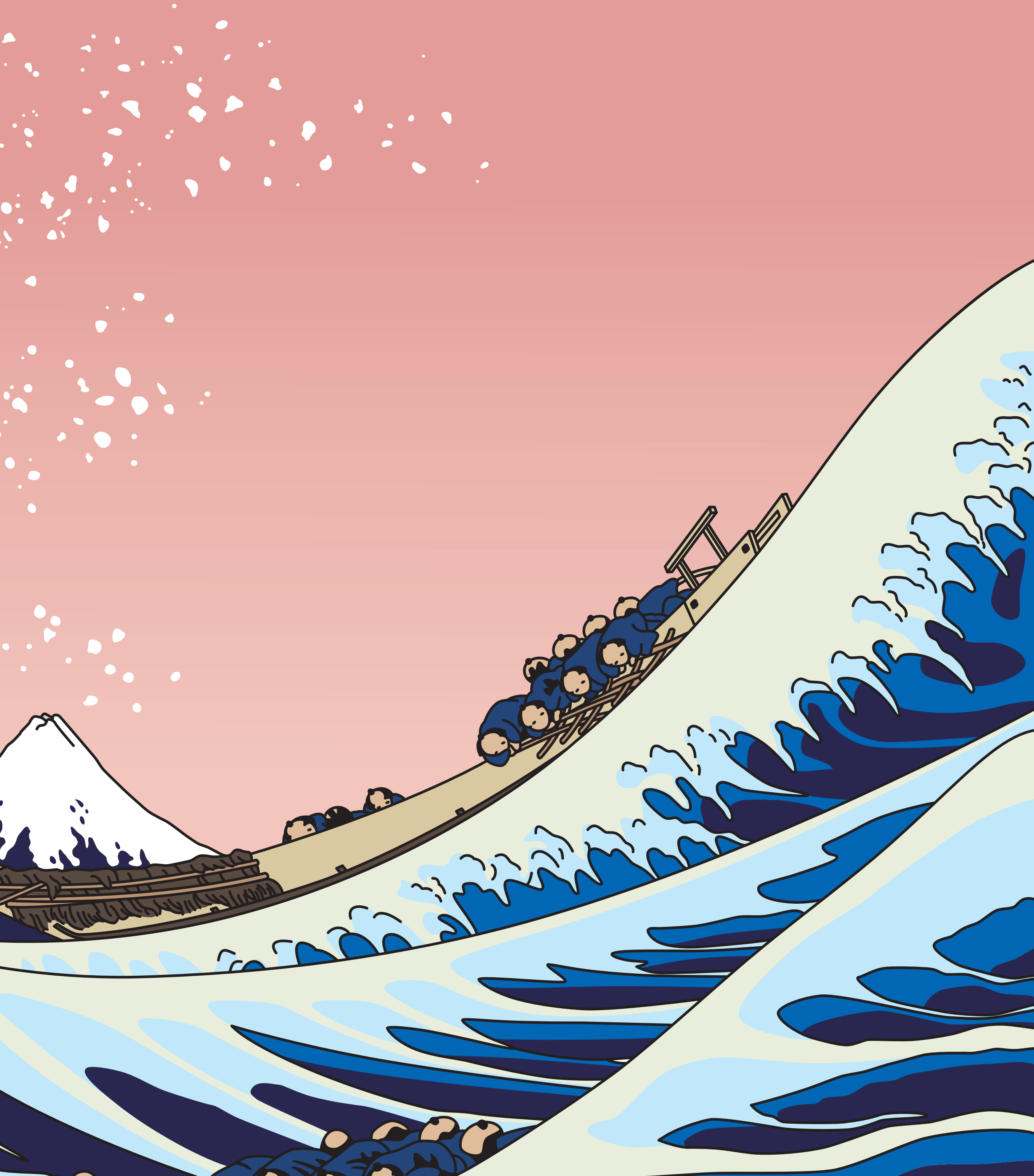
Apo-calypse now? Apolipoprotein profiling to reduce residual cardiovascular disease

Reijnders, E.

Citation

Reijnders, E. (2025, October 30). *Apo-calypse now?: Apolipoprotein profiling to reduce residual cardiovascular disease*.

CHAPTER 4



Quality Assurance for Multiplex Quantitative Clinical Chemistry Proteomics in Large Clinical Trials

---

E. Reijnders[1], F.P.H.T.M. Romijn[1], F. Arslan[1], J.J.J. Georges[1], M.M. Pieterse[1], E.R. Schipper[1], S. Didden-Buitendijk[1], M.C. Martherus-Bultman[1], N.P.M. Smit[1], N.M. Diederiks[1], M.M. Treep[1], J.W. Jukema[2,3], C.M. Cobbaert[1], L.R. Ruhaak[1]

1. Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Center, Leiden, The Netherlands
2. Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands
3. Netherlands Heart Institute, Utrecht, The Netherlands

Published in Journal of Applied Laboratory Medicine, Volume 9, Issue 6, 2024

ABSTRACT

Background: To evaluate the clinical performance and effectiveness of a multiplex apolipoprotein panel in the context of cardiovascular precision diagnostics, clinical samples of patients with recent ACS in the ODYSSEY OUTCOMES Trial were measured by quantitative clinical chemistry proteomics (qCCP). The ISO15189-accredited laboratory setting, including the Total Testing Process (TTP), served as a foundation for this study. Consequently, tailored quality assurance measures were necessary to be designed and implemented to suit the demands of a multiplex LC-MS/MS test.

Methods: Nine serum apolipoproteins were measured in 23,376 samples with a lab developed multiplex apolipoprotein test on four Agilent 6495 LC-MS/MS systems. A fit-for-purpose process was designed with tailored additions enhancing the accredited laboratory infrastructure and the TTP. Quality assurance was organized in three steps: system suitability testing (SST), internal quality control (IQC) evaluation with adjusted Westgard rules to fit a multiplex test, and interpeptide agreement analysis. Data was semi-automatically evaluated with a custom R-script.

Results: LC-MS/MS analyses were performed with the following between-run CVs: Apo(a) 6.2%, ApoA-I 2.3%, ApoA-II 2.1%, ApoA-IV 2.9%, ApoB 1.9%, ApoC-I 3.3%, ApoC-II 3.3%, ApoC-III 2.7%, and for ApoE 3.3% and an average interpeptide agreement Pearson R of 0.981.

Conclusions: This is the first study of its kind in which qCCP was performed at this scale. This research successfully demonstrates the feasibility of high-throughput LC-MS/MS applications in large clinical trials.

Clinical trial: NCT01663402

IMPACT STATEMENT

Quantitative Clinical Chemistry Proteomics allows for the in-house development of multiplexed lab developed tests for protein measurands. Quality assurance measures of these multiplex tests is challenging. We established a lab developed apolipoprotein test to address residual cardiovascular risk. To determine the clinical performance and clinical effectiveness of this test in a large randomized controlled clinical trial, we developed and evaluated a custom quality assurance strategy. The strategy ensured generation of high quality data. This study shows the feasibility of LC-MS/MS applications in clinical trials, a step towards cardiovascular precision diagnostics.

INTRODUCTION

There is an unmet clinical need to address residual cardiovascular risk beyond optimal lipid lowering therapy.[1] Even after low-density lipoprotein cholesterol (LDL-C) targets are met, a substantial risk of 70% of major adverse cardiovascular events (MACE) remains present.[2] To address this residual risk, a molecularly defined status of cardiovascular health and disease (CVD) is needed. The current lipid panel, including LDL-C, high-density lipoprotein cholesterol, total cholesterol, and triglycerides, is lacking this molecular definition and is not fit-for-purpose anymore.[3, 4] For example, LDL-C cannot be accurately measured (direct or indirect) at its low clinical target levels.[5, 6] Apolipoproteins, the functional proteins of the lipid metabolism, are considered potential candidates to fulfill this unmet clinical need as they are molecularly defined and can be measured directly with mass spectrometry.[3, 7]

In 2016, we developed a quantitative Clinical Chemistry Proteomics (qCCP) multiplex apolipoprotein panel test.[8] The test now comprises quantitation of apolipoproteins (a), A-I, A-II, A-IV, B, C-I, C-II, C-III and E as well as phenotyping of ApoE, and was analytically validated with stable performance over a longer period of time.[8-10] According to the test evaluation framework by the European Federation of Clinical Chemistry and Laboratory Medicine Working Group Test Evaluation[11], implementation of a new medical test requires assessment of the clinical performance and clinical effectiveness of the apolipoprotein panel for diagnosing and monitoring patients with CVD.

For prognostic biomarkers such as our apolipoprotein panel, clinical performance and effectiveness should ideally be proven in prognostic studies, in which a large number of individuals is followed over a longer period of time, until sufficient events have occurred to evaluate the prognostic and predictive value of a new test.[12] However, such a dedicated study is expensive, time-consuming and impractical.[11, 13] As an alternative, we leveraged an existing study, the ODYSSEY OUTCOMES Trial[14], which is a randomized controlled trial initially designed to evaluate the clinical efficacy of a proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitor alirocumab in patients with recent ACS. Measurement of our apolipoprotein panel in 23,376 samples of individuals from the ODYSSEY OUTCOMES Trial allows evaluation of the clinical effectiveness of this panel. In addition, twelve other biomarkers in the context of CVD were measured on Cobas and Diazyme analyzers.

The analysis of large numbers of samples using tests and technology that is not yet implemented in routine patient practice is challenging. In our general clinical chemistry laboratory setting, a total testing process (TTP) is in place comprising ISO15189-accredited quality management system (QMS), standard operating procedures (SOPs), a laboratory information management system (LIMS), and track-and-trace registration. This ensures accurate test results and fulfillment of predefined quality performance indicators, including total turn-around-times, analytical precision, accuracy, and sensitivity.[15] However, the apolipoprotein multiplex test is a research-based test originally not covered in the TTP. Therefore, concepts of the TTP were adapted and adopted for this test. Here we describe the design and implementation of the study process within our laboratory, with special attention for the evaluation of the quality assurance of LC-MS/MS analyses. Quality assurance in multiplex proteomics is challenging, prompting numerous published efforts[16-20] and the development of various tools[21-24]. However, at the start of the current study, publications addressing this, in particular in context of the classic clinical chemistry applications, were scarce. Even in the most recent clinical chemistry guidelines there is no clear consensus on quality assurance rules for multiplex tests.[25] Consequently, we designed our own quality assurance procedure tailored to our multiplex test. The presented study process may serve as an example for the implementation of high-throughput LC-MS/MS applications in large clinical trials.

MATERIALS AND METHODS

Apolipoprotein panel analyses

23,376 serum samples were received on dry ice, thawed, mixed, centrifuged, mixed, and divided into aliquots. Samples for LC-MS/MS analysis were stored at -80 °C and thawed prior to analysis (Supplemental Information and Supplemental Figure S1 for more details). The trial (NCT01663402) was approved by the institutional review board of each site, and all patients provided informed consent.

Serum apolipoprotein levels for Apo(a), ApoB, ApoA-I, ApoA-II, ApoA-IV, ApoC-I, ApoC-II, ApoC-III, ApoE including ApoE phenotypes were determined as published earlier for seven apolipoproteins.[8, 26] In brief, serum samples were 20x diluted in 96-wells plates and stable isotope labelled (SIL) peptides were added as internal standard (IS). Serum proteins underwent denaturation, reduction, alkylation and tryptic digestion. Subsequently, the reaction was quenched, and peptides were concentrated through solid phase extraction and measured on an 6495 QQQ-MS (6495A or 6495C) (Agilent, Santa Clara, CA). Sample preparation was performed semi-automated on a 96-channel BRAVO automated liquid handling platform (Agilent, Santa Clara, CA).

A single lot of sequencing grade trypsin (V5111)(Promega, Leiden, The Netherlands) was used. Additionally, one lot of five native serum calibrators, traceable to WHO-IFCC reference material, and one lot of IS mix containing stable isotope-labeled peptides were utilized. Bilevel internal quality control (IQC) native serum samples were measured in triplicate per batch. IQC target concentrations (Supplemental Table S1) were determined following CLSI protocols.[27] Two lots of IQC samples were used for this study.

A system suitability sample (SSS) containing synthetic peptides reflecting both endogenous (endo) and SIL peptides was prepared. Five replicate measurements of an SSS, followed by one blank, were run before and after sample measurement. Overall, two lots of SSS were used for this study.

Data validation and dataset creation

LC-MS/MS data was processed using Agilent MassHunter Quantitative Analysis. Concentrations, peak areas, ion ratios, relative responses, retention times, full width half maximum values, and peak symmetry results were exported as one csv file per batch for further data evaluation using a custom-made R-script (R version 4.2.2)[28] with RMarkdown and Knitr (version 1.33)[29] in RStudio (version 2023.6.1.524)[30]. Data was reported weekly semi-automated to the local principal investigator. Patient results were evaluated on three levels: 1st-line was the technical evaluation by the laboratory technician in Excel templates including monitoring of system suitability test (SST) results and IQC levels on single batch level, 2nd-line evaluation was performed using the R-script by the project leader who evaluated the data over multiple batches and 3rd-line evaluation and authorization was performed by the local principal investigator who evaluated the data from a clinical chemistry perspective on its clinical soundness. Results were transmitted to LIMS upon approval by all three lines. After completion of the full dataset, data validation comprised of consistency evaluation of retention times, ion ratios, and IS areas. The LC-MS/MS dataset was set up using the R-script.

RESULTS

This section describes the process, including the applied quality measures for multiplex qCCP testing, followed by an analysis of the results obtained from these measures.

Total Testing Process to support the ODYSSEY OUTCOMES Trial

The laboratory infrastructure with the TTP, operating under ISO15189 standards, including QMS, LIMS, and a team of proficient laboratory technicians, was extensively utilized with a carefully planned approach from sample receipt to data management (Supplemental Figure S1).[15] This infrastructure was essential for the LC-MS/MS analyses.

Quality assurance

To ensure high quality data, quality measures were applied. Because LC-MS/MS analysis is rather complex with semi-automated sample preparation and independent analytical instrumentation, special care was given to the quality assurance process. Specifically, a flow chart for data evaluation was constructed in collaboration with laboratory technicians involving: (1) SST, (2) IQC evaluation, and (3) interpeptide agreement (Figure 1). In addition, a RASCI matrix was constructed to delineate the different roles relating to responsibility-accountability-support-consultation-informed throughout the process. Data folders were organized following Findable, Accessible, Interoperable, and Reusable (FAIR) principles to allow track-and-trace.[31]

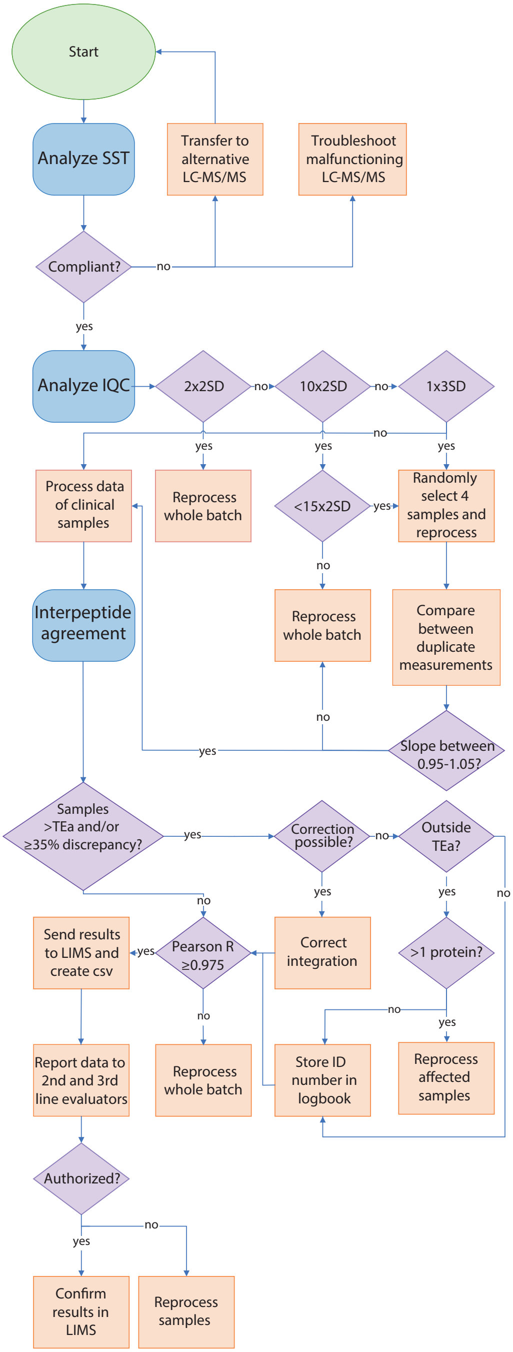
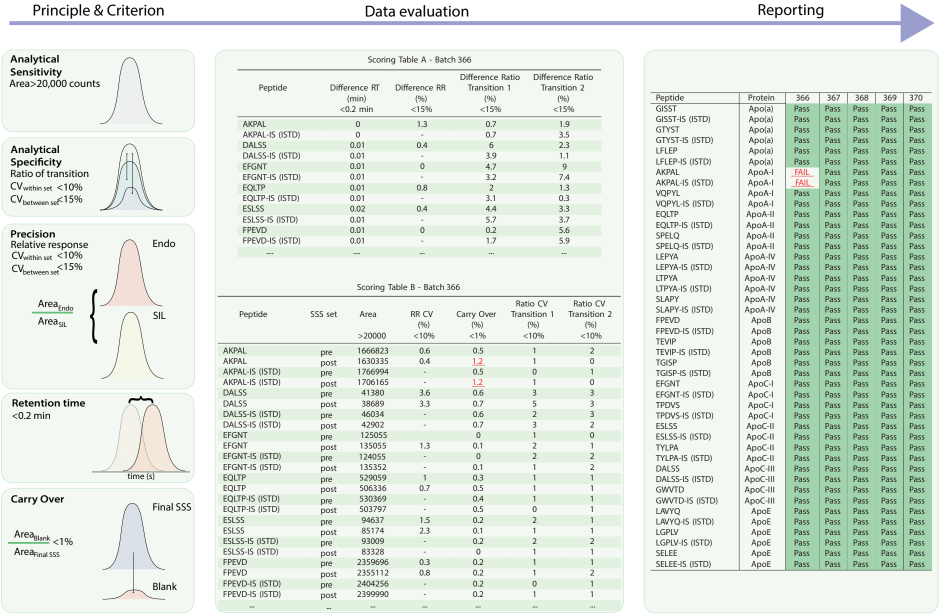


Figure 1: Quality assurance process of LC-MS/MS measurements. In rounded rectangles the different validation steps, in rectangles the actions and in diamond shapes the decisions or criteria. SST: system suitability testing, IQC: internal quality control, TEa: total allowable error, LIMS: laboratory information management system, csv: comma separated value.

System Suitability Testing

SST evaluated the performance of the LC-MS/MS instruments (Figure 2).[19, 32] Analytical sensitivity was assessed through a minimum threshold for both the IS and endo area of 20,000 counts. The variability of ion ratios and relative responses, which ensure analytical specificity and precision, respectively, was limited to 10% within five replicates and 15% between the SSS set before and after the run. Additionally, maximum deviation of retention times from the average within-run retention time was 12 s to ensure that the full peak is being detected. The maximum allowable carry-over for both endo and IS peptides is 1% and is determined by dividing the peptide area of the blank by the peptide area of the last SSS. If any of these criteria were not met for the first SSS set, the instrument was checked before running the clinical samples. Depending on the nature of the issue, either the HPLC or the MS underwent maintenance procedures, which might involve tasks such as cleaning the ion source or replacing the inlet filter of the HPLC.[19]



Scoring Table A - Batch 366

Peptide	Difference RT (min) <0.2 min	Difference RR (%) <15%	Difference Ratio Transition 1 (%) <15%	Difference Ratio Transition 2 (%) <15%
AKPAL	0	1.3	0.7	1.9
AKPAL-IS (ISTD)	0	-	0.7	3.5
DALSS	0.01	0.4	6	2.3
DALSS-IS (ISTD)	0.01	-	3.9	1.1
EFGNT	0.01	0	4.7	9
EFGNT-IS (ISTD)	0.01	-	3.2	7.4
EQLTP	0.01	0.8	2	1.3
EQLTP-IS (ISTD)	0.01	-	3.1	0.3
ESLSS	0.02	0.4	4.4	3.3
ESLSS-IS (ISTD)	0.01	-	5.7	3.7
FPEVD	0.01	0	0.2	5.6
FPEVD-IS (ISTD)	0.01	-	1.7	5.9
....	....	...	...	...

Scoring Table B - Batch 366

Peptide	SSS set	Area >20000	RR CV (%) <10%	Carry Over (%) <1%	Ratio CV Transition 1 (%) <10%	Ratio CV Transition 2 (%) <10%
AKPAL	pre	1666823	0.6	0.5	1	2
AKPAL	post	1630335	0.4	1.2	1	0
AKPAL-IS (ISTD)	pre	1766994	-	0.5	0	1
AKPAL-IS (ISTD)	post	1706165	-	1.2	1	0
DALSS	pre	41380	3.6	0.6	3	3
DALSS	post	38689	3.3	0.7	5	3
DALSS-IS (ISTD)	pre	46034	-	0.6	2	3
DALSS-IS (ISTD)	post	42902	-	0.7	3	2
EFGNT	pre	125055		0	1	0
EFGNT	post	135055	1.3	0.1	2	1
EFGNT-IS (ISTD)	pre	124055	-	0	2	2
EFGNT-IS (ISTD)	post	135352	-	0.1	1	2
EQLTP	pre	529059	1	0.3	1	1
EQLTP	post	506336	0.7	0.5	1	1
EQLTP-IS (ISTD)	pre	530369	-	0.4	1	1
EQLTP-IS (ISTD)	post	503797	-	0.5	0	1
ESLSS	pre	94637	1.5	0.2	2	1
ESLSS	post	85174	2.3	0.1	1	1
ESLSS-IS (ISTD)	pre	93009	-	0.2	2	2
ESLSS-IS (ISTD)	post	83328	-	0	1	1
FPEVD	pre	2359696	0.3	0.2	1	1
FPEVD	post	2355112	0.8	0.2	1	2
FPEVD-IS (ISTD)	pre	2404256	-	0.2	0	1
FPEVD-IS (ISTD)	post	2399990	-	0.2	1	1
...	...	...	...	...	...	...

Peptide	Protein	366	367	368	369	370
GISST	Apo(a)	Pass	Pass	Pass	Pass	Pass
GISST-IS (ISTD)	Apo(a)	Pass	Pass	Pass	Pass	Pass
GTYST	Apo(a)	Pass	Pass	Pass	Pass	Pass
GTYST-IS (ISTD)	Apo(a)	Pass	Pass	Pass	Pass	Pass
LFLEP	Apo(a)	Pass	Pass	Pass	Pass	Pass
LFLEP-IS (ISTD)	Apo(a)	Pass	Pass	Pass	Pass	Pass
AKPAL	ApoA-I	FAIL	Pass	Pass	Pass	Pass
AKPAL-IS (ISTD)	ApoA-I	FAIL	Pass	Pass	Pass	Pass
VQPYL	ApoA-I	Pass	Pass	Pass	Pass	Pass
VQPYL-IS (ISTD)	ApoA-I	Pass	Pass	Pass	Pass	Pass
EQLTP	ApoA-II	Pass	Pass	Pass	Pass	Pass
EQLTP-IS (ISTD)	ApoA-II	Pass	Pass	Pass	Pass	Pass
SPELQ	ApoA-II	Pass	Pass	Pass	Pass	Pass
SPELQ-IS (ISTD)	ApoA-II	Pass	Pass	Pass	Pass	Pass
LEPYA	ApoA-IV	Pass	Pass	Pass	Pass	Pass
LEPYA-IS (ISTD)	ApoA-IV	Pass	Pass	Pass	Pass	Pass
LTPYA	ApoA-IV	Pass	Pass	Pass	Pass	Pass
LTPYA-IS (ISTD)	ApoA-IV	Pass	Pass	Pass	Pass	Pass
SLAPY	ApoA-IV	Pass	Pass	Pass	Pass	Pass
SLAPY-IS (ISTD)	ApoA-IV	Pass	Pass	Pass	Pass	Pass
FPEVD	ApoB	Pass	Pass	Pass	Pass	Pass
FPEVD-IS (ISTD)	ApoB	Pass	Pass	Pass	Pass	Pass
TEVIP	ApoB	Pass	Pass	Pass	Pass	Pass
TEVIP-IS (ISTD)	ApoB	Pass	Pass	Pass	Pass	Pass
TGISP	ApoB	Pass	Pass	Pass	Pass	Pass
TGISP-IS (ISTD)	ApoB	Pass	Pass	Pass	Pass	Pass
EFGNT	ApoC-I	Pass	Pass	Pass	Pass	Pass
EFGNT-IS (ISTD)	ApoC-I	Pass	Pass	Pass	Pass	Pass
TPDVS	ApoC-I	Pass	Pass	Pass	Pass	Pass
TPDVS-IS (ISTD)	ApoC-I	Pass	Pass	Pass	Pass	Pass
ESLSS	ApoC-II	Pass	Pass	Pass	Pass	Pass
ESLSS-IS (ISTD)	ApoC-II	Pass	Pass	Pass	Pass	Pass
TYLPA	ApoC-II	Pass	Pass	Pass	Pass	Pass
TYLPA-IS (ISTD)	ApoC-II	Pass	Pass	Pass	Pass	Pass
DALSS	ApoC-III	Pass	Pass	Pass	Pass	Pass
DALSS-IS (ISTD)	ApoC-III	Pass	Pass	Pass	Pass	Pass
GWVTD	ApoC-III	Pass	Pass	Pass	Pass	Pass
GWVTD-IS (ISTD)	ApoC-III	Pass	Pass	Pass	Pass	Pass
LAVYQ	ApoE	Pass	Pass	Pass	Pass	Pass
LAVYQ-IS (ISTD)	ApoE	Pass	Pass	Pass	Pass	Pass
LGPLV	ApoE	Pass	Pass	Pass	Pass	Pass
LGPLV-IS (ISTD)	ApoE	Pass	Pass	Pass	Pass	Pass
SELEE	ApoE	Pass	Pass	Pass	Pass	Pass
SELEE-IS (ISTD)	ApoE	Pass	Pass	Pass	Pass	Pass

Figure 2: System suitability testing (SST) for the peptides of all apolipoproteins for five batches generated with the R-script that was run weekly. In case SST criteria are met a “pass” is generated, in case criteria are not met a “fail” is shown. In this case batch 366 was rejected and remeasured on another LC-MS/MS system.

Internal Quality Control

Bilevel native serum IQC samples were measured in triplicate per batch. Concentrations of all 22 peptides were evaluated with Levey-Jennings plots.[33, 34] Adjustments to the Westgard rules were needed to fit a multiplex test.[35, 36] The LC-MS/MS test includes 22 peptides yielding 132 IQC data points per batch for which the application of Westgard rules is too restrictive. For instance, for one analyte the 1x3SD rule provides a 0.27% chance of a false rejection. Translated to our multiplex test, the probability of a batch being falsely rejected based on a 3SD alarm within 132 datapoints is approximately 30%, which is too stringent. Therefore, three rules were applied to our multiplex test: (1) 2x2SD within the same IQC lot for the same peptide, (2) 10x2SD for the whole batch and (3) 1x3SD for the quantifying peptides only. All three rules were established based on our experience with this multiplex test and were applied per batch. The theoretic false rejection rate of the three rules was calculated in an independent manner: the 2x2SD rule would result in a false rejection rate of 23%, the 10x2SD rule and the 1x3SD rule both result in 13% of the cases. The 2x2SD rule identifies issues with specific peptides, potentially impacting reported concentrations of clinical samples as these concentrations cannot be confirmed. The 10x2SD rule was implemented to monitor overall data dispersion within the batch. The 1x3SD rule adheres to a classic Westgard rule but is solely applied to quantifying peptides to make it more suitable for a multiplex test. The 2x2SD violation resulted in an immediate rejection of the batch and all samples were reprocessed. In case of a 10x2SD violation there were two options: (1) in case there was violation of ≥15x2SD within the batch all samples of this batch were reprocessed, or (2) in case it was <15x2SD within the batch, results were confirmed by reprocessing four samples of this batch that were randomly selected as represents of the affected batch. Concentrations of all four samples of all peptides from the second measurement were plotted against the concentrations of the original measurement and compared with Deming regression (Supplemental Figure S2). A Deming slope between 0.95 and 1.05 was acceptable; otherwise, the entire batch was rejected, and all samples were reprocessed. In case the final rule, 1x3SD, was violated, four samples of that batch were randomly selected for reprocessing following the same procedure as described above.

Interpeptide agreement

The final step of quality assurance involves the interpeptide agreement assessment between the concentrations of quantifying and qualifying peptides. Disagreement could indicate sample preparation errors, such as inconsistent digestion kinetics, resulting in unreliable results. However, biological variations due to mutations on the measured peptide may also result in discordant concentrations between quantifying and qualifying peptides. Individual samples were reprocessed based on two criteria: (1) a concentration difference between quantifying and qualifying peptide exceeding the total allowable error (TE_a) for more than one protein and/or (2) a concentration difference of >35% between quantifying and qualifying peptide to identify possible mutations that affect protein quantification. The 35% rule was empirically established based on typical discrepancies observed for heterozygous and homozygous mutations, leading to biases of ~50% and ~100%, respectively. TE_a was defined based on biological variation for Apo(a), ApoA-I and ApoB resulting in 24.1%, 9.1% and 11.6%, respectively. For the other apolipoproteins a TE_a

of 20% was selected based on the state of the art.[19] To assess batch performance, a Pearson's R of ≥0.975 was applied as criterion. A coefficient <0.975 could suggest a potential batch effect. In such cases, all samples in that batch were reprocessed.

Data verification and validation

Daily/weekly data verification

1st-line evaluators processed the measurement data in Agilent MassHunter Quantitative Analysis, conducted the evaluation of SST and IQC results with Excel templates, and exported the raw data to a designated folder. A tailor-made R-script was run weekly on the CSV files. This R-script was developed for semi-automated quality evaluation, generating reports including SST, IQC evaluation and interpeptide agreement plots, as well as the concentrations of the apolipoproteins for weekly review and discussion by 2nd and 3rd-line evaluators.[15] Conclusions were automatically generated based on the predefined criteria. Inconsistencies in individual samples (e.g. caused by improper peak integration) were identified based on visual inspection and corrected if possible after which the sample results underwent the same process and review as other samples. Upon approval by 2nd and 3rd-line evaluators, results were transmitted to LIMS.

Final data validation

While quality assessment was performed daily, final data evaluation was performed after the study was completed. This included retention time checks between endo and IS peptides. If differences exceeded 0.1 min, data was visually inspected for integration errors. In addition, ion ratios of both endo and IS peptides, as well as IS areas were visually checked for outliers. If outliers were detected, integration was manually checked and corrected if needed.

Process performance

The entire process, spanning 74 weeks from sample reception to dataset completion, was monitored weekly using a dashboard constructed in RStudio (Supplemental Figure S3). Although LC-MS/MS throughput started relatively low, the expansion from two LC-MS/MS systems to four and from one BRAVO to three led to enhanced throughput. This resulted in handling 16 batches per week, translating to a total of 1,280 clinical samples per week.

System suitability testing

After measurements were conducted, the R-script generated an overview of the SST results of all peptides per batch. An example for five batches is presented in Figure 2 to illustrate the weekly R-output. 31 (9.6%) of the 322 batches were remeasured due to failed SST.

Internal Quality Control

IQC was evaluated semi-automated and results were reported weekly with the R-script. An example of the four batches left, after one batch failed the SST in previous section, is depicted in Figure 3 (left panel).

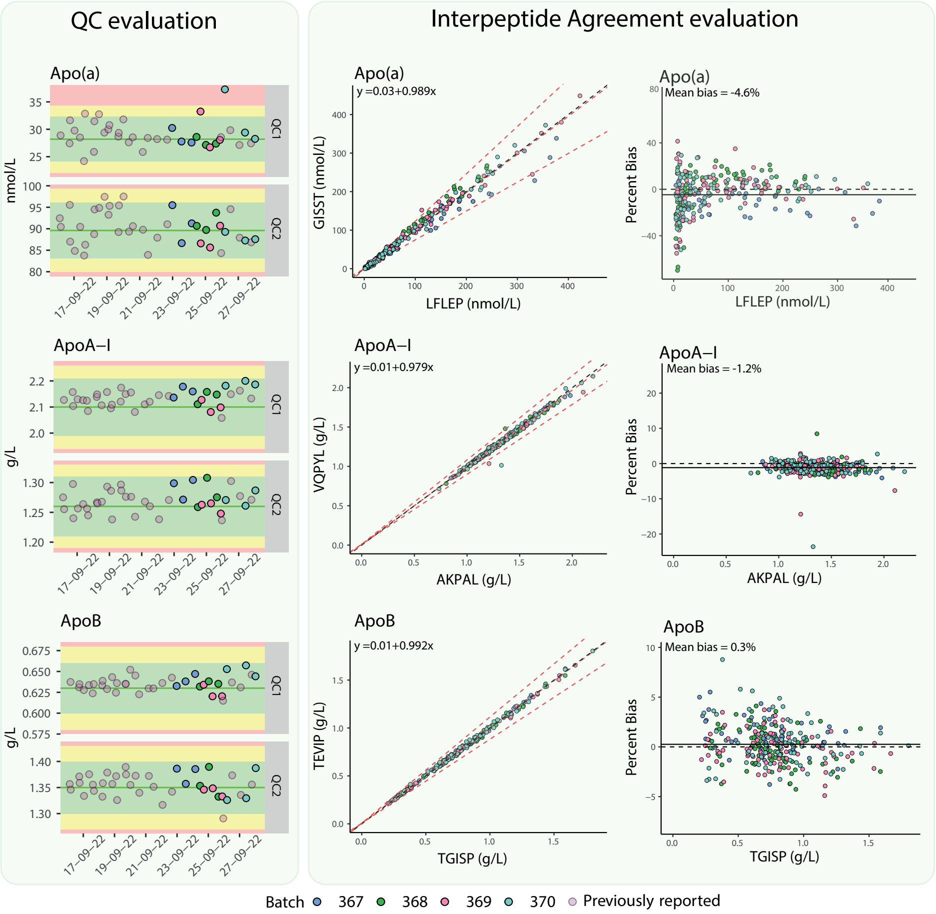


Figure 3: Example of QC evaluation and interpeptide agreement evaluation for four batches generated with the R-script that was run weekly. Left panel: Bilevel IQC monitoring for Apo(a) (quantifying peptide LFLEP), ApoA-I (quantifying peptide AKPAL), and ApoB (quantifying peptide TGISP) with 2SD (light-green), 3SD (yellow) and outside 3SD (red) borders and the target value is depicted as green line. Right panel: Interpeptide agreement evaluation of the same four batches with Deming regression plots (left) with the quantifying peptide on the x-axis and the qualifying peptide on the y-axis. On the right: Bland-Altman percentage plots, on the x-axis the concentration of quantifying peptide and on the y-axis the percent bias calculated as the difference between quantifying and qualifying concentrations divided by the quantifying concentration times 100. 0% bias depicted as black line and mean bias depicted as dashed black line. For mean bias calculations of Apo(a) concentrations below the limit of quantitation (3.8 nmol/L) were excluded from the calculation.

IQC evaluation based on multiplex-adjusted rules for LC-MS/MS

Violation of 2x2SD within the same IQC lot for the same peptide was true for 25 batches (322 batches total study (7.7%)). Most of these batches were immediately reprocessed except five, where upon visual inspection, it was decided that the affected peptide LFLEP concentration of this lot was so low (7 nmol/L) compared with the clinical decision limit (90 nmol/L), that it was clinically of limited importance. To prevent clinically less relevant alarms, IQC levels at more suitable concentrations were implemented. To establish whether the 2x2SD rule was effective, duplicate measurements for the full batches were compared retrospectively. For 16 of the 20 batches (5.0% of total study), concentrations between duplicate measurements were not comparable for at least one peptide as the Deming slope was not between 0.95-1.05 and therefore reprocessing of the samples was a correct decision.

Additionally, 4 (1.2% of total study) batches violated the 10x2SD rule. Four random samples were reprocessed for each of the batches. Comparison between results of all peptides from the four samples for the first and second preparation indicated equivalence (Deming slope 0.95-1.05) and showed no clinically relevant differences in concentrations between both measurements. Therefore, all 4 batches retrospectively passed IQC evaluation.

Violation of the 1x3SD rule for the quantifying peptides occurred in 7 batches (2.2%). Four randomly selected samples were reprocessed following the same procedure as described for the 10x2SD rule. All 7 batches retrospectively passed IQC evaluation demonstrating equivalence through comparison with a Deming slope ranging between 0.95 and 1.05 and showed no clinically relevant differences in concentrations between both measurements.

Overall internal quality control results

IQC concentrations were monitored for all 22 peptides with Levey-Jennings plots over a period of 14 months (Supplemental Figure S4). After study dataset completion, the average between-run coefficient of variation (CV) was calculated (Table 1). CVs vary among different levels of IQC, proteins and instruments. The average CVs per apolipoprotein were as follows: Apo(a) 6.2%, ApoA-I 2.3%, ApoA-II 2.1%, ApoA-IV 2.9%, ApoB 1.9%, ApoC-I 3.3%, ApoC-II 3.3%, ApoC-III 2.7%, and for ApoE 3.3% resulting in an overall between-run CV of 3.1%.

Table 1: Average between-run CV (%) per protein, per IQC lot (1A, 1B, 2A, 2B) and per LC-MS/MS type (Agilent QQQ-MS 6495A or Agilent QQQ-MS 6495C) for quantifying peptides.

Protein	Coefficient of variation %								
	IQC Level 1A		IQC Level 1B		IQC Level 2A		IQC Level 2B		Overall (%)
	6495A	6495C	6495A	6495C	6495A	6495C	6495A	6495C	
Apo(a)	14.3	13.3	5.4	4.3	7.1	6.6	3.2	3.0	3.2
ApoA-I	3.5	2.0	3.9	2.0	3.8	1.5	3.5	1.6	3.5
ApoA-II	3.2	2.0	2.9	2.1	2.6	1.7	2.4	1.6	2.4
ApoA-IV	4.6	2.3	5.2	2.2	4.5	2.1	4.2	1.9	4.2
ApoB	2.8	2.1	2.0	2.2	2.2	1.5	2.5	1.5	2.5
ApoC-I	5.2	2.8	5.8	3.1	4.0	2.4	4.3	2.7	4.3
ApoC-II	4.5	3.0	4.7	2.7	4.5	2.7	4.0	2.9	4.0
ApoC-III	3.1	2.6	4.0	2.6	3.3	2.0	4.0	2.3	4.0
ApoE	4.4	3.0	4.3	3.2	3.9	3.0	3.0	3.1	3.0

IQC: internal quality control, apo: apolipoprotein, 6495A: Agilent QQQ-MS 6495A, 6495C: Agilent QQQ-MS 6495C

Interpeptide agreement

An example of the four batches left, after all example batches passed IQC evaluation in previous section, is depicted in the right panel of Figure 3.

Samples that were annotated to be remeasured were gathered during the study. When 160 samples were reached, an interim analysis was performed by reprocessing these samples in two batches. Concentrations from the original and second measurement were compared with Deming regression. Comparison of concentrations per peptide and per sample led to the conclusion that second measurement of 152 samples were similar to the original measurement, confirming the observed discrepancies. For eight samples, no discrepancy was observed in a second measurement, indicating a false result in the first measurement. The most affected protein was ApoA-I in six of the cases. Consequently, the rule was adjusted from discrepancies for more than two proteins instead of three, except when the discrepancy is observed in ApoA-I as ApoA-I was overrepresented in the eight samples that were rejected correctly. An overview of the rejected plates per evaluation step is depicted in Figure 4.

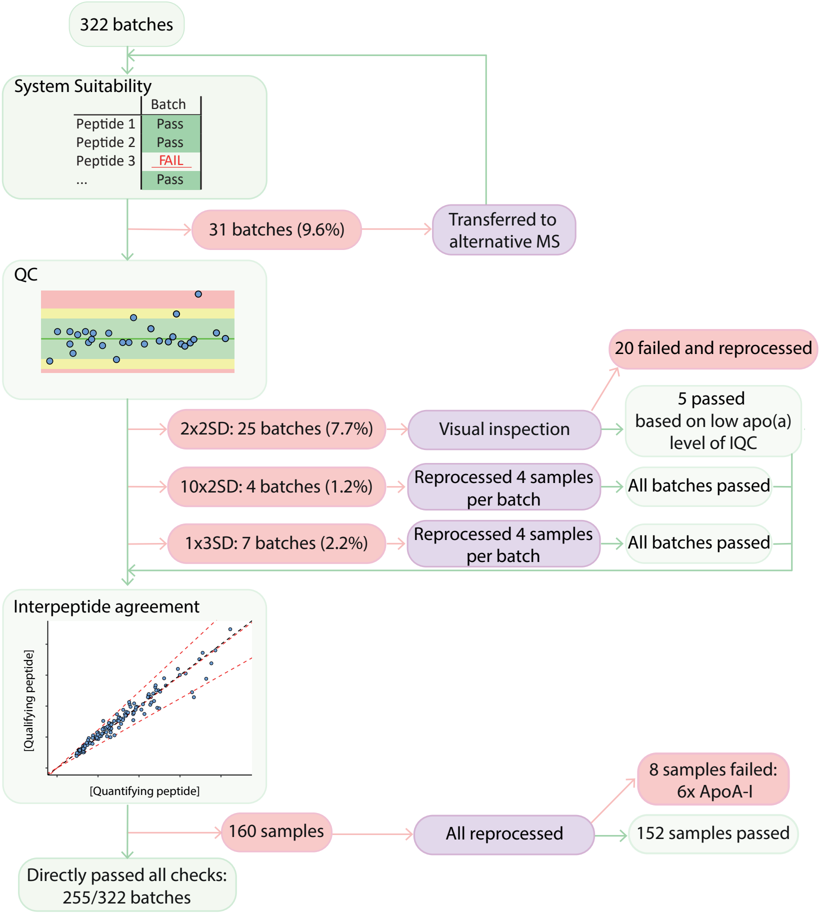


Figure 4: Overview of results of quality measures per evaluation step. A total of 322 was measured of which 31 failed the SST step. Batches were transferred to alternative LC-MS/MS and remeasured. A total of 36 batches failed QC initially, of which 20 were confirmed rejections upon further evaluation. A total of 160 samples failed the interpeptide agreement evaluation of which 8 were confirmed rejections upon further evaluation.

Overall interpeptide agreement

Interpeptide agreement plots for the complete study were created and the results for Apo(a), ApoA-I and ApoB are depicted in Figure 5 (the other apolipoproteins in Supplemental Table S2 and Supplemental Figure S5). Discrepancies of ±50% or ±100%, as observed for ApoB, could indicate the presence of a mutation on the measured peptide, either heterozygous or homozygous, respectively. There were no indications of batch effects for any of the batches during the weekly evaluation.

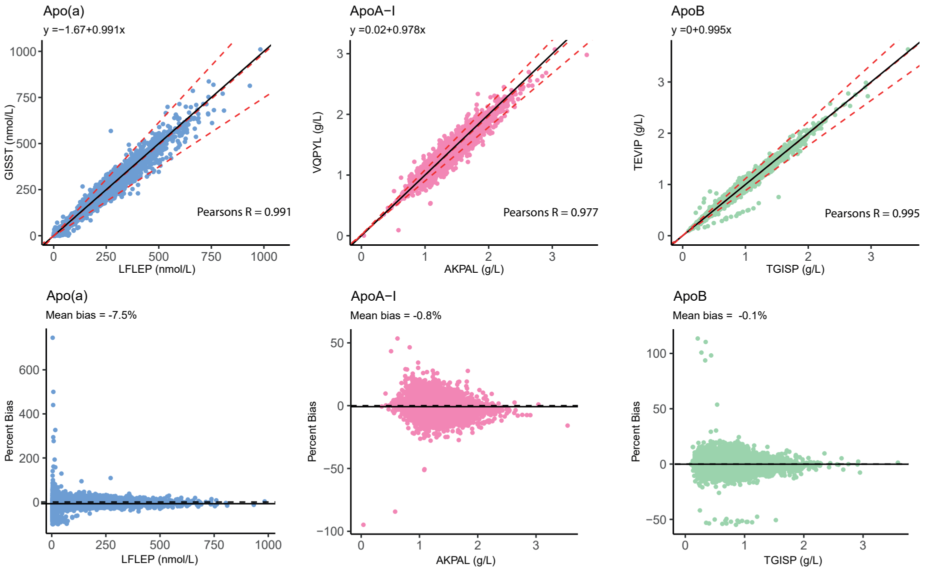


Figure 5: Interpeptide agreement plots of Apo(a), ApoA-I and ApoB. Top: Deming regression plots in which x-axis represent quantifying peptides and y-axis the qualifying peptide of the corresponding protein, including the Pearson's correlation coefficient (R). Black line represents line of identity, while the red dashed line represents the Deming regression and the total allowable error from the Deming regression. (n = 23,376) Bottom: Bland-Altman percentage plots, on the x-axis the concentration of quantifying peptide and on the y-axis the percent bias calculated as the difference between quantifying and qualifying concentrations divided by the quantifying concentration times 100. 0% bias depicted as black line and mean bias depicted as dashed black line. Discordances in interpeptide agreement between peptides could indicate the presence of a mutation in one of the peptides which can be observed for ApoB.

Final Data Validation

After completion of the study dataset, final data validation was performed. 30 samples had a retention time difference between the endo and IS peak of ≥0.1 min, which could be resolved by signal reintegration.

DISCUSSION

For quality assurance of routine laboratory tests Westgard rules are applied.[36] However, these rules cannot be transferred to a multiplex LC-MS/MS test in which a multitude of IQC data points is generated. Here, adjusted Westgard rules were developed and tested. The application of adjusted Westgard rules in a multiplex test proved to be challenging. In the total study, 11.1% of the batches were rejected based on the adjusted rules (Figure 4). Of the three conducted IQC rules, only the 2x2SD rule within the same peptide of the same IQC lot rejected batches correctly. The other two rules had no additional benefit as duplicate measurements yielded similar results.

An additional criterion for the Deming regression comparison in future applications could be that the mean bias of the four reprocessed samples must fall within twice the average CV of the IQCs. Upon retrospective evaluation, we determined that all batches we compared met this criterion, except one. In future measurements, it could be necessary to reprocess batches that do not meet this criterion. An important note is that the applicability of quality assurance rules in multiplex tests depend on multiple parameters, for example the number of IQC samples measured in a batch, the number of proteins, and therefore the number of quantifying peptides measured in a batch, as well as the total number of peptides measured. These rules should be fit-for-purpose for a specific multiplex test and cannot be universally applied to any multiplex test.

An interim analysis as part of the interpeptide agreement evaluation revealed that rejecting samples with discrepancies between peptides of at least two proteins was too strict. The rule was modified to evaluate discrepancies within the sample for more than three proteins instead of two, except for ApoA-I. Overall, the interpeptide agreement provides an additional layer of quality control at individual sample level in quantitative proteomics procedures.

The presence of a routine general clinical chemistry laboratory as well as a research laboratory facility within the same department was essential for the successful execution of the study. The procedure of the LC-MS/MS measurements required attention to ensure high quality data. Although the flowchart (Figure 1) seems complex, it was easy to adhere to and gave clear guidance to all stakeholders.

CONCLUSION

This is the first qCCP trial at this scale performed in a diagnostic clinical chemistry laboratory, which meets both the test process requirements as well as the predefined analytical performance criteria that make medical tests fit-for-clinical purpose. Anchoring tailored additions to the TTP ensured the generation of high-quality data. These achievements would not have been possible without the dedication of skilled laboratory technicians, whose contributions were crucial to the successful execution. In conclusion, we successfully demonstrated the feasibility of employing high-throughput clinical chemistry LC-MS/MS analyses in a large clinical trial setting.

FUNDING AND DISCLOSURES/CONFLICT OF INTEREST

The ODYSSEY OUTCOMES trial was funded by Sanofi and Regeneron Pharmaceuticals. Cobas biomarker tests were provided by Roche Diagnostics Int. Ltd., Switzerland, whereas the multiplex Quantitative Clinical Chemistry Proteomics test was a lab-developed-test from KCL/LUMC, Leiden, The Netherlands. This study was part of a research collaboration between LUMC and Agilent (Santa Clara, CA United States). J.W. Jukema /his department has received research grants from

and/or was speaker (with or without lecture fees) on a.o. (CME accredited) meetings sponsored/supported by Abbott, Amarin, Amgen, Athera, Biotronik, Boston Scientific, Dalcor, Daiichi Sankyo, Edwards Lifesciences, GE Healthcare Johnson and Johnson, Lilly, Medtronic, Merck-Schering-Plough, Novartis, Novo Nordisk, Pfizer, Roche, Sanofi Aventis, The Netherlands Heart Foundation, CardioVascular Research The Netherlands (CVON), The Netherlands Heart Institute and the European Community Framework KP7 Programme. C.M. Cobbaert reports to be chair of the IFCC Scientific Division and chair of the EFLM Task Force Regulatory Affairs. L.R Ruhaak reports to be chair of the IFCC working group for standardization of apolipoproteins by mass spectrometry. E. Reijnders, F.P.H.T.M. Romijn, F. Arslan, J.J.J. Georges, M.M. Pieterse, E.R. Schipper, S Didden-Buitendijk, M.C. Martherus-Bultman, N.P.M. Smit, N.M. Diederiks, M.M. Treep have no conflict of interest and have nothing to declare.

ACKNOWLEDGEMENTS

The authors express their gratitude towards the ODYSSEY OUTCOMES Trial steering committee, especially the cardiology professors G.G. Schwartz, P.G. Steg; the biostatistician M. Szarek; and from Sanofi, G. Garon. In addition, the authors would like to thank J.A.M. Aalbers-Geerdink, H. Ahmed, R. Bahi, W. van der Bent, M. Bourik, E. Dantuma, P.A.M. Does, W. Endlich, E. Galjaard, A.P.M. Giliams, S.M.D. van Gog, A. Grummels, D. Kolle, J.L.M. van der Kroft, A. van der Laarse, M.L Luiten, R.N. Malhoe Mishre-Lalai, J.A.L. Mambi, F.A. van der Meer, S. Meivis, R.T.J. Niesthoven, C. Oolbekkink, G.J. Pieters, M.J.A. Rijks, I. Sabbar, G.C.J. van Steen, R. Swillens, D.I. Tromp, C.J. Trompert, T.J. Trompert, and R.C. van Wissen for their contribution.

REFERENCES

1. Reijnders E, van der Laarse A, Jukema JW and Cobbaert CM. High residual cardiovascular risk after lipid-lowering: prime time for Predictive, Preventive, Personalized, Participatory, and Psycho-cognitive medicine. *Front Cardiovasc Med*. 2023;10:1264319.
2. Libby P. The forgotten majority: unfinished business in cardiovascular risk reduction. *J Am Coll Cardiol*. 2005;46:1225-8.
3. Ruhaak LR, van der Laarse A and Cobbaert CM. Apolipoprotein profiling as a personalized approach to the diagnosis and treatment of dyslipidaemia. *Ann Clin Biochem*. 2019;56:338-356.
4. Contois JH, Langlois MR, Cobbaert C and Sniderman AD. Standardization of Apolipoprotein B, LDL-Cholesterol, and Non-HDL-Cholesterol. *J Am Heart Assoc*. 2023;12:e030405.
5. Langlois MR, Nordestgaard BG, Langsted A, Chapman MJ, Aakre KM, Baum H, Borén J, Bruckert E, Catapano A, Cobbaert C, Collinson P, Descamps OS, Duff CJ, von Eckardstein A, Hammerer-Lercher A, Kamstrup PR, Kolovou G, Kronenberg F, Mora S, Pulkki K, Remaley AT, Rifai N, Ros E, Stankovic S, Stavljenic-Rukavina A, Sypniewska G, Watts GF, Wiklund O and Laitinen P. Quantifying atherogenic lipoproteins for lipid-lowering strategies: consensus-based recommendations from EAS and EFLM. *Clin Chem Lab Med*. 2020;58:496-517.
6. Langlois MR, Chapman MJ, Cobbaert C, Mora S, Remaley AT, Ros E, Watts GF, Borén J, Baum H, Bruckert E, Catapano A, Descamps OS, von Eckardstein A, Kamstrup PR, Kolovou G, Kronenberg F, Langsted A, Pulkki K, Rifai N, Sypniewska G, Wiklund O, Nordestgaard BG, for the European Atherosclerosis S, the European Federation of Clinical C and Laboratory Medicine Joint Consensus I. Quantifying Atherogenic Lipoproteins: Current and Future Challenges in the Era of Personalized Medicine and Very Low Concentrations of LDL Cholesterol. A Consensus Statement from EAS and EFLM. *Clinical chemistry*. 2018;64:1006-1033.
7. Reijnders E, van der Laarse A, Ruhaak LR and Cobbaert CM. Closing the gaps in patient management of dyslipidemia: stepping into cardiovascular precision diagnostics with apolipoprotein profiling. *Clinical Proteomics*. 2024;21.
8. van den Broek I, Romijn FP, Nouta J, van der Laarse A, Drijfhout JW, Smit NP, van der Burgt YE and Cobbaert CM. Automated Multiplex LC-MS/MS Assay for Quantifying Serum Apolipoproteins A-I, B, C-I, C-II, C-III, and E with Qualitative Apolipoprotein E Phenotyping. *Clin Chem*. 2016;62:188-97.
9. Ruhaak LR, Smit NPM, Suchiman HED, Pieterse MM, Romijn F, Beekman M and Cobbaert CM. MS-based proteomics: a metrological sound and robust alternative for apolipoprotein E phenotyping in a multiplexed test. *Clin Chem Lab Med*. 2019;57:e102-e104.
10. Ruhaak LR, Smit NPM, Romijn F, Pieterse MM, van der Laarse A, van der Burgt YEM and Cobbaert CM. Robust and Accurate 2-Year Performance of a Quantitative Mass Spectrometry-Based Apolipoprotein Test in a Clinical Chemistry Laboratory. *Clin Chem*. 2018;64:747-749.
11. Horvath AR, Lord SJ, StJohn A, Sandberg S, Cobbaert CM, Lorenz S, Monaghan PJ, Verhagen-Kamerbeek WD, Ebert C and Bossuyt PM. From biomarkers to medical tests: the changing landscape of test evaluation. *Clin Chim Acta*. 2014;427:49-57.
12. Mathes T and Pieper D. An algorithm for the classification of study designs to assess diagnostic, prognostic and predictive test accuracy in systematic reviews. *Syst Rev*. 2019;8:226.
13. Bossuyt PM, Reitsma JB, Linnet K and Moons KG. Beyond diagnostic accuracy: the clinical utility of diagnostic tests. *Clin Chem* 2012;58:1636-43.
14. Schwartz GG, Steg PG, Szarek M, Bhatt DL, Bittner VA, Diaz R, Edelberg JM, Goodman SG, Hanotin C, Harrington RA, Jukema JW, Lecorps G, Mahaffey KW, Moryusef A, Pordy R, Quintero K, Roe MT, Sasiela WJ, Tamby JF, Tricoci P, White HD, Zeiher AM, Committees OO and Investigators. Alirocumab and Cardiovascular Outcomes after Acute Coronary Syndrome. *N Engl J Med*. 2018;379:2097-2107.

15. Cobbaert C, Albersen A, Zwiers I, Schippers P and Gillis J. Designing a diagnostic Total Testing Process as a base for supporting diagnostic stewardship. *Clin Chem Lab Med*. 2020;59:473-489.

16. Tsantilas KA, Merrihew GE, Robbins JE, Johnson RS, Park J, Plubell DL, Huang E, Riffle M, Sharma V, MacLean BX, Eckels J, Wu CC, Bereman MS, Spencer SE, Hoofnagle AN and MacCoss MJ. A framework for quality control in quantitative proteomics. *bioRxiv*. 2024.

17. Bittremieux W, Meysman P, Martens L, Valkenborg D and Laukens K. Unsupervised Quality Assessment of Mass Spectrometry Proteomics Experiments by Multivariate Quality Control Metrics. *Journal of Proteome Research*. 2016;15:1300-1307.

18. Parker CE and Borchers CH. Mass spectrometry based biomarker discovery, verification, and validation — Quality assurance and control of protein biomarker assays. *Molecular Oncology*. 2014;8:840-858.

19. Smit NPM, Ruhaak LR, Romijn F, Pieterse MM, van der Burgt YEM and Cobbaert CM. The Time Has Come for Quantitative Protein Mass Spectrometry Tests That Target Unmet Clinical Needs. *J Am Soc Mass Spectrom*. 2021;32:636-647.

20. Smit NPM, van den Broek I, Romijn FPHTM, Haex M, Deelder AM, van der Burgt YEM, A. vdL and C.M. C. Quality requirements for quantitative clinical chemistry proteomics. *Translational Proteomics*. 2014;2:1-13.

21. Pichler P, Mazanek M, Dusberger F, Weilnböck L, Huber CG, Stingl C, Luider TM, Straube WL, Köcher T and Mechtler K. SIMPATIQCO: A Server-Based Software Suite Which Facilitates Monitoring the Time Course of LC-MS Performance Metrics on Orbitrap Instruments. *Journal of Proteome Research*. 2012;11:5540-5547.

22. Dogu E, Mohammad-Taheri S, Abbatiello SE, Bereman MS, MacLean B, Schilling B and Vitek O. MSstatsQC: Longitudinal System Suitability Monitoring and Quality Control for Targeted Proteomic Experiments*. *Molecular & Cellular Proteomics*. 2017;16:1335-1347.

23. Stanfill BA, Nakayasu ES, Bramer LM, Thompson AM, Ansong CK, Clauss TR, Gritsenko MA, Monroe ME, Moore RJ, Orton DJ, Piehowski PD, Schepmoes AA, Smith RD, Webb-Robertson B-JM and Metz TO. Quality Control Analysis in Real-time (QC-ART): A Tool for Real-time Quality Control Assessment of Mass Spectrometry-based Proteomics Data*. *Molecular & Cellular Proteomics*. 2018;17:1824-1836.

24. Taylor RM, Dance J, Taylor RJ and Prince JT. Metriculator: quality assessment for mass spectrometry-based proteomics. *Bioinformatics*. 2013;29:2948-2949.

25. Clarke W, Molinaro RJ, Bachmann LM and Cook J. *CLSI C62-ED2:2022 Liquid Chromatography-Mass Spectrometry Methods, 2nd Edition.* Second ed; 2022.

26. Diederiks NM, Ruhaak LR, Romijn F, Pieterse MM, Smit NPM and Cobbaert CM. An LC-MS-based designated comparison method with similar performance to the Lp(a) reference measurement procedure to guide molar Lp(a) standardization. *Clin Proteomics*. 2024;21:5.

27. CLSI. *Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions.* Fourth ed: Clinical and Laboratory Standards Institute; 2016.

28. R Core Team, R: A language and environment for statistical computing. [computer program]. Version 4.2.2. Vienna, Austria: R Foundation for Statistical Computing; 2014.

29. knitr: A General-Purpose Package for Dynamic Report Generation in R [computer program]. Version 1.33; 2021.

30. RStudio Team. RStudio: Integrated Development for R [computer program]. Version 2023.6.1.524. Boston, MA: RStudio, PBC; 2020.

31. Wilkinson MD, Dumontier M, Aalbersberg IJ, Appleton G, Axton M, Baak A, Blomberg N, Boiten J-W, da Silva Santos LB, Bourne PE, Bouwman J, Brookes AJ, Clark T, Crosas M, Dillo I, Dumon O, Edmunds S, Evelo CT, Finkers R, Gonzalez-Beltran A, Gray AJG, Groth P, Goble C, Grethe JS, Heringa J, ’t Hoen PAC, Hooft R, Kuhn T, Kok R, Kok J, Lusher SJ, Martone ME, Mons A, Packer AL, Persson B, Rocca-Serra P, Roos M, van Schaik R, Sansone S-A, Schultes E, Sengstag T, Slater T, Strawn G, Swertz MA, Thompson M, van der Lei J, van Mulligen E, Velterop J, Waagmeester A, Wittenburg P, Wolstencroft K, Zhao J and Mons B. The FAIR Guiding Principles for scientific data management and stewardship. *Sci Data*. 2016;3:160018.

32. Briscoe CJ, Stiles MR and Hage DS. System suitability in bioanalytical LC/MS/MS. *JPBA Open*. 2007;44:484-491.

33. Levey S and Jennings ER. The use of control charts in the clinical laboratory. *Am J Clin Pathol*. 1950;20:1059-66.

34. Anand U. The Levey–Jennings Plot. *Clinical chemistry*. 2013;59:865-866.

35. Westgard JO. Internal quality control: planning and implementation strategies. *Ann Clin Biochem*. 2003;40:593-611.

36. Westgard JO, Barry PL, Hunt MR and Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem* 1981;27:493-501.

SUPPLEMENTAL INFORMATION

Details of the process

The process from sample receipt upon data management has been schematically depicted in Figure S1. The first step in the process was the transfer of the samples from the central facilities (Covance Indianapolis, USA and Geneva, Switzerland) to our laboratory in The Netherlands. Four dedicated -80 °C freezers were put in place, three freezers were subdivided in two compartments each for 8 mL Sarstedt PP screw cap tubes, while the fourth freezer accommodated 96 trays. Custom panels were designed to accommodate boxes for the 8 mL tubes. Samples were shipped by DHL, and after a first test shipment of 500 samples, all samples were received in batches of up to 1000 samples on dry ice. A dedicated location for disposal of dry-ice and boxes was arranged for waste management.

Upon receival of each shipment, the number of boxes and the number of samples was determined, and per box, two samples were randomly selected for assessment of correct sample and box assignment. Then, sample lists were loaded into Sample Navigator, together with box location, for sample registration.

Prior to the start of the measurements, LIMS IDs were assigned to each of the samples, and labels containing LIMS IDs, barcodes and QR codes were printed for each of two aliquots in batches of 450 samples. Subsequently, lists for aliquoting were generated containing both the StudyID, LIMS ID, checkboxes, and comments sections. For aliquoting, samples were thawed per box of 36 or 25 samples (either 144 or 150 samples/day), mixed, centrifuged, mixed and aliquoted in one 1.2 mL LLG-Labware PP cryovial for LC-MS/MS analyses and one 5 mL Greiner Bio-One vacuette PET tube for Cobas and Diazyme analyses. Samples with low volume were marked, as well as samples containing fibrin clots. If needed, fibrin clots were removed with a wooden spatula. Samples were also visually checked on the presence of lipidemia and hemolysis. Samples for Cobas analyses were stored at 4 °C for analysis the following day, while samples from LC-MS/MS analyses were stored at -80 °C.

Cobas analysis was performed in batches of 144 or 150 samples per day on the day immediately following the aliquoting , and samples were subsequently stored at -80 °C. To ensure easy recovery of the samples from the freezer, a dedicated sample placement format was adhered to, resulting in a sample carousel system. LC-MS/MS analyses were performed in batches of 80 samples, independent of the aliquoting. For Diazyme, a subset of samples were collected from the frozen Cobas aliquots in batches of maximum 612 per day; samples were analyzed and refrozen at -80 °C on the same day. Barcodes and QR codes were used to ensure accurate sample identification during Cobas, LC-MS/MS and Diazyme measurements, and guaranteed track-and-trace throughout the process.

Cobas analyses

Methods

Eleven biomarkers in the context of CVD manufactured by Roche Diagnostics GmbH, Germany were determined in serum samples with a Roche Cobas 8000 comprising C502 and E602 modules (Supplemental Table S3). Quality controls (QCs) were run daily. Analyses were performed with Roche reagents following instructions for use provided by the supplier and SOPs. Data verification was performed daily following SOPS. Authorization of results was performed in LIMS. QCs were repeated if Westgard rules were violated. Recalibration was performed if repeated QCs failed to meet criteria.

Results

Levels of cystatin C, hs-TroponinT, NT-proBNP, hs-CRP, Lp(a), sd-LDL, GDF-15, SFlt-1, IL-6, insulin and homocysteine were measured in 23,376 samples on a Cobas Roche analyzer over 60 weeks. The coefficient of variation (CV) of the QCs over multiple lots ranged between 1.7% and 5.5% (Supplemental Table S4). No Westgard rules were violated, indicating excellent performance of instrument and applications. All Cobas biomarkers showed consistent results over time, with no lot-to-lot variations in the data.

Diazyme analyses

Methods

Oxidized phospholipids on apolipoprotein B-100 (OxPL-ApoB) were determined in a subset of 16,925 serum samples using a Diazyme DZ-Lite 3000 Plus analyzer. Four bilevel QC lots, four reagent lots, and four calibrator lots were used. QCs were run daily. The analysis was performed following instructions for use provided by the supplier and SOPs. Data verification was performed daily following SOPS. Authorization of results was performed in LIMS. QCs were repeated if Westgard rules were violated. Recalibration was performed if repeated QCs failed to meet criteria.

Results

OxPL-ApoB was determined in a subset of 16,925 samples on the Diazyme analyzer. Bilevel QCs were measured over 30 weeks with a mean value (CV%) of 6 nmol/L (10.0%) and 27 nmol/L (7.2%). OxPL-ApoB concentrations were also plotted over time and no inconsistencies implicated by lot-to-lot variations were observed.

Supplemental tables and figures

Table S1: Target values per peptide and IQC lot for LC-MS/MS measurements

Protein	Peptide	Unit	IQC Level 1A	IQC Level 1B	IQC Level 2A	IQC Level 2B
Apo(a)	GISST	nmol/L	7.2	39.7	28.2	89.6
Apo(a)	GTYST	nmol/L	7.3	42.8	32.7	91.1
Apo(a)	LFLEP	nmol/L	7.0	41.0	24.2	91.6
ApoA-I	AKPAL	g/L	1.29	1.28	2.10	1.26
ApoA-I	VQPYL	g/L	1.29	1.30	2.14	1.26
ApoA-II	EQLTP	mg/L	265	242	328	302
ApoA-II	SPELQ	mg/L	258	241	325	298
ApoA-IV	LEPYA	mg/L	294	227	245	231
ApoA-IV	LTPYA	mg/L	299	228	246	232
ApoA-IV	SLAPY	mg/L	298	227	245	233
ApoB	FPEVD	g/L	0.99	0.83	0.63	1.35
ApoB	TEVIP	g/L	0.98	0.83	0.63	1.35
ApoB	TGISP	g/L	0.96	0.82	0.62	1.35
ApoC-I	EFGNT	mg/L	21	18	26	21
ApoC-I	TPDVS	mg/L	22	18	27	20
ApoC-II	ESLSS	mg/L	46	35	34	39
ApoC-II	TYLPA	mg/L	46	35	34	39
ApoC-III	DALSS	mg/L	166	97	99	103
ApoC-III	GWVTD	mg/L	168	98	97	104
ApoE	LAVYQ	mg/L	50	29	26	35
ApoE	LGPLV	mg/L	50	29	26	35
ApoE	SELEE	mg/L	52	26	27	36

Table S2: Overall interpeptide agreement

Protein	Quantifying Peptide	Qualifying Peptide	Pearson *R*	Slope	Intercept
Apo(a)	LFLEP	GISST	0.991	0.991	-1.672
ApoA-I	AKPAL	VQPYL	0.977	0.978	0.016
ApoA-II	SPELQ	EQLTP	0.984	0.972	4.882
ApoA-IV	LEPYA	LTPYA	0.975	0.988	3.112
ApoB	TGISP	TEVIP	0.995	0.995	0.002
ApoC-I	EFGNT	TPDVS	0.944	1.064	-0.835
ApoC-II	ESLSS	TYLPA	0.990	0.988	0.505
ApoC-III	GWVTD	DALSS	0.991	1.01	-1.188
ApoE	SELEE	LGPLV	0.975	1.02	-0.502

Overall results of the interpeptide agreement per protein including Pearson’s correlation coefficient, slope of the Deming regression and intercept (n = 23,367).

Table S3: Lots per application for Cobas analyses in the ODYSSEY OUTCOMES Trial

Biomarker	Module	# bilevel QC lots	# Reagent lots	# Calibrator lots
Cystatin C	C502	3	4	2
TroponinT-hs	E602	2	2	1
NT-ProBNP	E602	3	3	3
hsCRP	C502	2	1	1
LPA	C502	2	3	1
sdLDL	C502	3	4	3
GDF-15	E602	3	5	2
SFlt-1	E602	3	3	2
IL-6	E602	3	3	1
Insulin	E602	2	3	1
Homocysteine	C502	3	3	3

Table S4: Between-run Coefficient of variation (CV%) for Cobas analyses

Biomarker	CV%		
	Level 1	Level 2	Level 3
Cystatin C	2.94	2.37	1.74
TroponinT-hs	2.76	3.25	
NT-ProBNP	3.62	3.33	
hsCRP	4.67	1.75	
Lp(a)	3.12	2.88	
sdLDL	3.35	3.07	
GDF-15	3.53	3.64	
SFlt-1	2.57	2.02	
IL-6	3.52	2.92	
Insulin	5.54	4.55	
Homocysteine	3.17	3.36	

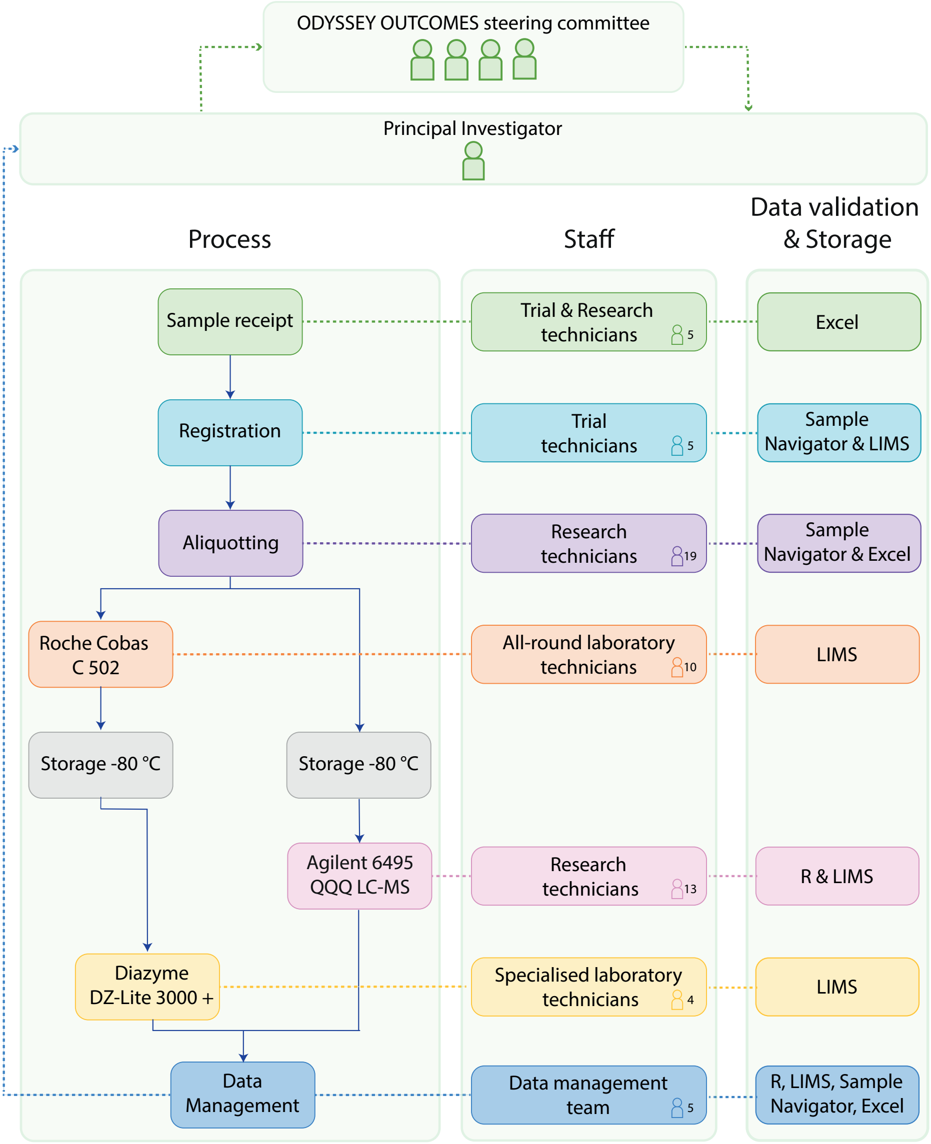


Figure S1: Process of measurements in the ODYSSEY OUTCOMES Trial. Process from sample receipt to data management of the ODYSSEY OUTCOMES trial. This includes the staff, quality assurance and data validation per step in the process. LIMS: laboratory information management system.

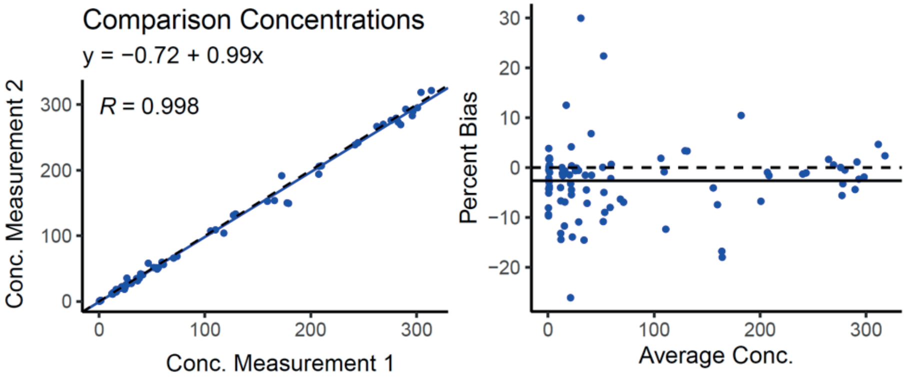


Figure S2. Comparison of measurement four random samples. Comparison of all concentrations of all peptides of four reprocessed samples. Left: Deming regression on x-axis the concentrations reported from the original measurement, on the y-axis the concentrations of the second measurement. R indicates the Pearson R coefficient. Right: Bias plot with the percent bias between the two concentrations of the measurements and on the x-axis the average concentration of the concentrations from two measurements.

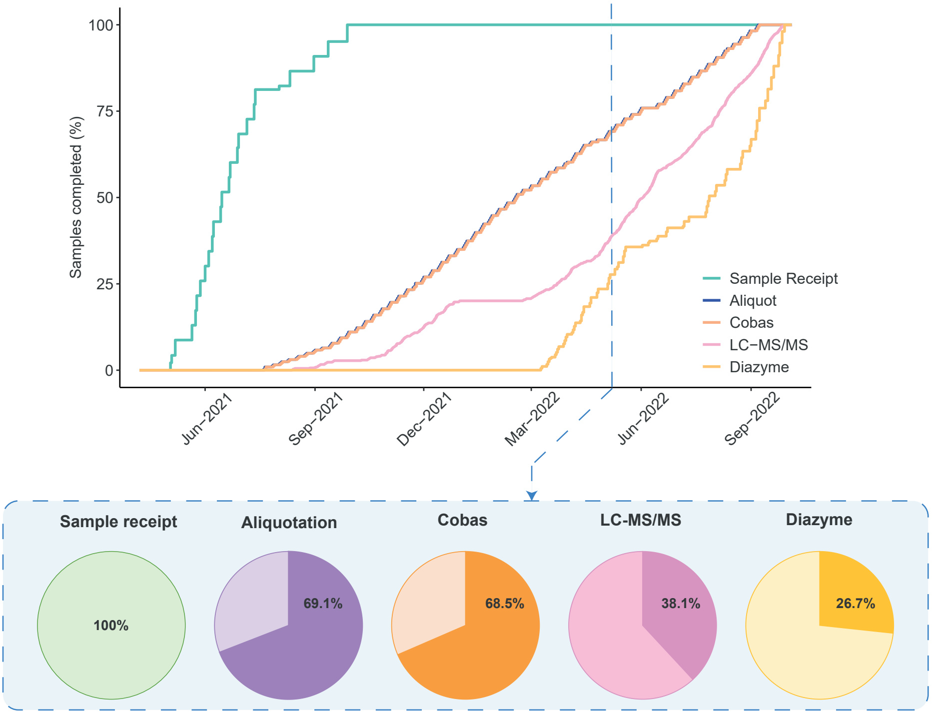
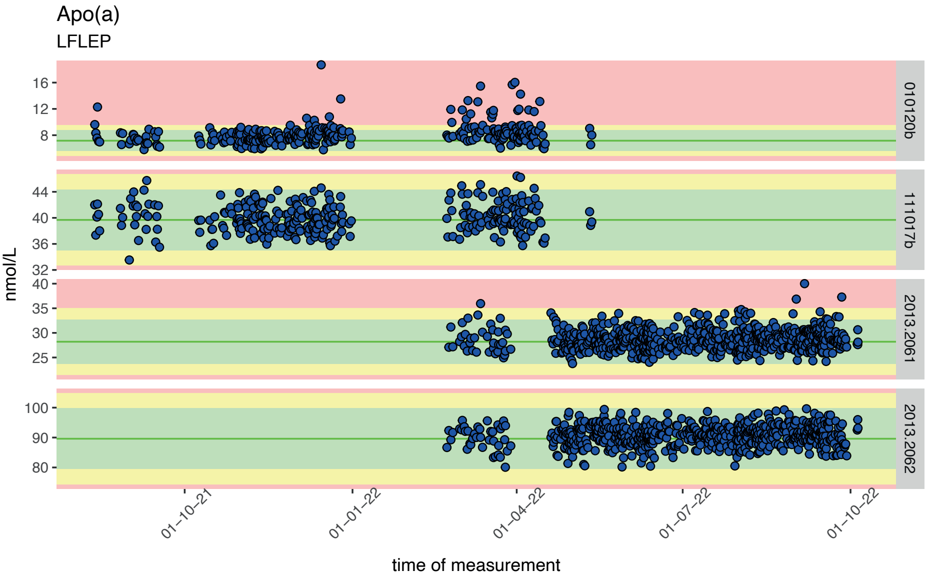
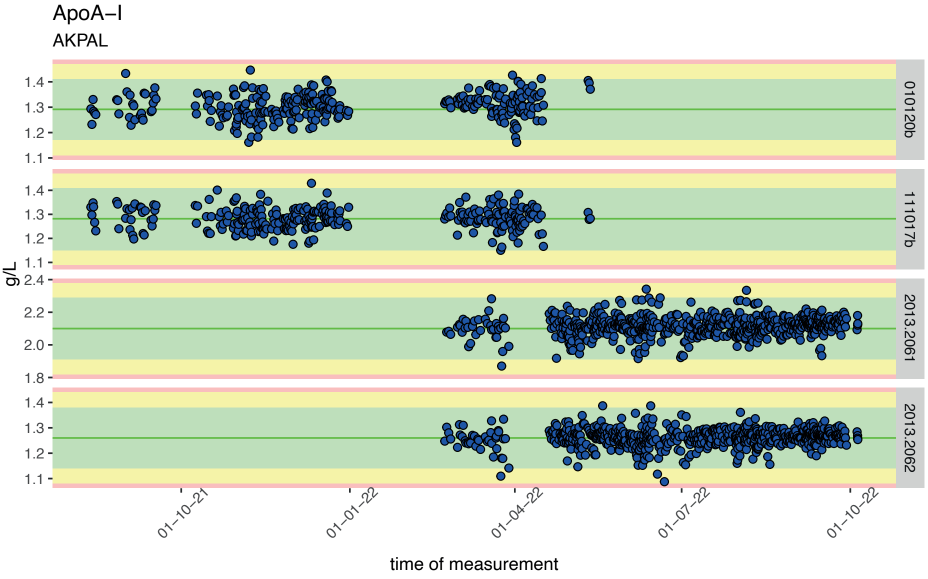
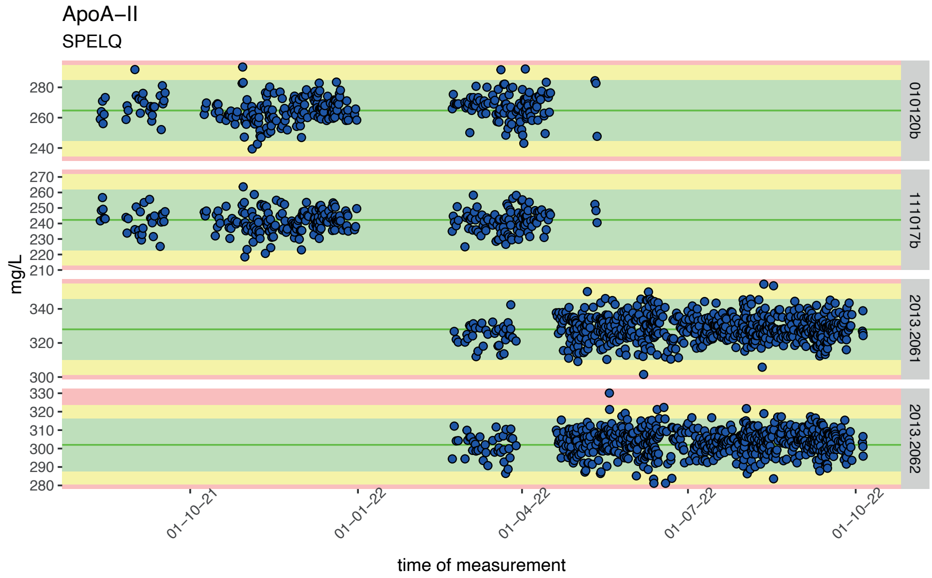
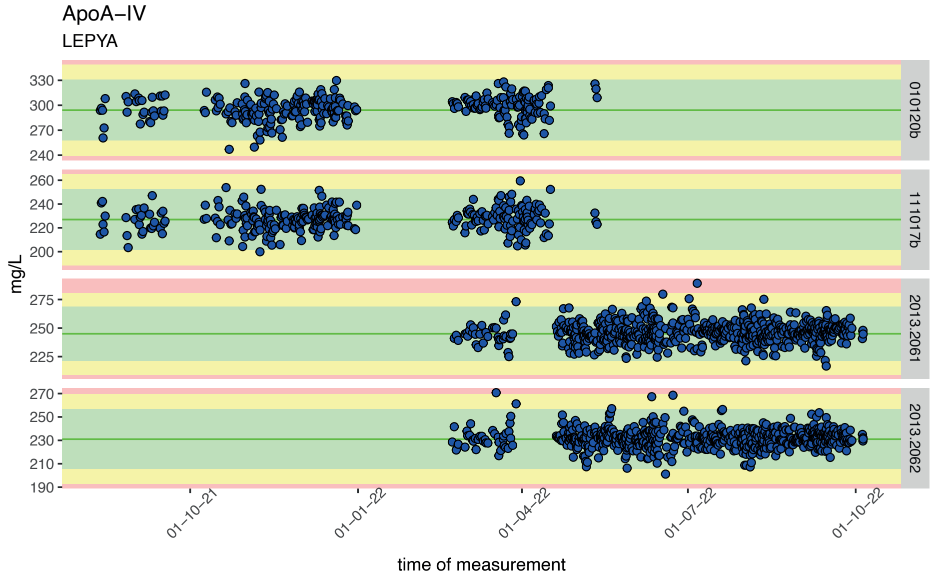


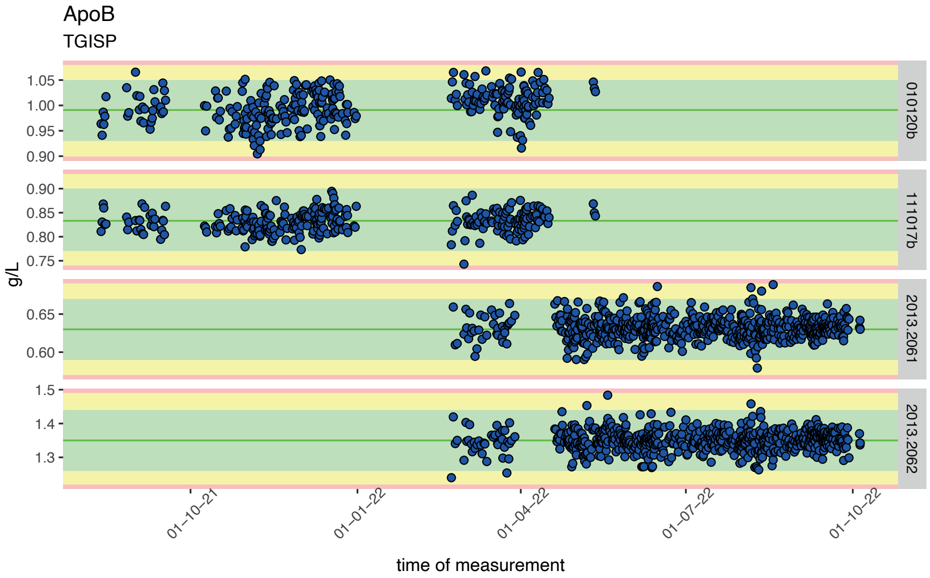
Figure S3. ODYSSEY OUTCOMES throughput. Sample throughput per step of the process including a snapshot of the dashboard on the 5th of May 2022.

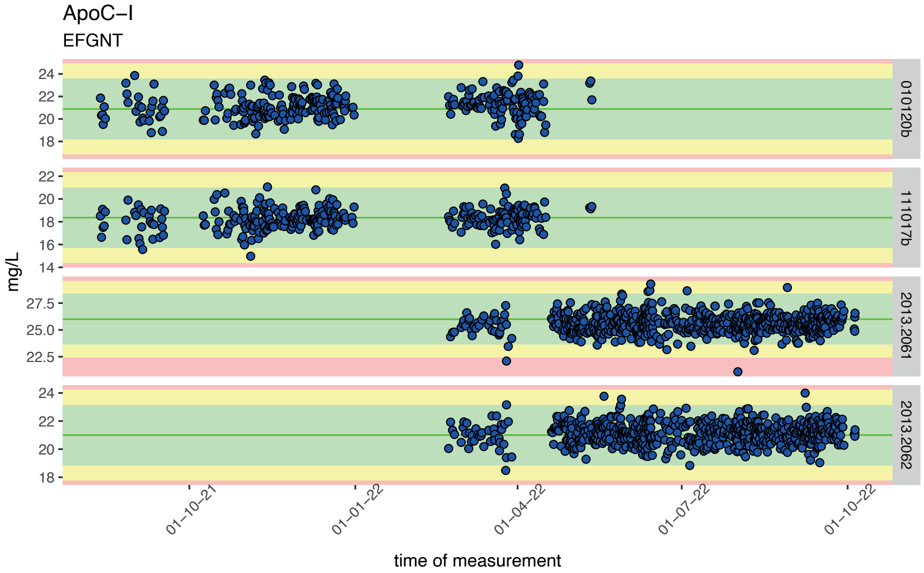

Apo(a)
LFLEP
16
12
8
44
40
36
32
40
35
30
25
100
90
80
nmol/L
010120b
111017b
2013.2061
2013.2062
01–10–21
01–01–22
01–04–22
01–07–22
01–10–22
time of measurement

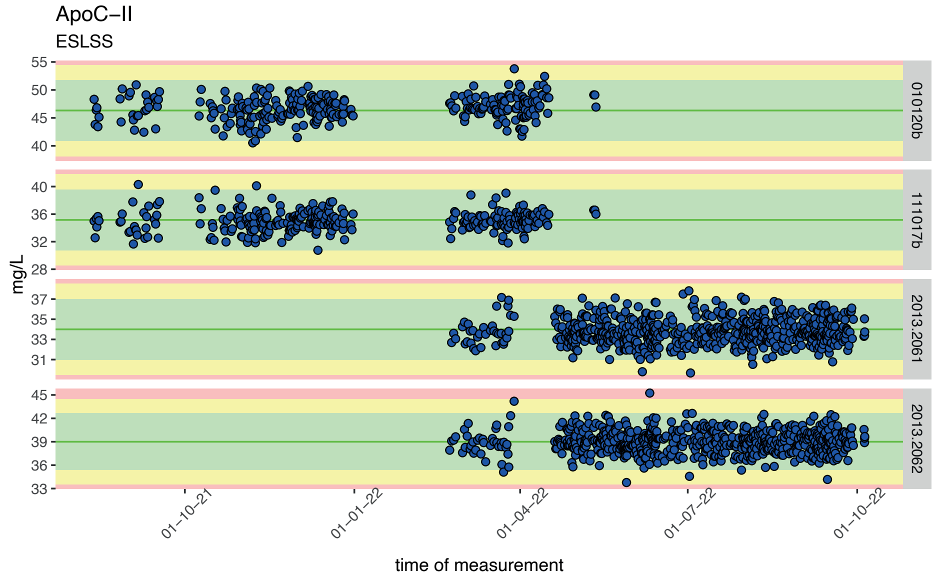

ApoA–I
AKPAL
1.4
1.3
1.2
1.1
1.4
1.3
1.2
1.1
2.4
2.2
2.0
1.8
1.4
1.3
1.2
1.1
g/L
010120b
111017b
2013.2061
2013.2062
01–10–21
01–01–22
01–04–22
01–07–22
01–10–22
time of measurement

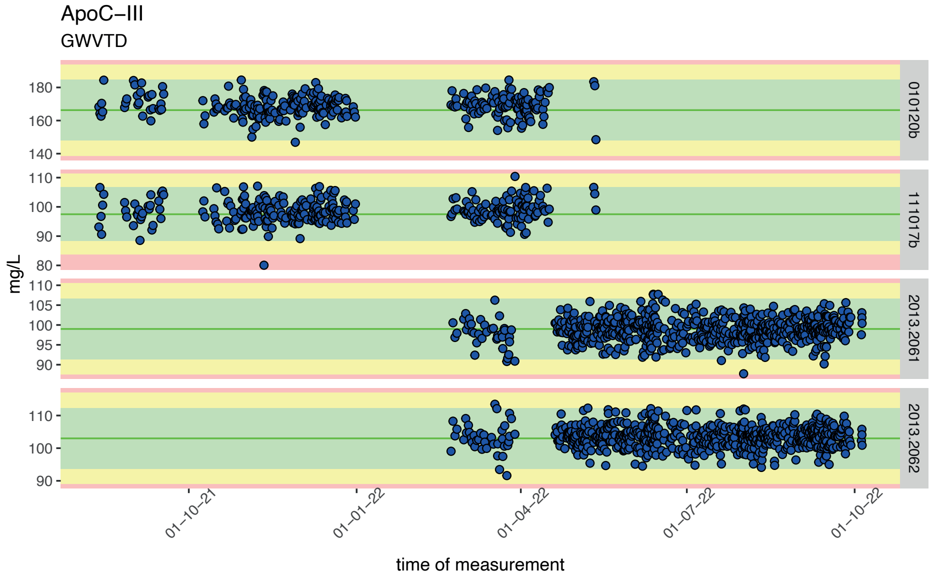

ApoA−II
SPELQ
mg/L
280
260
240
010120b
270
260
250
240
230
220
210
111017b
340
320
300
2013.2061
330
320
310
300
290
280
2013.2062
01−10−21
01−01−22
01−04−22
01−07−22
01−10−22
time of measurement


ApoA−IV
LEPYA
mg/L
330
300
270
240
010120b
260
240
220
200
111017b
275
250
225
2013.2061
270
250
230
210
190
2013.2062
01−10−21
01−01−22
01−04−22
01−07−22
01−10−22
time of measurement


ApoB
TGISP
g/L
1.05
1.00
0.95
0.90
010120b
0.90
0.85
0.80
0.75
111017b
0.65
0.60
2013.2061
1.5
1.4
1.3
2013.2062
01–10–21
01–01–22
01–04–22
01–07–22
01–10–22
time of measurement


ApoC–I
EFGNT
mg/L
24
22
20
18
010120b
22
20
18
16
14
111017b
27.5
25.0
22.5
2013.2061
24
22
20
18
2013.2062
01–10–21
01–01–22
01–04–22
01–07–22
01–10–22
time of measurement


ApoC–II
ESLSS
mg/L
55
50
45
40
010120b
40
36
32
28
111017b
37
35
33
31
2013.2061
45
42
39
36
33
2013.2062
01–10–21
01–01–22
01–04–22
01–07–22
01–10–22
time of measurement


ApoC–III
GWVTD
mg/L
180
160
140
010120b
110
100
90
80
111017b
110
105
100
95
90
2013.2061
110
100
90
2013.2062
01–10–21
01–01–22
01–04–22
01–07–22
01–10–22
time of measurement

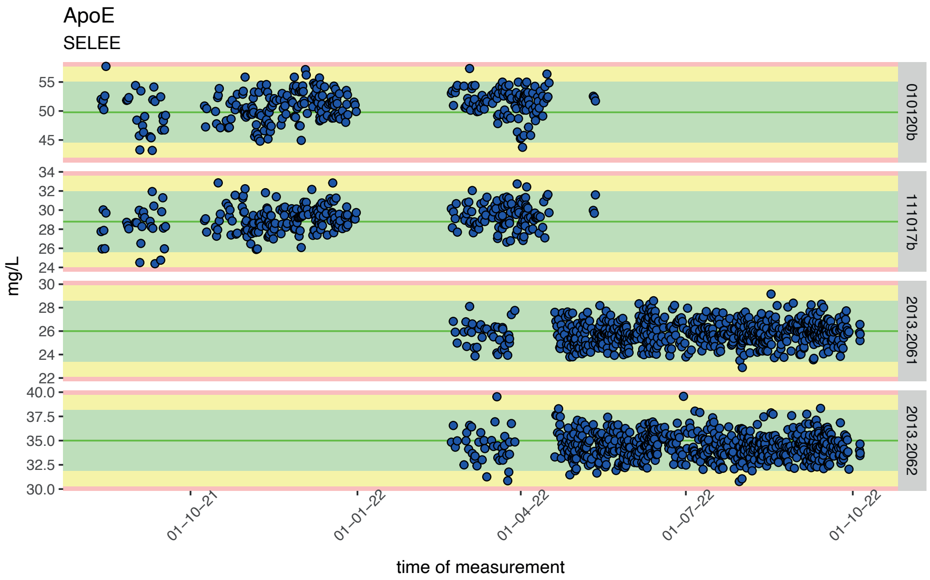
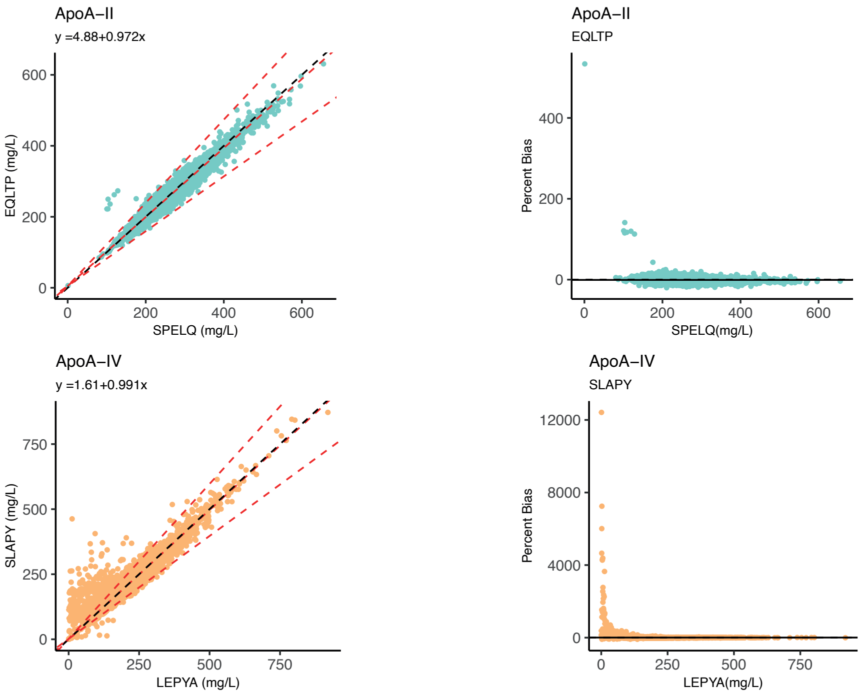


Figure S4. Long-term IQC LC-MS/MS analyses of the total ODYSSEY OUTCOMES Trial. Long-term IQC monitoring of four different IQC lots for nine apolipoproteins with 2SD (light-green), 3SD (yellow) and outside 3SD (red) borders. Target value is depicted as green line.



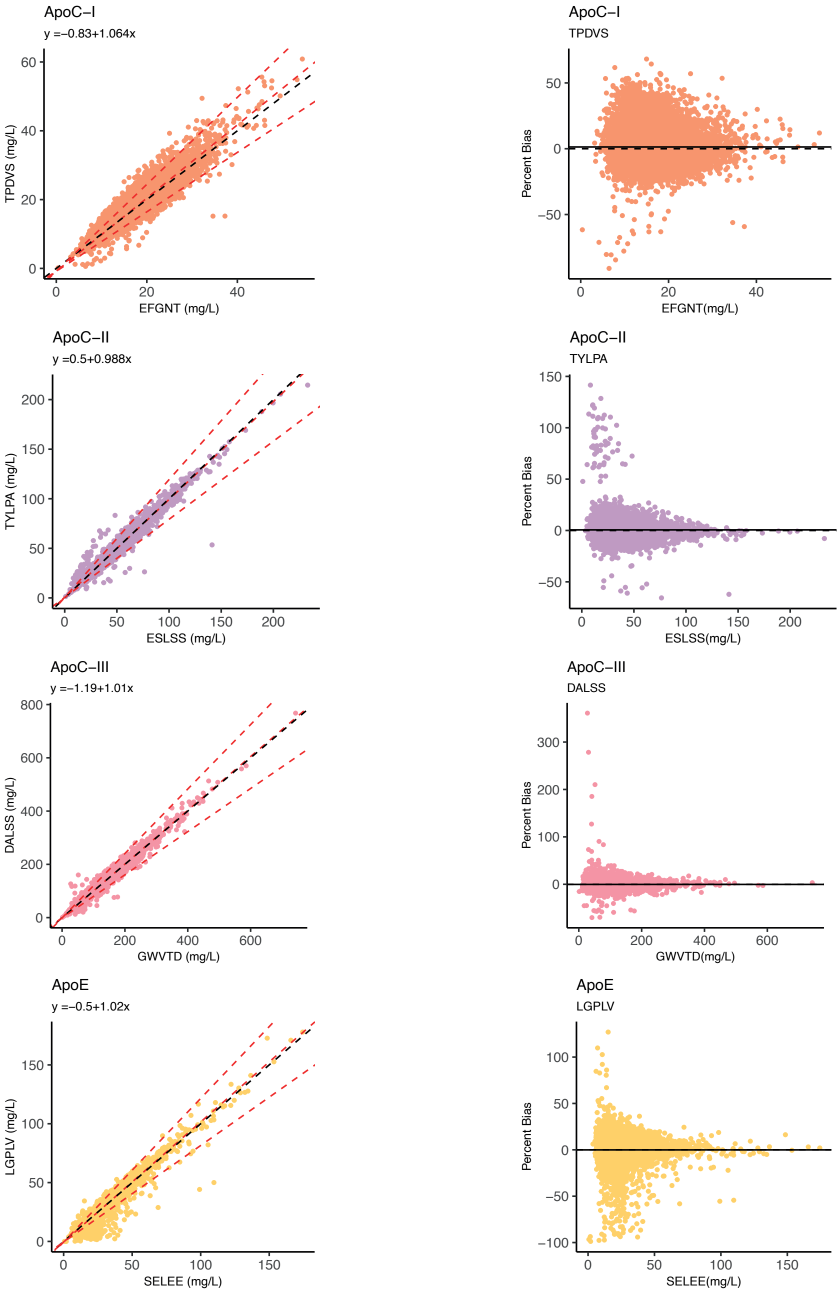


Figure S5. Interpeptide agreement plots of the apolipoproteins measured in the ODYSSEY OUTCOMES Trial. Interpeptide agreement plots. Left: Deming regression plots in which x-axis represent quantifying peptides and y-axis the qualifying peptide of the corresponding protein, including the Pearson's correlation coefficient (R). Black dashed line represents line of identity, while the red dashed line represents Deming regression and the total allowable error from the Deming regression. (n = 23,376) Right: Bland-Altman percentage plots, on the x-axis the concentration of quantifying peptide and on the y-axis the percent bias calculated as the difference between quantifying and qualifying concentrations divided by the quantifying concentration times 100. 0% bias depicted as black line and mean bias depicted as dashed black line.