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Novel biomarkers and their relation to clinical outcomes and pathophysiology in anticoagulated patients with atrial fibrillation

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Abstract
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Atrial fibrillation (AF) is a common arrhythmia and is associated with an increased risk of cardiovascular (CV) outcomes, including mortality and heart failure (HF).

The overall aim of this thesis was to evaluate the association of novel and established biomarkers with cardiovascular outcomes in patients with AF. Baseline levels of apolipoproteins A1 (ApoA1) and B (ApoB) were investigated in relation to CV outcomes. The geographic consistency of Growth differentiation factor 15 (GDF-15) and the ABC-AF risk scores in predicting bleeding and mortality were investigated. Proteomic analyses were employed to screen for novel biomarkers associated with CV death and hospitalization for HF in AF and biomarker profile differences between HF with reduced ejection fraction (HFrEF) and preserved ejection fraction (HFpEF) were also explored.

The study population consisted of patients with AF on anticoagulation included in the biomarker substudies from the large randomized clinical controlled trials RE-LY (n=6,187) and ARISTOTLE (n=14,954) with a median follow up of 2.0 and 1.9 years. Biomarker levels were measured at baseline.

Higher levels of ApoA1 were independently associated with a lower risk of ischemic events, whereas ApoB was not. Neither apolipoprotein was significantly associated with major bleeding. The predictive value of GDF-15 and the biomarker-based ABC-AF risk scores for bleeding and mortality across various geographic regions was consistent. In the screening investigation for novel markers, the biomarkers most strongly and consistently associated with CV death were: NT-proBNP, cTnT-hs, IL-6, GDF-15, FGF-23, uPAR, TFF3, TNFR1, TRAILR2 and CTSL1. The biomarkers most strongly associated with HF hospitalization were NT-proBNP, BNP, cTnT-hs, FGF-23, spon1, IGFBP-7, u-par, OPN, PTX3 and TR. In comparison of HFrEF versus HFpEF, levels of NT-proBNP, BNP, cTnT-hs, renin, ACE-2, GDF-15 and IL-6 were higher in HFrEF, whereas levels of SCF and leptin were higher in HFpEF.

In conclusion, this thesis underscores the pivotal role of biomarkers in better understanding AF and its complications. The insights from this thesis suggest potential therapeutic targets and strategies for personalized management in AF, possibly enhancing risk stratification and improving patient outcomes.

Keywords: Atrial Fibrillation, Biomarkers, Heart Failure

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Pol T**, Held C, Westerbergh J, Lindbäck J, Alexander JH, Alings M, Erol C, Goto S, Halvorsen S, Huber K, Hanna M, Lopes RD, Ruzyllo W, Granger CB, Hijazi Z. (2018) Dyslipidemia and Risk of Cardiovascular Events in Patients With Atrial Fibrillation Treated With Oral Anticoagulation Therapy: Insights From the ARISTOTLE (Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation) Trial. *J Am Heart Assoc*, 7(3):e007444

II. **Pol T**, Hijazi Z, Lindbäck J, Alexander JH, Bahit MC, De Caterina R, Eikelboom JW, Ezekowitz MD, Gersh BJ, Granger CB, Hylek EM, Lopes R, Siegbahn A, Wallentin L. (2021) Evaluation of the prognostic value of GDF-15, ABC-AF-bleeding score and ABC-AF-death score in patients with atrial fibrillation across different geographical areas. *Open Heart.* 8(1):e001471

III. **Pol T**, Hijazi Z, Lindbäck J, Oldgren J, Alexander JH, Connolly SJ, Eikelboom JW, Ezekowitz MD, Granger CB, Lopes RD, Yusuf S, Siegbahn A, Wallentin L. (2022) Using multimarker screening to identify biomarkers associated with cardiovascular death in patients with atrial fibrillation. *Cardiovasc Res.* 118(9):2112-2123

IV. **Pol T**, Lindbäck J, Oldgren J, Alexander JH, Granger CB, Lopes RD, Siegbahn A, Wallentin L, Hijazi Z. Screening of plasma biomarkers for heart failure hospitalizations and heart failure subtype in patients with atrial fibrillation. *In manuscript*

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Abbreviations

ABC-AF	Age, Biomarkers, Clinical history in AF
AF	Atrial fibrillation
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
cTn-hs	Cardiac troponin high-sensitivity
CV	Cardiovascular
ECG	Electrocardiogram
ESC	European Cardiology Society
GDF-15	Growth differentiation factor-15
HDL	High-density lipoprotein cholesterol
HFmrEF	Heart failure with mildly reduced ejection fraction
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
LDL	Low-density lipoprotein cholesterol
LVEF	Left ventricular ejection fraction
NOAC	Non-vitamin K antagonist
NT-proBNP	N-terminal fragment B-type natriuretic peptide
PEA	Proximity extension assay
SEE	Systemic embolism
TIA	Transient ischemic attack

Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia, increasingly contributing to morbidity, mortality and healthcare costs worldwide, with its prevalence expected to rise further due to an aging population[1]. AF is associated with a wide range of cardiovascular events and has been found to independently double the risk of ischemic stroke, increase all-cause mortality by up to 50% and raise the incidence of heart failure five-fold[2]. Even though stroke and stroke-related- death can largely be prevented by the use of anticoagulation[3], substantial morbidity and mortality remain and no complete curative treatment for AF exists.

Certain cardiovascular biomarkers have demonstrated improved prediction of adverse outcomes in AF and have been incorporated into several risk scores proposed for clinical use[4-9]. However the role of other traditional cardiovascular disease biomarkers, such as the components of dyslipidemia, is less clear in AF. Furthermore, is not known how the geographic variability of morbidity and mortality impacts the predictive performance of these biomarkers and biomarker-based risk scores across geographic regions.

In search of strategies to reduce the morbidity and non-stroke-related mortality and in patients with AF, the identification of new biomarkers could improve the understanding of the underlying disease processes that in turn could facilitate the identification of patients at risk for death, thus allowing for earlier intervention, optimization of secondary prevention, and possibly even for targeted therapy.

Background

Atrial fibrillation

1.1 Definition, clinical presentation and classification

AF is a supraventricular tachycardia with irregular, uncoordinated atrial activation. The diagnosis of AF can either be made with a standard 12-lead ECG or a single-lead ECG tracing of ≥30 seconds showing the typical appearance of irregular R-R intervals and the absence of distinct repeating p-waves[10]. The clinical presentation of AF varies widely with substantial individual variation spanning from being asymptomatic to hemodynamic instability. Patients with AF may commonly experience symptoms such as palpitations, dyspnea, fatigue, dizziness and chest pain[10]. Based on presentation, duration, spontaneous termination and therapeutic goals, AF traditionally has been classified into five categories: first diagnosed AF, paroxysmal AF, persistent AF, long-standing AF and permanent AF (Table 1).

Table 1. Classification of AF by disease persistence[10]

AF classification	**Definition**
First diagnosed AF	AF not diagnosed before, irrespective of duration and severity
Paroxysmal AF	AF episode that terminates spontaneously or with intervention within 7 days
Persistent AF	AF episode that is continuously sustained beyond 7 days, including those terminated by cardioversion after 7 or more days
Long-standing persistent AF	AF episode that lasts for ≥1 year and when it is decided to adopt a rhythm control strategy
Permanent AF	AF is accepted as permanent by both patient and physician with no intention to restore/maintain sinus rhythm

1.2 Pathophysiology

Despite significant advancements, our understanding of AF pathophysiology remains limited and involves a complex interplay of triggers (for the initiation) and substrates (for the perpetuation of the arrhythmia)[10].
Atrial alterations on both a macroscopic and microscopic level are key in the pathogenesis of AF. The structural changes seen in the atria consist of atrial

fibrosis, dilation and hypertrophy together with endothelial dysfunction and electrical and autonomic remodeling. These pathologic changes in the atria are called "atrial myopathy". Atrial myopathy contributes to the development and drives the progression of AF and some of its complications, like for example cardioembolic stroke[11]. Factors contributing to atrial myopathy include aging, hypertension, inflammation, heart failure, and atrial stretch due to volume overload[11]. Atrial dilation, often secondary to pressure or volume overload conditions, exacerbates the risk of AF by stretching atrial tissue, altering the geometry, and facilitating the maintenance of re-entrant circuits[12]. Similarly, fibrosis disrupts the normal electrical pathways, creating regions of slow conduction which also promoting re-entry circuits[13]. On a cellular level, structural atrial myocyte damage, ion channels changes and abnormal calcium handling further promote the development and persistence of AF[14]. The autonomic nervous system plays a crucial role in the initiation and maintenance of AF where both sympathetic and parasympathetic nervous systems are involved. Increased sympathetic activity can lead to enhanced automaticity and triggered activity via afterdepolarizations, whereas vagal stimulation shortens the atrial refractory period and promotes re-entry. The interplay between these autonomic influences can create a highly arrhythmogenic milieu[15]. Genetic predispositions also contribute to AF. Monogenic mutations in genes encoding ion channels, gap junction proteins, and other structural proteins have been identified in familial AF cases[16]. Inflammation and oxidative stress are emerging as significant contributors to AF and atrial biopsies reveal increased inflammatory cell infiltration and cytokine expression. Oxidative stress can induce atrial myocyte apoptosis, fibrosis, and electrical remodeling, further promoting AF[17]. The culmination of all these factors creates a substrate conducive to both focal ectopic activity and re-entrant circuits.

Ectopic foci, often originating from the pulmonary veins, can trigger AF episodes[18]. Re-entrant circuits, sustained by areas of fibrosis and altered electrophysiology, perpetuate the arrhythmia. The presence of multiple re-entrant circuits, with constantly changing activation patterns, underlies the characteristic irregular and rapid atrial activity seen in AF.

It is not clear which of these aforementioned mechanisms is the main initiator or driver of AF. What is known from the now famous goat experiments by Wijffels et al. is that "AF begets AF", namely that the longer the duration of atrial pacing on a goat heart (in this case rapid pacing, simulating AF), the longer the AF is maintained[19]. Thus AF itself is causing structural and electric changes to the heart which drives the progression of AF. The resulting irregular rhythm and fibrillary action of the atria reduces the cardiac output mainly by the loss of the "atrial kick", variable ventricular cycle lengths and possible atrioventricular valvular regurgitation[20, 21]. Other than the irregular rhythm itself, the AF-caused reduction in cardiac output in turn causes at least in part the symptoms generated by AF[22].

1.2.1 Dyslipidemia in AF

Traditional CV risk factors like obesity, diabetes mellitus and hypertension show clear associations with AF[23]. The link between dyslipidemia and AF is however less clear. Dyslipidemia is a condition characterized by abnormal levels of lipids in the blood, primarily elevated cholesterol and triglycerides. High levels of low-density lipoprotein (LDL) and low levels of high-density lipoprotein (HDL) and their main protein components apolipoprotein B (ApoB) and apolipoprotein A1 (ApoA1) respectively, are associated with CV disease like myocardial infarction (MI) and stroke[24-27]. Despite the association between dyslipidemia and the increased risk of coronary artery disease (which in turn may lead to an increased risk of AF) the association between lipid levels and incidence of AF has not been fully elucidated with even several studies suggesting that high levels of low-density lipoprotein cholesterol (LDL), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL) are associated with a lower risk of AF[28, 29]. Moreover the relation between dyslipidemia and cardiovascular outcomes and its role as a risk factor in patients with AF treated with oral anticoagulation therapy is also unclear.

1.3 Epidemiology

AF is considered a 21st-century cardiovascular disease epidemic with more than 59 million individuals living with AF in 2019[30, 31]. The prevalence of AF is rising markedly and is projected to continue doing so, reaching close to 18 million 2060 in Europe. However the true prevalence is likely underestimated due to the sometimes asymptomatic nature of AF[1, 32]. The rise of AF is probably a consequence of longer average life expectancy globally, as well as improved awareness and better access to diagnostic tools[1, 33]. The prevalence of AF is higher in the developed world, in males and increases substantially with age[34, 35]. In Sweden, the prevalence of AF in the adult population was at least 2.9% in 2013, not counting "silent AF"[36]. The calculated lifetime risk of AF with no cardiovascular (CV) risk factors is one in five and rises to one in three in individuals when having at least one CV risk factor[37].

1.4 Clinical complications

1.4.1 Stroke and systemic embolism

AF significantly increases the risk of stroke and transient ischemic attack (TIA), with strokes related to AF often resulting in severe disability or death[38, 39]. Stroke risk in AF is variable and is influenced by age, sex, previous stroke and cardiovascular comorbidities[40]. Additionally, non-paroxysmal AF is associated with an increase in thromboembolism compared with paroxysmal AF[41]. The mechanistic basis of stroke in patients with AF is not fully understood and is likely more complex than previously appreciated. Many factors are thought to be involved, including blood stasis related to impaired atrial contractility, endothelial dysfunction, and prothrombotic conditions[42].

Less is known about systemic embolism (SEE) compared to ischemic stroke in AF but the mechanisms are thought to be similar. SEE is much less common than stroke in AF, representing a minority of the clinically recognized embolic events. Common sites for SEE include the lower extremities and mesenteric circulation, with significant morbidity and mortality associated with SEE events[43].

1.4.2 Death

Patients with atrial fibrillation have a significantly increased risk of mortality[44, 45]. In contemporary anticoagulated AF populations, data from the large non-vitamin K antagonist (NOAC) trials suggests the majority of causes of death were due to CV causes rather than non-CV causes[46-48]. Specifically, the most common causes of CV death are sudden cardiac death followed by heart failure. Death by ischemic stroke remains one of the largest causes for death in AF, despite the use of anticoagulant therapy.

1.4.3 Heart failure

Heart failure (HF) is a clinical syndrome consisting of cardinal symptoms (e.g. dyspnea) that may be accompanied by signs (e.g. peripheral edema) due to a structural and/or functional abnormality of the heart that results in elevated intracardiac pressures and/or inadequate cardiac output at rest and/or during exercise[49]. HF is a common complication in patients with AF, affecting approximately 20-30% of AF patients[10]. Sharing common risk factors, AF and HF often coexist, or may exacerbate each other, resulting in significantly greater mortality than either condition alone[50]. The mechanism of HF in AF is not entirely clear but is thought to be multifactorial. AF can lead to an increase in resting heart rate and an exaggerated heart rate response to exercise resulting in shorter diastolic filling time, leading to a reduction in cardiac output and eventually, if high enough heart rate for a longer duration, a decrease in cardiac systolic function. Furthermore, the irregular systolic intervals in AF together with the loss of effective atrial contractile function also contribute to a decreased cardiac output. The resulting HF in turn promotes the development and maintenance of AF that further predispose to the exacerbation of HF in a vicious cycle[51].

According to the latest European Cardiology Society (ESC) guidelines for HF, HF is classified based on the left ventricular ejection fraction (LVEF); HF with reduced ejection fraction (HFrEF – LVEF ≤40%), HF with mildly reduced ejection fraction (HFmrEF – LVEF 41-49%) and HF with preserved EF (HFpEF – LVEF ≥50%)[52].

1.4.4 Other complications

AF is linked to an increased risk of cognitive impairment and dementia, both in patients with and without a history of clinical stroke[53, 54]. The risk of dementia is higher in younger patients with AF and increases with the duration of AF[54]. Potential mechanisms include asymptomatic cerebral infarctions and micro thromboembolism[10, 55]. Other notable AF-related complications also include depression[56].

1.5 Management

The main principles of AF management are centred around stroke prevention by use of anticoagulation, symptom relief by rate or rhythm control together with management of CV risk factors and lifestyle modification. The overall aim of these strategies is to prevent AF-related morbidity and mortality.

1.5.1 Rate and rhythm control

The decision between rate and rhythm control strategies is one of the most fundamental aspects of AF management and is influenced by a multitude of factors including patient symptoms, functional status, comorbidities, and the underlying cause of AF[10]. Rate control aims to decrease heart rate and avoid tachycardia, thus increase the cardiac output and improve AF-related symptoms. This approach is generally simpler, in some ways safer, and more cost-effective, as it reduces the need for frequent hospitalizations and intensive monitoring. However, rate control may not completely alleviate AF symptoms like palpitations and fatigue and address the underlying arrhythmia only to a limited extent.

On the other hand, rhythm control aims to restore and maintain normal sinus rhythm through the use of antiarrhythmic drugs, cardioversion, or ablation therapy. This strategy can provide significant symptom relief and improved quality of life, especially in symptomatic or younger patients, and may potentially reduce the long-term risk of thromboembolic events if sinus rhythm is maintained[41]. However, rhythm control comes with the drawbacks of adverse effects from antiarrhythmic drugs, including proarrhythmic risks, and requires more intensive monitoring and potentially invasive procedures.

The evidence on whether rate or rhythm control is preferable remains incomplete[10]. Previous trials, such as AFFIRM and CABANA, have not shown a definitive mortality benefit for rhythm control over rate control[57, 58]. However the more recent EAST-AFNET 4 trial indicated that *early* rhythm control therapy was associated with a lower risk of adverse cardiovascular outcomes, including mortality[59]. The choice between rate and rhythm control should thus be tailored to each patient's clinical scenario. Younger patients, those without significant comorbidities, and those with paroxysmal AF may derive the most benefit from rhythm control, particularly when maintaining sinus rhythm through ablation therapy[60].

1.5.2 Stroke prevention

At least 20% of all ischemic strokes are thought to be of cardioembolic nature, mainly due to AF[61, 62]. As previously mentioned, AF-related stroke is associated with substantial morbidity and mortality, thus one of the main focuses of the management of AF has been on prevention of strokes attributed to AF. Both aspirin and P_2Y_{12} inhibitors administered alone or in combination, have been evaluated in patients with AF but are significantly less effective than oral anticoagulants, even when combined[63-65]. The historical use of anticoagulants in AF has evolved significantly over the years. Historically warfarin, a vitamin K antagonist, has been the standard of stroke prevention in AF. Despite its effectiveness, warfarin's use is complicated by the need for regular blood monitoring and certain dietary cautions due to its narrow therapeutic window and numerous drug interactions[66]. To address these limitations, several new oral anticoagulants have been introduced in the last two decades. These include direct thrombin inhibitors (dabigatran) and factor Xa inhibitors (apixaban, rivaroxaban and edoxaban). Together, the non-vitamin K oral antagonists (NOACs) offer at least the same efficacy for stroke prevention as warfarin with a decreased risk of intracranial bleeding without the need for routine coagulation monitoring[67-70]. Mainly because of these benefits, NOACs are now recommended in preference to warfarin in most situations for stroke prevention in AF[10].

1.5.3 Risk assessment in AF

The risk of stroke in AF is individual, variable and depends in part on the presence of risk factors. Effective risk assessment helps identify patients at higher risk for stroke, benefiting from anticoagulation, as well as low-risk individuals where anticoagulation can result in a net harmful effect. Consequently, risk estimation tools have been developed to provide guidance for clinical decision making and the use of stroke prevention therapies. The most clinically widely used stroke risk score is the CHA_2DS_2-VASc score[40]. The CHA_2DS_2-VASc score summarizes common stroke risk fac-

tors/modifiers and include congestive HF, hypertension, age (≥65; 1 point, ≥75 years; 2 points), diabetes mellitus, stroke (2 points), vascular disease, sex category (female).
The use of anticoagulation for AF-related stroke prevention inherently carries the risk of increased bleeding, which can range from trivial to life-threatening. Therefore attempts to assess the bleeding risk in AF have been made; where the HAS-BLED score (uncontrolled hypertension, abnormal liver/renal function, stroke, history of bleeding, labile international normalized ratio, age ≥65 and drugs/excessive alcohol intake) traditionally has been the most popular[71].
Although the CHA_2DS_2-VASc and the HAS-BLED risk scores are simple to use in everyday practice, they perform at best modestly in predicting events[72, 73]. Furthermore, because they share many of the same components, they do not adequately separate stroke from bleeding risk. To address these limitations, the incorporation of biomarkers into risk scores has been suggested, in the form of the ABC (age, biomarkers, and clinical history) stroke and bleeding risk scores in AF[8, 74]. Both the ABC-AF-stroke (previous stroke, N-terminal fragment B-type natriuretic peptide (NT-proBNP) and cardiac troponin high-sensitivity (cTn-hs)) and ABC-AF-bleeding (history of bleeding, haemoglobin, cTn-hs, and growth differentiation factor-15 (GDF-15) or cystatin C) risk scores performed significantly better in regards to risk prediction as compared to the CHA_2DS_2-VASc and the HAS-BLED score respectively[8, 74]. In a similar way, the ABC-AF-death score (history of HF, NT-proBNP, cTn-hs and GDF-15) was developed to predict the mortality risk to allow a more complete risk assessment in AF[9].

1.6 Biomarkers in AF

According to the National Institutes of Health Biomarkers Definitions Working Group, a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”[75]. Among various types of biomarkers, blood-based biomarkers hold particular significance due to their accessibility and the wealth of information they can provide about an individual's health status. Several protein blood-based biomarkers reflecting different pathophysiological functions have been shown to be powerful predictors of CV death in AF. Examples include markers of myocardial damage (cardiac troponin T), myocardial stress (NT-proBNP), inflammation (interleukin 6 [IL-6] and growth differentiation factor 15 (GDF-15))[4-7]. Biomarkers also facilitate the understanding of key pathophysiologic processes underlying complications of AF and may help identify additional treatment targets, especially for patients with high residual CV risk despite oral anticoagulation therapy.

1.6.1 Cardiac biomarkers - cTn-hs and NT-proBNP

One of the most extensively studied biomarkers in AF are the cardiac proteins natriuretic peptides and cardiac troponins. NT-proBNP is a natriuretic peptide and an inactive fragment of the prohormone BNP, released by ventricular myocytes in response to increased wall stress and volume overload. The main functions of BNP include natriuresis and diuresis via the kidneys, reduction of vascular tone and inhibition of cardiac remodelling[76]. The most established use for natriuretic peptides is in the diagnostic algorithm of heart failure[52]. Higher levels of natriuretic peptides are seen with increasing age, impaired renal function and in females whereas lower levels can be seen in obesity[77, 78]. Higher natriuretic peptides concentrations are also observed in patients with AF as compared to those without AF, and these levels decrease significantly after the restoration of sinus rhythm via cardioversion[79-81]. The prognostic value of natriuretic peptides extends across various clinical scenarios, serving as powerful prognostic markers for CV outcomes and mortality in patients with and without HF and coronary artery disease[82-84]. In AF, elevated levels of NT-proBNP were strongly associated with incident AF and AF recurrence after cardioversion and catheter ablation[85-87]. Importantly, NT-proBNP has been shown to be a significant predictor of stroke and mortality in AF[5, 88].

Cardiac troponins like cTn-hs are regulatory proteins that control the calcium-mediated interaction of actin and myosin in cardiac muscle contraction. They are highly specific and sensitive biomarkers of myocardial injury, released into the bloodstream when myocardial cell damage occurs. Although elevated levels of cardiac troponins can be seen in a variety of disease states, they are primarily used to diagnose acute coronary syndromes[89]. The introduction of high-sensitivity assays for cardiac troponins has significantly improved the ability to detect even minor myocardial injury[90]. The majority of patients with AF have detectable levels of cTn-hs, especially in those with CV comorbidities, and troponin elevations in AF are thought to indicate myocardial stress and injury[4, 91]. Similar to NT-proBNP, cTn-hs is strongly associated with stroke and mortality but also bleeding in AF[4, 88]. Because of their excellent predictive abilities of CV events in AF, they have both been incorporated in the ABC-AF risk scores[8, 9, 74].

1.6.2 Markers of inflammation – IL-6, CRP and GDF-15

Inflammation plays an important role in the pathogenesis of AF. Various inflammatory markers, including Interleukin-6 (IL-6) and GDF-15, have been studied for their association with AF[17, 92, 93]. These markers are elevated in patients with AF and are implicated in the structural and electrical remodeling of the atria.

IL-6 is a multifunctional cytokine produced by various cells, such as T-cells, macrophages, and endothelial cells, in response to infections, trauma, and other stimuli. It plays a critical role in the immune response, inflammation, and hematopoiesis[94]. IL-6 exerts its effects mainly by either "classical signaling", that involves membrane-bound IL-6 receptor primarily on hepatocytes and various immune cells, or by "trans-signaling" by binding to soluble IL-6R that can interact with cells that do not express membrane-bound IL-6 receptors (most cells). While the "classical signaling" primarily is associated with immune response modulation, acute phase response and have anti-inflammatory proporties, "trans-signaling" drives systemic chronic inflammation[95, 96]. IL-6 has been implicated in chronic kidney disease, atherosclerotic disease and HF among many conditions[96]. Regarding AF, IL-6 levels are higher in patients with AF compared to those without and IL-6 contributes to atrial structural remodeling by promoting fibrosis and atrial dilation, making the atria more susceptible to arrhythmias[17, 92, 97]. Additionally, higher IL-6 concentrations are associated with an increased risk of stroke, major bleeding and mortality in AF[6].

C-reactive protein (CRP) is an acute-phase protein synthesized by the liver largely in response to IL-6. It is a member of the pentraxin family of proteins and plays an important role in the immune response[98]. CRP is routinely available and blood concentrations increase significantly during inflammatory conditions, making it a useful biomarker for diagnosing and monitoring inflammation and infections. In the context of cardiovascular diseases, elevated CRP levels are associated with an increased risk of CV disease, including atherosclerosis, coronary artery disease and incident HF[99-101]. Specifically, in AF, CRP is often elevated indicating an inflammatory component to the condition[102]. Elevated CRP levels have been linked to an increased risk of developing AF and patients with higher CRP levels are more likely to relapse into AF than those with lower serum levels[103]. However the causal effects of CRP on AF has not been supported by Mendelian randomization[104]. Elevated CRP levels are associated with an increased mortality risk in AF[6, 105].

GDF-15 is a member of the transforming growth factor-beta superfamily and is produced by various cells, including the placenta, cardiomyocytes, macrophages, and endothelial cells, in response to stress conditions such as inflammation, oxidative stress, and tissue injury[106]. GDF-15 acts as a cytokine with both pro-inflammatory and anti-inflammatory, anti-apoptotic and anti-fibrotic properties, influencing a wide range of physiological and pathological processes and even promotes weight loss[107-109]. In the context of CV diseases, GDF-15 has been linked to various adverse outcomes and mortality[107]. Elevated levels of GDF-15 are associated with worse prognosis in patients with heart failure, myocardial infarction, and other cardiac conditions. Additionally, GDF-15 is associated with mortality, and more importantly with bleeding but less with stroke in AF[7, 110]. These properties make GDF-15 an attractive candidate in the search for markers to better distinguishing between the risk of stroke and the risk of bleeding, thus also leading to its incorporation in the ABC bleeding risk score[8].

1.6.3 Renal biomarkers – Cystatin C

Cystatin C is a small protein produced by all nucleated cells and metabolized in the proximal tubule[111]. It plays a crucial role in regulating protease activity, thereby preventing excessive protein degradation and is considered a reliable biomarker for assessing kidney function as it is freely filtered by the kidneys and is less influenced by muscle mass and diet compared to the more frequently used measure of renal function, creatinine[112]. Cystatin C is increasingly recognized for its association with adverse outcomes in CV disease. Elevated plasma cystatin C levels are more strongly associated with CV events and mortality in the elderly, in the intensive care setting, in patients with coronary heart disease and in HF than creatinine[113-116]. In AF, worse renal function defined as elevated cystatin C is associated with increased risk of major bleeding and mortality and AF recurrence after catheter ablation[117-119].

2. Aims

The overall aim of this thesis is to evaluate the association of novel and established biomarkers with cardiovascular outcomes in patients with AF.

The specific aims were:

I. To assess the independent association of ApoA1, the main protein component of high-density lipoprotein HDL, and ApoB the main protein component of LDL, measured at baseline with clinical outcomes in AF.
II. To evaluate GDF-15 and the ABC-AF-bleeding and ABC-AF-death scores across geographical regions using data from both the ARISTOTLE trial and the RE-LY trial.
III. Use the PEA technology to comprehensively screen for biomarkers associated with CV death in order to improve the understanding of the processes that may be involved in CV death in anticoagulated patients with AF.
IV. Use PEA to comprehensively screen for biomarkers associated with hospitalization for HF and to examine baseline differences in biomarker profiles between HFrEF and HFpEF in order to improve the understanding of the processes that may be involved in heart failure in anticoagulated patients with AF.

3. Materials and methods

3.1 Study population

3.1.1 The ARISTOTLE trial

The Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE) trial was a multi-center, double-blind, non-inferiority study which enrolled and randomized 18,201 patients from 39 countries with non-valvular AF and at least 1 additional risk factor for stroke to receive either warfarin with target international normalized ratio (INR) 2.0-3.0 or apixaban for stroke prevention in a 1:1 fashion[68, 120]. Detail eligibility criteria of the ARISTOTLE trial can be found in Table 2. All participants were recruited between 2006 and 2010 and followed for approximately 2 years. Apixaban was not only shown to significantly reduce the primary endpoint of stroke or systemic embolism in comparison to warfarin, but also to significantly reduce major bleeding and all-cause mortality.

Table 2. Eligibility criteria of the ARISTOTLE trial

Inclusion Criteria	Exclusion Criteria
Age ≥ 18 years	AF or atrial flutter due to reversible causes
Permanent or persistent AF or atrial flutter on ECG at enrollment; or AF or atrial flutter documented by ECG or as an episode ≥1 min on rhythm strip, Holter monitor, or intracardiac recording on 2 separate occasions at least 2 wk apart in 12 mo before enrolment *One or more of the following risk factors for stroke*: Prior stroke, TIA, or systemic embolus	Clinically significant (moderate or severe) mitral stenosis
Symptomatic HF within 3 mo or LV dysfunction with LVEF ≤40% by echocardiography, radionuclide study, or contrast angiography	Increased bleeding risk believed to be a contraindication to oral anticoagulation (eg, previous intracranial hemorrhage)
Diabetes mellitus	Conditions other than AF that require chronic anticoagulation (eg, prosthetic mechanical heart valve)
	Persistent uncontrolled hypertension (SBP >180 mm Hg or DBP >100 mm Hg)
Women of childbearing potential must use contraception to avoid pregnancy during treatment period or for 2 wk after last dose of study medication, whichever is longer	Active infective endocarditis
All subjects must provide signed written informed consent	Planned major surgery
	Planned AF or atrial flutter ablation procedure
	Use of unapproved investigational drug or device in past 30 d
	Required aspirin >165 mg/d
	Simultaneous treatment with both aspirin and a thienopyridine (eg, clopidogrel, ticlopidine)
	Severe comorbid condition with life expectancy ≤1 y
	Recent stroke (within 7 d)
	Active alcohol or drug abuse or psychosocial reasons that make study participation impractical
	Severe renal insufficiency (serum creatinine level >2.5 mg/dL or calculated creatinine clearance <25 mL/min)
	ALT or AST >2 × ULN or a total bilirubin ≥1.5 × ULN (unless an alternative causative factor [eg, Gilbert's syndrome] is identified)
	Platelet count ≤100,000/mm3
	Hemoglobin level <9 g/dL
	Inability to comply with INR monitoring

ECG, Electrocardiogram; TIA, transient ischemic attack; HF, heart failure; LV, left ventricular, LVEF, left ventricular ejection fraction; SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine transaminase; AST, aspartate transaminase; ULN, upper limit od normal; wk, weeks; mo, months.

3.1.2 The RE-LY trial

The Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial was a non-inferiority multi-center study which randomized 18 113 patients from 44 countries with NVAF and at least one additional risk factor for stroke to either one of two blinded doses of dabigatran etexilate (150 or 110 mg twice daily) or open-label warfarin with target INR 2.0-3.0 for stroke prevention in a 1:1:1 ratio[67, 121]. All participants were recruited between 2005 and 2007 and the median follow-up time was 2 years. The trial included patients with documented AF and at least 1 additional risk factor for stroke. Detailed eligibility criteria of the RE-LY trial can be found in Table 3. The trial showed that compared to warfarin, dabigatran given in the 110mg dose was non-inferior for stroke or systemic embolism and superior for major bleeding. In addition, the dabigatran 150mg dosing was superior to warfarin with respect to stroke or systemic embolism and noninferior concerning major bleeding.

Table 3. Eligibility criteria of the RE-LY trial

Inclusion Criteria	Exclusion Criteria
There is ECG documented AF on the day of screening or randomization:	History of heart valve disorders (ie, prosthetic valve or hemodynamically relevant valve disease)
The patient has had a symptomatic episode of paroxysmal or persistent AF documented by 12-lead ECG within 6 m before randomization	Severe, disabling stroke within the previous 6 m, or any stroke within the previous 14 d
There is documentation of symptomatic or asymptomatic paroxysmal or persistent AF on 2 separate occasions, at least 1 day apart, one of which is within 6 m before randomization. In this case, AF may be documented by 12 lead ECG, rhythm strip, pacemaker/ICD electrogram, or Holter ECG. The duration of AF should be at least 30 s. Electrograms (not marker channels or mode switch episodes) from pacemakers and defibrillators can be used to document only 1 episode of paroxysmal or persistent AF	*Conditions associated with an increased risk of bleeding:*
In addition to documented AF, patients must have one of the following:	Major surgery in the previous month
History of previous stroke, TIA, or systemic embolism	Planned surgery or intervention in the next 3 m
Ejection fraction <40% documented by echocardiogram, radionuclide or contrast angiogram in the last 6 m	History of intracranial, intraocular, spinal, retroperitoneal or atraumatic intra-articular bleeding
Symptomatic heart failure, New York Heart Association class 2 or higher in the last 6 m	Gastrointestinal hemorrhage within the past year
Age ≥75 y	Symptomatic or endoscopically documented gastroduodenal ulcer disease in the previous 30 d
Age ≥65 y and one of the following:	Hemorrhagic disorder or bleeding diathesis
Diabetes mellitus on treatment	Need for anticoagulant treatment of disorders other than AF
Documented coronary artery disease (any of: prior myocardial infarction, positive stress test, positive nuclear perfusion study, prior CABG surgery or PCI, angiogram showing ≥75% stenosis in a major coronary artery	Fibrinolytic agents within 48 h of study entry
Hypertension requiring medical treatment	Uncontrolled hypertension (systolic blood pressure >180 mm Hg and/or diastolic blood pressure >100 mm Hg)
Age >18 y at entry	Recent malignancy or radiation therapy (≤6 m) and not expected to survive 3 y
Written, informed consent	Contraindication to warfarin treatment
	Reversible causes of atrial fibrillation (eg, cardiac surgery, pulmonary embolism, untreated hyperthyroidism).
	Plan to perform a pulmonary vein ablation or surgery for cure of the AF
	Severe renal impairment (estimated creatinine clearance ≤30 mL/min)
	Active infective endocarditis
	Active liver disease, including but not limited to:
	Persistent ALT, AST, Alk Phos >2× ULN
	Known active hepatitis C (positive HCV RNA)
	Active hepatitis B (HBs antigen +, anti HBc IgM+)
	Active hepatitis A
	Women who are pregnant or of childbearing potential who refuse to use a medic ally acceptable form of contraception throughout the study
	Anemia (hemoglobin level <100g/L) or thrombocytopenia (platelet count <100 × 109/L)
	Patients who have developed transaminase elevations upon exposure to ximelagatran.
	Patients who have received an investigational drug in the past 30 d
	Patients considered unreliable by the investigator or have a life expectancy less than the expected duration of the trial because of concomitant disease, or has any condition which in the opinion of the investigator, would not allow safe participation in the study (eg, drug addiction, alcohol abuse).

ALT, alanine aminotransferase; AST, aspartame aminotransferase; Alk phos, alkaline phosphatase; HCV, hepatitis C virus; HBs, hepatitis B surface; HBc, hepatitis B core; IgM, immunoglobulin M.

3.2 Ethics

Approval by the appropriate ethics committees was obtained at all sites and/or countries for the ARISTOTLE and RE-LY trials, including the prespecified substudies. Written informed consent was obtained from all study participants before randomization.

3.3 Outcomes

In both the ARISTOTLE and the RE-LY trial the primary efficacy endpoint was stroke or SEE and the primary safety endpoint was major bleeding[67, 68]. Other secondary efficacy outcomes were all-cause mortality, cardiovascular mortality and myocardial infarction.

Stroke was defined as the sudden onset of a focal neurologic deficit in a location consistent with the territory of a major cerebral artery and categorized as ischemic, hemorrhagic, or unspecified. Systemic embolism was defined as an acute vascular occlusion of an extremity or organ, documented by means of imaging, surgery, or autopsy. In both trials, death was classified as either vascular or non-vascular. Vascular death included cardiac death (e.g. death from heart failure, sudden cardiac death/arrhythmia, cardiac rupture) and other vascular deaths (e.g. all-cause stroke, pulmonary embolus, death from aortic disease and from non-stroke-related haemorrhage). In both trials, major bleeding was defined according to the ISTH criteria, as clinically overt bleeding accompanied by a decrease in the hemoglobin level of at least 2 g per decilitre or transfusion of at least 2 units of blood, or symptomatic bleeding occurring at a critical site, or resulting in death. Life-threatening bleeding was a subcategory of major bleeding that consisted of fatal bleeding, symptomatic intracranial bleeding, bleeding with a decrease in the hemoglobin level of at least 50 g per litre, or bleeding requiring transfusion of at least 4 units of blood or inotropic agents or necessitating surgery. All other bleeding was considered minor. All efficacy and safety endpoints mentioned above were adjudicated using standardised criteria by an independent, blinded, clinical events committee in both trials. The outcome hospitalisation for heart failure was registered in both trials if primary reason for admission, but was not adjudicated.

3.4 Study designs and statistical analyses

3.4.1 Study I

In the ARISTOTLE trial, a prespecified biomarker substudy was planned from as many of the 18,201 patients as possible with blood samples collected at randomization. The study population of paper I comprised of 14 884 patients, and the median length of follow-up was 1.9 years. Overall, the ARISTOTLE biomarker cohort was representative of the full study cohort[122].

This biomarker substudy assessed the associations between baseline levels of ApoA1 and ApoB and CV outcomes during follow up in anticoagulated patients with AF. It aimed to determine the predictive value of these apolipoproteins for the composite ischemic outcome (including ischemic stroke, systemic embolism, myocardial infarction, and cardiovascular death), all-cause mortality, and major bleeding after adjusting for CV risk factors and established biomarkers (NT-proBNP, cTn-hs, cystatin C, IL-6, CRP). Additionally, sensitivity analyses were conducted to assess the association of ApoB with cardiovascular outcomes in patients not on statin therapy.

Demographics and other baseline characteristics were summarized using frequencies for categorical variables and median and 25th and 75th centiles for continuous variables. For tests of differences among groups, the chi-square test was used for categorical variables and the Kruskal–Wallis test was used for continuous variables.

The association between baseline apolipoprotein levels and adverse outcomes was studied using multivariable Cox proportional-hazards models with apolipoprotein as continuous variables. Patients were followed until the respective event occurred or, if the event did not occur, were censored at end of study or at death (for nonfatal outcomes).

The multivariable analyses were adjusted for baseline characteristics and clinical risk factors; age, sex, body mass index, smoking status, systolic blood pressure, AF type, creatinine clearance, diabetes mellitus, heart failure, previous stroke/systemic embolism/transient ischemic attack, hypertension, use of warfarin within 7 days before randomization, randomized treatment (apixaban/warfarin), use of statin medication within 30 days before randomization, treatment at randomization with aspirin, treatment with angiotensin-converting enzyme inhibitors, or angiotensin II receptor blocker (model 1). The second Cox model (model 2) was further adjusted for other prognostic biomarkers, all log-transformed: C-reactive protein, IL-6, high-sensitivity cardiac troponin T, cystatin C, and N-terminal pro-B-type natriuretic peptide.

For the major bleeding outcome, additional adjustments were made for past bleeding and hemoglobin in model 2, and log-transformed growth differentiation factor 15 level in model 3.

All statistical tests were two-tailed and performed at the 0.05 significance level. Interaction between study treatment (apixaban or warfarin) and apolipoprotein level was analyzed using Cox proportional-hazards models including study treatment group, apolipoprotein, and treatment by apolipoprotein interaction as covariates. Given that statin therapy may affect ApoB levels substantially, sensitivity analyses were performed for the association of ApoB to outcomes in patients without any statin therapy at baseline (n=8420). Because all analyses were exploratory, there were no adjustments for multiple comparisons.

3.4.2 Paper II

The study population consisted of participants from the ARISTOTLE and the RE-LY trial:

In ARISTOTLE, the participating countries were categorised into pre-specified regions: Asia/Pacific, Europe, Latin America and North America. The patients included in this study were the first patients included that had all biomarker data was available (n=14 688). The median follow-up was 1.8 years. From RE-LY, the participating countries were similarly divided according to geographic regions: Asia, Europe, Latin America, North America and other. A total of 8402 patients had all biomarker data available from samples collected at randomisation and were included in this study. The median follow-up time was 2.0 years.

This study evaluated the consistency of GDF-15, ABC bleeding and ABC death risk scores across geographic regions. The association of GDF-15 with major bleeding and death and the performance of the ABC-AF-bleeding and ABC-AF-death scores, which incorporate GDF-15, in patients with AF, were assessed.

To assess the consistency over geographic regions regarding the association between time to major bleeding (or time to death) and GDF-15, a Cox-regression model including GDF-15, geographic region and their interaction was fitted. To assess the consistency of the ABC-AF scores, a corresponding model was fitted including the ABC-AF-bleeding (ABC-AF-death) score, here represented by the estimated 1-year risk for major bleeding (death), instead of GDF-15. In all models, GDF-15 and the ABC-AF scores were log-transformed (natural logarithm) and represented as restricted cubic splines with four knots placed at the 5th, 35th, 65th and 95th sample percentiles of the corresponding variable. The interaction term only included the linear part of GDF-15 or the ABC-AF score, respectively. Thus, the test of the hypothesis of no interaction between geographic region and GDF-15 (or the ABC-AF score) is the test for the regression coefficient of region×ln(GDF-15) (or region×the ABC-AF score) being zero in the model.

The associations are illustrated graphically by plotting the estimated relative hazard, with corresponding 95% confidence bands. For the models including GDF-15, an arbitrary reference was set at a GDF-15 value of 1500 ng/L for someone in Europe. For the ABC-AF scores, the reference point was set to an arbitrary value of 2% 1-year risk, also for someone in Europe. The horizontal range of the curves approximately represents the range of GDF-15 (ABC-AF score) after removal of the 10 lowest and 10 highest values.

Harrell's c-index was used to assess the discriminative ability of GDF-15 and the ABC-AF scores, both overall and within regions.

3.4.3 Paper III

This biomarker substudy consisted of patients from the ARISTOTLE and the RE-LY trial. Patients from the ARISTOTLE trial were included in the biomarker *identification* cohort, and patients from RE-LY comprised the *validation* cohort. The identification cohort was derived from the ARISTOTLE trial biomarker cohort where all biomarker data were available (n = 14 757) using a 1:4 case-cohort methodology. The identification cohort consisted of 517 cases with CV death during follow-up and 4057 randomly selected patients for comparison. The median length of follow-up was 1.8 years. The validation cohort was derived from the RE-LY biomarker cohort of patients where all biomarker data were available (n = 5533), also using a 1:4 case-cohort design. The validation cohort consisted of 277 cases with CV death during follow-up and 1042 randomly selected patients. The median length of follow-up was 1.9 years.

This biomarker substudy explored the association between 268 protein biomarkers and CV death in two cohorts of anticoagulated patients with AF. Both conventional immunoassays and the proximity extension assay (PEA) multiplex method were used to screen a large number of biomarkers, and multiple statistical methods were employed to ensure the robustness and reliability of the results.

The pairwise association between PEA biomarkers and established conventional biomarkers was assessed by the Spearman correlation. A Random Survival Forest algorithm was used to evaluate the simultaneous association between variables and CV death[123]. The evaluation included levels of 263 PEA markers, four conventional markers (NT-proBNP, cTnT-hs, GDF-15, and IL-6), renal function, and 13 clinical characteristics. The total of biomarkers analysed with the Random Survival Forest algorithm was therefore 268. The number of trees was 5000, splits were done according to a maximally selected statistic criterion, and the variables were ranked according to their permutation variable importance. Subjects with all PEA markers missing were excluded. An identical approach was used in the RE-LY evaluation, with a total of 184 PEA markers.

A Boruta algorithm was used for feature selection[124]. In short, repeated Random Survival Forests were run in which a permuted copy of each variable was added to the data. The permuted versions of the variables represent variables with the same distribution as the original variable but with no correlation with the outcome. Features were either rejected as not better, and were removed from subsequent forests, or confirmed better than these noise variables. The procedure continued until no more variables were undecided, or the maximum number of runs, set to 100, was reached. The remaining variables were labelled tentative.

Weighted Cox-regression analyses were performed including each of the established standard immunoassays (naturally log-transformed) and the PEA biomarkers, one at a time, assuming a linear association with the log hazard rate. According to the case-cohort design, the patients randomly selected were given a weight inversely proportional to the sampling probability, that is, 1/0.2946, and all cases were given a weight of 1.0. The Cox-regression analyses were performed in two steps, first (Cox model 1) adjusted for baseline, and second (Cox model 2) further adjusting for renal function (cystatin C in ARISTOTLE and CKD-EPI in RE-LY) and established biomarkers (NT-proBNP and cTnT-hs). Results were presented as the relative hazard for an inter-quartile difference of each marker with corresponding 95% confidence intervals and P-values. Thus, the hazard ratio can be interpreted as the relative hazard comparing the two biomarker values defining the inner 50% of the distribution, that is the third vs. the first quartile. On the inflammation panel, 16 of the proteins had more than 80% of the measurements below the limit of detection, and these were not included in the Cox-regression models. Therefore, the total amount of biomarkers included in the Cox analyses was 255. Due to the very large number of biomarkers evaluated, only biomarkers confirmed in the Boruta analysis and with significant association in the adjusted Cox-regression analysis (Cox model 2), in both the identification and validation cohorts, were considered to have confirmed association with the risk of CV death.

3.4.4 Paper IV

The study population was derived from the ARISTOTLE trial where all biomarker data was available (n = 14,757) from which a random sample representative of the trial population was selected (n = 4,205) that was enriched for cases not selected in the random sample (n = 420) (Figure 1). Thus, using this case-cohort design, the present substudy cohort consisted of 596 cases with hospitalizations for HF during follow-up and 4029 controls without hospitalization for HF during follow-up. In accordance with the case-cohort design, patients with events could be included in the random sample of controls. The median follow-up time was 1.9 years. Out of the random sample of 4,205, a total of 1,211 patients had additional information on history of HF and left ventricular systolic function at baseline, measured by echocardiography, contrast- or radionuclide ventriculography, or magnetic resonance imaging. This subset was used for the comparative analysis of biomarker profiles between HFpEF (n = 649) and HFrEF (n = 562), here using a definition of HFpEF; LVEF >40% (or if a numerical LVEF value was not available, a report of normal or only mildly reduced left ventricular systolic dysfunction) and HFrEF; LVEF ≤40% (or if a numerical LVEF value was not available, a report of moderately or severely reduced left ventricular systolic dysfunction) regardless of symptoms of HF.

Using a similar methodology to paper III, this study investigated the pathophysiological connections between AF and HF by exploring the association between baseline concentrations of 268 protein biomarkers levels at study entry and the occurrence of hospitalization for HF (HHF). Additionally, the study aimed to elucidate biomarker signatures differences between HFpEF and HFrEF status at baseline.

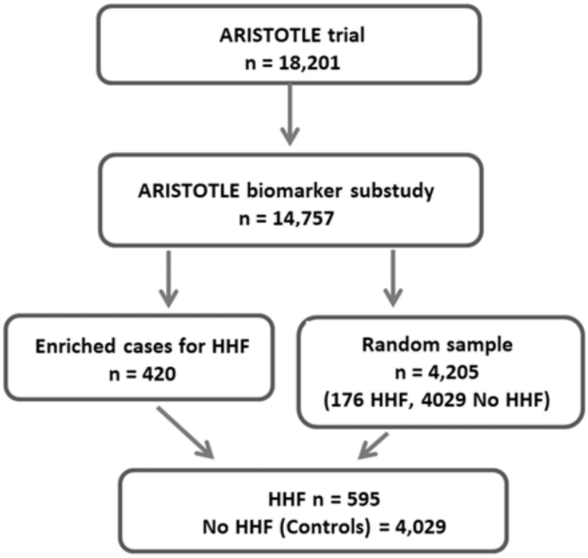

Figure 1. Study population selection for analyses of biomarkers associated with HHF in AF.

Baseline characteristics were summarized, within subgroups, by the sample median and interquartile range for continuous variables and proportions and counts for categorical variables.

The association between clinical variables and biomarkers, and the hazard of hospitalization for heart failure, was simultaneously evaluated by fitting a random survival forest. A permutation variable importance measure was used to rank the variables importance for predicting hospitalization for heart failure. The top-50 variables according to the variable importance are presented graphically.

Further, to evaluate each biomarker in isolation, Cox-regression analyses were done adjusting for clinical variables (age, sex, BMI, smoking, hypertension, diabetes, prior myocardial infarction, prior stroke/TIA, peripheral artery disease, heart failure, and randomized treatment) and renal function (cystatin C) in Cox model 1. Cox model 2 was further adjusted for the cardiac biomarkers NT-proBNP and cTnT-hs. The results are presented as hazard ratios comparing the third and first sample quartile of the respective biomarker to put them on somewhat equal scale. The top-50 biomarkers are presented according to the p-value for the respective biomarker.

To assess the association of the PEA markers and type of HF at baseline, i.e., HFrEF and HFpEF, a Wilcoxon-Mann-Whitney test was performed for each marker. A Bonferroni-Holm procedure was used for multiplicity adjustment of the p-values. For the markers passing the multiplicity adjustment ($p < 0.05$ after adjustment) the distribution was shown for the groups using empirical cumulative distribution function plots.

To evaluate the simultaneous association with HF group, a random forest with HF group as outcome was fitted. The top-50 biomarkers according to the variable importance were presented graphically.

3.5 Laboratory methods

Venous blood samples were obtained at randomization prior to study drug intake from enrolled patients in both trials. Plasma samples were collected in EDTA plasma tubes for NT-proBNP, cTn-hs, GDF-15, CRP, and cystatin C, and citrate tubes for IL-6 and were within 30 minutes centrifuged at 2000 g for 10 minutes at room temperature before frozen immediately -18 to -20 degrees Celsius and were within three months of collection stored in aliquots at -70 degrees Celsius. All plasma samples were sent to and centrally analysed at the Uppsala Clinical Research Center Laboratory, Uppsala, Sweden.

3.5.1 Traditional immunoassay analyses (Paper I-IV)

Plasma concentrations of ApoA1 and ApoB were analyzed using a particle-enhanced immunoturbidimetric assay (Abbott, Abbott Park, IL) with lower limits of detection of 0.16 g/L for ApoA1 and 0.11 g/L for ApoB, respectively, and a total coefficient of variation ≤3%. NT-proBNP and cTn-hs were both analysed using with sandwich immunoassays on the Cobas Analytics e601 Immunoanalyzer (Roche Diagnostics, Mannheim, Germany) according to manufacturer instructions[4, 5]. GDF-15 was analysed with the Elecsys GDF-15 precommercial assay kit P03 (Roche Diagnostics, Mannheim, Germany)[7]. Plasma concentrations of highly sensitive IL-6 were analysed using an ELISA technique, R&D Systems, Minneapolis, USA, and highly sensitive CRP using a particle enhanced immunoturbidimetric assay, Abbott, Abbott Park, Illinois, USA[6]. Cystatin C was analysed with the ARCHITECT system ci8200 (Abbott Laboratories, Abbott Park, IL, USA) using the particle-enhanced turbidimetric immunoassay (PETIA) from Gentian (Moss, Norway)[117].

3.5.2 Proteomics (Paper III-IV)

For the proteomics analyses, the proximity extension assay (PEA) technology, a new proteomics polymerase chain reaction (PCR)-based method, was used. This technique uses multiple unique oligonucleotide-labelled antibody pairs that bind to their respective target protein and can later be quantified by qPCR by the Fluidigm BioMark™ HD real-time PCR platform (Figure 2). In this way, multiple proteins can be analysed simultaneously with exceptionally high specificity, resulting in a time and sample-size effective screening method. Values are expressed as Normalized Protein Expression (NPX) where a high protein value corresponds to a high protein concentration, but not an absolute quantification. The PEA assays have shown high reproducibility and repeatability with low intra-assay, inter-assay, and inter-site variation[125]. Prior validation studies have also showed that biomarkers analysed with the PEA technique have an adequate concordance with conventional immunoassays[126].

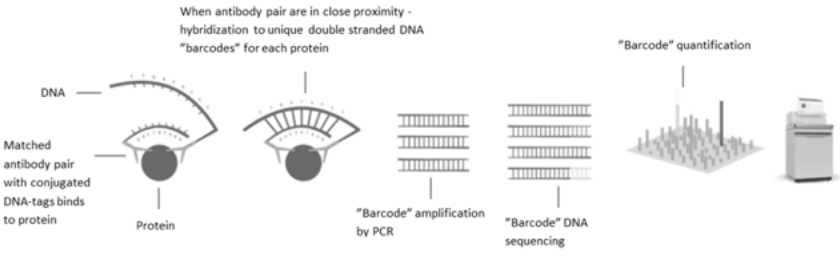

Figure 2. An overview of the Proximity extension assay[127]

In paper III and IV, the Proseek Multiplex PEA panels CVDII, CVDIII, and Inflammation were used (Olink Proteomics, Uppsala, Sweden; www. https://olink.com/products/olink-target-96) and analyzed at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University, Uppsala, Sweden. Within each panel, 92 biomarkers are measured simultaneously resulting in a total measurement of 276 pre-selected proteins associated with CV disease and inflammation on the three PEA panels. However, 10 biomarkers were analysed on more than one panel, resulting in duplicates and reducing the total number of analysed biomarkers. Therefore, 266 unique protein biomarkers were measured in total using PEA methodology.

In paper III, the PEA panels CVDII, CVDIII, and Inflammation were used in the identification cohort. Because of the comparatively low number of biomarkers with strong CV death association using the PEA inflammation chip in the initial analyses from the identification cohort and for the purpose of cost effectiveness, only the CVD II and III panel (and not the inflammation panel) was later used for biomarker analyses in the validation cohort. Thus, 192 biomarkers were measured in the validation group.

In paper IV, the proteomic analyses were performed identically to the identification (ARISTOTLE) cohort in paper III.

4. Results

4.1 Paper I

4.1.1 Baseline characteristics

The median (25th, 75th) ApoA1 concentration was 1.10 g/L (0.94, 1.30). For ApoB, the median (25th, 75th) was 0.70 g/L (0.55, 0.85). The median age was 70 years and ≈64% were male. Multivariable models indicated that low ApoA1 levels were significantly associated with male sex, higher IL-6 levels, and permanent or persistent atrial fibrillation. Conversely, high ApoA1 levels were associated to better renal function, older age, and higher hemoglobin levels. Low ApoB levels were significantly associated with statin therapy, male sex, higher growth differentiation factor 15 levels, and higher IL-6 levels. High ApoB levels were associated with better renal function and higher hemoglobin levels. Patient characteristics at baseline are summarized in Table 4 for ApoA1 and Table 5 for ApoB, whereas baseline characteristics with the strongest association on ApoA1 levels are shown in Table 6 and for ApoB in Table 7.

Table 4. Baseline characteristics of participants in relation to ApoA1 levels

	Apolipoprotein A1 level, g/L				
	≤0.94	>0.94-1.1	>1.1 - 1.3	>1.3	p-value*
n	3823	4521	3728	2812	
Age, Median (Q1,Q3)	70.0 (62.0, 76.0)	69.0 (62.0, 76.0)	70.0 (63.0, 76.0)	71.0 (64.0, 76.0)	<0.0001
Male	2823 (73.8%)	3085 (68.2%)	2329 (62.5%)	1347 (47.9%)	<0.0001
Weight, Kg, Median(Q1,Q3)	83.5 (70.4, 97.5)	84.0 (71.0, 98.0)	82.0 (70.0, 95.0)	78.5 (67.1, 90.0)	<0.0001
Permanent/persistent AF	3387 (88.6%)	3848 (85.2%)	3109 (83.4%)	2287 (81.3%)	<0.0001
Heart failure	1588 (41.5%)	1661 (36.7%)	1257 (33.7%)	833 (29.6%)	<0.0001
Hypertension	3327 (87.0%)	3957 (87.5%)	3277 (87.9%)	2467 (87.7%)	0.6897
Age ≥ 75 yrs	1142 (29.9%)	1329 (29.4%)	1169 (31.4%)	922 (32.8%)	<0.0001
Diabetes mellitus	1098 (28.7%)	1244 (27.5%)	806 (21.6%)	532 (18.9%)	<0.0001
Previous stroke or TIA	728 (19.0%)	860 (19.0%)	691 (18.5%)	514 (18.3%)	0.8122
Myocardial infarction	617 (16.1%)	575 (12.7%)	452 (12.1%)	269 (9.6%)	<0.0001
Previous PCI/CABG	594 (15.5%)	621 (13.7%)	490 (13.1%)	314 (11.2%)	<0.0001
Peripheral arterial disease	200 (5.2%)	227 (5.0%)	182 (4.9%)	115 (4.1%)	0.1714
Age 65-75 yrs	1467 (38.4%)	1767 (39.1%)	1448 (38.8%)	1153 (41.0%)	0.1606
CHA2DS2VASc– score	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)	0.0138
Aspirin	1289 (33.7%)	1441 (31.9%)	1087 (29.2%)	782 (27.8%)	<0.0001
ACEi or ARB	2766 (75.1%)	3224 (74.0%)	2636 (73.9%)	1941 (71.9%)	<0.0001
Beta blocker	2558 (69.4%)	2935 (67.4%)	2384 (66.9%)	1678 (62.2%)	<0.0001
Calcium channel blocker	1085 (29.4%)	1378 (31.6%)	1122 (31.5%)	962 (35.7%)	<0.0001
Digoxin	1374 (37.3%)	1494 (34.3%)	1105 (31.0%)	784 (29.1%)	<0.0001
Statin treatment	1644 (43.0%)	1941 (42.9%)	1633 (43.8%)	1246 (44.3%)	0.6069
Creatinine Clearance (ml/min)	74.8 (57.2, 97.1)	75.3 (57.3, 98.8)	74.4 (57.3, 95.3)	71.1 (54.4, 89.4)	<0.0001
CRP (mg/L)	2.2 (1.0, 5.2)	2.4 (1.1, 5.2)	2.1 (1.0, 4.4)	2.1 (1.0, 4.3)	<0.0001
Cystatin C (mg/L)	0.9 (0.7, 1.1)	1.0 (0.8, 1.2)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)	<0.0001
GDF-15 (ng/L)	1477.0 (996.5, 2266.5)	1414.0 (985.5, 2155.5)	1310.0 (957.0, 1901.0)	1331.5 (978.0, 1875.2)	<0.0001
IL-6 (ng/L)	2.8 (1.8, 5.0)	2.4 (1.6, 4.1)	2.1 (1.4, 3.5)	2.0 (1.3, 3.1)	<0.0001
NT-proBNP (ng/L)	695.0 (361.0, 1258.5)	728.0 (367.0, 1293.0)	716.0 (364.0, 1237.2)	711.5 (356.0, 1204.2)	0.5783
cTnT-hs (ng/L)	11.5 (7.7, 17.9)	11.2 (7.6, 16.8)	10.7 (7.4, 16.1)	10.4 (7.4, 15.4)	<0.0001

ACEi indicates angiotensin-converting enzyme inhibitor; AF, atrial fibrillation; ARB, angiotensin II receptor blocker; CABG, coronary artery bypass graft; CHD, congestive heart disease; CrCL, creatinine clearance; GDF-15, growth differentiation factor 15; IL-6, interleukin 6; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro B-type natriuretic peptide; PCI, percutaneous coronary intervention; Q, quartile; and TIA, transient ischemic attack and cTnT-hs, high-sensitivity cardiac troponin T. *Tests used: Pearson's x¬2 test for the CHA2DS2-VASc score and for statin treatment, all other by the Kruskal-Wallis test.

Table 5. Baseline characteristics of participants in relation to ApoB levels

	Apolipoprotein B level, g/L				
	≤0.55	>0.55-0.7	>0.7 - 0.85	>0.85	p-value*
n	3747	3951	3504	3682	
Age, Median (Q1,Q3)	72.0 (65.0, 78.0)	70.0 (64.0, 76.0)	69.0 (62.0, 75.0)	68.0 (60.0, 74.0)	<0.0001
Male	2511 (67.0%)	2583 (65.4%)	2195 (62.6%)	2294 (62.3%)	<0.0001
Weight, Kg	80.5 (68.0, 94.5)	82.0 (70.0, 95.3)	82.0 (70.0, 95.3)	83.5 (71.6, 97.0)	<0.0001
Permanent/persistent AF	3212 (85.7%)	3408 (86.3%)	2971 (84.8%)	3040 (82.6%)	<0.0001
Heart failure	1281 (34.2%)	1346 (34.1%)	1241 (35.4%)	1471 (40.0%)	<0.0001
Hypertension	3269 (87.2%)	3461 (87.6%)	3033 (86.6%)	3265 (88.7%)	0.0515
Age ≥ 75 yrs	1463 (39.0%)	1290 (32.6%)	977 (27.9%)	833 (22.6%)	<0.0001
Diabetes mellitus	1097 (29.3%)	1027 (26.0%)	823 (23.5%)	733 (19.9%)	<0.0001
Previous stroke or TIA	774 (20.7%)	775 (19.6%)	633 (18.1%)	613 (16.6%)	<0.0001
Myocardial infarction	647 (17.3%)	544 (13.8%)	354 (10.1%)	369 (10.0%)	<0.0001
Previous PCI/CABG	774 (20.7%)	636 (16.1%)	357 (10.2%)	252 (6.8%)	<0.0001
Peripheral arterial disease	211 (5.6%)	220 (5.6%)	140 (4.0%)	152 (4.1%)	0.0003
Age 65-75 yrs	1451 (38.7%)	1576 (39.9%)	1406 (40.1%)	1402 (38.1%)	<0.0001
CHA_2DS_2VASc– score	4.0 (3.0, 5.0)	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)	<0.0001
Aspirin	1657 (33.0%)	906 (30.7%)	1298 (29.9%)	733 (28.6%)	0.0003
ACEi or ARB	3577 (74.3%)	2060 (73.1%)	3087 (73.7%)	1831 (74.2%)	<0.0001
Beta blocker	3105 (64.5%)	1866 (66.2%)	2851 (68.0%)	1721 (69.8%)	<0.0001
Calcium channel blocker	1765 (36.7%)	896 (31.8%)	1242 (29.6%)	641 (26.0%)	<0.0001
Digoxin	1300 (27.0%)	909 (32.2%)	1503 (35.9%)	1043 (42.3%)	<0.0001
Statin treatment	2323 (62.0%)	1965 (49.7%)	1278 (36.5%)	898 (24.4%)	<0.0001
Creatinine clearance (ml/min)	70.0 (53.3, 90.0)	73.2 (56.3, 92.9)	75.4 (58.5, 96.7)	78.5 (60.1, 101.5)	<0.0001
CRP (mg/L)	1.6 (0.8, 3.8)	2.1 (1.0, 4.6)	2.4 (1.2, 5.2)	2.8 (1.4, 5.6)	<0.0001
Cystatin C (mg/L)	0.9 (0.7, 1.1)	1.0 (0.8, 1.2)	1.0 (0.8, 1.2)	1.0 (0.9, 1.2)	<0.0001

GDF-15 (ng/L)	1520.0 (1050.0, 2319.0)	1457.0 (995.0, 2118.0)	1330.0 (966.8, 1965.0)	1256.0 (915.0, 1836.8)	<0.0001
IL-6 (ng/L)	2.5 (1.6, 4.1)	2.3 (1.5, 3.9)	2.3 (1.5, 3.8)	2.3 (1.5, 3.8)	<0.0001
NT-proBNP (ng/L)	740.0 (381.0, 1299.0)	739.0 (382.0, 1290.8)	701.0 (365.5, 1221.0)	672.5 (331.0, 1183.0)	<0.0001
cTnT-hs (ng/L)	11.6 (7.9, 17.7)	11.2 (7.6, 17.0)	10.6 (7.3, 15.9)	10.4 (7.3, 15.7)	<0.0001

ACEi indicates angiotensin-converting enzyme inhibitor; AF, atrial fibrillation; ARB, angiotensin II receptor blocker; CABG, coronary artery bypass graft; CHD, congestive heart disease; CrCL, creatinine clearance; GDF-15, growth differentiation factor 15; IL-6, interleukin 6; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro B-type natriuretic peptide; PCI, percutaneous coronary intervention; Q, quartile; TIA, transient ischemic attack and cTnT-hs, high-sensitivity cardiac troponin T. *Tests used: Pearson's x¬2 test for the CHA2DS2-VASc score and for statin treatment, all other by the Kruskal-Wallis test.

Table 6. Baseline characteristics with the strongest association on ApoA1 level.

Variable	Comment	Ratio of geometric means (95% CI)	P-value
Age	10 year increase	1.039 (1.034, 1.045)	<0.0001
Atrial Fibrillation	Permanent versus persistent	0.962 (0.952, 0.973)	<0.0001
Creatinine clearance	100 % increase	1.172 (1.158, 1.186)	<0.0001
Hemoglobin (g/dl)	Per 1 g/dL increase	1.024 (1.022, 1.027)	<0.0001
IL-6	100 % increase	0.954 (0.950, 0.958)	<0.0001
Sex	Male vs female	0.857 (0.850, 0.865)	<0.0001

The analysis is based on a model including all variables shown in Table 4. IL-6 indicates interleukin 6.

Table 7. Baseline characteristics with the strongest association on ApoB level.

Variable	Comment	Ratio of geometric means (95% CI)	P-value
Creatinine clearance	100 % increase	1.105 (1.088, 1.123)	<0.0001
GDF-15	100 % increase	0.952 (0.945, 0.959)	<0.0001
Hemoglobin (g/dl)	Per 1 g/dL increase	1.051 (1.048, 1.055)	<0.0001
IL-6	100 % increase	0.959 (0.954, 0.964)	<0.0001
Sex	Male vs female	0.906 (0.896, 0.917)	<0.0001
Statin treatment	Yes vs no	0.856 (0.848, 0.865)	<0.0001

The analysis is based on a model including all variables shown in Table 5. GDF-15 indicates growth-differentiation factor-15 and IL-6, interleukin 6.

4.1.2 Baseline biomarker level in relation to outcome

During follow-up there were a total of 397 strokes or systemic embolisms, 149 myocardial infarctions, 543 CV deaths, 1068 all-cause deaths, 883 events of the composite ischemic outcome consisting of ischemic stroke, systemic embolism, myocardial infarction (MI), and CV death and 702 major bleeding events.

4.1.2.1 ApoA1

Cumulative hazard rates for the composite ischemic outcome are shown in Figure 3. Higher baseline levels of ApoA1 were found to be independently associated with a lower risk of composite ischemic outcomes when adjusting for established clinical risk factors (Model 1) but also when further adjusting for other prognostic biomarkers: CRP, IL-6, cTn-hs, cystatin C, and NT-proBNP (Model 2). Specifically, in the fully adjusted analyses, for each interquartile increase in ApoA1 levels, the HR for these outcomes was 0.81 (95% confidence interval [CI], 0.73–0.90; P<0.0001). When examining the risk of stroke or systemic embolism alone, higher ApoA1 levels were also associated with a reduced risk, with an HR of 0.84 (95% CI, 0.72–0.98; P=0.0248). Although there was a lower risk of MI with higher baseline levels of ApoA1, however not statistically significant in any model. Regarding mortality, higher ApoA1 levels were significantly associated with a reduced risk of both all-cause and cardiovascular death. The HR for all-cause mortality was 0.77 (95% CI, 0.70–0.85; P<0.0001), and for cardiovascular mortality, the HR was 0.78 (95% CI, 0.68–0.89; P=0.0002). For major bleeding, there was lower risk of major bleeding with higher ApoA1 levels in Model 1. However after further adjustment for biomarkers (Model 2), ApoA1 did not remain significantly associated with the outcome (Table 8).

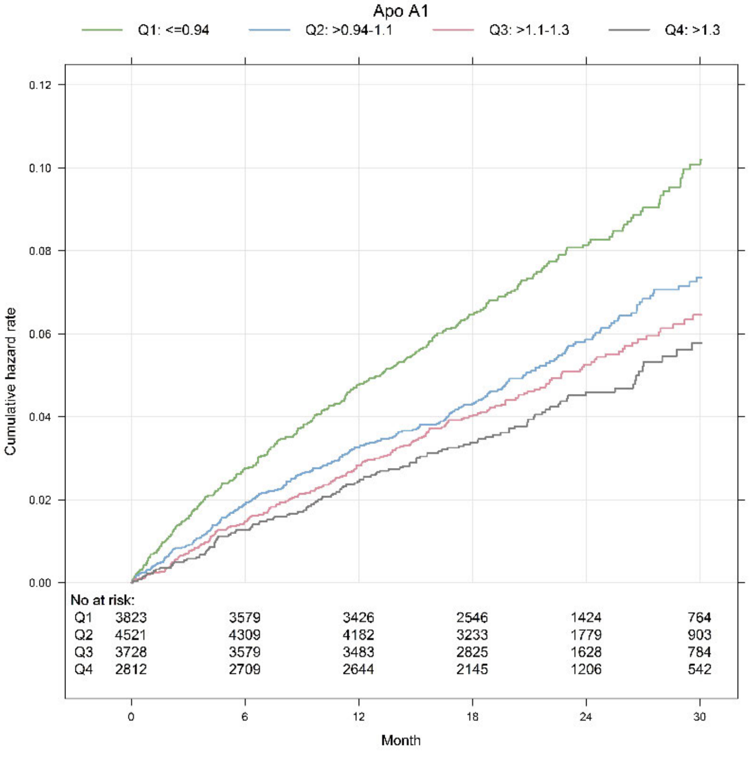

Figure 3. Cumulative hazard rates for the composite ischemic outcome by quartiles of ApoA1.

Table 8. The association of ApoA1 at baseline with outcomes according to continuous levels of ApoA1

			Unadjusted		Adjusted clinical risk factors		Adjusted clinical + biomarkers	
Outcome	n	Events	HR (95 % CI)	p-value	HR (95 % CI)	p-value	HR (95 % CI)	p-value
Ischemic composite outcome	14884	883 (3.13)	0.75 (0.69 - 0.82)	<0.0001	0.80 (0.72 - 0.87)	<0.0001	0.81 (0.73 - 0.90)	<0.0001
Stroke or systemic embolism	14884	397 (1.41)	0.83 (0.73 - 0.95)	0.0080	0.83 (0.73 - 0.96)	0.0094	0.84 (0.72 - 0.98)	0.0248
Myocardial infarction	14884	149 (0.52)	0.85 (0.68 - 1.06)	0.1522	0.89 (0.71 - 1.12)	0.3200	0.86 (0.67 - 1.10)	0.2356
Major bleeding	14853	702 (2.74)	0.87 (0.78 - 0.96)	0.0065	0.91 (0.82-1.01)	0.0768	0.90 (0.80 - 1.01)	0.0724
Cardiovascular death	14884	543 (1.88)	0.69 (0.61 - 0.77)	<0.0001	0.75 (0.66 - 0.84)	<0.0001	0.78 (0.68 - 0.89)	0.0002
Death	14884	1068 (3.69)	0.69 (0.64 - 0.75)	<0.0001	0.73 (0.67-0.80)	<0.0001	0.77 (0.70 - 0.85)	<0.0001

Three different proportional hazards model have been used, one without any adjustment, one adjusted for randomized treatment, demographic and clinical risk factors and one adjusted for randomized treatment, demographic and clinical risk factors plus biomarkers. The demographic and clinical risk factors used were; age, sex, BMI, smoking status, systolic BP, AF type, creatinine clearance, diabetes, heart failure, previous stroke/SEE/TIA, hypertension, randomized treatment, use of warfarin within 7 days of randomization and use of statin medication within 30 days before randomization, treatment at randomization with aspirin, treatment with ACE inhibitors or ARB. The used biomarkers markers were cTnT-hs, NT-proBNP, cystatin C, CRP, and IL-6. For major bleeding, prior bleeding and hemoglobin were added to model 1, and GDF-15 to model 2. Cox models based on continuous biomarker levels showing hazard ratio per interquartile change (e.g. Q3 vs Q1).

4.1.2.2 ApoB

Cumulative hazard rates for the composite ischemic outcome are shown in Figure 4. For ApoB, the study found differing associations with cardiovascular outcomes compared to ApoA1. Higher baseline levels of ApoB were independently associated with an increased risk of myocardial infarction. Specifically, each interquartile increase in ApoB levels corresponded to an HR of 1.33 (95% CI, 1.06–1.68; P=0.0144). In contrast, ApoB levels did not show a significant association with the ischemic composite outcome, stroke or systemic embolic events and cardiovascular death. Lower ApoB levels were paradoxically associated with an increased risk of all-cause mortality, with an HR of 0.84 (95% CI, 0.76–0.92; P=0.0002) (Table 9).

Sensitivity analyses for ApoB were performed to explore whether the association with death was influenced by statin therapy. These revealed that the associations with cardiovascular outcomes remained consistent even in patients not on statin therapy at baseline (Table 10). Additionally, the increased all-cause mortality associated with lower ApoB levels was primarily driven by non-cardiovascular deaths, such as malignancy and infection (Table 11).

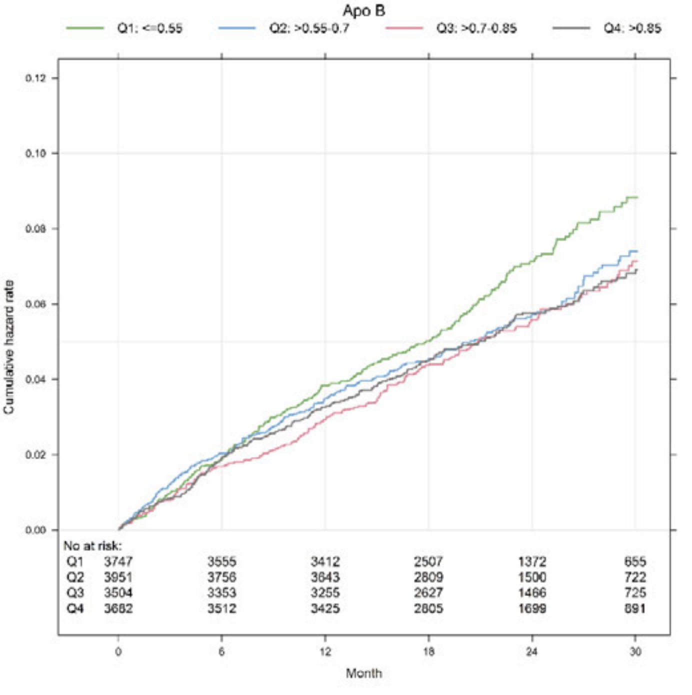

Figure 4. Cumulative hazard rates for the composite ischemic outcome by quartiles of ApoB

Table 9. The association of ApoB at baseline with outcomes according to continuous levels of ApoB

			Unadjusted		Adjusted clinical risk factors		Adjusted clinical + biomarkers	
Outcome	n	Events	HR (95 % CI)	p-value	HR (95 % CI)	p-value	HR (95 % CI)	p-value
Ischemic composite outcome	14884	883 (3.13)	0.94 (0.86 - 1.03)	0.1609	1.00 (0.91 - 1.10)	0.9841	1.01 (0.92 - 1.12)	0.8240
Stroke or systemic embolism	14884	397 (1.41)	0.94 (0.83 - 1.07)	0.3722	1.02 (0.89 - 1.18)	0.7798	1.05 (0.90 - 1.21)	0.5564
Myocardial infarction	14884	149 (0.52)	1.10 (0.89 - 1.35)	0.3685	1.37 (1.10 - 1.71)	0.0055	1.33 (1.06 - 1.68)	0.0144
Major bleeding	14853	702 (2.74)	0.80 (0.72 - 0.88)	<0.0001	0.98 (0.87- 1.10)	0.7562	0.98 (0.86 - 1.11)	0.7100
Cardiovascular death	14884	543 (1.88)	0.87 (0.78 - 0.98)	0.0170	0.88 (0.78 - 0.99)	0.0357	0.89 (0.79 - 1.02)	0.0839
Death	14884	1068 (3.69)	0.81 (0.74 - 0.88)	<0.0001	0.84 (0.77- 0.92)	0.0002	0.84 (0.76 - 0.92)	0.0002

Three different proportional hazards model have been used, one without any adjustment, one adjusted for randomized treatment, demographic and clinical risk factors and one adjusted for randomized treatment, demographic and clinical risk factors plus biomarkers. The demographic and clinical risk factors used were; age, sex, BMI, smoking status, systolic BP, AF type, creatinine clearance, diabetes, heart failure, previous stroke/SEE/TIA, hypertension, randomized treatment, use of warfarin within 7 days of randomization and use of statin medication within 30 days before randomization, treatment at randomization with aspirin, treatment with ACE inhibitors or ARB. The used biomarkers markers were cTnT-hs, NT-proBNP, cystatin C, CRP, and IL-6. For major bleeding, prior bleeding and hemoglobin were added to model 1, and GDF-15 to model 2. Cox models based on continuous biomarker levels showing hazard ratio per interquartile change (e.g. Q3 vs Q1).

Table 10. The association of ApoB at baseline with outcomes according to continuous levels of ApoB showing the hazard ratio per interquartile change in patients without statin treatment

Outcome			Unadjusted		Adjusted clinical risk factors		Adjusted clinical + biomarkers	
	n	Events	HR (95 % CI)	p-value	HR (95 % CI)	p-value	HR (95 % CI)	p-value
Ischemic composite outcome	8420	515 (3.22)	0.88 (0.78 – 0.99)	0.0327	0.95 (0.85 - 1.09)	0.4161	0.96 (0.85 - 1.09)	0.5601
Stroke or systemic embolism	8420	220 (1.37)	0.87 (0.73 - 1.04)	0.1178	0.95 (0.79 - 1.14)	0.5757	0.98 (0.81 - 1.19)	0.8310
Myocardial infarction	8420	70 (0.43)	1.29 (0.96 - 1.73)	0.0908	1.36 (1.01 - 1.84)	0.0447	1.29 (0.94 - 1.78)	0.1161
Major bleeding	8409	365 (2.52)	0.82 (0.71 - 0.94)	0.0051	0.99 (0.86-1.15)	0.9290	0.98 (0.84 - 1.16)	0.8403
Cardiovascular death	8420	343 (2.09)	0.79 (0.68 - 0.91)	0.0015	0.86 (0.74 - 0.99)	0.0372	0.87 (0.74 - 1.02)	0.0783
Death	8420	655 (3.99)	0.74 (0.67 - 0.82)	<0.0001	0.81 (0.73-0.91)	0.0002	0.81 (0.72 - 0.90)	0.0002

Three different proportional hazards model have been used, one without any adjustment, one adjusted for randomized treatment, demographic and clinical risk factors and one adjusted for randomized treatment, demographic and clinical risk factors plus biomarkers. The demographic and clinical risk factors used were; age, sex, BMI, smoking status, systolic BP, AF type, creatinine clearance, diabetes, heart failure, previous stroke/SEE/TIA, hypertension, randomized treatment, use of warfarin within 7 days of randomization and use of statin medication within 30 days before randomization, treatment at randomization with aspirin, treatment with ACE inhibitors or ARB. The used biomarkers markers were cTnT-hs, NT-proBNP, cystatin C, CRP, and IL-6. For major bleeding, prior bleeding and hemoglobin were added to model 1, and GDF-15 to model 2. Cox models based on continuous biomarker levels showing hazard ratio per interquartile change (e.g. Q3 vs Q1).

Table 11. The association of ApoB at baseline with non-CV death according to continuous levels of ApoB showing the hazard ratio per interquartile change in all patients and in patients without statin treatment

			Unadjusted		**Adjusted clinical risk factors**		**Adjusted clinical + biomarkers**	
	n	Events	HR (95 % CI)	p-value	HR (95 % CI)	p-value	HR (95 % CI)	p-value
All	14884	525 (1.82)	0.74 (0.66 – 0.84)	<0.0001	0.81 (0.71 - 0.92)	0.0015	0.79 (0.69 - 0.90)	0.0005
Without statin medication	8420	312 (1.90)	0.69 (0.59 - 0.80)	<0.0001	0.77 (0.66 - 0.91)	0.0015	0.75 (0.63 - 0.88)	0.0006

4.1.2.3 Outcomes according to dyslipidemia and randomized treatment

There was no significant interaction between study treatment (apixaban or warfarin) and apolipoprotein levels for composite ischemic outcomes. For major bleeding, apixaban showed a greater relative risk reduction in those with high ApoB levels (p=0.0234), with a similar result for ApoA1 (p=0.0584) (Figure 5).

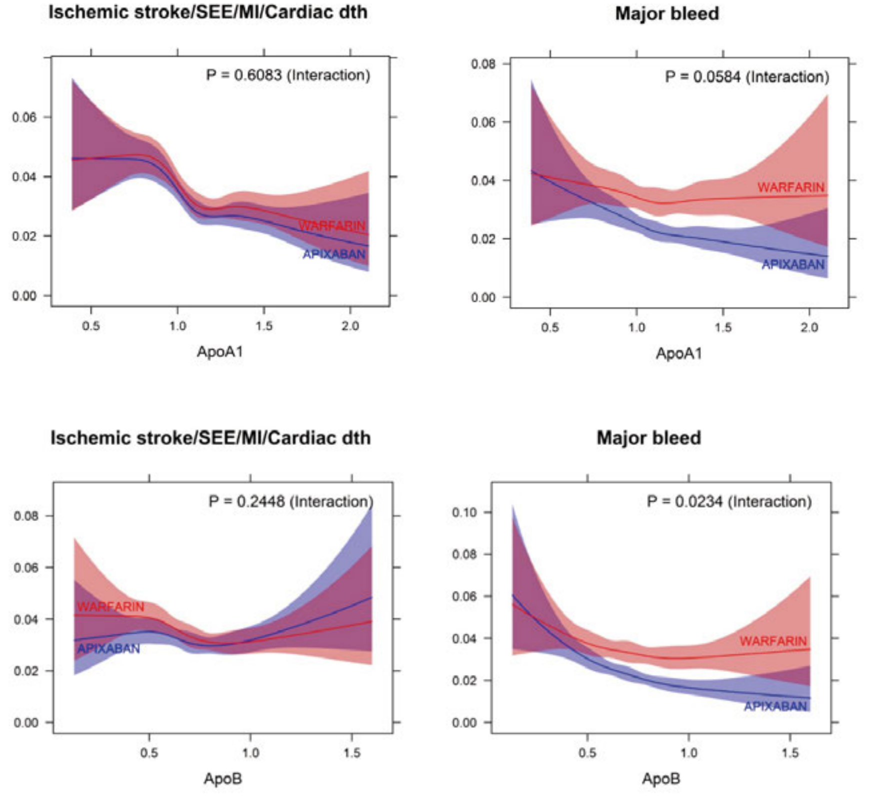

Figure 5. One-year event rates for continuous level of ApoA1 (top) and ApoB (bottom) according to randomized treatment

4.2 Paper II

4.2.1 Baseline characteristics

The median age in the ARISTOTLE trial cohort was 70 (IQR: 63, 76) years and 64% were men, while in the RE-LY cohort, the median age was 72 (IQR: 67, 77) years with the same proportion of men, 64%. The largest proportion of patients was from Europe in both cohorts, 40% in ARISTOTLE and 54% in RE-LY. The number of patients and events in each trial as well as baseline demographics and biomarker levels according to the pre-specified geographic regions of both trials are shown in Table 12 and 13.

Table 12. Baseline characteristics of the 14949 patients in the ARISTOTLE cohort according to study region

	ASIA/PACIFIC *N* = 2389	**EUROPE** *N* = 6027	**LATIN AMERICA** *N* = 2959	**NORTH AMERICA** *N* = 3574
Age, Median (Q1 - Q3)	69.0 (61.0 - 75.0)	69.0 (62.0 - 75.0)	70.0 (64.0 - 77.0)	72.0 (65.0 - 78.0)
Female	35.7% (853)	37.1% (2235)	38.0% (1125)	31.0% (1109)
BMI, Median (Q1 - Q3)	25.1 (22.6 - 28.0) [7]	29.1 (26.1 - 32.8) [21]	28.7 (25.4 - 32.6) [24]	30.2 (26.5 - 35.0) [19]
Systolic blood pressure Median (Q1 - Q3)	130.0 (120.0 - 140.0) [8]	133.0 (123.0 - 143.0) [12]	130.0 (120.0 - 140.0) [2]	128.0 (117.0 - 138.0) [10]
Diabetes	24.7% (589)	22.7% (1370)	20.1% (595)	31.7% (1133)
Hypertension	81.0% (1936)	89.8% (5414)	88.8% (2629)	86.9% (3105)
Smoker	8.4% (200) [1]	9.9% (593) [9]	5.6% (166) [4]	7.2% (258) [0]
Permanent or persistent AF	89.6% (2141) [0]	80.8% (4867) [1]	91.5% (2706) [1]	83.2% (2973) [1]
Stroke/TIA	26.7% (637)	19.0% (1148)	15.3% (454)	15.8% (565)
Prior bleeding	15.8% (377)	12.1% (732)	13.2% (392)	26.1% (932)
Prior anemia	3.8% (90) [0]	4.9% (298) [3]	3.5% (104) [6]	14.4% (514) [2]
Congestive heart failure	24.9% (596)	40.8% (2458)	33.4% (989)	16.8% (601)
Myocardial infarction	8.2% (196) [0]	15.0% (906) [0]	9.5% (280) [1]	15.2% (542) [0]
Peripheral arterial disease	2.5% (59) [0]	5.3% (317) [0]	3.7% (109) [1]	6.8% (244) [0]
CKD-EPI (mL/min/1.73m2), Median (Q1 - Q3)	59.0 (47.2 - 72.8) [1]	56.5 (46.4 - 67.8) [7]	57.0 (45.5 - 70.5) [0]	52.4 (42.1 - 63.2) [0]
GDF-15 (ng/L), Median (Q1 - Q3)	1476.5 (1049.8 - 2167.5) [29]	1263.0 (919.5 - 1865.0) [88]	1362.0 (973.0 - 2012.0) [34]	1559.0 (1074.5 - 2297.5) [31]
Haemoglobin (g/dL), Median (Q1 - Q3)	14.1 (13.0 - 15.3) [13]	14.3 (13.3 - 15.3) [13]	14.4 (13.4 - 15.5) [26]	14.1 (13.0 - 15.0) [18]
NT-proBNP (ng/L), Median (Q1 - Q3)	725.0 (377.8 - 1220.2) [13]	695.0 (339.2 - 1244.0) [57]	751.0 (397.0 - 1325.0) [10]	706.0 (370.0 - 1217.8) [8]
hs-cTnT (ng/L), Median (Q1 - Q3)	9.8 (7.0 - 14.7) [12]	10.8 (7.4 - 16.3) [53]	11.4 (7.9 - 17.6) [9]	11.7 (7.9 - 17.6) [9]
Outcomes				
Death	152 (6%)	330 (5%)	332 (11%)	245 (7%)
Major bleeding	130 (5%)	206 (3%)	138 (5%)	195 (5%)
Missing GDF-15	28 (1%)	88 (1%)	34 (1%)	31 (1%)

AF indicates atrial fibrillation; BMI, body mass index; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration equation; Q, quartile; TIA, transient ischemic attack and number in brackets represents number of missing.

Table 13. Baseline characteristics of the 9369 patients in the RE-LY cohort according to study region

	ASIA *N* = 891	**EUROPE** *N* = 5024	**LATIN AMERICA** *N* = 629	**NORTH AMERICA** *N* = 2082	**OTHER** *N* = 743
Age, Median (Q1 - Q3)	69.0 (63.0 - 75.0)	72.0 (66.0 - 77.0)	73.0 (67.0 - 78.0)	74.0 (68.0 - 79.0)	74.0 (69.0 - 79.0)
Female	32.9% (293)	36.9% (1855)	40.1% (252)	34.1% (711)	40.0% (297)
BMI, Median (Q1 - Q3)	24.3 (22.0 - 27.0) [3]	28.1 (25.4 - 31.4) [1]	27.9 (25.3 - 31.6) [0]	29.0 (25.7 - 33.3) [3]	28.4 (25.3 - 31.6) [0]
Systolic blood pressure, Median (Q1 - Q3)	130.0 (120.0 - 140.0) [2]	132.0 (120.0 - 145.0) [8]	131.0 (120.0 - 144.0) [0]	128.0 (118.0 - 140.0) [2]	130.0 (120.0 - 143.8) [1]
Diabetes	25.3% (225)	20.4% (1024)	16.2% (102)	24.4% (509)	29.5% (219)
Hypertension	71.6% (638)	78.7% (3954)	82.0% (516)	81.4% (1694)	79.3% (589)
Smoker	8.4% (75)	8.6% (432)	4.8% (30)	6.3% (132)	7.0% (52)
Permanent or persistent AF	69.7% (621) [0]	71.3% (3580) [0]	87.3% (549) [0]	55.2% (1147) [4]	58.7% (436) [0]
Stroke/TIA	29.4% (262)	18.8% (944)	14.9% (94)	16.8% (349)	23.4% (174)
Anemia	22.2% (198)	10.2% (513)	7.5% (47)	14.0% (291)	18.0% (134)
Congestive heart failure	34.9% (311) [0]	33.8% (1696) [0]	33.9% (213) [0]	16.0% (334) [1]	21.1% (157) [0]
Myocardial infarction	10.2% (91)	17.2% (864)	7.5% (47)	19.6% (408)	24.1% (179)
Peripheral arterial disease	0.4% (4) [0]	3.8% (193) [0]	2.2% (14) [0]	5.0% (105) [0]	3.9% (29) [1]
CKD-EPI (mL/min/1.73m2), Median (Q1 - Q3)	70.7 (58.5 - 85.4) [0]	65.5 (54.5 - 76.9) [75]	57.9 (47.5 - 68.3) [0]	64.2 (53.3 - 76.4) [8]	63.1 (52.2 - 75.2) [8]
GDF-15 (ng/L), Median (Q1 - Q3)	1606.0 (1181.5 - 2407.5) [76]	1436.0 (1060.0 - 2067.0) [365]	1545.0 (1142.2 - 2081.2) [19]	1608.0 (1182.0 - 2332.0) [171]	1744.5 (1231.5 - 2605.8) [87]
Hemoglobin (g/dL), Median (Q1 - Q3)	13.9 (12.7 - 15.1) [3]	14.4 (13.4 - 15.4) [103]	14.4 (13.4 - 15.5) [4]	14.1 (13.1 - 15.1) [29]	14.0 (12.8 - 15.0) [20]
NT-proBNP (ng/L), Median (Q1 - Q3)	807.0 (400.5 - 1576.5) [3]	837.0 (396.0 - 1477.0) [35]	819.0 (453.0 - 1410.0) [8]	748.0 (368.5 - 1354.0) [19]	854.0 (372.5 - 1406.5) [8]
hs-cTnT (ng/L), Median (Q1 - Q3)	10.8 (7.1 - 18.0) [39]	12.2 (7.7 - 19.5) [385]	12.9 (8.3 - 20.0) [21]	12.0 (7.6 - 19.0) [121]	13.8 (8.5 - 22.8) [84]
Outcomes					
Death	66 (7%)	341 (7%)	54 (9%)	104 (5%)	42 (6%)
Major bleeding	38 (4%)	210 (4%)	29 (5%)	151 (7%)	46 (6%)
Missing GDF-15	76 (9%)	365 (7%)	19 (3%)	171 (8%)	87 (12%)

AF indicates atrial fibrillation; BMI, body mass index; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration equation; Q, quartile; TIA, transient ischemic attack and number in brackets represents number of missing.

4.2.2 Geographic evaluation of GDF-15 and ABC scores

4.2.2.1 GDF-15

In both trial cohorts, GDF-15 showed a robust association with major bleeding and death across all geographic regions (p<0.0001 for both outcomes and cohorts). Although the overall risk of major bleeding and death differed among regions in both cohorts, the interaction between GDF-15 levels and specific regions was not statistically significant (Figure 6).

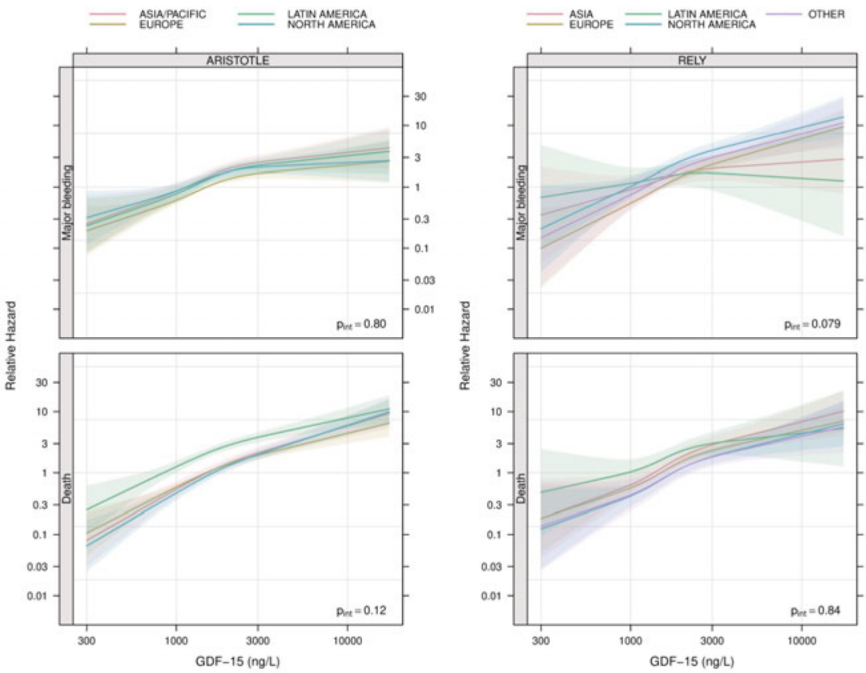

Figure 6. Relative hazard of major bleeding (top panels) and death (bottom panels) in relation to levels of GDF-15 among patients from different regions in the ARISTOTLE (left panels) and RE-LY (right panels) trials. An arbitrary reference point is set at a GDF-15 value of 1500 ng/L in Europe. The p-value in each panel is for a test of no interaction between region and GDF-15. ARISTOTLE, Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation; GDF-15, growth differentiation factor 15; RE-LY, Randomized Evaluation of Long-Term Anticoagulation Therapy.

4.2.2.2 ABC bleeding risk score

The ABC-bleeding score was associated with major bleeding across all regions in both trial cohorts (p=0.0001 for both). There was no significant interaction between the ABC-AF-bleeding score and specific regions in the ARISTOTLE cohort and this finding was similar in the RE-LY trial cohort (Figure 7). Comparison of the discrimination of the ABC bleeding score showed consistency across all studied geographic regions in both trial cohorts (Table 14).

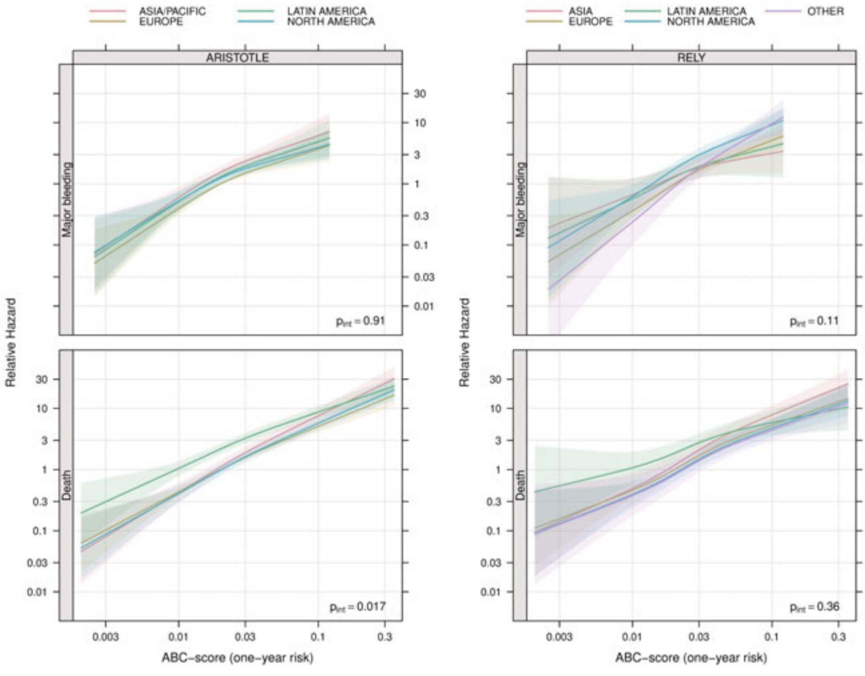

Figure 7. Relative hazard of major bleeding (top panels) and death (bottom panels) in relation to predicted ABC-AF-bleeding (top) and ABC-AF-death (bottom) one-year risks among patients from different regions in the ARISTOTLE (left panels) and RE-LY (right panels) trials. An arbitrary reference point is set at a predicted ABC-AF-risk value of 0.02 in Europe. The p-value in each panel is for a test of no interaction between region and predicted ABC-AF-risk.

Table 14. C-indices with 95% confidence interval for the ABC-AF-bleeding risk score in ARISTOTLE and RELY comparing geographic regions

	ARISTOTLE		RE-LY	
	C-index	95% CI	C-index	95% CI
Major bleeding				
All	0.677	0.657 - 0.698	0.708	0.684 - 0.731
Asia/Pacific	0.684	0.639 - 0.728	0.649	0.558 - 0.739
Europe	0.680	0.641 - 0.719	0.701	0.664 - 0.739
Latin America	0.674	0.629 - 0.719	0.657	0.561 - 0.754
North America	0.659	0.620 - 0.699	0.710	0.672 - 0.749
Other			0.760	0.698 - 0.822

4.2.2.3 ABC death risk score

The ABC-AF-death score was associated with all-cause mortality across all studied regions in both the ARISTOTLE and the RE-LY trial cohorts ($p<0.0001$ for both). In both cohorts, we observed a higher mortality at lower ABC-AF-death scores in Latin America than in the other regions leading to a significant quantitative interaction with region in the ARISTOTLE cohort (interaction $p=0.018$) although not in the RE-LY cohort ($p=0.365$) as shown in Figure 7. The discrimination of the ABC-AF-death risk score was similar among geographic regions in both trial cohorts (Table 15).

Table 15. C-indices with 95% confidence interval for the ABC-AF-death risk score in ARISTOTLE and RELY comparing geographic regions

	ARISTOTLE		RE-LY	
	C-index	95% CI	C-index	95% CI
Death				
All	0.743	0.728 - 0.759	0.741	0.721 - 0.761
Asia/Pacific	0.765	0.725 - 0.805	0.806	0.760 - 0.852
Europe	0.740	0.712 - 0.767	0.735	0.707 - 0.762
Latin America	0.715	0.686 - 0.743	0.677	0.606 - 0.749
North America	0.763	0.733 - 0.793	0.743	0.693 - 0.792
Other			0.734	0.662 - 0.806

4.3 Paper III

4.3.1 Baseline characteristics

Patients that died from a CV cause during follow-up were older, were more likely to have had a previous CV event including heart failure, and had higher levels of established CV biomarkers. Baseline characteristics of the identification and validation cohorts are shown in Table 16.

Table 16. Baseline characteristics and concentration of established biomarkers in the identification cohort

	Identification cohort		Validation cohort	
Baseline characteristics	Random sample N=4057	CV-Death N=517	Random sample N=1042	CV-Death N=277
Age (years)	70 (72,76)	73 (65,69)	72 (67,77)	75 (67, 80)
Females	1491 (36.8%)	148 (28.6%)	392 (37.7%)	99 (34.5%)
Body mass index	28.6 (25.4, 32.7)	27.2 (24.1, 31.4)	27.9 (25.0, 31.2)	27.4 (23.8, 31.2)
Current smoker	355 (8.8%)	60 (11.6%)	76 (7.3%)	23 (8.0%)
Hypertension	3550 (87.5%)	441 (85.3%)	826 (79.5%)	220 (76.7%)
Diabetes	1022 (25.2%)	130 (25.1%)	218 (21.0%)	82 (28.6%)
Prior myocardial infarction	511 (12.6%)	122 (23.6%)	169 (16.3%)	85 (29.6%)
Prior stroke/TIA	733 (18.1%)	123 (23.8%)	208 (20.0%)	54 (18.8%)
Peripheral arterial disease	190 (4.7%)	47 (9.1%)	39 (3.8%)	15 (5.2%)
Heart failure	1232 (30.4%)	258 (49.9%)	293 (28.2%)	152 (53.0%)
CRP	2.2 (1.0, 4.6)	2.8 (1.2, 6.3)	2.5 (1.2, 5.5)	3.7 (1.6, 8.1)
Cystatin C (mg/L)	1.0 (0.8, 1.2)	1.1 (0.9, 1.4)	1.0 (0.8, 1.2)	1.2 (1.0, 1.5)
GDF-15 (ng/L)	1356.0 (961.0, 2038.0)	1780.0 (1292.0, 2868.0)	1460.0 (1086.0, 2127.0)	1997.0 (1438.5, 3191.5)
eGFR, CKD-EPI (ml/min)	74.7 (57.6, 96.0)	62.7 (45.2, 84.7)	65.1 (54.3, 74.2)	58.8 (48.4, 69.7)
Haemoglobin (g/dL)	14.2 (13.1, 15.2)	14.0 (12.9, 15.2)	14.2 (13.1, 15.3)	13.7 (12.7, 15.0)
IL-6 (ng/L)	2.3 (1.5, 3.9)	3.2 (2.1, 6.0)	2.3 (1.5, 3.8)	3.6 (2.1, 5.8)
NT-proBNP (ng/L)	683.0 (362.0, 1219.8)	1289.0 (709.0, 2435.0)	800.0 (375.5, 1436.0)	1568.0 (925.0, 2805.0)
cTnT-hs (ng/L)	10.7 (7.4, 16.2)	17.6 (12.0, 27.0)	11.8 (7.6, 18.9)	21.1 (14.2, 33.6)

Continuous variables presented as median (Q1, Q3). Categorical variables presented as numbers (percentage). CABG, coronary artery bypass graft; CRP, C-reactive protein; GDF-15, growth differentiation factor 15; eGFR, estimated glomerular filtration rate; IL-6, interleukin 6; NT-proBNP, N-terminal pro-B-type natriuretic peptide; cTnT-hs, cardiac troponin T measured with high-sensitivity assay; PCI, percutaneous coronary intervention; Q, quartile; and TIA, transient ischemic attack

4.3.2 Random Survival Forest analyses

In both cohorts, cTnT-hs was identified as having the strongest association with CV death according to the Random Survival Forest analysis and was followed by NT-proBNP (Figure 8). Due to the vast numbers of biomarkers consistently associated with the outcome, additional Boruta analyses were performed. According to the Boruta analysis, 32 biomarkers in the identification cohort (Table 17) and 29 biomarkers in the validation cohort (Table 18) had confirmed importance for CV death. In total, 15 biomarkers were consistently confirmed in both the identification and validation cohorts according to the Boruta analysis: cTnT-hs, NT-proBNP, BNP, fibroblast growth factor 23 (FGF-23), insulin-like growth factor-binding protein 2 (IGFBP2), fibrinolysis (suPAR), trefoil factor 3 (TFF3), renin (REN), tumour necrosis factor receptor 1 (TNFR1), IL-6, TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2), insulin-like growth factor-binding protein 7 (IGFBP7), GDF-15, junctional adhesion molecule A (JAM-A), and cathepsin L1 (CTSL1).

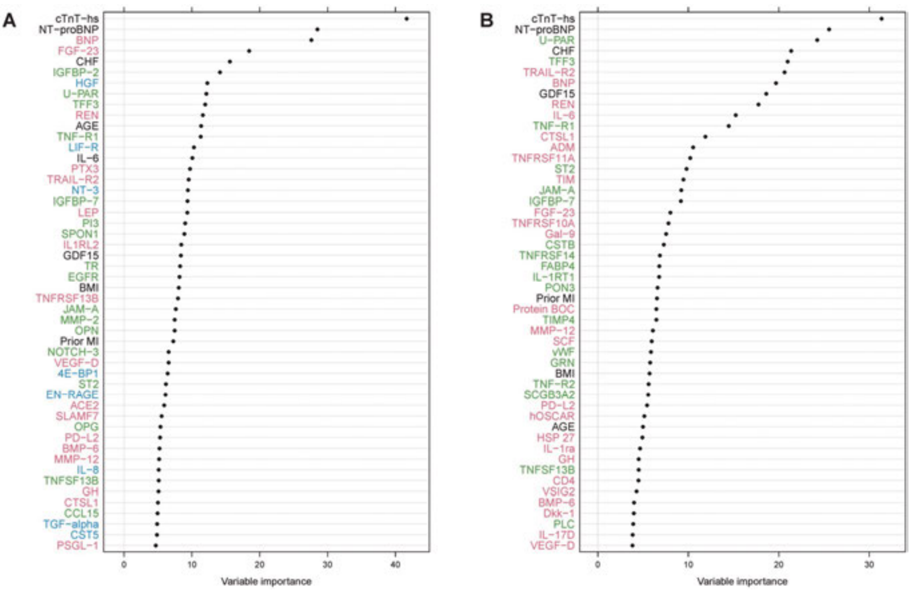

Figure 8. Variable importance for CV-death according to the Random Survival Forest analyses in the identification cohort (left-A) and validation cohort (right-B).

Variable importance for CV death according to the Random Survival Forest analyses in (A) the identification cohort and (B) the validation cohort. Red colour indicates biomarkers analysed on CVD II panel, green colour CVD III, and blue colour inflammation panel. Biomarkers listed in black were analysed with conventional immunoassays. Only the top 50 variables are shown. The evaluation included 263 PEA markers, five conventional markers [N-terminal pro-B-type natriuretic peptide (NT-proBNP), troponin T-hs, growth differentiation factor 15 (GDF-15), cystatin C, and interleukin-6 (IL-6)] and 13 clinical characteristics.

Table 17. Summary of biomarkers with the highest association with CV-death from the identification cohort

Biomarker	RF Ranking	Model 1		Model 2		Boruta
		Hazard ratio (95% CI)	**p-value**	**Hazard ratio (95% CI)**	**p-value**	
cTnT-hs	**1**	**1.890 [1.630, 2.193]**	**<10e-16e**	**1.596 [1.355, 1.880]**	**2.174e−08**	**Confirmed**
NT-proBNP	**2**	**2.081 [1.772, 2.444]**	**<10e-16e**	**1.628 [1.373, 1.931]**	**2.179e−08**	**Confirmed**
BNP	3	2.016 [1.718, 2.365]	<10e-16e	1.361 [1.047, 1.769]	2.148e−02	Confirmed
FGF.23	**4**	**1.451 [1.341, 1.571]**	**<10e-16e**	**1.209 [1.096, 1.332]**	**1.408e−04**	**Confirmed**
IGFBP.2	5	1.788 [1.499, 2.133]	1.099e−10	1.288 [1.077, 1.540]	5.509e−03	Confirmed
HGF	6	1.268 [1.183, 1.358]	1.835e−11	1.085 [0.986, 1.194]	9.345e−02	Confirmed
U.PAR	**7**	**1.783 [1.550, 2.050]**	**5.551e−16**	**1.378 [1.158, 1.641]**	**3.105e−04**	**Confirmed**
TFF3	**8**	**1.528 [1.376, 1.697]**	**2.109e−15**	**1.269 [1.101, 1.462]**	**9.769e−04**	**Confirmed**
REN	9	1.168 [1.000, 1.366]	5.061e−02	1.152 [0.976, 1.360]	9.456e−02	Confirmed
TNF.R1	**10**	**1.598 [1.395, 1.831]**	**1.336e−11**	**1.207 [1.008, 1.447]**	**4.087e−02**	**Confirmed**
LIF.R	11	1.616 [1.410, 1.852]	5.666e−12	1.229 [1.055, 1.433]	8.316e−03	Confirmed
IL−6	**12**	**1.575 [1.410, 1.760]**	**9.992e−16**	**1.288 [1.126, 1.474]**	**2.338e−04**	**Confirmed**
PTX3	13	1.555 [1.357, 1.781]	2.031e−10	1.261 [1.091, 1.459]	1.733e−03	Confirmed
TRAIL.R2	**14**	**1.357 [1.249, 1.474]**	**4.613e−13**	**1.183 [1.043, 1.343]**	**9.037e−03**	**Confirmed**
NT.3	15	1.228 [1.123, 1.343]	6.769e−06	1.103 [0.993, 1.225]	6.652e−02	Confirmed

IGFBP.7	16	1.430 [1.284, 1.593]	8.415e−11	1.204 [1.068, 1.358]	2.487e−03	Confirmed
LEP	17	0.783 [0.652, 0.939]	8.545e−03	0.833 [0.686, 1.010]	6.275e−02	Confirmed
PI3	18	1.238 [1.110, 1.380]	1.219e−04	1.153 [1.024, 1.298]	1.904e−02	Confirmed
IL1RL2	20	0.764 [0.667, 0.876]	1.170e−04	0.794 [0.689, 0.914]	1.305e−03	Confirmed
GDF−15	**21**	**1.749 [1.532, 1.996]**	**1.110e−16**	**1.243 [1.050, 1.471]**	**1.142e−02**	**Confirmed**
TR	22	1.476 [1.308, 1.665]	2.637e−10	1.299 [1.141, 1.479]	8.092e−05	Confirmed
EGFR	23	0.831 [0.757, 0.912]	9.443e−05	0.861 [0.774, 0.959]	6.415e−03	Confirmed
TNFRSF13B	24	1.289 [1.176, 1.414]	6.732e−08	1.185 [1.064, 1.320]	1.949e−03	Confirmed
JAM.A	25	1.333 [1.210, 1.469]	5.954e−09	1.167 [1.031, 1.320]	1.423e−02	Confirmed
MMP.2	26	1.304 [1.133, 1.501]	2.211e−04	1.033 [0.904, 1.181]	6.340e−01	Confirmed
4E.BP1	30	1.331 [1.181, 1.499]	2.555e−06	1.202 [1.056, 1.369]	5.497e−03	Confirmed
EN.RAGE	32	1.267 [1.111, 1.444]	3.973e−04	1.147 [0.991, 1.327]	6.511e−02	Confirmed
ACE2	33	1.396 [1.234, 1.580]	1.211e−07	1.148 [1.000, 1.318]	5.018e−02	Confirmed
SLAMF1	34	1.237 [1.116, 1.372]	5.327e−05	1.112 [0.987, 1.251]	8.000e−02	Confirmed
TNFSF13B	40	1.257 [1.118, 1.412]	1.262e−04	1.097 [0.975, 1.235]	1.242e−01	Confirmed
CTSL1	**42**	**1.365 [1.207, 1.544]**	**7.328e−07**	**1.220 [1.069, 1.392]**	**3.173e−03**	**Confirmed**
TGFA	44	1.384 [1.214, 1.579]	1.210e−06	1.115 [0.983, 1.266]	9.156e−02	Confirmed

All biomarkers confirmed by Boruta analysis included, ranked according to Random Survival Forest variable importance. Biomarkers in bold indicate biomarkers p≤0.05 in adjusted Cox regression model 2 and Confirmed in Boruta analyses in both cohorts. Model 1: Cox-regression analysis model adjusted for clinical characteristics - Age, gender, BMI, smoking, hypertension, diabetes, prior MI, prior stroke/TIA, prior PAD, prior CHF, randomized treatment. Model 2: same as Model 1 and also adjusted for marker for renal function, Cystatin C and cardiac markers NT-proBNP + hs-cTnT.

Table 18. Summary of biomarkers with the highest association with CV-death from the validation cohort

Biomarker	RF Ran-king	Model 1		Model 2		Boruta
		Hazard ratio (95% CI)	**p-value**	**Hazard ratio (95% CI)**	**p-value**	
cTnT−hs	**1**	**1.753 [1.500, 2.047]**	**1.464e−12**	**1.528 [1.305, 1.788]**	**1.307e−07**	**Confir-med**
NT−proBNP	**2**	**2.959 [2.297, 3.813]**	**<10e-16e**	**2.256 [1.758, 2.894]**	**1.555e−10**	**Confir-med**
U.PAR	**3**	**2.193 [1.824, 2.636]**	**1.110e−16**	**1.624 [1.300, 2.028]**	**1.931e−05**	**Confir-med**
TFF3	**4**	**1.793 [1.492, 2.154]**	**4.581e−10**	**1.518 [1.236, 1.865]**	**7.028e−05**	**Confir-med**
TRAIL.R2	**5**	**1.343 [1.196, 1.508]**	**6.012e−07**	**1.256 [1.121, 1.407]**	**8.435e−05**	**Confir-med**
BNP	6	2.191 [1.673, 2.870]	1.201e−08	1.073 [0.741, 1.555]	0.70972	Confirmed
GDF−15	**7**	**1.980 [1.613, 2.431]**	**6.749e−11**	**1.383 [1.100, 1.739]**	**5.506e−03**	**Confir-med**
REN	8	1.825 [1.421, 2.343]	2.385e−06	1.772 [1.380, 2.274]	7.224e−06	Confirmed
IL6	**9**	**1.556 [1.379, 1.757]**	**8.954e−13**	**1.310 [1.152, 1.490]**	**3.729e−05**	**Confir-med**
TNF.R1	**10**	**2.153 [1.771, 2.617]**	**1.377e−14**	**1.544 [1.207, 1.974]**	**5.339e−04**	**Confir-med**
CTSL1	**11**	**1.684 [1.415, 2.004]**	**4.241e−09**	**1.330 [1.107, 1.599]**	**2.364e−03**	**Confir-med**
ADM	12	1.681 [1.133, 2.494]	9.867e−03	1.092 [0.889, 1.343]	0.40156	Confirmed
TNFRSF11A	13	2.062 [1.691, 2.513]	7.775e−13	1.529 [1.210, 1.931]	3.759e-04	Confirmed
ST2	14	1.622 [1.357, 1.940]	1.127e−07	1.266 [1.056, 1.517]	1.065e-02	Confirmed

TIM1	15	1.584 [1.317, 1.904]	1.023e−06	1.317 [1.096, 1.582]	3.317e-03	Confirmed
JAM.A	16	1.425 [1.245, 1.632]	2.819e−07	1.168 [0.981, 1.391]	8.162e-02	Confirmed
IGFBP.7	17	1.560 [1.361, 1.788]	1.606e−10	1.178 [0.996, 1.394]	5.635e-02	Confirmed
FGF.23	**18**	**1.476 [1.288, 1.690]**	**1.907e−08**	**1.183 [1.023, 1.368]**	**2.335e-02**	**Confirmed**
TNFRSF10A	19	1.844 [1.519, 2.238]	6.347e−10	1.344 [1.078, 1.677]	8.663e−03	Confirmed
GAL.9	20	1.966 [1.610, 2.399]	3.012e−11	1.529 [1.212, 1.930]	3.419e−04	Confirmed
CSTB	21	1.679 [1.432, 1.968]	1.574e−10	1.357 [1.113, 1.653]	2.474e−03	Confirmed
FABP4	23	2.159 [1.697, 2.747]	3.749e−10	1.743 [1.347, 2.254]	2.343e−05	Confirmed
IL.1RT1	24	1.521 [1.260, 1.836]	1.286e−05	1.200 [0.999, 1.441]	5.066e−02	Confirmed
PON3	25	0.684 [0.572, 0.818]	3.159e−05	0.794 [0.651, 0.969]	0.02328	Confirmed
BOC	26	1.470 [1.197, 1.806]	2.372e−04	1.353 [1.123, 1.631]	1.491e-03	Confirmed
MMP12	28	1.550 [1.274, 1.886]	1.212e−05	1.322 [1.080, 1.619]	6.845e-03	Confirmed
TNF.R2	32	1.415 [1.188, 1.685]	9.948e−05	1.198 [1.009, 1.422]	3.896e-02	Confirmed
CD4	40	1.799 [1.513, 2.139]	2.923e−11	1.385 [1.125, 1.704]	2.139e-03	Confirmed
IGFBP2	48	2.117 [1.621, 2.764]	3.652e−08	1.290 [0.981, 1.695]	6.796e−02	Confirmed

All biomarkers confirmed by Boruta analysis included, ranked according to Random Survival Forest variable importance. Biomarkers in bold indicate biomarkers p≤0.05 in adjusted Cox regression model 2 and Confirmed in Boruta analyses in both cohorts. Model 1: Cox-regression analysis model adjusted for clinical characteristics - Age, gender, BMI, smoking, hypertension, diabetes, prior MI, prior stroke/TIA, prior PAD, prior CHF, randomized treatment. Model 2: same as Model 1 and also adjusted for marker for renal function, Cystatin C and cardiac markers NT-proBNP + hs-cTnT.

4.3.3 Cox-regression analyses

Out of the 255 biomarkers evaluated by Cox analyses, adjusted solely for clinical characteristics (Cox model 1), 64% (n = 163) of the biomarkers were statistically associated with CV death in the identification cohort. In the validation cohort, the proportion was identical, and 64% (n = 121) out of 188 biomarkers were analysed. When further adjusted for renal function (Cystatin C) and for the two established biomarkers for CV death (NT-proBNP and cTnT-hs) (Cox model 2), 24% (n = 62) of the biomarkers in the identification cohort and 26% (n = 49) in the validation remained significantly associated with CV death cohort (top 50 shown in Figure 9).

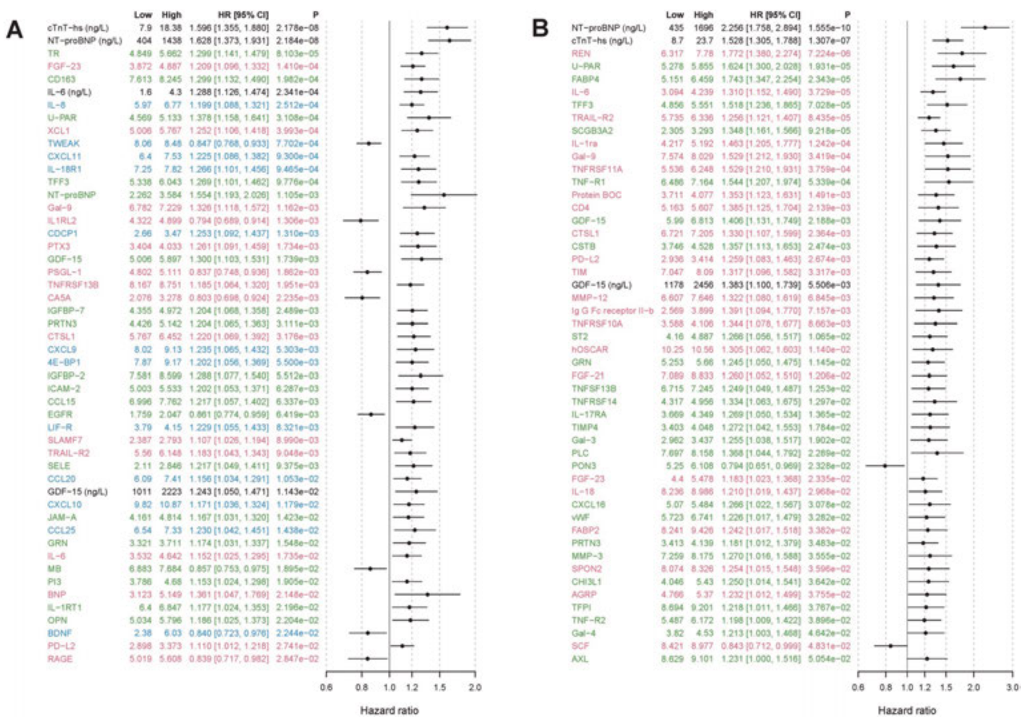

Figure 9. Forest plot of the top 50 biomarkers associated with CV-death according to adjusted Cox-regression analysis in the identification cohort (left-A) ant the validation cohort (right-B) by p-value.

All biomarkers confirmed by Boruta analysis included, ranked according to Random Survival Forest variable importance. Biomarkers in bold indicate biomarkers p≤0.05 in adjusted Cox regression model 2 and Confirmed in Boruta analyses in both cohorts. Model 1: Cox-regression analysis model adjusted for clinical characteristics - Age, gender, BMI, smoking, hypertension, diabetes, prior MI, prior stroke/TIA, prior PAD, prior CHF, randomized treatment. Model 2: same as Model 1 and also adjusted for marker for renal function, Cystatin C and cardiac markers NT-proBNP + hs-cTnT.

4.3.4 Biomarker selection

Out of the 15 biomarkers that were confirmed in the Boruta analysis in both the identification and validation cohorts, 10 were also determined statistically significant in the fully adjusted Cox analyses (model 2) in both cohorts: cTnT-hs, NT-proBNP, FGF-23, suPAR, TFF3, TNFR1, IL-6, TRAILR2, GDF-15, and CTSL1. A summary of the top candidate biomarkers according to the performed statistical analyses in both cohorts are presented in Tables 19 and 20. The correlation between these top biomarkers and traditional cardiovascular markers (NT-proBNP and cTnT-hs), marker for renal function (cystatin C) and for inflammation (CRP and IL-6), is shown in Table 21. suPAR, TFF3, TNFR1, IL-6, GDF-15, and TRAILR2 did all moderately correlate with renal function (rho >0.5). Beyond that, no strong patterns of correlation were seen (rho <0.5).

Table 19. Summary of biomarkers with the highest association with CV-death from the identification cohort

Biomarker		RF Ranking	Model 1		Model 2		Boruta
			Hazard ratio (95% CI)	**p-value**	**Hazard ratio (95% CI)**	**p-value**	
cTnT-hs		**1**	**1.890 [1.630, 2.193]**	**<10e-16e**	**1.596 [1.355, 1.880]**	**2.174e−08**	**Confirmed**
NT-proBNP		**2**	**2.081 [1.772, 2.444]**	**<10e-16e**	**1.628 [1.373, 1.931]**	**2.179e−08**	**Confirmed**
BNP		3	2.016 [1.718, 2.365]	<10e-16e	1.361 [1.047, 1.769]	2.148e−02	Confirmed
FGF.23		**4**	**1.451 [1.341, 1.571]**	**<10e-16e**	**1.209 [1.096, 1.332]**	**1.408e−04**	**Confirmed**
IGFBP.2		5	1.788 [1.499, 2.133]	1.099e−10	1.288 [1.077, 1.540]	5.509e−03	Confirmed
HGF		6	1.268 [1.183, 1.358]	1.835e−11	1.085 [0.986, 1.194]	9.345e−02	Confirmed
U.PAR		**7**	**1.783 [1.550, 2.050]**	**5.551e−16**	**1.378 [1.158, 1.641]**	**3.105e−04**	**Confirmed**
TFF3		**8**	**1.528 [1.376, 1.697]**	**2.109e−15**	**1.269 [1.101, 1.462]**	**9.769e−04**	**Confirmed**

REN		9	1.168 [1.000, 1.366]	5.061e−02	1.152 [0.976, 1.360]	9.456e−02	Confirmed
TNF.R1		**10**	**1.598 [1.395, 1.831]**	**1.336e−11**	**1.207 [1.008, 1.447]**	**4.087e−02**	**Confirmed**
LIF.R		11	1.616 [1.410, 1.852]	5.666e−12	1.229 [1.055, 1.433]	8.316e−03	Confirmed
IL−6		**12**	**1.575 [1.410, 1.760]**	**9.992e−16**	**1.288 [1.126, 1.474]**	**2.338e−04**	**Confirmed**
PTX3		13	1.555 [1.357, 1.781]	2.031e−10	1.261 [1.091, 1.459]	1.733e−03	Confirmed
TRAIL.R2		**14**	**1.357 [1.249, 1.474]**	**4.613e−13**	**1.183 [1.043, 1.343]**	**9.037e−03**	**Confirmed**
NT.3		15	1.228 [1.123, 1.343]	6.769e−06	1.103 [0.993, 1.225]	6.652e−02	Confirmed
IGFBP.7		16	1.430 [1.284, 1.593]	8.415e−11	1.204 [1.068, 1.358]	2.487e−03	Confirmed
LEP		17	0.783 [0.652, 0.939]	8.545e−03	0.833 [0.686, 1.010]	6.275e−02	Confirmed
PI3		18	1.238 [1.110, 1.380]	1.219e−04	1.153 [1.024, 1.298]	1.904e−02	Confirmed
IL1RL2		20	0.764 [0.667, 0.876]	1.170e−04	0.794 [0.689, 0.914]	1.305e−03	Confirmed
GDF−15		**21**	**1.749 [1.532, 1.996]**	**1.110e−16**	**1.243 [1.050, 1.471]**	**1.142e−02**	**Confirmed**
TR		22	1.476 [1.308, 1.665]	2.637e−10	1.299 [1.141, 1.479]	8.092e−05	Confirmed
EGFR		23	0.831 [0.757, 0.912]	9.443e−05	0.861 [0.774, 0.959]	6.415e−03	Confirmed
TNFRSF13B		24	1.289 [1.176, 1.414]	6.732e−08	1.185 [1.064, 1.320]	1.949e−03	Confirmed
JAM.A		25	1.333 [1.210, 1.469]	5.954e−09	1.167 [1.031, 1.320]	1.423e−02	Confirmed
MMP.2		26	1.304 [1.133, 1.501]	2.211e−04	1.033 [0.904, 1.181]	6.340e−01	Confirmed
4E.BP1		30	1.331 [1.181, 1.499]	2.555e−06	1.202 [1.056, 1.369]	5.497e−03	Confirmed
EN.RAGE		32	1.267 [1.111, 1.444]	3.973e−04	1.147 [0.991, 1.327]	6.511e−02	Confirmed
ACE2		33	1.396 [1.234, 1.580]	1.211e−07	1.148 [1.000, 1.318]	5.018e−02	Confirmed

SLAMF1		34	1.237 [1.116, 1.372]	5.327e−05	1.112 [0.987, 1.251]	8.000e−02	Confirmed
TNFSF13B		40	1.257 [1.118, 1.412]	1.262e−04	1.097 [0.975, 1.235]	1.242e−01	Confirmed
CTSL1		**42**	**1.365 [1.207, 1.544]**	**7.328e−07**	**1.220 [1.069, 1.392]**	**3.173e−03**	**Confirmed**
TGFA		44	1.384 [1.214, 1.579]	1.210e−06	1.115 [0.983, 1.266]	9.156e−02	Confirmed

All biomarkers confirmed by Boruta analysis included, ranked according to Random Survival Forest variable importance. Biomarkers in bold indicate biomarkers p≤0.05 in adjusted Cox regression model 2 and Confirmed in Boruta analyses in both cohorts. Model 1: Cox-regression analysis model adjusted for clinical characteristics - Age, gender, BMI, smoking, hypertension, diabetes, prior MI, prior stroke/TIA, prior PAD, prior CHF, randomized treatment. Model 2: same as Model 1 and also adjusted for marker for renal function, Cystatin C and cardiac markers NT-proBNP + hs-cTnT.

Table 20. Summary of biomarkers with the highest association with CV-death from the validation cohort

Biomarker	RF Ranking	Model 1		Model 2		Boruta
		Hazard ratio (95% CI)	**p-value**	**Hazard ratio (95% CI)**	**p-value**	
cTnT−hs	**1**	**1.753 [1.500, 2.047]**	**1.464e−12**	**1.528 [1.305, 1.788]**	**1.307e−07**	**Confirmed**
NT−proBNP	**2**	**2.959 [2.297, 3.813]**	**<10e-16e**	**2.256 [1.758, 2.894]**	**1.555e−10**	**Confirmed**
U.PAR	**3**	**2.193 [1.824, 2.636]**	**1.110e−16**	**1.624 [1.300, 2.028]**	**1.931e−05**	**Confirmed**
TFF3	**4**	**1.793 [1.492, 2.154]**	**4.581e−10**	**1.518 [1.236, 1.865]**	**7.028e−05**	**Confirmed**
TRAIL.R2	**5**	**1.343 [1.196, 1.508]**	**6.012e−07**	**1.256 [1.121, 1.407]**	**8.435e−05**	**Confirmed**

BNP	6	2.191 [1.673, 2.870]	1.201e−08	1.073 [0.741, 1.555]	0.70972	Confirmed
GDF−15	**7**	**1.980 [1.613, 2.431]**	**6.749e−11**	**1.383 [1.100, 1.739]**	**5.506e−03**	**Confirmed**
REN	8	1.825 [1.421, 2.343]	2.385e−06	1.772 [1.380, 2.274]	7.224e−06	Confirmed
IL6	**9**	**1.556 [1.379, 1.757]**	**8.954e−13**	**1.310 [1.152, 1.490]**	**3.729e−05**	**Confirmed**
TNF.R1	**10**	**2.153 [1.771, 2.617]**	**1.377e−14**	**1.544 [1.207, 1.974]**	**5.339e−04**	**Confirmed**
CTSL1	**11**	**1.684 [1.415, 2.004]**	**4.241e−09**	**1.330 [1.107, 1.599]**	**2.364e−03**	**Confirmed**
ADM	12	1.681 [1.133, 2.494]	9.867e−03	1.092 [0.889, 1.343]	0.40156	Confirmed
TNFRSF11A	13	2.062 [1.691, 2.513]	7.775e−13	1.529 [1.210, 1.931]	3.759e-04	Confirmed
ST2	14	1.622 [1.357, 1.940]	1.127e−07	1.266 [1.056, 1.517]	1.065e-02	Confirmed
TIM1	15	1.584 [1.317, 1.904]	1.023e−06	1.317 [1.096, 1.582]	3.317e-03	Confirmed
JAM.A	16	1.425 [1.245, 1.632]	2.819e−07	1.168 [0.981, 1.391]	8.162e-02	Confirmed
IGFBP.7	17	1.560 [1.361, 1.788]	1.606e−10	1.178 [0.996, 1.394]	5.635e-02	Confirmed
FGF.23	**18**	**1.476 [1.288, 1.690]**	**1.907e−08**	**1.183 [1.023, 1.368]**	**2.335e-02**	**Confirmed**
TNFRSF10A	19	1.844 [1.519, 2.238]	6.347e−10	1.344 [1.078, 1.677]	8.663e−03	Confirmed
GAL.9	20	1.966 [1.610, 2.399]	3.012e−11	1.529 [1.212, 1.930]	3.419e−04	Confirmed
CSTB	21	1.679 [1.432, 1.968]	1.574e−10	1.357 [1.113, 1.653]	2.474e−03	Confirmed
FABP4	23	2.159 [1.697, 2.747]	3.749e−10	1.743 [1.347, 2.254]	2.343e−05	Confirmed
IL.1RT1	24	1.521 [1.260, 1.836]	1.286e−05	1.200 [0.999, 1.441]	5.066e−02	Confirmed
PON3	25	0.684 [0.572, 0.818]	3.159e−05	0.794 [0.651, 0.969]	0.02328	Confirmed
BOC	26	1.470 [1.197, 1.806]	2.372e−04	1.353 [1.123, 1.631]	1.491e-03	Confirmed
MMP12	28	1.550 [1.274, 1.886]	1.212e−05	1.322 [1.080, 1.619]	6.845e-03	Confirmed
TNF.R2	32	1.415 [1.188, 1.685]	9.948e−05	1.198 [1.009, 1.422]	3.896e-02	Confirmed
CD4	40	1.799 [1.513, 2.139]	2.923e−11	1.385 [1.125, 1.704]	2.139e-03	Confirmed
IGFBP2	48	2.117 [1.621, 2.764]	3.652e−08	1.290 [0.981, 1.695]	6.796e−02	Confirmed

All biomarkers confirmed by Boruta analysis included, ranked according to Random Survival Forest variable importance. Biomarkers in bold indicate biomarkers p≤0.05 in adjusted Cox regression model 2 and Confirmed in Boruta analyses in both cohorts. Model 1: Cox-regression analysis model adjusted for clinical characteristics - Age, gender, BMI, smoking, hypertension, diabetes, prior MI, prior stroke/TIA, prior PAD, prior CHF, randomized treatment. Model 2: same as Model 1 and also adjusted for marker for renal function, Cystatin C and cardiac markers NT-proBNP + hs-cTnT.

Table 21. Spearman correlation between the top candidate PEA biomarkers and established biomarkers

Biomarker	NT-proBNP	cTnT-Hs	Cystatin C	Il-6	CRP
cTnT-hs	0.38	-	0.48	0.31	0.14
NT−proBNP	-	0.38	0.41	0.26	0.14
IL-6	0.26	0.31	0.28	-	0.52
GDF-15	0.35	0.50	0.52	0.36	0.17
uPAR	0.33	0.40	0.55	0.38	0.27
FGF-23	0.11	0.18	0.21	0.20	0.16
TRAILR2	0.39	0.49	0.64	0.37	0.20
TNFR1	0.29	0.45	0.64	0.33	0.24
TFF3	0.34	0.44	0.59	0.24	0.12
CTSL1	0.14	0.18	0.20	0.17	0.17

Correlation values presented from the identification cohort.

4.4 Paper IV

4.4.1 Baseline characteristics

Patients with HHF exhibited a higher proportion of a history of HF, diabetes, atherosclerotic disease and treatment with ACE-inhibitors and the age distribution was similar between the group with HHF and the controls. Additionally, patients with HHF had higher baseline levels of cardiac markers (cTnT-hs and NT-proBNP), markers of inflammation (C-reactive protein (CRP) and interleukin-6 (IL-6)) and GDF-15. Baseline characteristics of patients in the random sample and patients with HHF during follow up are detailed in Table 22, and for subgroups defined by baseline HFpEF, HFrEF, and no HF are shown in Table 23.

Table 22. Baseline characteristics and concentration of established biomarkers

Baseline characteristics	Controls N=4029	Hospitalization for Heart failure N=596
Age (years)	70.0 (63,76)	71.0 (64,77)
Females	1477 (36.7%)	218 (36.6%)
Body mass index	28.6 (25.3,32.7)	28.5 (25.0,33.4)
Current smoker	352 (8.7%)	58 (9.7%)
Hypertension	3526 (87.5%)	521 (87.4%)
Diabetes	1003 (24.9%)	197 (33.1%)
Prior myocardial infarction	489 (12.1%)	153 (25.7%)
Prior stroke/TIA	737 (18.3%)	118 (19.8%)
Periferal arterial disease	190 (4.7%)	51 (8.6%)
Heart failure	1197 (29.7%)	346 (58.1%)
Beta blockers	2586 (64.2%)	422 (70.8%)
ACE inhibitors	1965 (48.8%)	375 (62.9%)
NT-proBNP (ng/L)	681.0 (358.0, 1206.0)	1373.5 (811.8, 2301.2)
cTnT-hs (ng/L)	10.6 (7.4, 16.2)	16.6 (11.0, 26.1)
GDF-15 (ng/L)	1352.0 (962.0, 2037.0)	1905.0 (1321.5, 3025.8)
Cystatin C (mg/L)	1.0 (0.8, 1.2)	1.1 (0.9, 1.4)
IL-6 (ng/L)	2.3 (1.5, 3.9)	3.6 (2.1, 5.8)
CRP	2.2 (1.0, 4.6)	3.3 (1.5, 7.3)
CKD-EPI (CysC, mL/min)	75.5 (57.6, 97.1)	62.1 (45.2, 80.6)

Continuous variables presented as median (Q1, Q3). Categorical variables presented as numbers (percentage). CRP, C-reactive protein; GDF-15, growth differentiation factor 15; eGFR, estimated glomerular filtration rate; IL-6, interleukin 6; NT-proBNP, N-terminal pro-B-type natriuretic peptide; cTnT-hs, cardiac troponin T measured with high-sensitivity assay; Q, quartile; and TIA, transient ischemic attack

Table 23. Comparison of baseline characteristics between HFpEF and HFrEF with no HF.

Variable	No HF (N = 1917)	HFpEF (N = 649)	HFrEF (N = 562)
Age (years)	70.0 (63.0, 76.0)	69.0 (62.0, 74.0)	67.0 (59.0, 73.0)
Females	705 (36.8%)	291 (44.8%)	116 (20.6%)
Body mass index	28.7 (25.5, 32.7)	29.4 (25.8 , 33.6)	28.0 (24.9, 32.6)
Current smoker	158 (8.2%)	89 (9.1%)	71 (12.6%)
Hypertension	1732 (90.3%)	566 (87.2%)	438 (77.9%)
Diabetes	504 (26.3%)	157 (24.2%)	142 (25.3%)
Prior myocardial infar-ction	203 (10.6%)	104 (16.0%)	135 (24.0%)
Prior stroke/TIA	368 (19.2%)	108 (16.6%)	80 (14.2%)
Periferal arterial disease	90 (4.7%)	40 (6.2%)	34 (6.0%)
Heart failure	0 (0.0%)	649 (100.0%)	374 (66.5%)
Beta blockers	1241 (64.7%)	450 (69.3%)	432 (76.9%)
ACE inhibitors	829 (43.2%)	376 (57.9%)	378 (67.3%)
NT-proBNP (ng/L)	618.0 (323.5, 1057.0)	746.0 (399.5, 1340.5)	1060.5 (571.8, 2052.2)
cTnT-hs (ng/L)	9.9 (7.1, 14.7)	11.1 (7.7, 17.3)	14.1 (9.5, 22.1)
GDF-15 (ng/L)	1315.0 (946.0, 1939.0)	1370.0 (951.0, 2052.0)	1579.0 (1080.2, 2429.8)
Cystatin C (mg/L)	0.9 (0.8, 1.1)	1.0 (0.8, 1.2)	1.1 (0.9, 1.3)
IL-6 (ng/L)	2.1 (1.4, 3.5)	2.4 (1.5, 4.2)	2.9 (1.8, 5.0)
CRP	2.0 (0.9, 4.2)	2.6 (1.1, 6.0)	2.4 (1.1, 5.1)
CKD-EPI (CysC, mL/min)	77.9 (60.4, 98.8)	74.5 (54.9, 93.5)	67.2 (50.8, 88.9)

Continuous variables presented as median (Q1, Q3). Categorical variables presented as numbers (percentage). CRP, C-reactive protein; GDF-15, growth differentiation factor 15; eGFR, estimated glomerular filtration rate; IL-6, interleukin 6; NT-proBNP, N-terminal pro-B-type natriuretic peptide; cTnT-hs, cardiac troponin T measured with high-sensitivity assay; Q, quartile; and TIA, transient ischemic attack

4.4.2 Biomarkers associated with hospitalization for HF

4.4.2.1 Random Survival Forest analyses

A total of 268 biomarkers and 13 clinical variables were examined for association with HHF. NT-proBNP was identified as having the strongest association with HHF, followed by BNP, history of HF, cTnT-hs, fibroblast growth factor 23 (FGF-23), spondin 1 (spon1) and TNF-related apoptosis-inducing ligand receptor 2 (trail-r2). The strongest associations according to the Random Survival Forest analysis are shown in Figure 10. The importance of these biomarkers was also confirmed in the corresponding Boruta analysis. In total, the Boruta analysis confirmed the importance of 42 biomarkers (Table 24).

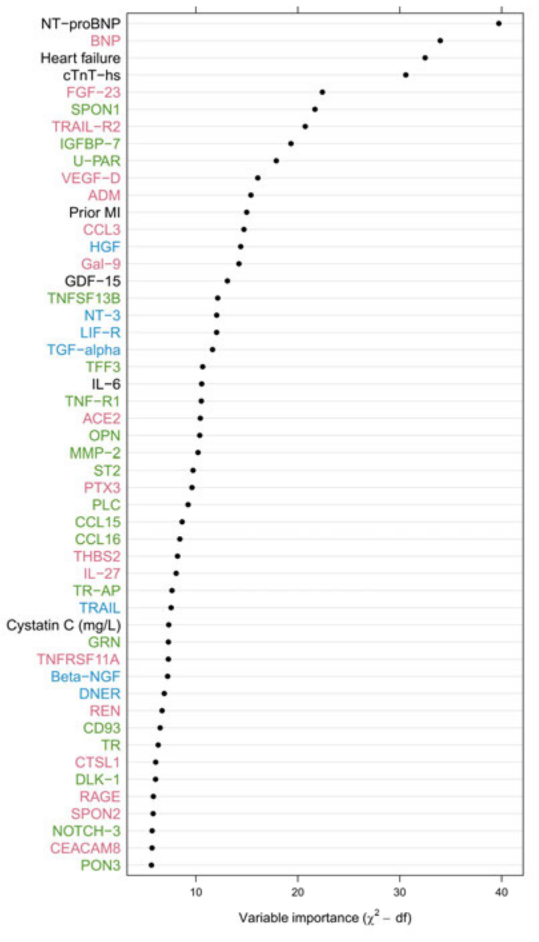

Figure 10. Variable importance of the top 50 biomarkers associated with HHF according to the Random Survival Forest analysis.

Table 24. Summary of biomarkers with the highest association with HHF

Biomarker	RF Ranking	Model 1		Model 2		Boruta
		Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value	
NT-proBNP	*1*	*2.325 [1.977, 2.735]*	*<1.0e-16*	*2.068 [1.759, 2.432]*	*<1.0e-16*	*Confirmed*
BNP	*2*	*2.256 [1.948, 2.613]*	*<1.0e-16*	*1.572 [1.246, 1.982]*	*1.3e−04*	*Confirmed*
cTnT−hs	*3*	*1.644 [1.418, 1.905]*	*4.5e−11*	*1.346 [1.148, 1.579]*	*2.5e−04*	*Confirmed*
FGF-23	*4*	*1.434 [1.319, 1.558]*	*<1.0e-16*	*1.264 [1.153, 1.385]*	*5.5e−07*	*Confirmed*
SPON1	*5*	*1.385 [1.273, 1.507]*	*3.6e−14*	*1.211 [1.099, 1.334]*	*1.0e−04*	*Confirmed*
TRAIL-R2	6	1.292 [1.175, 1.421]	1.1e−07	1.190 [1.070, 1.323]	1.3e−03	Confirmed
IGFBP-7	*7*	*1.443 [1.307, 1.594]*	*4.6e−13*	*1.378 [1.127, 1.393]*	*2.9e−05*	*Confirmed*
U-PAR	*8*	*1.716 [1.465, 2.011]*	*2.4e−11*	*1.406 [1.194, 1.655]*	*4.2e−05*	*Confirmed*
VEGF-D	9	1.707 [1.450, 2.008]	1.2e−10	1.284 [1.080, 1.526]	4.6e−03	Confirmed
ADM	10	1.524 [1.241, 1.873]	5.9e−05	1.202 [1.032, 1.399]	1.8e−02	Confirmed
CCL3	11	1.194 [1.115, 1.278]	4.0e−07	1.147 [1.062, 1.238]	4.6e−04	Confirmed
HGF	12	1.265 [1.174, 1.363]	7.2e−10	1.152 [1.056, 1.257]	1.4e−03	Confirmed
GAL-9	13	1.407 [1.175, 1.686]	2.1e−04	1.295 [1.084, 1.547]	4.4e−03	Confirmed
GDF-15	14	1.624 [1.399, 1.885]	1.9e−10	1.289 [1.103, 1.398]	1.7e−03	Confirmed
TNFRSF13B	15	1.096 [0.960, 1.251]	1.8e−01	1.242 [0.993, 1.225]	3.4e−04	Confirmed
NT3	16	1.232 [1.104, 1.375]	1.9e−04	1.122 [1.022, 1.233]	1.6e−02	Confirmed

LIF-R	17	1.583 [1.391, 1.802]	3.4e−12	1.260 [1.093, 1.451]	1.4e−03	Confirmed
TGFA	18	1.298 [1.155, 1.459]	1.3e−05	1.163 [1.024, 1.322]	2.0e−02	Confirmed
TFF3	19	1.299 [1.151, 1.467]	2.3e−05	1.188 [1.041, 1.355]	1.0e−02	Confirmed
IL-6	20	1.419 [1.275, 1.580]	1.7e−10	1.188 [1.056, 1.337]	4.1e−03	Confirmed
TNF-R1	21	1.325 [1.113, 1.577]	1.6e−03	1.132 [0.958, 1.338]	1.5e−01	Confirmed
ACE2	22	1.420 [1.271, 1.586]	4.9e−10	1.218 [1.077, 1.378]	1.7e−03	Confirmed
OPN	*23*	*1.496 [1.302, 1.718]*	*1.2e−08*	*1.313 [1.139, 1.514]*	*1.8e−04*	*Confirmed*
MMP2	24	1.644 [1.418, 1.905]	4.5e−11	1.286 [1.116, 1.482]	5.2e−04	Confirmed
ST2	25	1.482 [1.319, 1.665]	3.9e−11	1.215 [1.083, 1.364]	8.8e−04	Confirmed
PTX3	*26*	*1.502 [1.333, 1.693]*	*2.7e−11*	*1.298 [1.146, 1.469]*	*4.0e−05*	*Confirmed*
PLC	27	1.370 [1.145, 1.639]	5.7e−04	1.127 [0.952, 1.334]	1.7e−01	Confirmed
CCL15	28	1.239 [1.095, 1.402]	6.8e−04	1.164 [1.021, 1.327]	2.3e−02	Confirmed
CCL16	29	1.118 [0.999, 1.252]	5.3e−02	1.096 [0.977, 1.229]	1.2e−01	Confirmed
THBS2	30	1.498 [1.273, 1.763]	1.2e−06	1.175 [0.979, 1.411]	8.4e−02	Confirmed
IL27	31	1.383 [1.221, 1.567]	3.5e−07	1.213 [1.058, 1.391]	5.6e−03	Confirmed
TRAP	32	0.772 [0.693, 0.860]	2.6e−06	0.865 [0.766, 0.977]	2.0e−02	Confirmed
TRAIL	33	0.736 [0.652, 0.831]	7.6e−07	0.820 [0.721, 0.932]	2.4e−03	Confirmed
CYSTATIN C	34	1.663 [1.468, 1.883]	1.2e−15	1.130 [0.980, 1.302]	9.3e−02	Confirmed

GRN	35	1.326 [1.166, 1.508]	1.7e−05	1.272 [1.113, 1.453]	4.1e−04	Confirmed
DNER	38	0.804 [0.720, 0.898]	1.0e−04	0.861 [0.762, 0.974]	1.7e−02	Confirmed
REN	39	1.108 [0.963, 1.274]	1.5e−01	1.228 [1.062, 1.419]	5.6e−03	Confirmed
TR	*41*	*1.376 [1.231, 1.539]*	*2.2e−08*	*1.262 [1.120, 1.421]*	*1.3e−04*	*Confirmed*
CTSL1	42	1.302 [1.150, 1.474]	3.0e−05	1.184 [1.034, 1.354]	1.4e−02	Confirmed
PON3	48	0.838 [0.757, 0.928]	6.7e−04	0.860 [0.764, 0.968]	1.3e−02	Confirmed
GIF	49	0.809 [0.727, 0.900]	1.0e−04	0.845 [0.754, 0.948]	3.9e−03	Confirmed
TWEAK	51	0.882 [0.801, 0.971]	1.0e−02	0.899 [0.813, 0.995]	3.9e−02	Confirmed

Biomarkers confirmed by Boruta analysis included, ranked according to Random Survival Forest variable importance. Biomarkers in bold indicate biomarkers p≤0.05, and biomarkers in italics indicate biomarkers p=≤0.00027 (multiplicity adjusted) in adjusted Cox regression model 2 and confirmed in Boruta analyses in both cohorts. Biomarkers in Model 1: Cox-regression analysis model adjusted for clinical characteristics - Age, gender, BMI, smoking, hypertension, diabetes, prior MI, prior stroke/TIA, prior PAD, prior CHF, randomized treatment. Model 2: Model 1 + adjustment for renal function and NT-proBNP + hs-cTnT.

4.4.2.2 Cox-regression analyses

In the Cox-regression analyses adjusted for clinical characteristics and renal function (Cox model 1), out of 255 biomarkers, 46% (n=116) were associated with HHF. When further adjusting for the two cardiac biomarkers (NT-proBNP and cTnT-hs) (Cox model 2), 27% (n=68) of the biomarkers remained significantly associated with HHF. Among the 42 biomarkers confirmed in the Boruta analysis, 37 biomarkers were significantly associated with the outcome (Table 24). When adjusting for multiplicity, ten biomarkers remained significant (p<=0.0002665); NT-proBNP, BNP, cTnT-hs, FGF-23, spon1, insulin like growth factor binding protein 7 (IGFBP-7), urokinase receptor (u-par), osteopontin (OPN), pentraxin-related protein (PTX3) and transferrin receptor protein 1 (TR).

4.4.3 Biomarker profiles in HFpEF versus HFrEF

4.4.3.1 Random Forest analyses

The biomarkers that differed the most between HFpEF and HFrEF at baseline were NT-proBNP, renin (ren), cTnT-hs, brain natriuretic peptide (BNP), Pulmonary surfactant-associated protein D (PSP-D), stem cell factor (SCF), GDF-15, angiotensin-converting enzyme 2 (ACE2). The relative importance of biomarkers for differentiating between HFpEF and HFrEF at baseline is shown in Figure 11.

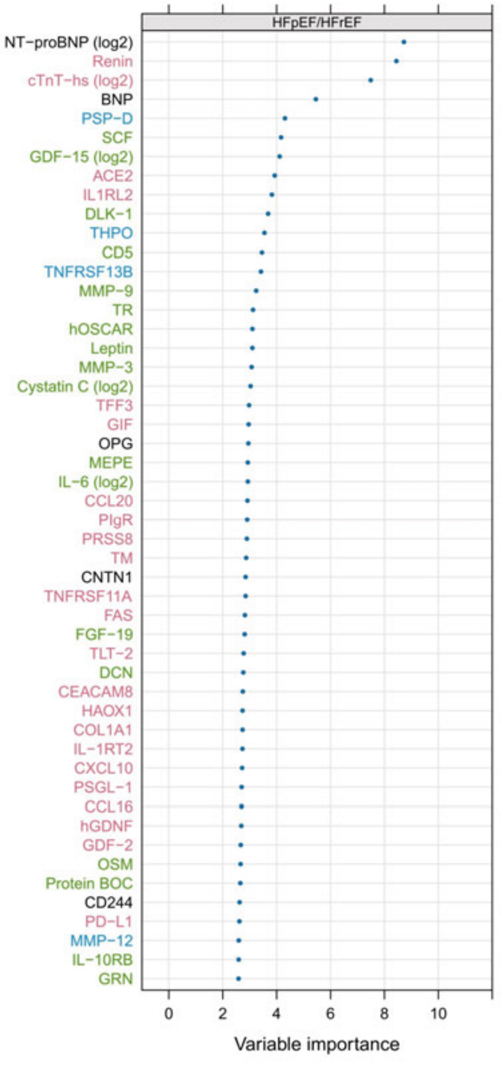

Figure 11. Variable importance for biomarker difference between HFpEF and HFrEF according to the Random Forest analysis - top 50 biomarkers shown

4.4.3.2 Wilcoxon−Mann−Whitney test

Out of 268 biomarkers, 53 biomarkers differed significantly between HFpEF and HFrEF. After adjustment for multiplicity, nine biomarkers were still significantly different (p>0.05) between HFpEF and HFrEF (Figure 12). In HFpEF, the proteins levels of SCF and leptin were higher than in HFrEF, whereas levels of NT-proBNP, BNP, cTnT-hs, renin, GDF-15, ACE-2 and interleukin-6 (IL-6) were higher in HFrEF. In sensitivity analyses, the results for renin and ACE2 were materially unaltered when adjusted for treatment with ACE inhibitors (ACEi)/angiotensin receptor blockers (ARB) and diuretics within 30 days before randomization. A summary of these biomarkers according to the variable importance from the Random Forest analyses is shown in Table 25.

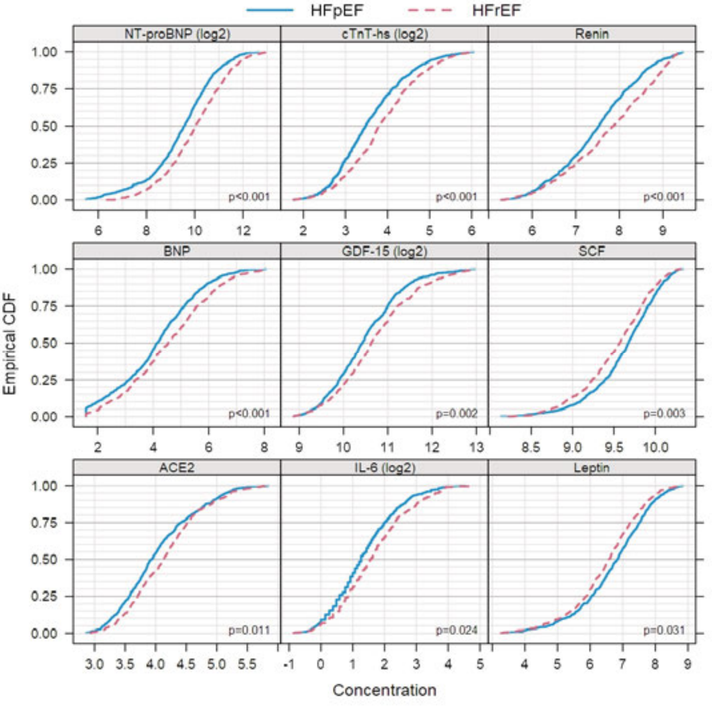

Figure 12. Empirical cumulative distribution function of biomarkers according to HF subtype

Table 25. Summary of biomarkers with the strongest difference between HFpEF and HFrEF.

Protein	RF ran-king	Median HFpEF	Median HFrEF	Relative median HFpEF	Relative median HFrEF	p-value	p-value adjusted
NT-proBNP (log2)	1	9,53527538	10,0505249	1	1,42924131	2,5E-12	6,8E-10
Renin	2	7,51158293	7,87313518	1	1,28480752	1,6E-07	4,2E-05
cTnT-hs (log2)	3	3,47248777	3,82272114	1	1,27476682	1,0E-09	2,7E-07
BNP	4	4,10283075	4,56128965	1	1,37407323	2,4E-07	6,5E-05
SCF	6	9,67360325	9,56611021	1	0,92819958	1,2E-05	0,0032
GDF-15 (log2)	7	10,4199602	10,6247943	1	1,15255382	5,7E-06	0,0015
ACE2	8	3,92957549	4,12990073	1	1,14895734	4,1E-05	0,011
Leptin	17	6,84624058	6,57601232	1	0,82918835	0,00012	0,031
IL-6 (log2)	24	1,26303441	1,5360529	1	1,20833333	9,0E-05	0,0236

5. Discussion

In this thesis, both novel and established biomarkers and their associations with CV events and mortality were investigated in two large cohorts of anticoagulated patients with AF. The overarching aims were to better understand the risks and pathophysiologic processes that may be involved with CV outcomes in AF and to understand how GDF-15 and its related risk scores perform universally.

5.1 Main findings

5.1.1 Paper I

Higher levels of ApoA1 were associated with lower risk of composite ischemic outcomes, stroke, and death. Higher levels of ApoB were associated with higher rates of MI only, but not with the other cardiovascular outcomes. In contrast, lower levels of ApoB were associated with an increased risk of all-cause death. Importantly, both apolipoproteins remained statistically significantly associated with these outcomes even after adjustment for baseline characteristics and other prognostic biomarkers.

5.1.2 Paper II

GDF-15 and the ABC-AF-bleeding and ABC-AF-death scores were consistently associated with major bleeding and mortality across all studied geographic regions. There was no significant interaction between GDF-15 in regard to outcomes by regions in either trial cohort. Results were similar for the ABC-AF-risk scores although there seemed to be a quantitative interaction between ABC-AF-death score and region for Latin America. The ABC-AF-bleeding and ABC-AF-death risk scores were consistent regarding their discriminative ability when comparing geographic regions in both cohorts.

5.1.3 Paper III

Using proteomics to screen for prognostic importance of biomarkers associated with CV death in AF, 10 biomarkers were found to have a strong and consistent association with CV death in AF. Of these, four were previously known: cTnT-hs, NT-proBNP, IL-6, and GDF-15, and six biomarkers novel in regard to their association with CV death in AF: FGF-23, suPAR, TFF3, TNFR1, TRAILR2, and CTSL1, and seem to reflect a spectrum of different pathological processes.

5.1.4 Paper IV

Using proteomics to screen for prognostic importance of biomarkers associated with HHF in AF, 10 biomarkers were strongly associated with HHF: NT-proBNP, BNP, cTnT-hs, FGF-23, spon1, IGFBP-7, u-par, OPN, PTX3 and TR. There were significant biomarker differences between HFrEF and HFpEF. Specifically, we observed higher levels of SCF and leptin in HFpEF patients, while NT-proBNP, BNP, cTnT-hs, renin, GDF-15, ACE-2, and IL-6 levels were notably elevated in HFrEF patients.

5.2 Apolipoproteins in relation to CV events in AF

The known associations between dyslipidemia and AF have unfortunately been limited to incident AF. This study provided new insights by demonstrating that plasma levels of apolipoproteins are independently associated with major CV events and mortality in anticoagulated patients with AF. High baseline ApoA1 levels were associated with lower risk of stroke or SEE, reduced risk for cardiac and all-cause mortality, and similar association to composite ischemic outcomes. High levels of ApoB on the other hand, were associated with increased the risk of myocardial infarction. These associations, similar to that of dyslipidemia in coronary artery disease, might be explained by the atherogenic properties of dyslipidemia[128-131].

Another finding in this study is the association between dyslipidemia and mortality for both ApoA1 and ApoB. Similar to low HDL levels, low ApoA1 conferred higher risk of death. However, for ApoB, the all-cause mortality rate was paradoxically higher in patients with lower ApoB concentrations. Similar findings have been presented in other cohorts as well[132, 133]. In a large prospective observational study of patients following an acute myocardial infarction, an increased risk of all-cause mortality was shown in the group with the lowest levels of LDL cholesterol[132]. Increased rate of all-cause mortality has also been associated with low levels of LDL cholesterol in the elderly[133]. Plasma cholesterol levels decline with age, malnutrition, chronic disease and even with inflammation[133-136]. This “lipid paradox” in which patients with the lowest levels of LDL cholesterol, or in this case ApoB, are at an increased risk for all-cause mortality could thus possibly be explained, at least in part, by a higher disease burden and frailty. Patients in this study in the lowest ApoB quartile were in fact more comorbid, as illustrated by higher CHA2DS2–VASc scores, and were therefore likely at an increased risk for death.

The relationship between dyslipidemia and stroke is complex and few studies have evaluated dyslipidemia in relation to stroke risk in an AF population on oral anticoagulation. In a small cohort of AF patients without anticoagulant therapy, high LDL cholesterol was found to be an independent predictor of ischemic stroke[137]. In the present study, no such findings were observed, which may be attributable to a relatively lower event rate due to the fact that all patients received oral anticoagulants. Low ApoA1 levels were on the other hand associated with higher stroke/SEE rates and even more so to the composite ischemic outcome. This could indicate that ApoA1 indeed may play a role in the pathophysiology of ischemic outcomes in AF. Furthermore, a greater relative risk reduction of major bleedings was seen with apixaban compared to warfarin in patients with higher levels of apolipoprotein. In these individuals, apixaban may be an even more attractive choice than warfarin. The underlying mechanism for the treatment interaction is however unclear and warrants further investigation.

The findings from the present study indicate that dyslipidemia, a traditional cardiovascular risk factor, may play a role in AF-related adverse outcomes. Therapeutic interventions for dyslipidemia could therefore prove beneficial effects in reducing the risk of these complications. In clinical practice, however, most drugs that increase ApoA1/HDL cholesterol (such as niacin and fibrates) have so far not been able to show further reduction in cardiovascular events[138, 139]. It is possible that newer agents, such as cholesteryl ester transfer protein (CETP) inhibitors, may be more favourable[140]. The medications, however, have not been studied specifically in patients with AF and any potential beneficial effect of these agents on AF burden and associated outcomes need to be tested in future prospective trials and may possibly also need stratification according to genetic variances[141]. Another issue that warrants mentioning is that only half (51.6%) of the patients with AF and a traditional indication for statin therapy (e.g. established vascular disease or diabetes) was on statin treatment in the study cohort. Better adherence to existing guidelines for management of dyslipidemia may thus also be an important factor in the efforts to improve outcomes in AF.

5.3 Geographical evaluation of GDF-15, ABC bleeding and ABC death risk score

The understanding of regional geographic influences on health outcomes and biomarkers are limited but significant differences have been observed. For instance, when compared with other regions, AF patients from Asia have both a higher risk of ischemic stroke and bleeding[142-145]. This variability may be due to factors such as genetic variation and regional treatment differences. Geographic differences could thus impact the predictive ability of biomarkers and risk scores, potentially reducing their reliability in certain regions. Evaluating geographic variation could be beneficial as part of the validation process when introducing new biomarkers or risk scores for widespread use, ensuring their efficacy and reliability across diverse populations.

In paper II, there was a strong association between GDF-15, the ABC-AF-bleeding score and ABC-AF-death risk score with major bleeding and death independent of geographic regions and countries. There was a statistically significant interaction between the ABC-AF-death score and all-cause mortality for geographic regions in the ARISTOTLE cohort. This interaction could be explained by a somewhat weaker association between the ABC-AF-death score and death in Latin America where patients had a generally higher mortality risk as compared with the other regions, especially for those having a low ABC-AF-death score. Although the interaction was not statistically significant, a similar pattern was seen in the RE-LY cohort suggesting a possibly weaker association between the ABC-AF-death score and mortality in Latin America compared with the other regions. However, as the discriminatory ability of the ABC-AF-death score was consistent across geographic regions, this eventual interaction is likely to be less relevant from a clinical perspective. Otherwise, the variability between the regions concerning the association with the outcomes for GDF-15 and the ABC-AF scores were almost negligible in comparison with the strong associations with outcomes within the regions. The findings from this study therefore indicate an overall consistency of the performance of GDF-15 and the ABC-AF-risk scores across geographic regions.

Despite convincing documentation, the ABC-AF scores currently are not routinely used. Part of that may be due to additional time and cost to obtain biomarker levels, but another component might have been the lack of specific validation across geographic areas which are now eliminated. The findings from this study thus add novel information regarding the consistency of GDF-15 and the ABC-AF-risk scores in AF and strengthen their role as a risk refinement tool applicable for a wide international usage.

5.4 Biomarkers in AF associated with CV death

In paper III, our comprehensive study emphasized NT-proBNP and cTnT-hs as the biomarkers with the strongest association with CV-death in both trial cohorts, even when compared to all other 266 markers of inflammation and CV disease and 13 clinical risk factors including age. As have been demonstrated earlier, IL-6 and GDF-15 were also confirmed to be among the top biomarkers for CV-death, signifying their respective pathophysiologic importance in AF [6, 7].

Six other biomarkers less studied in AF; FGF-23, suPAR, TFF3. TNFR1, TRAILR2, and CTSL1, were identified as potential prognostic biomarkers with strong independent association with CV-death in the present study:

FGF-23 is a circulating peptide hormone that regulates phosphate and, indirectly, calcium balance[146]. In AF, FGF-23 has been shown to be associated with all-cause mortality in patients with end stage renal disease and several studies have also found an association between FGF-23 with incident AF[147-149]. By displaying an association even after adjustment for cardio-renal markers, our findings extend upon these results and for the first time show a significant importance of FGF-23 for CV-death in AF suggesting that FGF-23 plays an important role in cardiovascular disease and in AF in particular. The exact mechanism of how FGF-23 is linked to CV-death in AF is yet to be established but could include induction of left ventricular hypertrophy and cardiac remodeling, a mechanism that might in fact be reversible by therapeutic interventions [150, 151].

A novel finding was the independent association of suPAR with CV-death in AF. uPAR is an important component of the fibrinolytic system and is involved in cell migration and matrix degradation[152]. suPAR has been shown to be associated with cardiovascular disease, cancer and renal failure[153, 154]. In AF, suPAR has been explored as a predictor for incident AF however with diverging results[155, 156]. Our data indicate suPAR as having an important role in regards to mortality in AF - something that perhaps might be explained by the role of suPAR in the development of myocardial fibrosis and/or atherosclerosis, previously demonstrated in animal models[157, 158].

Another biomarker that was strongly associated with mortality in AF was TFF3, a member of the trefoil family. Data regarding the involvement of TFF3 in AF and heart disease is scarce but in experimental models TFF3 seems to be elevated during myocardial ischemia, possibly enhancing ischemic myocardial resistance[159]. Whether TFF3 is an indirect marker of myocardial ischemia or part of a novel pathophysiological process that leads to mortality in AF needs to be further examined.

TNFR1 is one of the receptors to which Tumor necrosis factor alpha (TNF alpha) binds mainly leading to necrosis or apoptosis[160]. TNFR1 seems to be increased in heart failure patients compared to controls but does not in our material strongly correlate with NT-proBNP, another marker of heart failure[161]. Evidence suggests that TNFR1 may participate in the pathophysiology of heart failure by mediating adverse remodeling[162]. Furthermore, TNFR1 has been has been shown to be a predictor for incident heart failure, in particular for the heart failure with preserved ejection fraction (HFpEF) subtype, a condition that often co-exists with AF[163]. The strong association between TNRF1 and mortality in AF has to our knowledge not previously been described. Our finding suggests the TNF alpha/TNFR1 system as a possible target for therapy for improving outcomes in patients with AF.

Similar to TNFR1, TRAILR2 is a marker of apoptosis and belongs to the tumor necrosis factor receptor superfamily[164]. TRAILR2 concentrations do not seem affected by the presence of AF compared with sinus rhythm[148]. TRAILR2 has previously been associated with heart failure and AF incidence and was also shown to predict mortality in patients after an acute myocardial infarction perhaps reflecting inflammation and apoptotic activity[148, 165, 166]. Our results extend the latter finding to the AF population suggesting that TRAIL-R2 is not specific for AF but rather a marker that rises in several disease states indicating poor prognosis.

CTSL1 is a lysosomal protease expressed in the heart and is involved in turnover and degradation of intra- and extracellular proteins[167]. It is thought to help maintain normal cardiac function and morphology, something that was demonstrated by showing that CTSL1 knockout mice developed a dilated cardiomyopathy-like syndrome[168]. Additionally, CTSL1 has in other animal models been shown to contribute to the repair and remodeling post myocardial infarction as well as exerting cardioprotective properties following pressure overload[169, 170]. There are limited data regarding CTSL1 in AF but the levels of CTSL1 in patients with AF in sinus rhythm is thought to be higher compared to patients without known history of AF[171]. In the present study CTSL1 emerged as an independent biomarker with strong association with CV-death in AF. However further research is needed to explore weather CTSL1 contributes to or protects against the deleterious processes leading to death from CV-causes.

Many of these newly identified biomarkers with the highest association with CV-death showed a correlation to renal function. However, in the present study, Cystatin C, an established renal function marker, did not show strong independent association with CV-death in the Random Survival Forest analyses in comparison to the other biomarkers, nor after adjustment for NT-proBNP and cTnT-hs in the Cox-analysis. This suggests that the association with CV-death of these newly identified biomarkers was independent of renal function.

In search for strategies to reduce the non-stroke related mortality in patients with AF, exploring the underlying disease processes are of prime importance as understanding them better could facilitate the identification of patients at risk for death, thus allowing for earlier intervention, optimization of secondary prevention, and possibly even for targeted therapy to reduce AF-related mortality. This study identified several novel candidate biomarkers that reflect separate, although in many cases potentially overlapping biological pathways involved with CV-death in AF; cardiac remodeling, cardio-renal dysfunction, inflammation, cell death, disturbances in calcium phosphate balance, fibrinolysis and oxidative stress (Figure 13). The present study provides valuable insights into important processes involved with CV-death in patients with AF, further studies are however needed for exploration of causal relationships and potential therapeutic interventions.

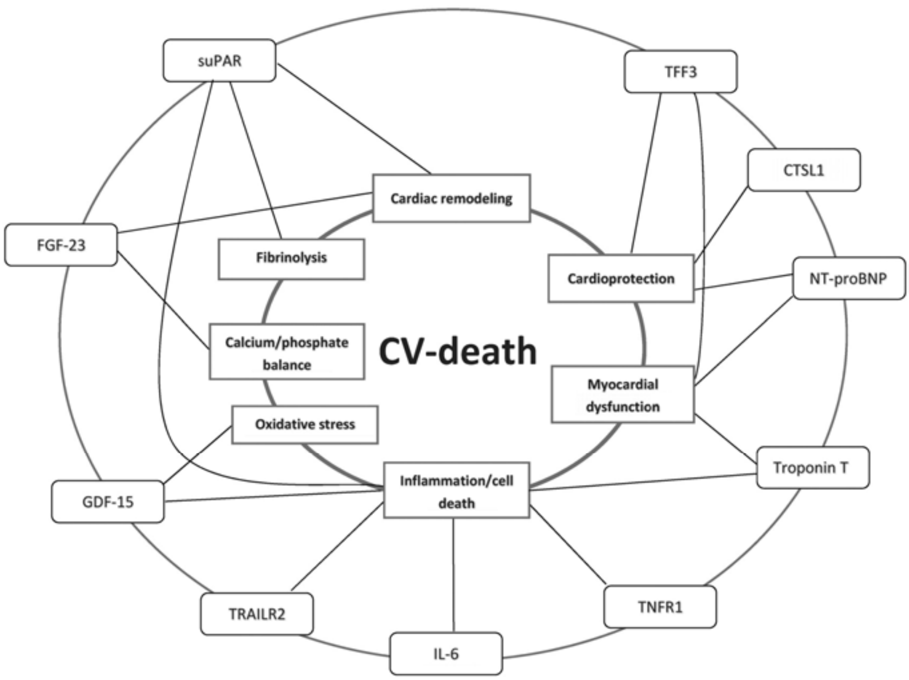

Figure 13. Conceptual figure showing top biomarkers and their associated processes in relation to CV death in AF

The present study adds to knowledge by using mass screening for identification of candidate biomarkers associated with CV-death in AF, confirmed with validation in an independent dataset. The vast amount of proteins screened from two contemporary AF cohorts, the large number of events and sample size and the use of multiple statistical methods including both a linear and non-linear evaluation are some of the strengths of this study.

5.5 Biomarkers in AF in relation to HHF

5.5.1 Biomarkers associated with HHF in AF

Not surprisingly, in paper IV similar to paper III, because the most common cause of death in ARISTOTLE was death due to HF, the biomarkers with the strongest association with HHF were the cardiac markers NT-proBNP, BNP and cTnT-hs[48]. These are the most extensively investigated and clinically used cardiac markers for assessing HF diagnosis, worsening and prognosis[49].

Notably higher levels of six biomarkers; CD40 ligand (TRAP), Tumor necrosis factor ligand superfamily member 10 (TRAIL), Delta and Notch-like epidermal growth factor-related receptor (DNER), Serum paraoxonase/lactonase 3 (PON3), Cobalamin binding intrinsic factor (GIF) and Tumor necrosis factor ligand superfamily member 12 (TWEAK) exhibited lower risk for HHF, however they did not reach statistical significance after multiplicity adjustment (Table 24).

The seven other biomarkers, FGF-23, Spon1, IGFBP-7, uPAR, OPN, PTX3, and TR, which displayed the strongest association with HHF have consistently demonstrated similar strong associations in several other studies examining incident HF and worsening HF mainly in populations with a low degree of AF[165, 172-174]. Furthermore there is data from Mendelian randomization studies suggesting the inflammatory markers Spon1, IGFBP-7, and the peptide hormone FGF-23, which regulates phosphate and calcium balance, to have a causal relationship with HF. Specifically, Spon1 and IGFBP-7 have a potential causal association with left ventricular size and function while FGF-23 possibly exerts protective effects instead[174, 175]. Our results from this multimarker study extend previous findings to an AF population. Considered together, our findings strengthen the evidence for the involvement of these biomarkers and their corresponding processes in HF.

5.5.2 Biomarker profiles in HFpEF versus HFrEF

Although natriuretic peptides can be used as a diagnostic tool in HFpEF and HFrEF, HFpEF patients generally exhibit lower levels of natriuretic peptides compared to HFrEF patients[176]. This is believed to result from a lesser degree of myocardial wall stress in HFpEF[177]. Similarly, higher levels of cardiac troponins have been seen in patients with HFrEF, signifying myocardial damage and loss of cardiomyocytes[178, 179]. The results of the present study confirm these findings with NT-proBNP, BNP, and cTnT-hs levels being significantly higher in HFrEF patients.

GDF-15 is expressed in states of inflammatory and oxidative stress, exerts anti-inflammatory actions and plasma levels does not seem to be influenced by AF[107, 180]. Higher levels of GDF-15 are associated with both incident HFpEF and HFrEF in a cohort were only a minority had AF[181]. In our study, significantly higher levels of GDF-15 were seen in HFrEF as compared to HFpEF. In contrast, prior data did not show any difference between the two HF subtypes[181]. The larger sample size and the international patient population of the present study might account for some differences between the results of the two studies.

Another marker of inflammation is IL-6 which in prior studies has been associated with both HFpEF and HFrEF[182, 183]. Our study revealed higher levels of IL-6 in HFrEF, consistent with earlier findings of elevated IL-6 concentrations in this HF subtype[172].

Renin acts as an activator, and ACE-2 as a counter-regulator, of the renin-angiotensin-aldosterone system (RAAS) that regulate volume and electrolyte balance, the systemic vascular resistance and is central in HF pathophysiology[184]. While HF and some antihypertensive medications can up-regulate renin via renal feedback mechanisms, they do not seem to affect ACE-2 levels[185, 186]. Renin levels were significantly higher in HFrEF patients, even when adjusted for HF medication, and perhaps signify a stronger RAAS activation in HFrEF. Similarly, higher ACE-2 activation have previously been observed in HFrEF and our study further demonstrates higher ACE-2 levels in HFrEF compared to HFpEF[187].

The two biomarkers that were significantly higher in HFpEF were SCF and leptin. SCF play an important role in vasculogenesis and tissue repair and elevated SCF concentrations have been associated with lower risk of cardiovascular disease, including lower risk for HF[188, 189]. On the other hand, increased leptin levels are seen in obesity as it acts as a satiety hormone while also inducing proinflammatory molecules[190]. Leptin concentrations were in a prior study increased in both HFpEF and HFrEF and after adjustment for background variables, there was no leptin concentration difference between the two HF groups[191]. Our study had a comparatively larger population, thus providing more adequate power for detecting group differences. Other possible explanations for the disparities between the two studies could be related to type of study population, comorbidities and severity of disease. To our knowledge the present study is the first to identify elevated SCF and leptin levels in HFpEF compared to HFrEF, shedding new light on their importance in these conditions.

Taken together, our results provide valuable insights into the pathophysiological processes involved in HHF as well as the differing mechanisms underlying HFpEF and HFrEF. These might provide a better understanding of HF, particularly in the setting of patients with AF.

5.6 Limitations

The studies of the present thesis have limitations to be considered when interpreting the results. A general limitation concerns a possible lack of generalization to other populations, especially non-anticoagulated patients with AF. Furthermore, the observational nature of these studies show associations and does not permit any deductions concerning causal relationships between biomarkers and outcomes. In some of the studies, lowering the number of events by creating subgroups reduces the statistical power to detect true differences. Even though the statistical analyses were adjusted for a variety of cardiovascular risk factors, patient background characteristics and cardiovascular biomarkers, residual confounding cannot be excluded. Another limitation is the lack of data regarding biomarker level change over time that could provide a better comprehension of the temporal dynamics and their potential impact on CV outcomes and pathophysiological processes.

In paper II and III, in order to increase the certainty of the results and to minimise the risk of random findings, we used data from two cohorts as any inconsistency appearing in both trials would lower the risk of it being a chance finding.

For paper III and IV, the use of a conservative statistical approach applying two statistical methods for biomarker selection could result in an overly strict selection process and thereby fail to identify other potentially important biomarker candidates. However, the use of this approach adds robustness to the screening process and increases the certitude to the selection of candidate biomarkers. In paper III and IV, our study focused solely on protein biomarkers which might overlook potential contributions from other biomolecule classes such as lipids or nucleic acids, which could provide a more comprehensive understanding of the pathophysiology of CV death and HHF in AF.

The absence of adjudication for HHF in paper IV could somewhat reduce outcome certainty. In the same paper, even without adjusting the analyses for baseline heart rhythm, previously published data have shown that while individual biomarker levels may be influenced by rhythm status, the relationship between biomarkers and cardiovascular outcomes remains unaffected[192]. It's also worth noting that AF can complicate the diagnosis of HHF thus making the results potentially less reliable, as NT-proBNP, a marker used to determine worsening of HF, is elevated in both conditions. Additionally, there was no standardization of LVEF measurement in the ARISTOTLE trial, allowing for potential HFpEF/HFrEF misclassification. Because of original trial protocol, heart failure with mildly reduced ejection fraction (HFmrEF) was included in the HFpEF category in this biomarker substudy. Although HFpEF definition has varied through the years and some mechanistic similarities exist between the two, mechanistic differences are likely to be present which could impact the interpretation of our findings.

While appropriate for protein screening purposes, the PEA analytic method provides relative protein concentrations only and thus in further evaluation of clinical usefulness quantitative assays should be preferred.

6. General conclusions

The aim of this thesis has been to evaluate the association of novel and established biomarkers with cardiovascular outcomes in patients with AF.

Based on the findings in this thesis, we can conclude that in patients with AF with anticoagulation therapy:

I. Higher levels of ApoA1 were associated with lower risk of composite ischemic outcomes, stroke, and death. Higher levels of ApoB were associated with higher rates of MI only, but not with the other cardiovascular outcomes. In contrast, lower levels of ApoB were associated with an increased risk of all-cause death. Dyslipidemia may therefore play a role in CV outcomes.

II. The associations between GDF-15 and the ABC-AF-bleeding and ABC-AF-death risk scores and the outcomes major bleeding and death, are consistent across geographic regions. Accordingly, the discriminatory abilities of the ABC-AF-bleeding and ABC-AF-death scores to prognosticate major bleeding and death are consistent and similarly clinically useful across global geographic regions. The assessment of geographic variation might be helpful as part of future validation processes.

III. NT-proBNP, cTnT-hs, IL-6, and GDF-15 from previous studies and six additional novel biomarkers such as FGF-23, suPAR, TFF3, TNFR1, TRAILR2, and CTSL1, were identified as the most important out of 268 biomarkers for CV death. These findings highlight essential pathophysiologic pathways that may be involved in CV death.

IV. NT-proBNP, BNP, cTnT-hs, FGF-23, spon1 factor, IGFBP-7, u-par, OPN, PTX3 and TR as being the most strongly associated with HHF. HFrEF was more strongly associated with cardiorenal dysfunction and inflammation markers, while HFpEF was associated with adipose metabolism and tissue repair proteins. Our findings highlight the most important pathophysiological pathways involved in new or worsening HF and add valuable knowledge to the understanding of the underlying differing biological mechanisms in the HF subtypes HFrEF and HFpEF in AF.

7. Clinical implications and future perspectives

Despite improvements in management of AF, mortality and morbidity remain high and the disease and its adverse outcomes are still poorly understood. The findings from this thesis need to be confirmed in future studies but provide a potential way for improving outcomes and understanding of the disease.

Further investigation of the role of dyslipidemia in AF, its use as a risk predictor and studying therapeutic interventions for dyslipidemia specifically on AF burden and outcomes in AF could pave the way for targeted therapies, ultimately improving patient outcomes. The ABC-AF scores, here validated for global use, provide a means to improve risk prediction in AF. To truly demonstrate superiority over existing risk stratification methods however, prospective evaluation of the ABC scores is currently underway in the form of the randomized clinical trial – the ABC-AF trial (NCT03753490). The results of this study will be available in the near future.

Proteomic studies, like those performed in this thesis, allow for the identification of new mechanistic pathways involved in AF, HF and HF-subtypes and their adverse outcomes. These findings can subsequently be utilized to highlight therapeutic targets and identify patients at higher risk for mortality and morbidity for whom upstream or early therapy might be useful.

To assess potential causality between the identified proteins and their corresponding outcomes, Mendelian randomization studies could be performed[193]. Future studies should include longitudinal biomarker measurements to assess changes over time and their correlation with disease progression and outcomes. This can help in understanding the dynamic nature of these biomarkers and their role in AF and HF. Furthermore, incorporating multi-omic approaches and other populations are warranted to validate and expand upon our observations, ultimately paving the way for individual optimised therapies.

8. Summary in Swedish (sammanfattning på svenska)

Förmaksflimmer är den vanligaste ihållande rytmrubbningen i världen. Man uppskattar att mer än 59 miljoner individer hade förmaksflimmer 2019 och prevalensen spås fortsätta att öka. Förutom att förmaksflimmer i sig kan ge besvär i främst i form av hjärtklappning och andfåddhet, så ökar det kraftigt risken för stroke, hjärtsvikt och död. Risken för att drabbas av dessa komplikationer varierar mellan individer och beror på faktorer som ålder, tidigare stroke och andra kardiovaskulära riskfaktorer. Dagens behandling av förmaksflimmer fokuserar på behandling av symptom, reglering av hjärtfrekvens samt förebyggande av stroke med hjälp av antikoagulation. Tyvärr medför antikoagulantiabehandling en ökad risk för blödningar, i värsta fall hjärnblödning. Dessutom kvarstår en viss risk för stroke och död trotts antikoagulantiabehandling. Det senaste decenniet har det introducerats nya antikoagulantia som har ersatt Waran i de flesta fall då dessa nya antikoagulantia visat sig vara minst lika effektiva på att minska risken för stroke med minskat antal blödningar, inklusive allvarliga sådana. Två av de nya antikoagulanita, Dabigatran (Pradaxa) och Apixaban (Eliquis), jämfördes mot Waran i de stora kliniska studierna RE-LY respektive ARISTOLE som var av en inkluderade drygt 18,000 patienter. Denna avhandling baseras på en del av studiedeltagarna i dessa två studier där blodprover sparades för analyser.

I den kliniska vardagen används enkla riskskattningsinstrument för att hjälpa identifiera vilka patienter med förmaksflimmer som har hög risk för stroke och blödning. Dessa instrument som används idag har stora begränsningar gällande riskskattning på individnivå och den individuella risken för hjärtsvikt och död vid förmaksflimmer tas inte i beaktande. Biomarkörer, i detta fall proteiner i blodet, kan förbättra och individualisera riskstratifiering och i förlängningen behandling vid förmaksflimmer. Biomarkörer kan också förbättra kunskapen om de processer som är involverade i stroke, blödning, hjärtsvikt och död vid förmaksflimmer. Syftet med denna avhandling har därför varit att utvärdera nya och traditionella biomarkörer med kardiovaskulära händelser vid förmaksflimmer. De traditionella biomarkörerna som undersöktes var apolipoprotein A1 och B, som speglar blodfetterna HDL respektive LDL. Deras relation med ett sammanslaget ischemisk utfall (ischemisk stroke, systemisk embolism, hjärtinfarkt och kardiovaskulär död), de individuella komponenterna av det sammanslagna ischemiska utfallet samt blödning och död bland ca 15,000 deltagare i ARISTLOTLE studien (arbete I). Resultatet från arbete I visade att förhöjda nivåer av apolipoprotein A1 var starkt kopplade till lägre risk för det sammanslagna ischemiska utfallet, stroke, systemisk embolism och död. Förhöjda nivåer av Apolipoprotein B var kopplade till ökad risk för hjärtinfarkt och paradoxalt nog var lägre nivåer kopplade till ökad risk för död.

I arbete II utvärderades geografiska skillnader av biomarkören GDF-15, som tidigare visat sig vara bra markör för blödning och död vid förmaksflimmer, samt de biomarkörbaserade riskskattningsinstrumenten ABC bleeding och ABC death score i både RE-LY och ARISTOTLE studien. I detta arbete var GDF-15 och ABC score (death och bleeding) tydligt associerade med blödning och död i alla geografiska regioner. Det fanns ingen signifikant variation i dessa kopplingar till utfall baserat på region. Patienter i Latinamerika hade dock högre dödlighet vid lägre ABC death score värde, vilket kan indikera vissa regionala skillnader. Studien bekräftar att GDF-15 och ABC score på ett tillförlitligt sätt förutsäger komplikationer från förmaksflimmer över hela världen.

I arbete III användes proximity extension assay, en analysteknik som gör det möjligt undersöka en stor mängd proteiner i en liten volym blod, för att hitta biomarkörer kopplade till, och därmed bättre förstå, kardiovaskulär död vid förmaksflimmer. Av totalt 268 biomarkörer var de mekanismerna starkast kopplade till kardiovaskulär död vid förmaksflimmer; hjärt- och njurdysfunktion (NT-proBNP, cTnT-hs, CTSL1, TFF3, FGF-23), oxidativ stress (GDF-15), inflammation (IL-6 och GDF-15), fibrinolys (suPAR) och apoptos (TNFR1, TRAILR2).

I arbete IV studerades på liknande sätt som i arbete III biomarkörer kopplade till sjukhusvård för hjärtsvikt hos patienter med förmaksflimmer från ARISTOTLE studien. Dessutom undersöktes skillnader mellan biomarkörer av de två huvudformerna av hjärtsvikt; hjärtsvikt med bevarad pumpförmåga (heart failure with preserved ejection fraction – HFpEF) och hjärtsvikt med sänkt pumpförmåga (heart failure with reduced ejection fraction – HFrEF) hos patienter med förmaksflimmer. I detta arbete var mekanismerna starkast kopplade till sjukhusvård för hjärtsvikt; hjärt- och njurdysfunktion (NT-proBNP, BNP, cTnT-hs, FGF-23), cellbindning (spon1), tillväxtfaktorer (IGFBP-7), fibrinolys (u-par), cellstruktur (OPN), inflammation (PTX3) och järnmetabolism (TR). HFrEF var starkare kopplat till markörer för hjärt- och njurdysfunktion (NT-proBNP, BNP, cTnT-hs), renin–angiotensin–aldosteron systemet (renin, ACE-2) och oxidative stress/inflammation (GDF-15, IL-6). HFpEF var starkare kopplat till högre fettvävnadsmetabolism (leptin) samt tillväxtfaktorer(SCF).

Sammanfattningsvis studerades biomarkörer där ett flertal visade sig ha en stark koppling till kardiovaskulära utfall vid förmaksflimmer. Resultaten stöder användning av biomarkörer och biomarkörbaserade riskskattningsverktyg vid förmaksflimmer. Vidare identifierades biomarkörer som kan vara involverade i processer som leder till död och försämring av hjärtsvikt.

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