via its sulphur content using inductively coupled plasma-triple-quadrupole-mass spectrometry (ICP-QQQ-MS), as the protein sequence of cTn has sulphur-containing cysteines and methionines. However, it turned out that the background and LOD for these elements was too high in ICP-MS. Serial dilution of cTn was linear up to the lowest observable concentration of around 1 mg/L in buffer for the sulphur content, which is far from the clinically relevant LOD.

Therefore, a new approach using lanthanide containing labels to modify cTn was developed including an immunoenrichment. For lanthanides very low LODs and low backgrounds can be achieved with ICP-MS. For quantifing the cTn as intact protein different commercially available Labeling Kits and an inhouse produced label were investigated. Two different main strategies were pursued: Either direct labelling of the protein or “indirect” labelling, where the cTn specific antibodies are labelled with the lanthanide and then bound to cTn. Two different antibodies were used for labelling and enrichment targeting two different epitopes in the cTnI sequence. Both antibodies were chosen to neither bind in the contact region to another subunit nor in the degradable part of the protein. The successful binding of the label to the antibody could be verified both by native SDS page and peptide specific LC-MS. One of the commercially available labelling kit resulted in broad peaks, indicating a high variability in the labelling efficiency. The second kit adding a polymeric label to specific cysteine residues in the antibody proved to be more reproducible. Binding of the labelled antibody to the cTnI could be verified using a native SDS page. However, during the chromatographic separation using SEC the formed antibody troponin complexes could not be recovered completely from the sample. In addition, extraction and enrichment procedures of these complexes did not lead to repeatable results. Future investigations are necessary to find a suitable method for the quantification of the intact cTn.

Simultaneously another approach was pursued in which cTnI was labelled with the lanthanide containing complex produced in-house, followed by digestion and quantification of the resulting specific peptides. For this purpose, a complex of 2-[4,7-bis(carboxymethyl)-10-[2-[2-(2,5-dioxopyrrol-1-yl)ethylamino]-2-oxoethyl]-1,4,7,10-tetrazacyclododec-1-yl]acetic acid (DOTA-Mal) with a lanthanide (figure 7) was produced and cleaned

up by fractionation on high-performance liquid chromatography (HPLC) using a column with octadecyl carbon chain (C18)-bonded silica as stationary phase (C18 column). The purity of the complex was verified with ESI-TOF-MS. With this complex, synthetic peptides specific for cTn could be successfully labelled.

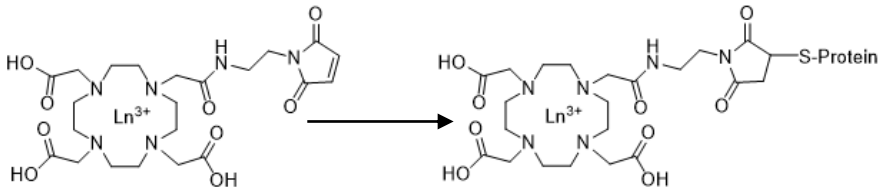


Fig 7. 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid DOTA)-complex used to label cTn for the quantification with LC-ICP-MS.

As the endoproteinase GluC (GluC) complex binds to sulfhydryl groups, cysteine-containing peptides have to be chosen. Therefore, protease GluC was used, because the digestion with GluC results in two cysteine containing peptides of cTnI. The cleaving specificity of GluC depends on the used buffer. In ammonium bicarbonate and ammonium acetate (NH_4Ac), the enzyme specificity is higher at the glutamic residues. In phosphate buffers, cleavage occurs favourable at the aspartic and glutamic residue. The in-silico digest for phosphate buffer proposed two cysteine-containing peptides KGRALSTRCQPLE (KE13) and LCRQLHARVDKVDEE (LE15) (figure 8). A Blast search with UniProt shows no agreement of these sequences with other human proteins.

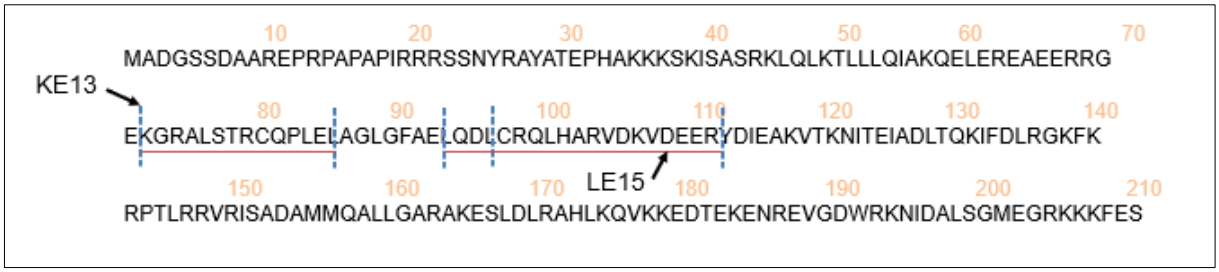


Fig 8. Amino acid sequence of cTnI (TNNI3-human), with desired cleaving sides

Recombinant cTnI from GUF was digested under different conditions and measurements were performed with Orbitrap-LC-MS. The results of data analysis with proteome discoverer showed the two target peptides but also some miss cleaved peptides around LE15. The highest yield of the desired peptide could be achieved after two days of digestion with a yield of 80 %, which could not be increased further.
To investigate the labelling yield of synthetic peptides, the peptides were labelled in 50 mM NH_4Ac under different conditions. It could be shown that after reduction of the cysteines with 50 mM tris(2-carboxyethyl)phosphine) (TCEP) for 30 min at 37 °C followed by labelling with 10x excess of label, no unlabelled peptide could be detected.
Different chromatographic columns were tested for the separation of the labelled peptides from the matrix. As the LOD did not change with column, a C18 column was chosen. Serial dilution of labelled peptides results in a LOD for cTnI of about 24 µg/L. The disadvantage using the DOTA complex, the reaction with the peptides leads to diastereomers which have different elution times from the column.
After ensuring complete labelling of the peptides, the recombinant cTnI from GUF was labelled. SEC measurement confirmed the labelling of the whole protein. To prove the completeness of the reaction, recombinant cTnI was reduced by TCEP and labelled with DOTA-Mal-praseodymium for 2 hours. Afterwards the sample was treated with TCEP and iodoacetamide (IAA) before it was digested with 0.4 µg GluC. The measurement performed with HPLC coupled to trapped ion mobility spectrometry time-of-flight mass spectrometry (HPLC-TIMS-TOF) detected no unlabelled protein.
As the desired LOD could not be reached without enrichment, different strategies using magnetic beads were tested. Finally, Protein G magnetic beads (Pierce) were chosen. For the enrichment procedure magnetic beads were washed, resuspended and incubated at room temperature with anti cTnI antibody 19C7 (Hytest). After washing with buffer again, the resuspended beads are added to 1mL of the cTnI containing sample. Digestion and analysis (Orbitrap-LC-MS) of the incubated and washed beads showed specific peptides for immunoglobulin G (IgG) and cTnI verifying a successful enrichment. Optimal conditions for incubation time

and amount of protein load were tested. After 30 min incubation time no increase in intensities from the peptide peaks could be detected (Figure 9).
For optimal digestion efficiency different parameters were tested. Next to different denaturation agents such as acetonitrile, urea, Rapigest and heat also different protease to protein ratios ranging from 1:1 to 1:200 and digestion with and without alkylation agent was tested. Despite all the different conditions, the digest did not seem to be complete after overnight digestion. Therefore, the course of the digest yield over time with digestion up to three days was performed with GluC addition after 6 h, 12 h, 24 h, and 48 h. Measurement was done with HPLC-TIMS-TOF and evaluation of the peptide content was done via signal intensities. There seems to be no point in time where both peptides were fully cleaved before degradation starts. As a large part of LE15 seems to part of a miss cleaved peptide, it was decided to quantify only via one peptide (KE13). This method was then applied to cTnI from Hytest and the recombinant cTnC-I and cTnC-T-I-complex from GUF. The measurements show similar intensities.

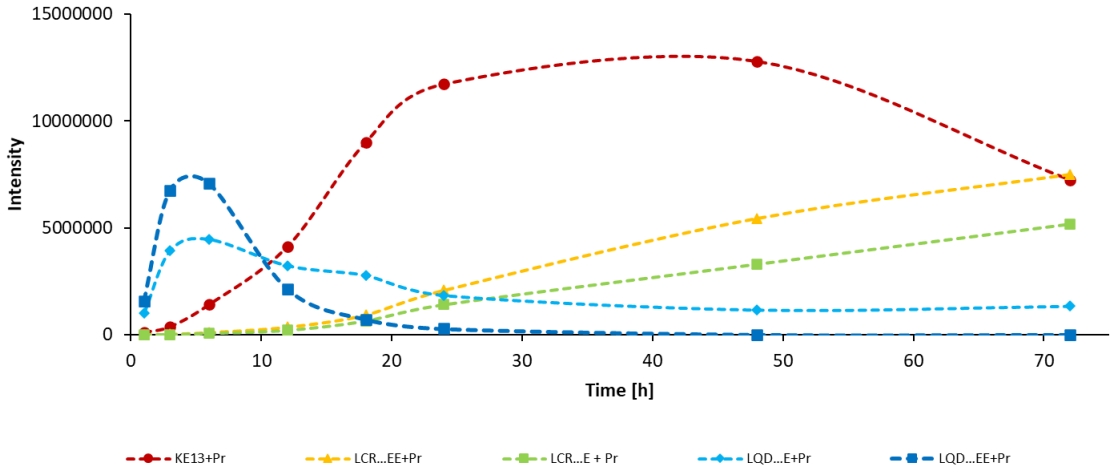


Fig 9. Course of the digest yield over time for the digest of enriched and labelled cTnI. Shown are specific peptides with and without missed cleavages.

A calibration curve of first labelled and then enriched cTnI (GUF) shows a LOD of 10 µg/L for cTnI in buffer. For serum samples with enrichment before labelling the LOD with 125 µg/l is higher (figure 10).

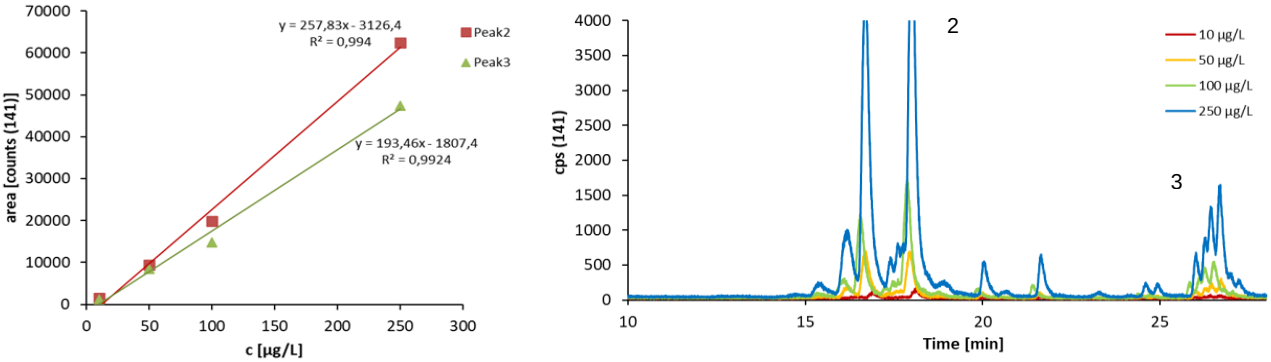


Fig 10. Calibration curve and chromatograms of enriched cTnI (Peak 2 is peptide KE13 and Peak 3 is LE15)

Development of a cTnI quantification method using immunoaffinity enrichment ID-LC-MS/MS

Another approach is the quantification of cTnI in human serum using an immunoaffinity enrichment strategy and isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) method. The isolation of cTnI from plasma using anti-cTnI antibody attached to magnetic nanoparticles (Nanomag®-D, 130 nm –

COOH) followed by an enzymatic digestion with trypsin and the simultaneous quantitation of multiple cTnI peptides constitute the key steps in the workflow.

For cTnI quantification by ID-LC-MS/MS, the two signature peptides NITEIADLTQK and TLLLQIAK were selected to act as surrogate peptides primarily based on high peptide-response and with no known post-translational modifications. ISADAMMQALLGAR and NIDALSGMEGR produced high signal responses but were not selected because of their possible post-translational modifications. Both labelled and non-labelled peptide sequences TLLLQIAK, NITEIADLTQK and anti-cTnI peptide DLPSPIER were successfully detected via protein immunoaffinity enrichment method using ID-LC-MS/MS analysis. cTnI was quantified at a concentration range of 1.8-24 ng/mL. The LC-MS/MS chromatograms of cTnI tryptic peptides and the isotopically labelled internal standards in serum are shown in figure 11. Linear and reproducible calibration curve could be obtained in this range. The analytical run was assessed regarding linearity and recovery. Both peptides, TLLLQIAK and NITEIADLTQK, showed a linear response in the range of 1.8-24 ng/mL. LOD values for cTnI in serum using peptides NITEIADLTQK and TLLLQIAK were calculated as 2.4 and 1.8 ng/mL, respectively. The correlation coefficient (r) of the calibration curve was found to be higher than 0.995.

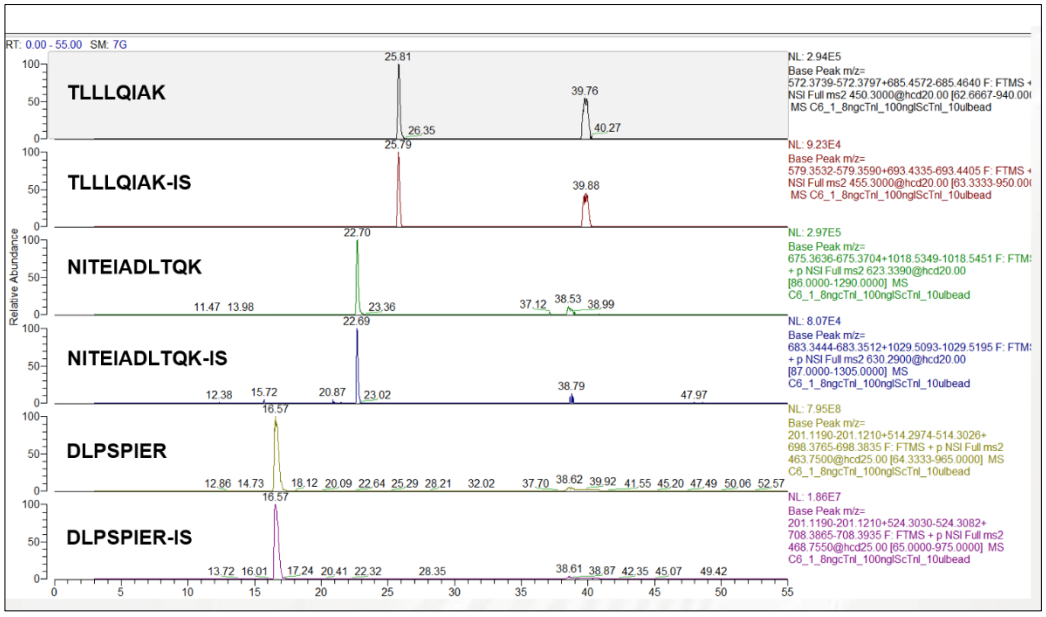


Fig 11. Extracted ion chromatograms of tryptic cTnI peptides screened for arallel reaction monitoring analysis

Possible degradation processes, which can affect the results, was investigated in the prepared samples during measurement and storage using the developed method. The results of the study are presented in figure 12. It could be shown that the calibrant is stable for at least 4 weeks during storage at -80 °C.

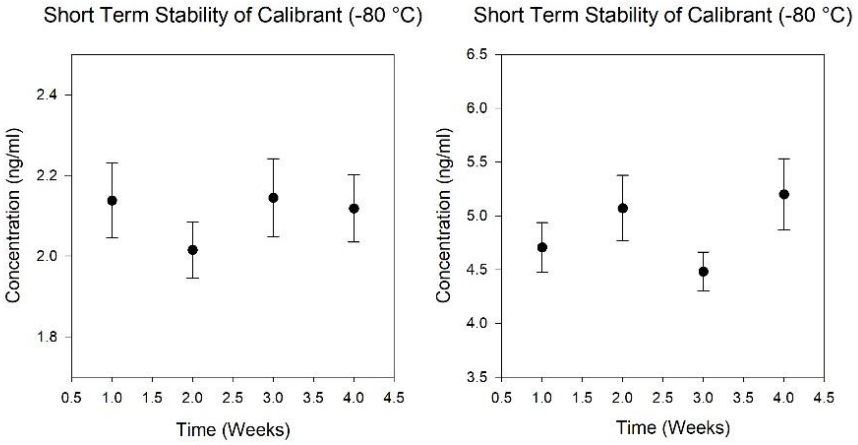


Fig 12. Short term stability study of the calibrant during storage at -80 ℃

National Institute of Standards and Technology standard reference material 2921 (NIST SRM 2921) spiked with isotopically labelled cTnI protein was used as a quality control sample for immunoenrichment recovery studies. The accuracy of the analysed quality control was between ≥ 95.0 % and ≤ 110.0 %. The recovery was between 94.8 % and 106.1 %.

Method comparison of the developed quantification method of cTn

Because of the unavailability of clinical patient samples for a method comparison, cTnI was spiked into cTnI-depleted serum, was aliquoted into subsamples and then send by PTB to BAM and TUBITAK for quantification of the cTnI with their developed methods. At BAM, two ELISAs developed in-house using different capture and detection antibody combinations have been applied. TUBITAK used the immunoaffinity enrichment ID-LC-MS/MS method and PTB the quantification method based on specific peptides of cTnI and LC-ICP-QQQ-MS. The determined recoveries vary between 73.9 % and 118.1 %, suggesting that there may have been problems with homogeneity of the different subsamples. This will be investigated in future experiments.

Collaboration:

The cooperation within this consortium enabled the production of reference and spike material as well as the development of different quantification methods for cTn than one institution alone would be able to deliver within this timeframe and with available resources. These methods enable a complementary quantification of cTn and a cross-validation of the methods. The production of reference and spike materials was done by GUF. Methods for quantification of cTnI via ELISA was developed by BAM, via IDMS of specific peptides of cTn by PTB and TUBITAK and for the intact cTnI also by PTB.

Key outputs and conclusions:

This objective was only partly completed. Reference and spike materials for troponin were produced and are available on demand. A quantification method based on ELISA was developed with a LOD of 4 ng/L. Potential RMPs based on immunoaffinity enrichment strategy followed by either ID-MS/MS or ID- ICP-QQQMS via the lanthanide labelled peptides were developed for the quantification of cTnI with LODs in serum of 2.4 µg/L and 122 µg/L, respectively. Further optimisations are necessary to reach the targeted LOQ of 3-4 ng/L in future. The target uncertainty of < 15 % could be achieved in spiked serum samples. The methods based on specific peptides were completely validated. The results represent an important step to ensure traceability in clinical tests for cTnI in serum based on a RMS including both RMPs and matrix matched calibration and reference materials.

However, application to patients' samples was not possible in the lifetime of the project due to delays in the ethical approval of clinical samples from UPP and also due to the currently still high LODs in serum matrix.

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.

4.3 Development of a biosensor capable of fast (one measurement per 10 minutes) and quasi continuous monitoring of cardiac biomarkers

To achieve a fast and reliable determination of cTnI, a biosensor was developed within the present project. The biosensor used to detect cTnI is based on chemiluminescence detection to reduce background and improve sensitivity. The principle is shown in figure 13. First, cTnI-specific antibodies labelled with horseradish peroxidase are mixed with the sample potentially containing cTnI. The mixture then passes an affinity column consisting of a borosilicate glass monolith, which was functionalised with a surrogate peptide of the cTnI epitope targeted by the antibody. If the sample contains cTnI, the antibody labelled with the enzyme is saturated and cannot bind to the peptide on the column (competition) and passes through to the detector. This results in a signal by a chemiluminescence substrate reaction, and light is emitted. In contrast, if no cTnI is present in the sample, the labelled antibody will bind to the surrogate peptide on the column. No enzyme-labelled antibody will exit the column and no signal can be detected. Different antibody and peptide combinations were tested as a proof-of-concept to achieve the best sensitivity and selectivity for cTnI.

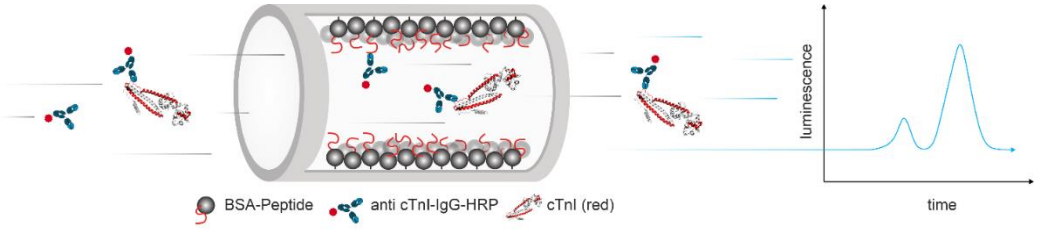


Fig 13. Biosensor principle: If cTnI is present in the sample, anti-cTnI-IgG-HRP binds the antigen cTnI. Since the antibody cannot interact with the epitope-functionalised column, the antigen-antibody complex elutes and causes a chemiluminescence signal by substrate mixing. An optical detector (CMOS) can measure the concentration-dependent chemiluminescence intensity.

Biosensor Design and Microfluidic Setup

The optical detection of the chemiluminescence is based on the commercially available camera QHY174M-GPS with a CMOS sensor and a custom-designed flow cell. The camera uses the highly sensitive Sony IMX174 front-side illuminated CMOS sensor with 2.3 megapixels (1920x1200 pixels) and a pixel pitch of 5.86 µm. A black enclosure shields the camera and the connected flow cell to avoid the influence of ambient light. The flow cell made of polyoxymethylene (POM) was designed and precision manufactured in-house (figure 14). The cover plate was additively manufactured and consisted of acrylonitrile-butadiene-styrene (ABS), retaining the planoconvex lens (12 mm focal length) with a diameter of 9 mm.

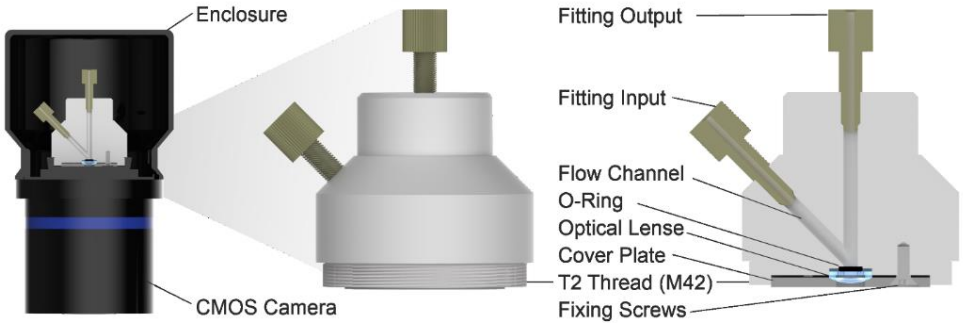


Fig 14. Flow cell design for chemiluminescent detection.

Initially, for equilibration and to remove all air bubbles inside the tubings and the flow cell, the whole microfluidic setup was flushed with filtered (0.2 µm) and degassed running buffer (phosphate buffered saline (PBS) with 0.05 % Tween 20 and 0.1 % bovine serum albumin (BSA)). Subsequently, the running buffer and chemiluminescence substrate (SuperSignal West Pico Plus) were injected with a high-precision syringe pump from Chemyx (Fusion 4000X dual). The buffer flow was directed through a six-way valve, which was used to inject 500 µl samples via a sample loop to the monolithic affinity column (figure 15). After passing the monolithic column, the chemiluminescence substrate was mixed with the sample flow with a microfluidic pearl-chain mixer. In this process, the sample flow was diluted and mixed 1:1 with the chemiluminescence substrate and incubated in an incubation loop (figure 16). The chemiluminescence signal was detected via a CMOS sensor connected to the flow cell. The exit flow was discarded.

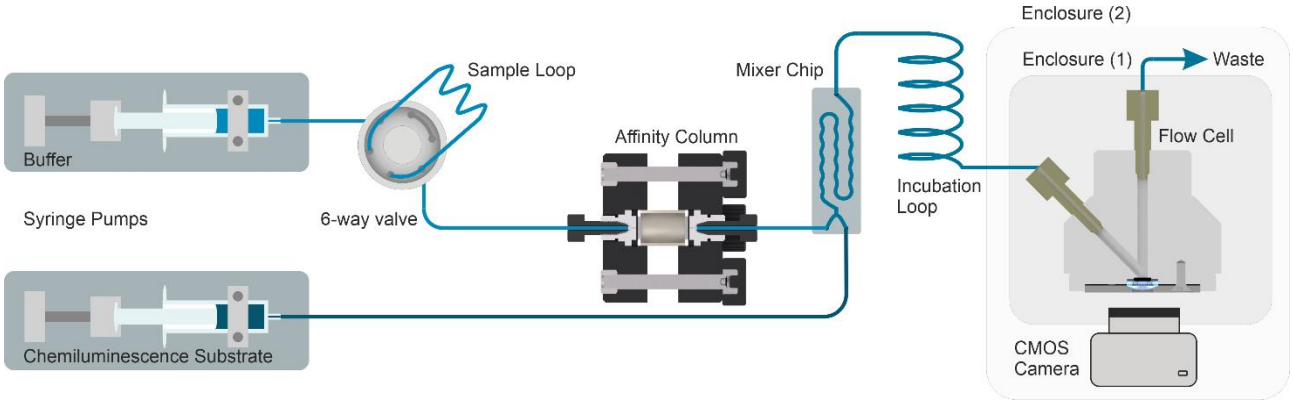


Fig 15. Microfluidic setup for the detection of cTnI. The sample injection was performed by a 6-way valve and a continuous flow of running buffer and chemiluminescence substrate. After passing the peptide-BSA affinity column, the sample and substrate were mixed 1:1 inside a mixer chip. This was followed by an incubation loop for preincubation and the flow cell for optical detection.

For the detection, a sequence of images was captured by the CMOS camera with an exposure time of 15 seconds each. The sensor was cooled to -5 °C during the measurement to improve the noise performance further. Due to the temperature-dependent nature of the enzymatic reaction, the temperature inside and outside the enclosure was closely monitored continuously by temperature sensors.

To capture the sensor data, the Software SharpCap 3.2 was used. Images were saved as 12-bit FITC-raw files. The gain was set to zero, 2x2 pixel binning was selected (final image size 960x600 px). Image evaluation was performed by a custom Python script with a fixed region of interest (ROI) of 640x600 px and exported as text (txt) files.

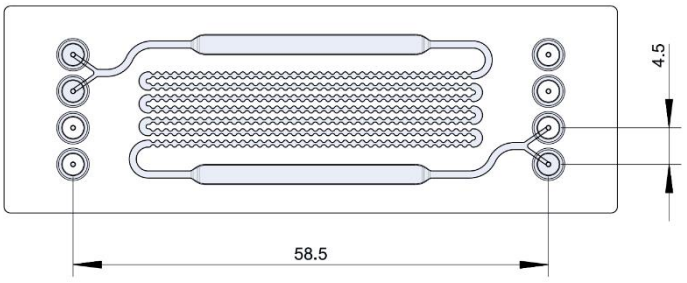


Fig 16. Drawing of the pearl-chain mixer chip (658) from microfluidic ChipShop (Reproduced with courtesy of microfluidic ChipShop).

For the synthesis of the peptide conjugate, the bifunctional crosslinker succinimidyl-3-(bromoacetamido)propionate (SBAP) was chosen. In the first step, SBAP was conjugated to some of the primary amines of BSA (lysine side chains) via N-hydroxysuccinimide (NHS) ester reaction leading to SBAP-BSA. In the second step, after adding peptide188-199, the free bromoacetyl groups on the BSA reacted with the N-terminal cysteines of the peptide. The conjugate (Pep188-199-BSA) was analysed by matrix

assisted laser desorption/ionisation time-of-flight MS (MALDI-TOF-MS). Approximately twenty molecules of SBAP and five peptides per BSA were bound on average (figure 17).

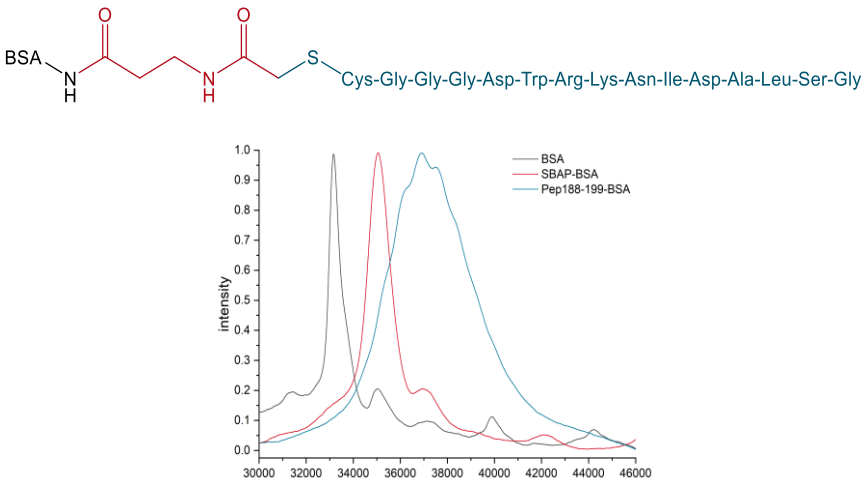


Fig 17. Above, MALDI-TOF-MS of doubly charged species [M+H]2+ of BSA (black), SBAP-BSA (red), and the final Pep188-199-BSA conjugate (blue). Below, a schematic structure of the conjugate is shown. Due to the shift in the spectrum towards higher mass to charge ratio (m/z), an average of about five peptides per BSA could be determined.

Functionality Test and Measurement Speed

In order to confirm the functionality of the peptide-BSA conjugate and the specificity of the antibody-HRP conjugate, the performance of the functionalised affinity column was tested. Therefore, the highly diluted Mab9707-HRP conjugate was injected via a six-way valve and a sample loop (1:60,000 dilution). The relative chemiluminescence was measured with and without an inserted affinity column. The signal difference provides information about the binding interaction of the antibody with the affinity column. By inserting the affinity column, the signal was reduced by up to 94 %, meaning that this antibody-HRP conjugate fraction was bound to the peptide-BSA column. Subsequently, the antibody-HRP conjugate was injected six times onto the column, shown in figure 18. A stable baseline was obtained, and the peak area of each blank was integrated, resulting in a relative standard deviation of 4.4 %.

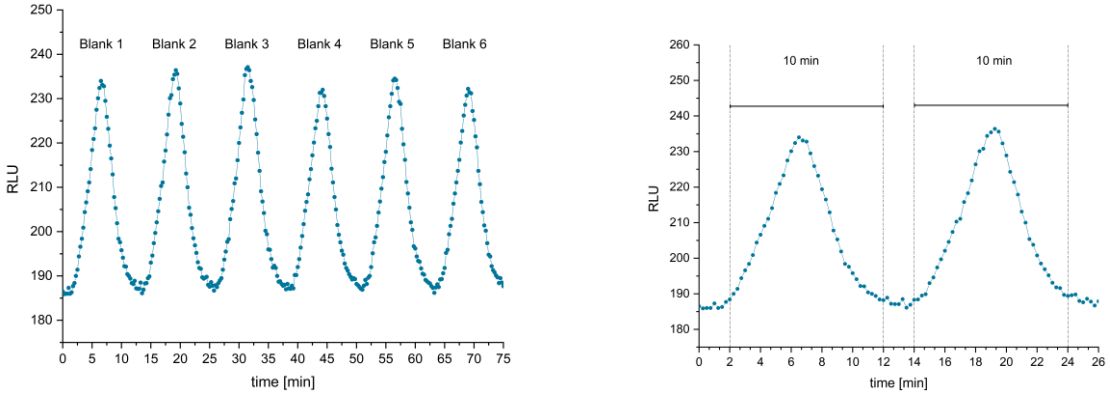


Fig 18. The injection of a sample series with six blanks achieved a standard deviation of 4.4 % using peak integration. A peak width of about 10 minutes was achieved.

Test of Specificity (Cross-reactivity)

To explore the antibody specificity and to rule out interference of the analyte storage buffer, different samples were tested in duplicates: cTnC, cTnT, cTnI, and the sample buffer. In addition, the blank (running buffer) was also measured as a reference (figure 19).

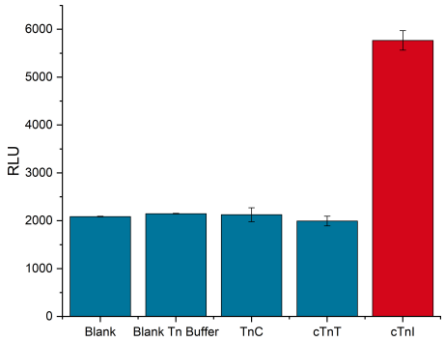


Fig 19. Antibody specificity test for cTnI: The biosensor detected cTnI (red column). No cross-reactivity with cTnT, cTnC, or any influence of the sample buffer was observed. Chemiluminescence was quantified by integration of the peak area. Error bars represent standard deviations.

For the specificity test of the biosensor, each troponin species was diluted to 16 µg/L, considering the same dilution factor for the blank buffer. For all samples but the cTnI, the chemiluminescence signals at the background level were obtained, the same as the negative control. The integrated cTnI signal is significantly higher than the signals for the other troponin species and negative controls. This experiment underlines the excellent selectivity of the system.

Linearity Test

The linearity and LOD of the biosensor were investigated. Therefore, various concentrations of cTnI were measured, ranging from 0 to 16 µg/L, as shown in figure 20.

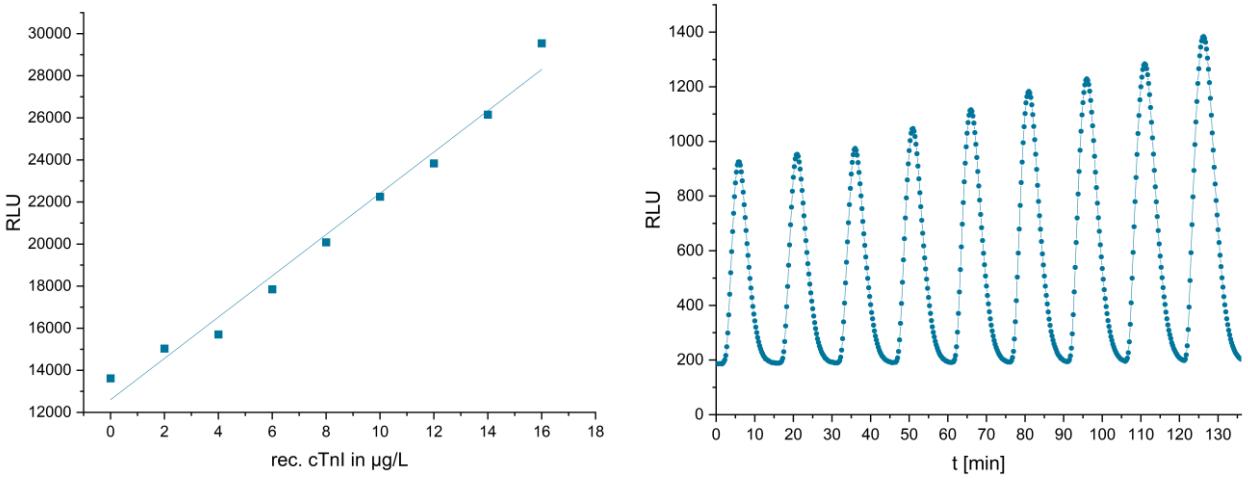


Fig 20. Calibration with a linear fit for Troponin I in the lower range of 0, 2, 4, 6, 8, 10, 12, 14, 16 µg/L and appropriate calibration curve after peak integration.

The LOD of 2.8 µg/L was calculated based on three standard deviations of the blank measurement series (4.4 %) (shown in figure 18).

Validation and Measurement of human cTnI

Recombinantly produced cTnI (provided by project partner Goethe University Frankfurt, GUF) expressed and purified from *E. coli* was used for the biosensor measurements. In addition, a calibration with commercial recombinant cTnI (HyTest Ltd.) was performed, and for this purpose, a sandwich-ELISA was established. Subsequently, the concentration of the stock solution could be determined and used for the measurements (figure 21, right).

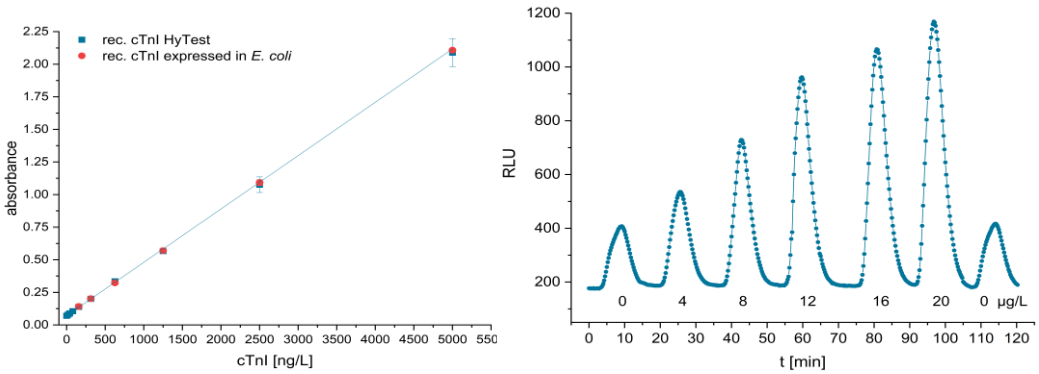


Fig 21. Left: Quantification of cTnI expressed in *E. coli* by using commercially available cTnI (Hytest 8RTI7), which was used to calibrate the sandwich ELISA. Right: Measurement of human plasma samples spiked with recombinant cTnI in a range between 0 and 20 µg/L diluted 1:40 in running buffer (in the Figure, final concentrations are given).

Finally, the biosensor was tested with real sample matrices. Recombinant cTnI was spiked into human plasma with defined cTnI concentrations as well as human serum (figure 22). The samples were diluted forty-fold with the running buffer to reduce matrix effects. The chemiluminescence intensity increases with higher concentrations of cTnI in a linear range between 0 and 800 µg/L in the sample or between 0 and 20 µg/L final concentration (figure 22). A LOD of 40 µg/L for plasma and 140 µg/L for serum and a LOQ of 70 µg/L for plasma and 260 µg/L serum were obtained based on the blank value series with a standard deviation of 4.4 %.

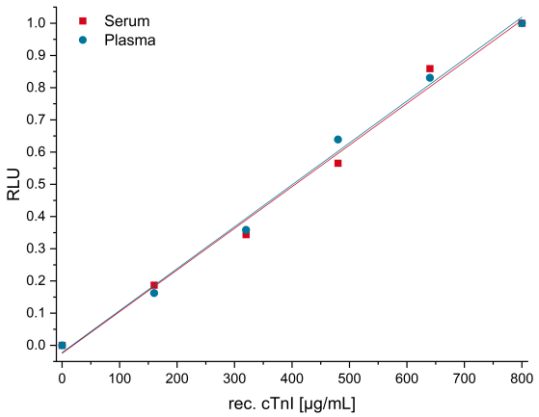


Fig 22. Linearity of the cTnI detection in spiked human plasma and purified human serum (complex matrix). The samples were diluted 1:40 in the running buffer. Here we present the proof of principle for a chemiluminescence biosensor, which enables the measurement of cTnI in blood plasma and serum within 10 minutes. A LOQ of 70 µg/L for plasma and 260 µg/L serum was achieved. In addition, recombinant cTnI, expressed in *E. coli* by GUF, was successfully tested compared to commercially available cTnI from HyTest Ltd (Purity > 95 % by SDS-PAGE).

Collaboration:

Based on BAM's experience in developing microfluid biosensor, they developed a biosensor for cTnI using the recombinant cTnI material provided by GUF.

Key outputs and conclusions:

This objective was partly completed. A biosensor for monitoring cTnI was developed. Measurements of serum samples within 10 minutes are possible. Test of functionality, measurement speed, specificity, linearity and validation were performed. LOQs of 70 µg/L for plasma samples and 260 µg/L for serum samples were achieved. However, to give results comparable to the currently established high sensitivity assays used in clinical laboratories, a LOQ of 5 ng/L is required. So further optimisation is necessary. 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.

4.4 Development of a reference measurement procedure for the quantification of heart failure biomarkers such as brain natriuretic peptides (BNP)

The aim of the project was to develop RMPs for the measurements of natriuretic peptides (NPs) in patient serum samples, to assist in the standardisation of EQA schemes and NP clinical assays. One aim was to improve understanding of the discrepancy of the brain natriuretic peptide (1-32 BNP) measurement results from commercially available immunoassays through a better definition of the analytical and clinically relevant measurand. A further aim was the development of a RMP for *N*-terminal proBNP (NT-proBNP) and its application to EQA schemes and clinical samples. Procedures for the calculation of measurement uncertainty within EQA schemes should be defined, based on the data and inform about the requirements of commutable NT-proBNP EQA materials. This will facilitate the comparison of the results and provide additional information on sample requirements.

Optimisation of the method for the quantification of BNP and its circulating forms

A reference measurement procedure targeting intact 1-32 BNP in plasma with a LOQ of 15 fmol/g was developed by Torma et al. (Clin- Chem Lab. Med. 2017, 55(9), 1397). This method was optimised to monitor total BNP, including intact 1-32 BNP and its most relevant truncated forms. The method has been optimised by applying a tryptic digestion step to monitor a peptide “ISSSSGLGCK” specific for almost all truncated BNP forms identified in human plasma such as 1-32 BNP, 3-32 BNP, 4-32 BNP, 5-32 BNP, 2-31 BNP, 3-27 BNP, 4-27 BNP, 4-30 BNP, 4-31 BNP, 5-31 BNP, 6-32 BNP, 5-31 BNP, 6-32 BNP, 5-31 BNP.

A peptide primary calibrator was characterised, and a LC-MS/MS method was developed to monitor ISSSSGLGCK. However, evidence suggests that BNP is being used less often by clinical laboratories than NT-proBNP as the preferred marker in the diagnosis of heart failure (HF). This reduction in use may be due to the introduction of NT-proBNP methods on multiple analytical systems or due to the short half-life of BNP in serum. **Error! Reference source not found.**23 shows trends the participation in EQA schemes for BNP and NT-proBNP for Abbott and Siemens systems from 2017 to 2022 (UK NEQAS Cardiac Markers organised by GGHB). These two manufacturers started offering NT-proBNP routinely in 2018. Therefore, focus was placed on the development and validation of a NT-proBNP quantification method based on ID-LC-MS/MS including the characterisation of a NT-proBNP primary calibrator.

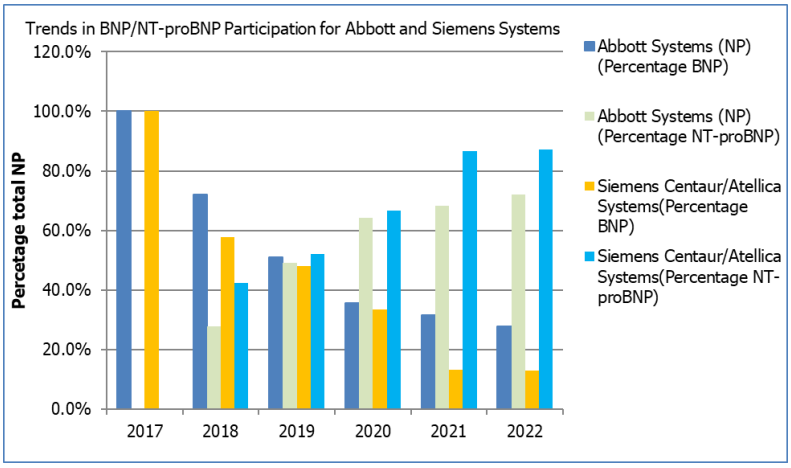


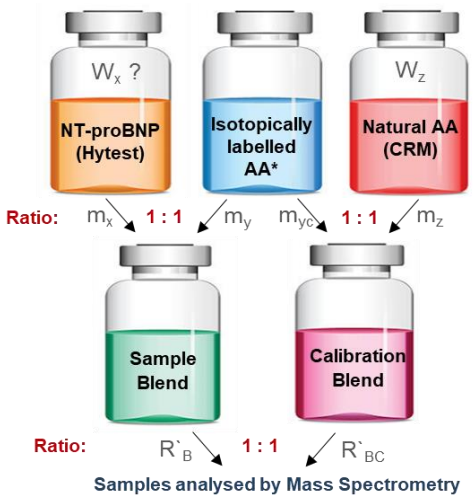
Fig 23. Trends in BNP/NT-proBNP participation for Abbott and Siemens systems.

NT-proBNP primary calibrator characterisation

For the development of a RMP for NT-proBNP, the use of a well characterised primary calibrator is essential. Primary calibrators are traceable to the SI and are characterised very precisely and accurately with the lowest measurement uncertainty using primary reference methods. A very well characterised primary reference method to quantify peptides and smaller proteins is based on amino acid analysis and double exact matching IDMS (EM-IDMS). The principle and the equation of EM-IDMS is shown in **Error! Reference source not found.**4.

As a candidate primary calibrator, an aqueous solution of NT-proBNP (material supplied by Hytest) was prepared, with a concentration estimated assuming complete release of the amino acids from the protein (glycine, leucine, alanine, valine, lysine, isoleucine and proline). The actual concentration of the amino acid released from NT-proBNP to be quantified is represented by W_x in the EM-IDMS equation. Natural and isotopically labelled amino acid standard solutions, containing the expected molar concentrations of the amino acids released from NT-proBNP, were used as calibrants. NT-proBNP primary calibrator quantification was realised by using certified natural amino acids from the National Metrology Institute of Japan (NMIJ; Tsukuba, Japan). W_z describes the concentration of the pure amino acid certified reference materials in the standard solution. The calibration blends (CB) were prepared by mixing the natural and isotopically labelled amino acid solutions in equimolar amounts. To form sample blends (SB), NT-proBNP samples were spiked with the isotopically labelled amino acid solution. All blends were prepared gravimetrically.

A



B

$$w_x = w_z \frac{m_y}{m_x} \frac{m_z}{m_{yc}} \frac{R'_B}{R'_{BC}}$$

Ratio: 1 : 1 : 1

Fig 24. Principle of double exact matching isotope dilution mass spectrometry (EM-IDMS) (A) and simplified EM-IDMS equation (B). W_x is the amount content of the amino acid (AA) released from the peptide or protein to be quantified (µg/g). W_z is the amount content of the pure standard material in the standard solution (µg/g). m_z and m_{yc} is the mass of the natural standard and the isotopically labelled standard used to prepare the calibration blend (g). m_y and m_x is the mass of the isotopically labelled standard and the peptide or protein to be quantified in the sample blend (g). $R`_B$ and $R`_{BC}$ are the measured ratios of the natural and labelled signals in the sample and calibration blend. CRM represents certified reference material)

The amino acids were released from the samples by using microwave assisted acid hydrolysis and analysed by gas chromatography coupled to MS (GC-MS). As amino acid solutions and all blends are prepared to result in mass ratios and measured signal ratios of 1:1, the unknown primary calibrator concentration is directly linked to the amount of the amino acid in the certified reference material. Experimental and instrument bias are, thus, minimised. The standard and combined uncertainties were calculated in accordance with ISO *Guide to the Expression of Uncertainty in Measurement* and EURACHEM guidelines. The uncertainty (u) of the individual amino acid results were calculated and combined with the uncertainty arising from sample variability and hydrolysis. Finally, the expanded uncertainty was calculated using a coverage factor value k=2 to give the final uncertainty (U) at the 95 % confidence interval. The results for each amino acid obtained for NT-proBNP were combined to provide a value for the mass fraction of NT-proBNP of (140.6 ±9.1) nmol/g (k=2) corresponding to the 95 % confidence interval (**Error! Reference source not found.**).

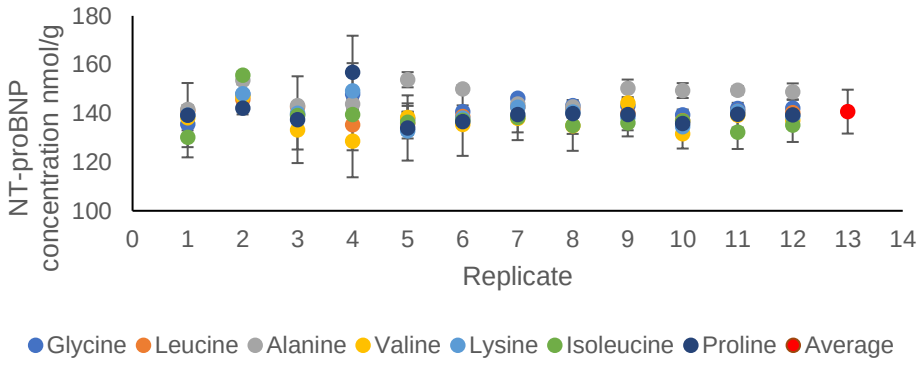


Fig 25. Mass fraction of NT-proBNP where each point represents a single amino acid value for each sample blend converted to nmol/g of protein along with the average of all results. The error bars represent the standard uncertainty calculated by Error! Reference source not found. **for individual amino acid results and the expanded uncertainty (*k*=2) for the average value.**

NT-proBNP quantification with electrospray ionisation liquid chromatography tandem MS (ESI-LC-MS/MS)

The principle of EM-IDMS explained in **Error! Reference source not found.**4 can be applied to quantify endogenous NT-proBNP in serum using the developed and validated RMP to provide value assigned materials either to be used in EQA schemes or as reference materials. In that case, the characterised NT-proBNP primary calibrator is used as a calibrant and an isotopically labelled NT-proBNP material is needed to prepare a calibration blend. The NT-proBNP sample to be quantified is spiked with the isotopically labelled NT-proBNP to prepare the sample blend.

The characterised NT-proBNP primary calibrator and the purchased $^{13}C^{15}N$ labelled NT-proBNP (Promise) was used for the development of a NT-proBNP quantification method based on ID-LC-MS/MS with a proposed target LOQ of 200 ng/g – 400 ng/g and a target uncertainty of 15 %. In a first step, the NT-proBNP spiked serum samples are cleaned up by solid phase extraction separating NT-proBNP from some of the serum matrixl. After drying the NT-proBNP elution fraction in a vacuum centrifuge, the sample is resuspended in an appropriate buffer for NT-proBNP proteolysis using the enzyme GluC. The proteolysis with GluC releases three specific NT-proBNP peptides from the native and labelled protein with the following peptide sequences: “QRNHLQGKLSE”, “QTSLEPLQE” and “SPRPTGVWKSRE”. The digested samples were analysed with a triple quadrupole MS coupled to a capillary flow LC system (Waters) and the three specific peptides are monitored by LC-MS/MS via a developed multiple reaction monitoring (MRM) method.

The NT-proBNP quantification method in serum has been evaluated regarding method sensitivity, efficiency of proteolysis of NT-proBNP, NT-proBNP stability and optimal equilibration time of native and labelled proteins. The LOD for NT-proBNP is 0.2 pmol/g (1.717 ng/g) for all three specific NT-proBNP peptides (figure 26). The sensitivity of 23-48 pmol/g (200-400 ng/g) proposed at the start of the project is easily achievable with the developed NT-proBNP method. However, the clinically relevant NT-proBNP threshold is 23-48 fmol/g (0.2- 0.4 ng/g), which is about 10 times lower than the reached LOD of 0.2 pmol/g using the developed NT-proBNP quantification method. The sensitivity of the NT-proBNP method has to be improved in the future by introducing additional sample clean-up procedures such as chromatography to separate peptides from the complex sample matrix.

Furthermore, the efficiency of NT-proBNP proteolysis in human serum using the enzyme GluC was analysed by monitoring the intensity of the three native NT-proBNP peptides QRNHLQGKLSE, SPRPTGVWKSRE and QTSLEPLQE during the time course of 0.5-30 h. It could be shown that the NT-proBNP proteolysis in serum proceeds very quickly and seems to be complete after 30 min incubation as the intensity of all three peptides decreases logarithmically even after the addition of fresh GluC after an 8 h incubation. Therefore, samples can be analysed after 0.5-1 h incubation time with GluC to retain the intensity of the fully released NT-proBNP peptides. The ratio of native to labelled peptides is very consistent during the time course with an average ratio of all peptides and time points of 0.88 ± 0.03 (±3.49 %). The stability of NT-proBNP and the equilibration time

needed to reach homogeneity of native and labelled NT-proBNP in the serum sample was tested in a time range of 5-90 min. The results suggest that NT-proBNP is stable up to 20 min in serum without protease inhibitor treatment, whereas once labelled protein is spiked to serum, the sample needs to be equilibrated for at least 20 min to reach homogeneity.

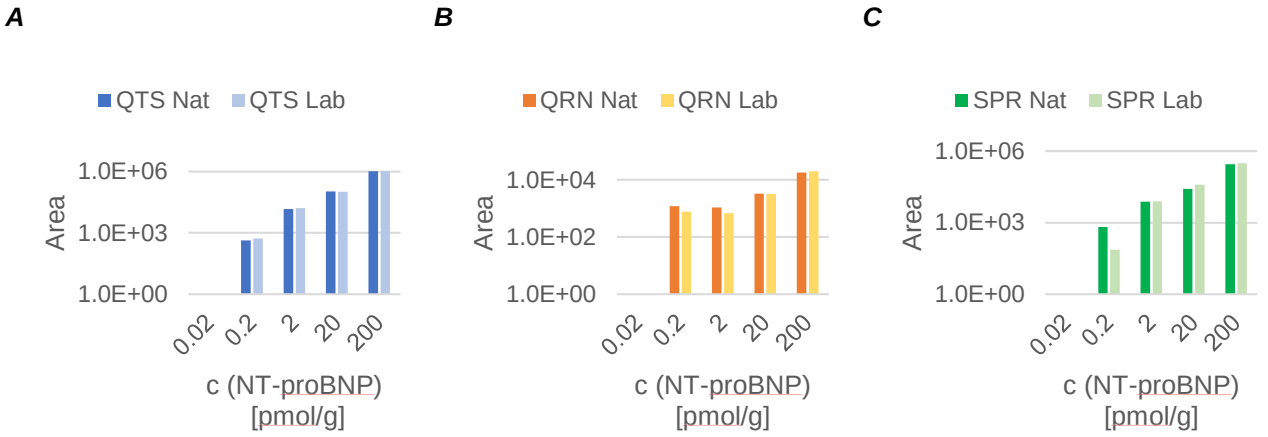


Fig 26. Sensitivity of NT-proBNP quantification in a concentration range of 0.02-200 pmol/g measured by LC-MS/MS for native and labelled peptides QTSLEPLQE (A), QRNHLQGKLSE (B) and SPRPTGVWKSRE (C).

Comparison of EQA schemes for NT-proBNP measurements

As described, the development of the RMP was delayed for various reasons. Therefore, accuracy-based evaluation of EQA and patient samples could not be realised within this project.

Since GGHB in the scheme UK NEQAS Cardiac Markers (UK NEQAS-CM) and SPMD-RfB have collected a large amount of EQA results for NT-proBNP in recent years, the partner analysed data including results from a two-year period of time within the project lifetime in order to facilitate pinpoint planning, conduction and evaluation of future studies based on the application of the NT-proBNP-RMP to EQA and patient samples. All data were analysed regarding statistical characteristics as well as measurement uncertainty calculation. To this end, two approaches for calculating measurement uncertainties from proficiency testing data described in the NORDTEST Report TR 537 have been applied. Between-laboratory and, where appropriate, within-laboratory comparison data have been included. In spite of differences in design of the EQA schemes, there is a very good coherence between the two. Extended relative measurement uncertainties for the methods and concentration ranges investigated vary approximately between 7 and 15 %.

Additionally, UK NEQAS-CM of GGHB and LGC investigated the influence of O-glycosylation of NT-proBNP on the performance of routine immunoassays via issuing samples with four different synthetically produced O-glycosylated forms in two of their EQA schemes. Results returned on three of the four glycosylated forms give similar method distribution of EQA results to the recombinant material used by UK NEQAS-CM. However, the form glycosylated at the S44 site of NT-proBNP gave lower results regarding NT-proBNP concentrations. Measurement uncertainty calculation for all glycosylated forms of NT-proBNP gave comparable results to the normally used recombinant material.

As both EQA schemes are not accuracy-based, all calculations regarding bias could only be based on mean values per method collective. It also means, random or systematic effects due to the character of the spike material used could not be investigated based on accuracy of results. Consequently, further investigations need to be conducted as soon as a RMP and/or a suitable CRM is in place. The results found with the glycosylated forms strongly indicate that glycosylation should be considered for the design of commutable calibrators, control materials and EQA samples. They also imply that the definition of the measurand needs to be reviewed for some routine assays.

Collaboration

The cooperation within the consortium enabled the investigation of different aspects and forms of NT-proBNP. While LGC developed a method for NT-proBNP quantification based on IDMS, GGHB in UK NEQAS-CM and SPMD-RfB compared EQA schemes for NT-proBNP measurements to give indications about comparability of

results and measurement uncertainty. Furthermore, the impact of O-glycosylation on the performance of immunoassays was analysed by GGHB in UK NEQAS-CM using glycosylated materials provided and quantified by LGC using amino acid analysis and EM-IDMS.

Key outputs and conclusions

A NT-proBNP quantification method based on LC-MS/MS could be developed including the full characterisation of an appropriate NT-proBNP primary calibrator. The method has a LOD of 0.2 pmol/g NT-proBNP in serum and reaches the proposed target sensitivity of 23-48 pmol/g easily. In the future, sensitivity needs to be improved by a factor of ten to reach the clinically relevant NT-proBNP concentration of 23-48 fmol/g. Therefore, the developed NT-proBNP method was only partially validated in terms of proteolysis efficiency, NT-proBNP stability in serum and optimal equilibration time of sample and standards. The published BNP RMP was further optimised to monitor total BNP including intact BNP and its truncated forms. The method has not been further validated as BNP is being used less and less by clinical laboratories with NT-proBNP being the preferred marker in the diagnosis of HF now.

The comparison of results of EQA schemes for NT-proBNP measurements indicated that the laboratory performances are comparable between the different schemes. However, an accuracy-based assessment applying the NT-proBNP-RMP on the EQA samples issued is still highly needed. With that assessment complete, a more adequate measurement uncertainty calculation for data based on the approach established in this project will be possible.

The experiments regarding O-glycosylation of NT-proBNP indicate that glycosylation should be considered for the design of commutable calibrators, control materials and EQA samples. They also imply that the definition of the measurand needs to be reviewed for some routine assays.

The data and results presented achieved during this project are a sound foundation for further investigations on measurement uncertainty of measurement procedures for NT-proBNP and commutability of materials used in standardisation of this analyte. 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.

5 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.

6 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/

7 Contact details

Dr. Claudia Swart

Physikalisch-Technische Bundesanstalt

Bundesallee 100

38108 Braunschweig, Germany

Email: claudia.swart@ptb.de

Phone: +49-(0)531-5923220

Project public website: https://www.ptb.de/empir2019/cardiomet/home/