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

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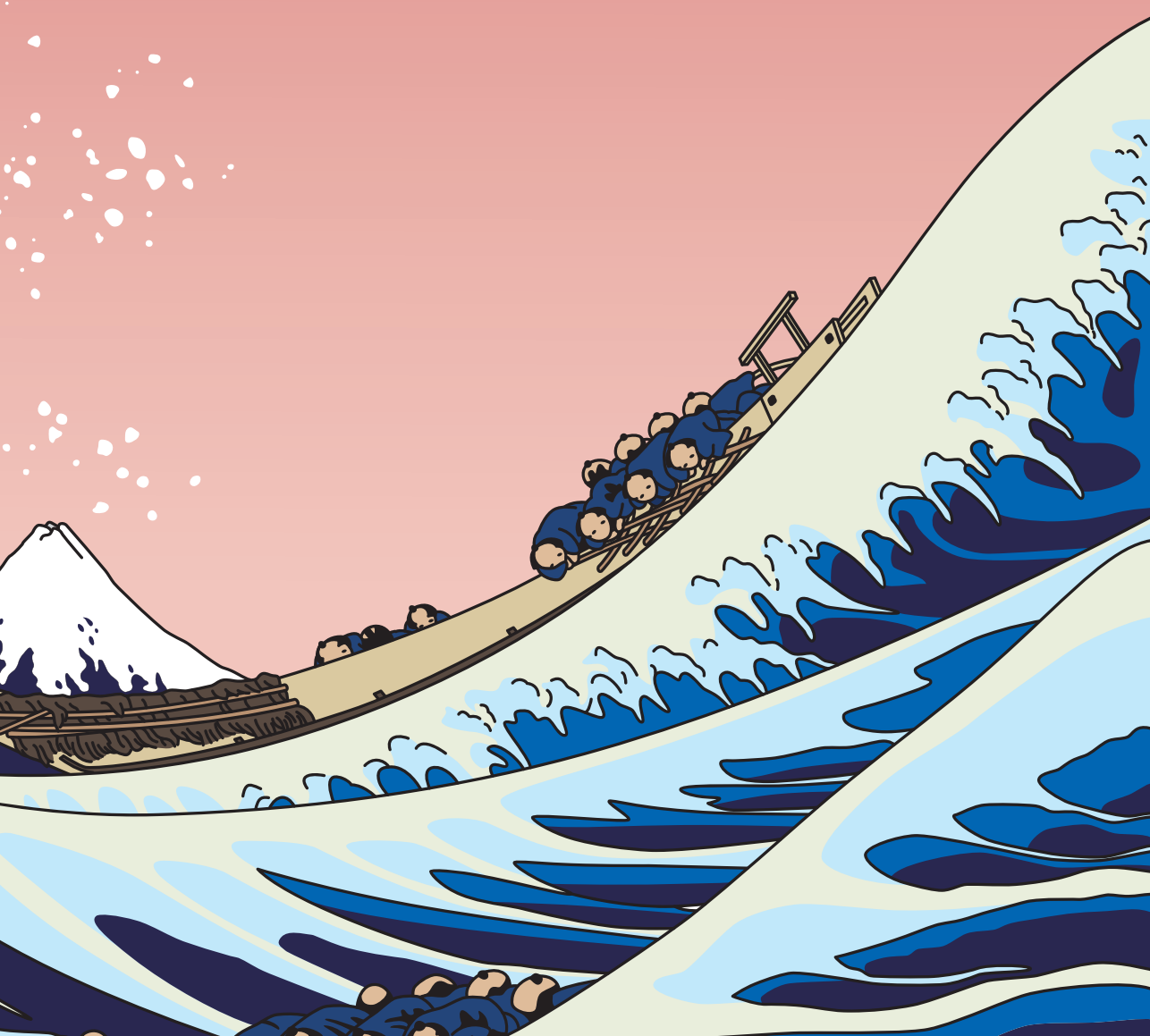
CHAPTER 8



General Discussion and Conclusion

Apolipoproteins and their clinical relevance

Every new diagnostic test intended for clinical use should address an unmet clinical need. According to the EFLM test evaluation framework, key factors of test evaluation: analytical performance, clinical performance, clinical effectiveness, cost-effectiveness, and broader impact, must all be assessed to transform a promising biomarker into a clinically valuable and reimbursed test (Figure 1).[1] These five aspects of test evaluation are interconnected, each revolving around the clinical care pathway, and the starting point is always the unmet clinical need. In this thesis, we sought to address the unmet clinical need for more tailored management of residual cardiovascular risk by implementing a more refined, molecularly defined diagnostic approach.

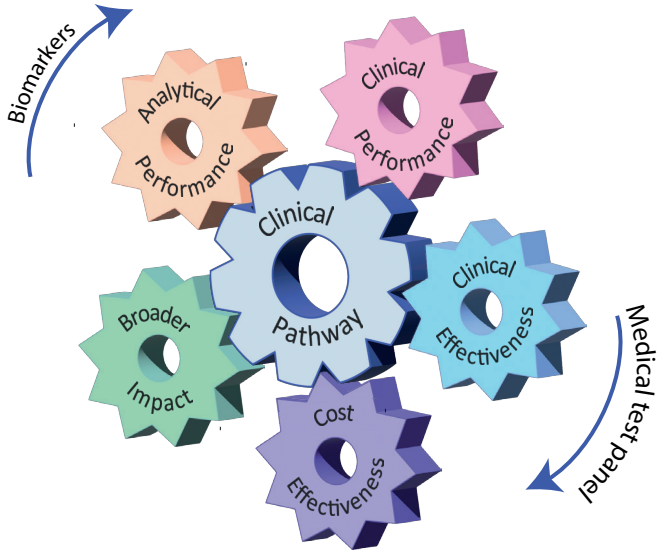


Figure 1: Test evaluation framework for multiplex apolipoprotein testing in cardiovascular patient management. Framework adapted from Horvath et al.[1]

Residual cardiovascular risk cannot be eliminated

In our search for residual cardiovascular risk factors we found out that residual cardiovascular risk is a rather undefined term without general consensus (**Chapter 1**).[2] The most comprehensive definition of residual CVD risk is "*the risk of CVD of an individual who is given proper therapy for hyperlipidemia, hypertension, hyperglycemia, and advice for healthy lifestyle, and who is checked for proinflammatory and procoagulant factors.*" However, in practice it is usually defined as a more practical definition e.g., the risk of cardiovascular events after optimized lipid-lowering therapy with for example, statins. In our search for risk factors addressing this cardiovascular risk we found that a plethora of risk factors including conventional risk factors such as diabetes,

smoking, high blood pressure and high BMI, contributes to this residual cardiovascular risk but also less traditional factors like household air pollution, depression, and low education level. It is evident that not all risk factors can be treated to completely eliminate the residual risk of an individual.

Residual cardiovascular risk can be a result of a combination of several risk factors for which a patient might see different clinical or health care experts, for example general practitioners, endocrinologists, immunologists, dieticians, and cardiologists. Multidisciplinary meetings between these experts might help with addressing the multifactorial causes of the residual risk a patient is facing. In addition, cardiovascular-kidney-metabolic (CKM) health is increasingly recognized, as transcending the boundaries between clinical specialties, aimed to address the risk of cardiovascular disease. This has also been recently emphasized in a Presidential Advisory from the American Heart Association[3] emphasizing the need for a clear definition of CKM syndrome, as well as coordinated, interdisciplinary care strategies that address the shared risk factors and outcomes. In addition to the clinicians and health experts, the patient themselves should play an active role in implementing proactive P5 medicine encompassing Predictive, Preventive, Personalized, Participatory, and Psycho-cognitive medicine.[4, 5] Instead of a "one-size-fits-all" approach, healthcare is gradually shifting towards personalized strategies that take into account interindividual diversity and tailor care to each individual.[6]

To assess cardiovascular risk in a comprehensive way, clinicians need biologically meaningful biomarkers that reflect the individual's (patho)physiological state. Novel diagnostic tests that improve detection, classification and risk stratification are essential for more precise, personalized risk management. This refined approach integrates biomarkers, lifestyle factors, family history of cardiovascular disease, and the management of comorbidities, enabling personalized medical decisions based on each patient's unique characteristics. Despite substantial progress in reducing traditional risk factors, newer contributors—such as Lp(a), inflammation, genetic influences, and psychosocial factors—contribute to explaining residual cardiovascular risk. Managing cardiovascular disease is increasingly complex, as the state of science reveals a multifaceted interplay of traditional and novel cardiovascular risk factors. As residual cardiovascular risk remains high despite optimized conventional therapies, more effort must be made to better address the forgotten majority. Through personalized medicine and with the addition of emerging clinically relevant biomarkers, significant progress can be made in addressing residual cardiovascular risk and improving cardiovascular health.

Relevance of apolipoproteins in the context of cardiovascular disease

Current cardiovascular risk prediction relies biochemically on the classic lipid panel, including LDL-C, HDL-C, TC, and triglycerides. To enhance risk prediction and address residual cardiovascular risk, we aimed to adopt a more refined approach utilizing biologically relevant biomarkers. Apolipoproteins, as key proteins of lipid metabolism, may better reflect an individual's dyslipidemic state compared to lipids.[7-10] We selected nine apolipoproteins for our investigation: Apo(a), ApoA-I, ApoA-II, ApoA-IV, ApoB, ApoC-I, ApoC-II, ApoC-III and ApoE and ApoE phenotype. The biochemical rationale for selecting these nine apolipoproteins is discussed in **Chapter 2**. Some of these apolipoproteins have demonstrated their clinical performance and clinical effectiveness in the context of cardiovascular disease. For example, ApoB has been shown to outperform the conventional benchmark, LDL-C, in the context of cardiovascular disease.[8, 11, 12] Mendelian randomization studies further support that ApoB is associated with coronary heart disease, coronary artery disease, ACS, and heart failure.[13-15] Additionally, Apo(a), the characteristic apolipoprotein of Lp(a), is valuable for quantifying Lp(a). Although the exact function of Lp(a) remains unclear, it is an established causal genetically determined risk factor for ASCVD and aortic valve stenosis.[16-18] Others have documented the predictive value of baseline 13 plasma apolipoproteins of which ApoC-I, ApoC-III, and ApoE were most strongly associated with the risk of CHD the PROCARDIS study.[19] The clinical performance and clinical effectiveness of the apolipoproteins as part of a panel required further investigation. It became evident that apolipoproteins are closely interrelated and operate collectively within an integrated biochemical network. This supports the idea that apolipoproteins should be measured as a comprehensive apolipoprotein panel rather than individual biomarkers for cardiovascular risk prediction. However, it should be noted that once the refined diagnosis is made for other intended uses beyond cardiovascular risk prediction such as therapy monitoring, measuring single apolipoproteins may be sufficient. For instance, as therapeutics directly targeting Lp(a), such as pelacarsen in the Lp(a) HORIZON[20] trial and olpasiran in the OCEAN(a) trial[21], are on the horizon, measuring only Apo(a) levels may be adequate for future therapy monitoring. Similarly, monitoring ApoC-III levels could be relevant when using volanesorsen, an antisense oligonucleotide designed to selectively inhibit ApoC-III production, which has shown to be promising in treating LPL deficiency.[22]

Apolipoproteins and cardiovascular outcomes

The scientific validity and clinical relevance of the apolipoprotein panel were discussed in **Chapter 2**. To further assess its clinical performance and effectiveness in predicting patient outcomes, we measured the apolipoprotein panel in two distinct populations, following the test evaluation framework (Figure 1). The first population is from the NEO study, a large observational study primarily involving overweight individuals centered in the Netherlands (**Chapter 5**).[23] The second

population is from the ODYSSEY OUTCOMES trial, a randomized controlled trial of patients with recent ACS on optimized statin therapy, designed to test the efficacy of alirocumab, a PCSK9 inhibitor (**Chapter 6**).[24] These two populations are notably different: the NEO study could be considered a cohort for primary prevention, while the ODYSSEY OUTCOMES trial includes patients with a history of cardiovascular events, assessing their risk for additional events in a secondary prevention context.

In the NEO population, the apolipoprotein panel showed to improve prediction of cardiovascular events beyond the classic lipid panel in a predominantly overweight population without preexisting cardiovascular disease. In the ODYSSEY OUTCOMES population, apolipoproteins demonstrated prediction of MACE events or all-cause death only when included as a comprehensive panel (**Chapter 6**). None of the individual apolipoproteins showed significant predictive performance on their own. Additionally, when used as an add-on to the classic lipid panel, the apolipoprotein panel significantly improved cardiovascular risk prediction in a secondary prevention population. Despite the fact that this highly selected ACS population was predominantly hypercholesterolemic, not hypertriglyceridemic, and optimally treated, the apolipoprotein panel still showed added value. This suggests that the apolipoprotein panel could serve as a valuable add-on test for cardiovascular risk management within the clinical care pathway. An interesting finding in the ODYSSEY OUTCOMES trial was that the baseline levels of apolipoproteins could identify individuals who would benefit from alirocumab treatment (**Chapter 6**). Although alirocumab does not directly target apolipoproteins—it inhibits PCSK9 to enhance LDL particle clearance—the interconnected role of apolipoproteins in lipid metabolism allows baseline apolipoprotein levels to identify which patients are likely to respond well to this therapy.

The future of apolipoprotein profiling

Apolipoprotein profiling holds great potential in future precision diagnostics with various intended uses, one of which is cardiovascular risk prediction. The apolipoprotein panel demonstrated a significant improvement in prediction of cardiovascular events beyond the classic lipid panel in two very distinct populations: (1) a predominantly overweight, white population without a significant history of cardiovascular disease, representing a primary prevention setting, and (2) a population of patients who had recently experienced an ACS event on optimized statin therapy, representing a secondary prevention setting.

A second intended use of the apolipoprotein panel is to identify patients at risk for CVD who would benefit from specific therapies, ultimately aiding in therapy selection. In our research, we investigated the apolipoprotein panel's ability to predict the treatment benefit of a PCSK9 inhibitor in a highly selected hypercholesterolemic ACS cohort on optimized statin therapy,

yielding promising results. We anticipate that the apolipoprotein panel will also predict treatment benefit for other therapies, especially those that directly target apolipoproteins. Ultimately, the panel may help identify the therapy with the highest potential benefit for each individual patient enabling personalized medicine. Personalized therapy improves effectiveness of treatment and reduces side effects at the individual level, while lowering costs, optimizing resource use, and supporting better health outcomes. This personalized therapy selection could help shift healthcare from a "one-size-fits-all" approach to one that maximizes outcomes at both individual and population level, ultimately leading to a more effective, efficient, and sustainable healthcare system.

A third intended use would be therapy monitoring, especially for therapies based on siRNAs or ASOs which directly hinder the formation and secretion of targeted apolipoproteins such as ApoC-III or Apo(a). For this test purpose, monitoring the entire apolipoprotein panel stays relevant because of the apolipoprotein interdependencies, despite the targeted treatment approach. For example, pelacarsen and olpasiran target Apo(a) to reduce Lp(a) levels. Monitoring Apo(a) levels will help to assess the effectiveness of these therapies, and to guide treatment decisions.

A fourth intended use of the apolipoprotein panel is the refinement of dyslipidemia classification, a concept we began to explore in **Chapter 5** for type III hyperlipidemia, metabolic syndrome (MetS) and hypertriglyceridemia (HTG). In addition, the apolipoprotein panel combined with liver and kidney function tests should also be explored for the staging of Cardiovascular-Kidney-Metabolic (CKM) syndrome, which is characterized by five evolving phases: Stage 0 involves no known cardiovascular risk factors; Stage 1 is characterized by the presence of overweight or obesity without additional risk factors; Stage 2 involves the addition of metabolic risk factors such as HTG, hypertension, MetS, diabetes, or chronic kidney disease (CKD); Stage 3 progresses to subclinical cardiovascular disease besides metabolic or kidney issues; and Stage 4 results in clinical cardiovascular disease.[3] By incorporating apolipoprotein profiles alongside traditional risk markers, we may gain deeper insights into an individual's metabolic lipid state for example to diagnose MetS in a more refined way, potentially enhancing risk stratification and management of CKM.

Having discussed the intended uses of the apolipoprotein panel test, it is also important to address its test role and thus the position in the clinical care pathway. In our current research, we aimed to determine whether the apolipoprotein panel could serve as an add-on test to the classic lipid panel in cardiovascular risk management. For this test role, the apolipoproteins need to provide additional predictive value beyond the classic lipids alone, and indeed, as we demonstrated in two distinct populations, they do enhance cardiovascular prediction. The current clinical care pathway for CVD can thus be refined by using the apolipoprotein panel test as an add-on test which enables

a more personalized and refined approach, less residual cardiovascular risk, identify patients at increased cardiovascular risk, guides therapy selection and enables targeted treatment and therapy monitoring (Figure 2). In contrast, for certain metabolic disorders, such as those related to ApoC-III or Apo(a), the apolipoprotein panel may serve as the primary diagnostic tool and therapeutic target, rather than an additional test. The clinical performance and effectiveness of the apolipoprotein test have been demonstrated in two populations. A subsequent step in the test evaluation framework would be to assess the cost-effectiveness of the apolipoprotein test and evaluate its broader impact. In the case of hyperlipoproteinemia(a), the broader impact is evident: 20% of the population exceeds the clinical decision limit of 125 nmol/L. For an index patient with premature CVD, the entire family should be investigated, as recommended in the EAS 2022 consensus.[36]

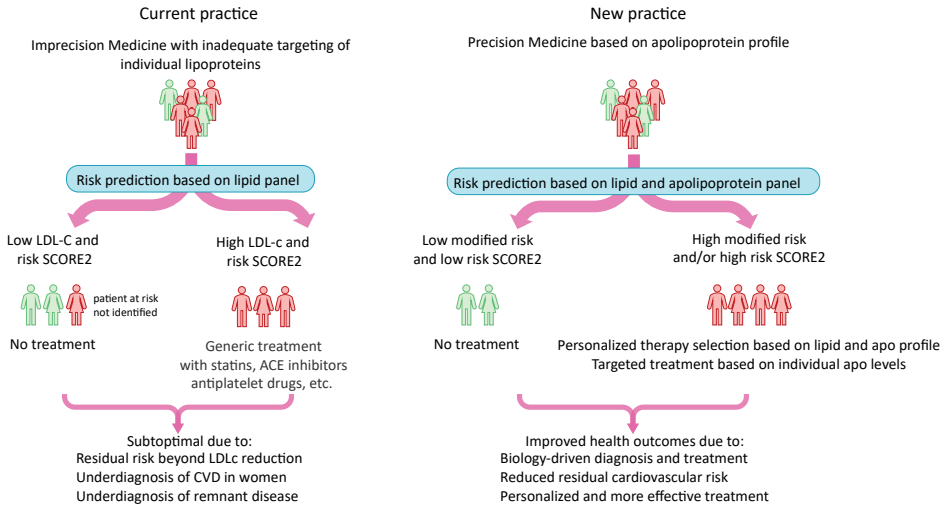


Figure 2: Current and future clinical care pathway in cardiovascular risk management

Mass spectrometry as tool for precision diagnostics and personalized medicine

Our literature review and clinical research have shown that apolipoproteins are interrelated, functioning as a highly-integrated network. This underscores the need to measure apolipoproteins as a multiplex panel, which is best achieved through mass spectrometry (MS). Unlike immunoassays, MS enables multiplex testing through the direct measurement of proteotypic peptides, without relying on monoclonal or polyclonal antibodies. For instance, quantification of Lp(a) through Apo(a) measurement by immunoassays has shown inaccuracies due to the heterogeneity of Apo(a) isoforms in both patient samples and calibrators.[25-28] Immunoassays

often use latex-bound polyclonal antibodies that detect Apo(a)'s repeating kringle IV type 2 domain, resulting in large between-method variations.[29] This can be mitigated within allowable measurement uncertainty with the use of multiple calibrators. In contrast, our MS-based test is designed to be isoform-independent by selecting Apo(a) peptides that are not present on KIV2, thus offering high analytical specificity and eliminating confounding by heterogenous apo(a) isoforms. Quantitative protein MS offers substantial potential to overcome issues of test non-selectivity and standardization challenges due to its capacity for direct measurement of molecularly defined measurands.

Metrological traceability of apolipoprotein tests

Standardization and metrological traceability are essential for global exchangeability of test results, including apolipoprotein results. Ideally, medical tests should yield results that are directly comparable across different suppliers, countries, and continents. The ISO 17511:2020 standard addresses this by emphasizing the need to anchor test results to internationally recognized reference materials (RMs) and reference measurement procedures (RMPs) through an unbroken chain of materials and methods. MS offers a promising approach as RMP, as it enables molar concentration measurements with high specificity, traceable to molecular standards. Implementing this RMP across a network of calibration laboratories and running certification programs for in vitro diagnostics (IVD)-manufacturers could make test results exchangeable and sustainable in time and space. The current intermethod variability in Lp(a) levels reported by different suppliers further underscores the need for effective standardization.[28, 30] Recent work has outlined a reference measurement system (RMS) for apolipoproteins, including Lp(a), which shall contribute to ensuring consistency in clinical testing and increase the reliability of findings in epidemiological research and clinical trials.[31] Global efforts are being made by the IFCC Working Group on Apolipoproteins by Mass Spectrometry to develop a RMS for Apo(a), ApoA-I, ApoB, ApoC-I, ApoC-II, ApoC-III and ApoE and ApoE phenotype.[28, 31] The metrological traceability of current and new apolipoprotein tests is being established following the latest metrology guidelines set out in ISO 17511:2020.

Quality assurance in multiplex testing

An important key element of the test evaluation framework is the analytical performance of a diagnostic test. The analytical performance of the multiplex apolipoprotein panel has been demonstrated.[32] Sustainability of the analytical performance of a medical test in time and space, including its longitudinal accuracy, should be guaranteed with a Quality Assurance program. Traditionally, Westgard rules have been used to assess the quality of clinical tests by monitoring the accuracy and precision of mostly single-biomarker tests.[33, 34] These rules were developed to

flag potential issues like random errors or systematic biases. However, when applied to multiplex tests, such as the apolipoprotein panel test, Westgard rules can generate false, unnecessary alarms. This is because multiplex tests measure multiple analytes simultaneously. As a result, applying these traditional rules to multiplex tests may lead to inaccurate conclusions regarding the test's performance, necessitating alternative or adjusted quality control strategies for multiplex assays to ensure their reliability. In **Chapter 4**, we encountered this challenge while analyzing samples from the ODYSSEY OUTCOMES trial. Applying typical Westgard rules would have resulted in a high rejection rate (39.6%), based purely on statistical thresholds. After adjusting the rules, we obtained a more realistic rejection rate of 10.5%. A review of the rejected samples revealed that only a small proportion warranted rejection, indicating that the adjusted rules were too stringent as well. This illustrates that quality assurance rules should be tailored to the specific application of the test. An important note is that the applicability of quality assurance rules in multiplex tests depends on multiple parameters, such as the number of IQC samples measured in a batch, the number of multiplexed 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. Nevertheless, it should be noted that the quality control rules we implemented resulted in an excellent average between-run coefficient of variation of 3.1% across nine apolipoproteins, measured in 23,000 clinical samples over one year, highlighting the reproducibility and precision of our tailored approach.

Mass Spectrometry enables personalized medicine

Given that MS can measure well-defined analytes directly without relying on antibodies, we investigated its practical application by comparing it to other commercially available tests in **Chapter 7**. We focused on one of the apolipoproteins included in our panel, Apo(a), the characteristic and extremely heterogenous apolipoprotein of Lp(a). In 11,970 individuals from the ODYSSEY OUTCOMES study, Apo(a) levels measured by MS in our multiplex test were compared to Lp(a) measurements on the Siemens (immunonephelometric) and Roche (immunoturbidimetric) analyzers, alongside an analysis of the impact on patient outcomes. At the population level, all three tests predicted patient outcomes comparably, despite the inferior analytical performance of the immunoassay-based tests due to KIV2 size polymorphism compared to the MS-based test.

Initially, more pronounced differences were expected among the three evaluated Lp(a) tests due to the heterogeneity of Apo(a). Apo(a) shows size polymorphisms due to variable KIV2 repeats. Polyclonal immunoassays that recognize Apo(a) epitopes may under- or overestimate Lp(a) concentrations depending on isoform size, although the use of selected calibrators with varied isoforms, like the Roche test, mitigates this bias.[35] MS-based quantification of Lp(a) through its

proteotypic peptides theoretically offers an advantage by measuring Apo(a) independently of kringle size. Furthermore, reporting Lp(a) levels in molar units is necessary for Lp(a) due to the clear association between Lp(a) particle number and atherogenicity, as supported by international consensus statements.[36, 37] For predicting MACE and all-cause death, the clinical effectiveness of the immunoassay-based Siemens test reporting in mass units, the immunoassay-based Roche test reporting in molar units, and the MS-based test reporting in molar units was equally useful regardless the Lp(a) test used. Yet, at the individual level, significant discrepancies were observed. For example, in one patient, MS measured an Apo(a) level of 983 nmol/L, while the Roche immunoturbidimetric test showed 148 nmol/L, a difference substantial enough to lead to misdiagnosis or mistreatment, especially as treatments specifically targeting elevated Lp(a) levels become available in the nearby future. Depending on the clinical trial design, the test purpose, test role, and the study population, the test may work on average but can hide important interindividual differences in patients. In this precision medicine era, we aim to diagnose, treat, and monitor patients as individuals, not as an average person, underscoring the potential of MS to enable personalized medicine.

Lab developed tests and the challenges of implementation with In Vitro Diagnostic Regulations (IVDR)

The apolipoprotein panel shows great clinical potential. However, being a lab-developed test (LDT), it does face challenges in its implementation in clinical care. LDTs are diagnostic tests created and used within a single clinical laboratory, with the purpose to meet specialized needs or to offer innovative diagnostics not yet available from commercial manufacturers. Traditionally, LDTs have been less regulated than commercially available tests, allowing clinical laboratories flexibility in developing and implementing tailored diagnostics. However, the implementation of the European Union's In Vitro Diagnostic Regulation (IVDR) EU IVDR 2017/746, which came into full effect in 2022, presents new challenges for LDTs.[38, 39] The IVDR aims to regulate market entry of commercial tests, quality, safety, and adequate performance of IVD tests across the EU. Under the IVDR, LDTs are exempted in article 5.5. Yet, LDTs must now meet stringent documentation, validation, and post-market surveillance requirements similar to those for commercially manufactured tests. For many clinical laboratories, meeting these requirements involves substantial administrative and technical burdens, especially given the resources and expertise required for regulatory compliance. In addition, when an equivalent commercial alternative becomes available, laboratories have to justify why they want to keep the LDT in routine practice.[39] Demonstrating the required clinical evidence is resource intensive and makes the development of new LDTs less attractive. Yet, in academic environments this is very important for translation to the clinic, and flexibility of laboratories is essential for addressing specific patient needs, as well

as for orphan diagnostics, rare metabolic diseases and other specialized diagnostics. As clinical laboratories adapt to these changes, clear frameworks for LDT test evaluation will be crucial to maintain high standards while preserving the ability to meet unique diagnostic needs and encourage innovation in healthcare.

CONCLUSION

In conclusion, this thesis aimed to identify clinically relevant risk factors contributing to residual cardiovascular risk and established the rationale for measuring apolipoproteins in the context of cardiovascular disease. We assessed both the clinical performance and clinical effectiveness of the multiplex apolipoprotein test, quantified by MS, in two distinct populations. Furthermore, our findings demonstrate that LC-MS/MS can measure apolipoproteins in large clinical trials or observational studies, generating high-quality data.

Adding the multiplex apolipoprotein panel to traditional lipid testing enhances precision diagnostics by revealing the patients with e.g. elevated Lp(a) and/or other apo deficiencies, supports personalized therapy selection, and, in the future, may enable therapy monitoring as apolipoprotein-targeting treatments become available. Together, these advancements lay a foundation for P5 medicine to reduce residual cardiovascular risk. The broader implementation of apolipoproteins is already recommended in the EFLM-EAS consensus for ApoB[10], and in the 2022 EAS consensus for Apo(a).[36] Furthermore, implementation of the apolipoprotein panel may enhance the diagnosis and staging of CKM syndrome, as described in the AHA presidential advisory on preventing or retarding the progression of CKM syndrome.[3] Nevertheless it remains essential to address the test panel's cost-effectiveness and the broader impact. This marks the dawn of the APOcalypse of the multiplex apolipoprotein panel revealing its potential for cardiovascular precision medicine. The significant improvement in clinical performance and clinical effectiveness of the apolipoprotein panel for MACE and all-cause death on top of the serum lipid profile, marks a new era in tackling residual cardiovascular risk.

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