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ORIGINAL ARTICLE
Special populations

NT-proBNP and stem cell factor plasma concentrations are independently associated with cardiovascular outcomes in end-stage renal disease hemodialysis patients

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Aims

End-stage renal disease (ESRD) treated by chronic hemodialysis (HD) is associated with poor cardiovascular (CV) outcomes, with no available evidence-based therapeutics. A multiplexed proteomic approach may identify new pathophysiological pathways associated with CV outcomes, potentially actionable for precision medicine.

Methods and results

The AURORA trial was an international, multicentre, randomized, double-blind trial involving 2776 patients undergoing maintenance HD. Rosuvastatin vs. placebo had no significant effect on the composite primary endpoint of death from CV causes, nonfatal myocardial infarction or nonfatal stroke. We first compared CV risk-matched cases and controls ($n = 410$) to identify novel biomarkers using a multiplex proximity extension immunoassay (276 proteomic biomarkers assessed with Olink™). We replicated our findings in 200 unmatched cases and 200 controls. External validation was conducted from a multicentre real-life Danish cohort [Aarhus-Aalborg (AA), $n = 331$ patients] in which 92 Olink™ biomarkers were assessed. In AURORA, only *N*-terminal pro-brain natriuretic peptide (NT-proBNP, positive association) and stem cell factor (SCF) (negative association) were found consistently associated with the trial's primary outcome across exploration and replication phases, independently from the baseline characteristics. Stem cell factor displayed a lower added predictive ability compared with NT-ProBNP. In the AA cohort, in multivariable analyses, BNP was found significantly associated with major CV events, while higher SCF was associated with less frequent CV deaths.

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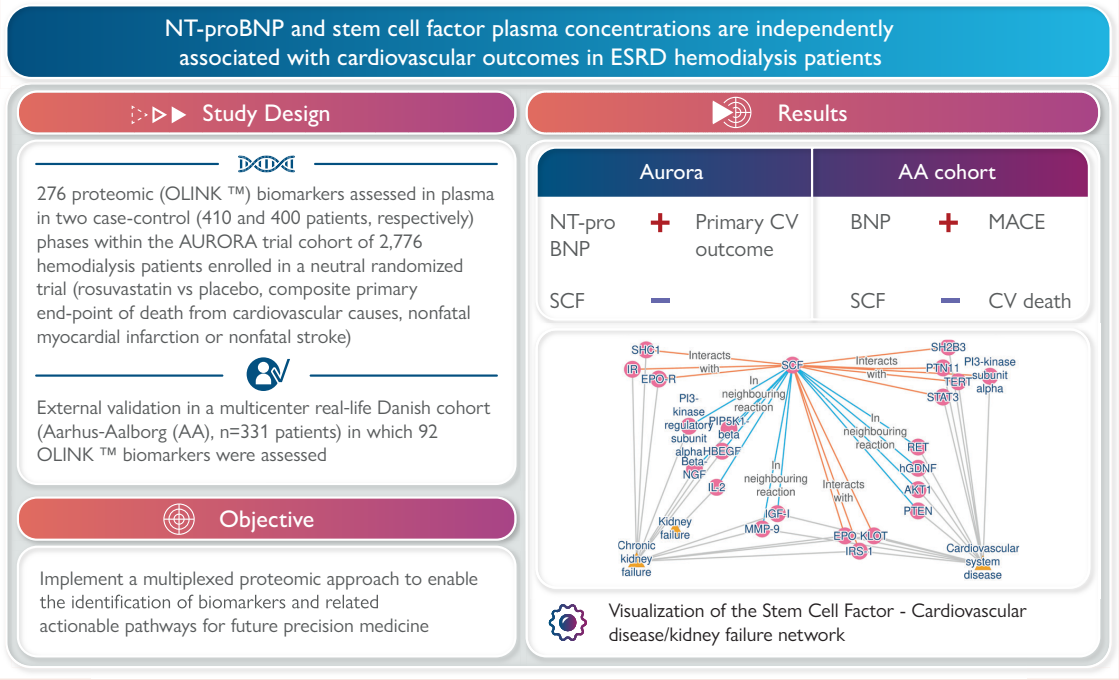
Conclusions Our findings suggest that NT-proBNP and SCF may help identify ESRD patients with respectively high and low CV risk, beyond classical clinical predictors and also point at novel pathways for prevention and treatment.

Graphical Abstract



Keywords Stem cell factor • NT-proBNP • Chronic hemodialysis • Prognosis • Cardiovascular

Highlights

- Of the 256 evaluable proteins (from the three Olink panels ontologically associated with CV diseases and/or inflammation), only two proteins were consistently found associated with the AURORA trial's composite primary endpoint of death from cardiovascular causes, nonfatal myocardial infarction or nonfatal stroke, independent from the clinical and biological features at baseline.
- Increased NT-proBNP was associated with worse outcomes, while increased SCF was associated with better outcomes.
- *N*-terminal pro-brain natriuretic peptide and stem cell factor may help identify ESRD patients with respectively high and low CV risk, beyond classical clinical predictors.

Introduction

Both traditional (Framingham) and non-traditional cardiovascular (CV) risk factors occur in patients with chronic kidney disease. The latter comprise a long list of uraemia-induced changes such as anaemia, inflammation, and disturbances of lipoprotein metabolism, resulting in pathophysiological mechanisms for CV disease, which differ from those in the general population.[1,2] End-stage renal disease (ESRD) is associated with premature CV ageing, and a 3- to 10-fold increased risk of CV events when compared with the general population.[3] Cardiac disease is the leading cause of death among patients with ESRD, representing 42% of all-cause mortality.[4] Despite these alarming observations, there is no proven intervention in ESRD. Patients on hemodialysis (HD) are usually excluded from CV prevention trials.[1,5–7] Where specific interventions have been assessed—such as statin therapy, the results have been neutral.[8,9] However, neutral trials such as the AURORA trial (a Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Hemodialysis: An Assessment of Survival and Cardiovascular Events)[9] have created resources allowing to seek novel risk factors and pathophysiological mechanisms,[1] and to assess the importance of biomarkers that have been proposed as non-traditional risk factors.[2]

In the present study, we have used the AURORA biobank in a comprehensive analysis that includes both established and novel biomarkers in order to seek new pathophysiological pathways associated with CV outcomes. This indeed may inform a future 'pharmacophenomics' approach[10] combining biomarker-guided treatment strategies and individually targeted pharmacological treatments.

Methods

Because of the sensitive nature of the data collected for this study, requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to the corresponding author.

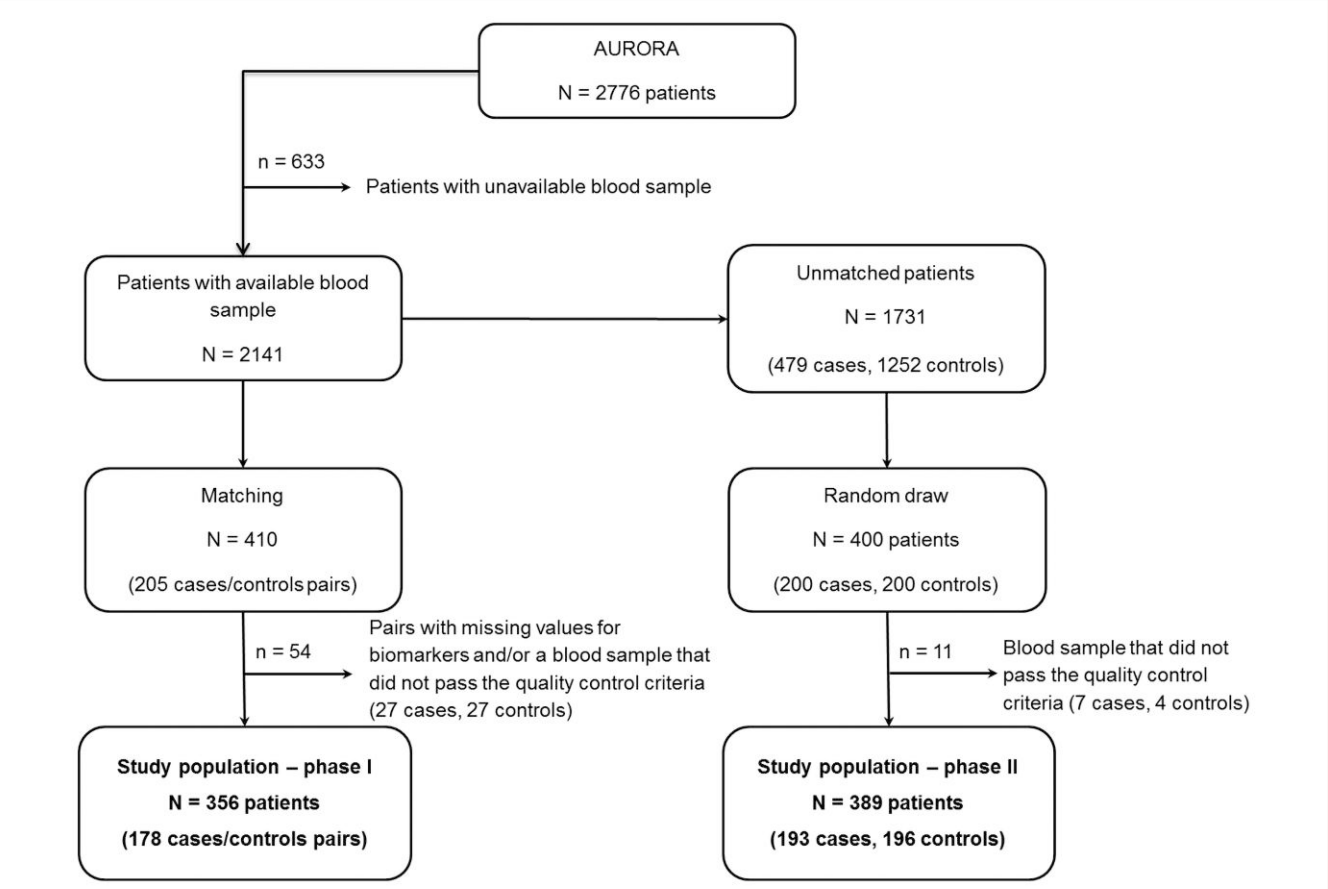


Figure 1 AURORA subcohorts flow-chart.

Study population

The AURORA trial (NCT00240331) was an international, multicentre, randomized, double-blind trial involving 2776 patients, 50–80 years of age, receiving maintenance HD. Patients were randomly assigned to receive rosuvastatin, 10 mg daily, or placebo, and followed-up for a median of 3.8 years. Rosuvastatin had no significant effect on the composite primary endpoint of death from CV causes, nonfatal myocardial infarction or nonfatal stroke.[9]

In the derivation discovery step (Phase I), 205 patients with primary endpoint (cases) were matched with 205 patients without primary endpoint (controls) for age, sex, geographical region, body mass index, KT/V, and AURORA risk score[11] including age, albumin, high sensitivity C-reactive protein (hsCRP), prior CV disease, and diabetes mellitus. Twenty-seven pairs of patients were excluded due to incomplete biomarker data and/or because samples did not pass the quality control for biomarker analyses.

In the replication step (Phase II), 200 cases and 200 controls were randomly drawn in previously unmatched AURORA patients. Eleven patients (seven cases, four controls) were excluded since these proteomic analyses did not pass the quality control (*Figure 1*).

The independent replication analysis used the Aarhus-Aalborg (AA) prospective cohort, which enrolled adult chronic HD patients (age > 18 years) from December 2010 to March 2011 with 5 years of follow-up at five HD units in Jutland, Denmark. After excluding patients with missing or low-quality proteomics protein data, 331 HD patients were included in the final analysis. Two endpoints were considered for this cohort: major adverse CV event (MACE; see supplemental material online for a complete definition of MACE) and CV death.

Biomarker analysis

Three panels, each consisting of 92 protein biomarkers ontologically associated with CV and/or inflammation (CVD II, III, and INF), were assessed using the Olink proximity extension assay (PEA) in EDTA plasma samples stored at −80°C. The result format for Olink PEA Multiplex technology is normalized protein eXpression (NPX). NPX is an arbitrary unit on log2 scale meaning that an increase in one NPX corresponds to a doubling of the concentration. PEA results are reported as relative values; hence, even if two different proteins have the same NPX values, their actual concentration may differ. Quality control was performed using the Olink NPX Manager software. Samples that deviated >0.3 NPX from the median of all samples in one of two control assays for incubation and detection were flagged and excluded from the analysis. In both phases, values below the limit of detection (LOD) were set at the LOD. All biomarkers with more than 25% of values below the LOD in Phase I and/or Phase II were excluded from statistical analyses: 254 biomarkers were ultimately assessed. A complete list of biomarkers measured in each panel is presented in Supplementary material online, *Table S1*.

Statistical analysis

All analyses were performed using R software (the R foundation for Statistical Computing). The two-tailed significance level was set at $P < 0.05$. No adjustment for multiple comparisons was used for the identification of proteins associated with the primary endpoint by a univariate analysis. Continuous variables are described as medians (interquartile range), categorical variables as frequencies (percentages). Comparison of baseline characteristics was carried out using the non-parametric Wilcoxon test for continuous variables and Fisher's exact test or χ^2 test for categorical variables. To assess the association between biomarkers and primary endpoint, time-to-event analyses were performed using a Cox regression model. Standardized hazard ratios (sHR) are presented with their 95% confidence intervals as sHR (CI 95%). sHR represent the increase or

decrease in risk if the variable is increased by 1 SD. For all protein biomarkers, values were reported in NPX unit and sHR were based on NPX data.

A two-step selection [least absolute shrinkage and selection operator (LASSO),[12,13] followed by backward stepwise regression] using the Cox model was implemented in order to identify the biomarkers associated with the risk of primary endpoint.[14] LASSO was used for the selection of a first subset of variables. This method penalizes the sum of the absolute values of the regression coefficients leading to some coefficients shrinking to zero and thus to a concomitantly performed variable selection. LASSO requires the estimation of a tuning parameter which was chosen by cross-validation using the minimization of the partial likelihood deviance as rule. Stepwise backward selection, starting from the subset of LASSO-selected variables, with *P*-to-remove set at 0.05 (based on the Wald test), was used to retain variables that were significantly associated with the risk of primary endpoint. To establish the sensitivity of the variable selection to the cross-validation process, the two-step selection was repeated 1000 times with different cross-validation folds randomly drawn between 3 and 10. All biomarkers selected more than 50% of the time were finally retained. Matching variables were also considered as candidates variables in this two-step selection. All biomarkers and continuous variables used for the matching were modelled as linear in Cox models. Furthermore, missing values were encountered in Phase II for KT/V, body mass index, albumin and hsCRP and given their small number, a simple median imputation was used. As the LASSO approach provides a variable selection based on a repeated procedure with cross-validation, and the biomarkers identified in Phase I were further validated in Phase II, we did not use a false-discovery rate approach. Importantly, the cross-validation procedure and the retention of biomarkers selected more than 50% of the time in the LASSO procedure, limits the likelihood of false discovery. Then, multivariable Cox model was fitted using the subset of variables retained by the 'LASSO/backward' selection process as explanatory variables.

Continuous net reclassification improvement (NRI)[15,16] and integrated discrimination improvement (IDI)[15,16] were calculated to assess the reclassification performance and improvement in discrimination caused by the addition of a biomarker (i) on top of biomarkers/clinical variables retained by the 'LASSO/backward' selection process or (ii) on top of the previously published AURORA risk score (11).

The same methodology was applied in the AA cohort (additional details provided in the supplemental material online), with a single 92 biomarker Olink panel. All data are included in the submission/manuscript file.

Networks

A complex network analysis involving protein–disease and protein–protein relationships is available through the fight heart-failure graph knowlegde box (FHF-GKBox) as a Neo4J graph database where nodes represent entities like proteins or diseases and edges correspond to relationships between these entities. The main goals of the FHF-GKBox are to facilitate contextualization and to allow the grouping of several proteins, either via their direct interactions or through an intermediary, or via the diseases in which they are involved.[17] An easy way to obtain such context is to identify the most direct relationships between a list of proteins and diseases, which correspond to all the shortest paths between the protein and the disease nodes.

For this, FHF-GKBox is composed of data extracted from public databases: 20 214 protein nodes were imported from Uniprot[18] and 26 428 disease nodes from disease ontology (DO).[19] DO is a standardized ontology that aims to provide consistent, reusable and sustainable description of human disease terms and phenotypes. Protein–protein relationships and gene–disease associations were retrieved from STRING (v10.5)[20] or Reactome (release 61)[21] or WikiPathways[22] and DisGenet (19 July 2017)[23] data sources, respectively. Association properties, such as STRING confidence score for protein–protein interactions or DisGenet score for protein–disease associations, were also retrieved and mapped to the corresponding edges.

Disease nodes representing CV diseases and kidney failure were selected from DO.[19] To cover the three studied outcomes with a minimal number of terms and to reduce the graph complexity without losing information, CV diseases were mapped to the unique general term: 'cardiovascular system disease' (DOID:1287) describing diseases which occur in the blood, heart, blood vessels, or the lymphatic system. Kidney failure was mapped to the two terms: 'kidney failure' (DOID:1074) defined in DO as 'a kidney disease characterized by the failure of the kidneys to adequately filter waste products from the blood' and 'chronic kidney failure' (DOID:784) corresponding to 'a kidney failure that is characterized by the gradual loss of kidney function' in DO. It should be noted that the group of genes associated with a group of diseases such as 'cardiovascular system disease' in DisGenet does not encompass all of the genes associated with all of the distinct, more specific CV diseases.

The shortest paths between these disease nodes and the proteins of interest [NPPB—for brain natriuretic peptide or stem cell factor (SCF)] were extracted from FHF-GKBox by the 'allShortestPaths' Neo4J function. Filters were also applied, as follow, on the retrieved edged to ensure their quality:

- Protein—protein relationships have a STRING confidence score >800.
- Gene—disease associations have[24] a DisGenet score >0.001.

Queries on the FHF-GKBox and network visualization were executed with Cytoscape[24] and its cyNeo4j App adapter.[25]

Pathway enrichment

Pathway enrichment for the proteins connecting the diseases to the protein of interest was calculated by the Reactome analysis function.[21–28] A FDR *q*-value threshold of 0.05 was applied to retain only significant enrichment. Pathways annotating more than 500 genes were discarded to avoid the most generic pathways.

Results

Baseline patient characteristics within the AURORA derivation and replication subcohorts are presented in *Table 1*. Per our study design, cases and matched controls in the derivation (Phase I) cohort did not statistically differ. In contrast, in the replication (Phase II) cohort, cases were older and sicker than controls. A total of 254 biomarkers were ultimately detectable. Biomarker concentrations in Phases I—II are presented as online Supplementary material online, *Table S2*.

In univariable analyses (*Figure 2*), 22 biomarkers were found associated in both phases with a *P*-value lower than 5% using a Cox model with the primary outcome of death from CV causes, nonfatal myocardial infarction or nonfatal stroke. Among these, there was a single biomarker with a $P < 0.0001$, namely *N*-terminal pro-brain natriuretic peptide (NT-proBNP); two biomarkers with $P < 0.001$: BNP and angiotensin-converting enzyme 2 ; four biomarkers with $P < 0.01$: SCF, interleukin-10 (IL-10), growth/differentiation factor 15, and CUB domain-containing protein 1; and 15 biomarkers with $P < 0.05$.

In the derivation subcohort, five biomarkers (SCF, VSIG2, CCL22, NT-proBNP, and IL-10) were selected from the 'LASSO + backward' selection, whereas five biomarkers (SCF, HSP 27, OPN, NT-proBNP, and LIR-R) and three clinical variables (age, hsCRP, and CV disease) were selected in the replication subcohort. In multivariable models fitted on these selected subsets of variables, only higher NT-proBNP (positive association) and lower SCF (negative association) concentrations were found consistently associated with the trial primary outcome across the two phases, independent from the clinical and biological features at baseline (*Table 2*).

With regard to the added predictive ability of NT-proBNP (on top of the other retained biomarkers or conditions), the NRI and IDI were, respectively, 21.4 (6.0–30.7), $P < 0.0001$ and 3.8 (1.1–7.7), $P < 0.0001$ in Phase I and 19.2 (8.4–30.5), $P < 0.0001$ and 3.6 (0.7–7.1), $P < 0.0001$ in Phase II. The added predictive ability of SCF (on top of the other retained biomarkers or conditions) was lower, with the NRI and IDI being 9.3 (−0.6 to 21.3), $P = 0.053$ and 2.2 (0.2–4.9), $P = 0.020$ in Phase I and 15.0 (−4.3 to 26.3), $P = 0.086$ and 1.5 (−0.1 to 4.6), $P = 0.066$ in Phase II, respectively. Consistent findings were observed on top of the AURORA risk score (see Supplementary material online, *Table S3*).

Table 1 Comparison of matching variables between cases and controls within each study phase (derivation and replication) and between the two phases

	Phase I—Derivation subcohort				Phase II—Replication subcohort				*P*-value		
	n	Global (*n* = 356)	Controls (*n* = 178)	Cases (*n* = 178)	*n*	Global (*n* = 389)	Controls (*n* = 196)	Cases (*n* = 193)	cases vs. controls Phase I	cases vs. controls Phase II	Phase I vs. Phase II
Age (years)	356	67 (59–74)	67 (59–74)	67 (59–74)	389	65 (57–72)	62 (55–70)	67 (59–74)	0.96	**<0.0001**	**0.017**
Sex	356				389				1.00	1.00	**<0.0001**
Male		282 (79.2%)	141 (79.2%)	141 (79.2%)		225 (57.8%)	113 (57.7%)	112 (58.0%)			
Female		74 (20.8%)	37 (20.8%)	37 (20.8%)		164 (42.2%)	83 (42.3%)	81 (42.0%)			
Region	356				389				1.00	**0.0006**	**<0.0001**
Western Europe		220 (61.8%)	110 (61.8%)	110 (61.8%)		207 (53.2%)	113 (57.7%)	94 (48.7%)			
Eastern Europe		108 (30.3%)	54 (30.3%)	54 (30.3%)		79 (20.3%)	35 (17.9%)	44 (22.8%)			
South America		4 (1.1%)	2 (1.1%)	2 (1.1%)		36 (9.3%)	18 (9.2%)	18 (9.3%)			
Asia		0 (0.0%)	0 (0.0%)	0 (0.0%)		16 (4.1%)	14 (7.1%)	2 (1.0%)			
Other		24 (6.7%)	12 (6.7%)	12 (6.7%)		51 (13.1%)	16 (8.2%)	35 (18.1%)			
Caucasian	356	340 (95.5%)	169 (94.9%)	171 (96.1%)	389	333 (85.6%)	160 (81.6%)	173 (89.6%)	0.80	**0.030**	**<0.0001**
Body mass index (kg/m^2)	356	24.9 (22.7–27.0)	25.0 (22.7–26.9)	24.8 (22.8–27.2)	383	24.4 (21.8–28.1)	24.2 (21.9–27.7)	24.9 (21.6–28.9)	0.86	0.33	0.68
Systolic BP (mmHg)	355	140 (123–155)	135 (120–150)	142 (130–160)	389	140 (120–150)	134 (120–150)	140 (126–154)	**0.003**	**0.019**	0.46
Diastolic BP (mmHg)	355	80 (70–85)	80 (70–84)	80 (70–86)	389	78 (68–84)	79 (68–84)	76 (67–82)	0.76	0.32	0.17
Current smoker	356	62 (17.4%)	23 (12.9%)	39 (21.9%)	389	62 (15.9%)	31 (15.8%)	31 (16.1%)	**0.035**	1.00	0.62
KT/V	356	1.10 (0.98–1.26)	1.09 (0.97–1.27)	1.13 (0.99–1.25)	370	1.22 (1.04–1.38)	1.23 (1.01–1.40)	1.21 (1.05–1.36)	0.34	0.51	**<0.0001**
Total Cholesterol (mg/dL)	355	171 (145–199)	171 (147–198)	173 (145–199)	385	169 (146–200)	171 (146–200)	165 (145–200)	1.00	0.56	0.72
LDL Cholesterol (mg/dL)	355	98 (78–123)	97 (78–119)	99 (75–123)	385	97 (74–121)	98 (75–120)	94 (73–121)	0.81	0.77	0.47
HDL Cholesterol (mg/dL)	355	42 (35–53)	43 (35–51)	41 (35–53)	385	43 (35–53)	44 (35–55)	41 (35–53)	0.72	0.24	0.26
Triglycerides (mg/dL)	355	124 (92–185)	125 (93–185)	124 (91–185)	385	119 (92–171)	121 (92–173)	118 (91–168)	0.71	0.99	0.32
hsCRP (mg/L)	356	6.4 (2.8–17.5)	6.8 (2.6–19.4)	6.2 (2.9–15.4)	384	4.7 (1.7–14.9)	4.0 (1.4–9.0)	5.9 (2.1–19.9)	0.71	**0.001**	**0.002**
Hemoglobin (g/dL)	335	11.9 (10.9–12.8)	11.8 (11.0–12.8)	11.9 (10.9–12.7)	373	11.6 (10.4–12.6)	11.6 (10.4–12.6)	11.6 (10.4–12.6)	0.67	0.77	**0.016**
Albumin (g/L)	356	40 (37–41)	39 (37–41)	40 (38–42)	382	40 (37–42)	40 (38–42)	39 (37–41)	0.074	**0.003**	0.78
Calcium (mg/dL)	356	9.2 (8.9–9.7)	9.2 (8.8–9.7)	9.2 (8.9–9.7)	382	9.3 (8.8–9.9)	9.3 (8.8–9.9)	9.3 (8.7–9.8)	0.94	0.45	0.75
Phosphate (mg/dL)	356	5.5 (4.6–6.6)	5.3 (4.5–6.3)	5.7 (4.7–7.1)	382	5.3 (4.3–6.4)	5.3 (4.3–6.2)	5.4 (4.4–6.5)	**0.003**	0.46	0.052
Duration of treatment with hemodialysis (years)	356	2.3 (0.9–4.5)	1.9 (0.9–3.8)	2.8 (0.9–5.2)	389	2.1 (1.1–4.6)	2.1 (1.0–4.5)	2.2 (1.1–4.7)	0.11	0.69	0.69
Duration of dialysis sessions	355				389				0.67	0.60	0.12
<12 h/week		67 (18.9%)	32 (18.0%)	35 (19.8%)		93 (23.9%)	51 (26.0%)	42 (21.8%)			
12 h/week		221 (62.3%)	115 (64.6%)	106 (59.9%)		214 (55.0%)	104 (53.1%)	110 (57.0%)			
>12 h/week		67 (18.9%)	31 (17.4%)	36 (20.3%)		82 (21.1%)	41 (20.9%)	41 (21.2%)			
Cause of end-stage renal disease	356				389				**0.003**	0.082	**0.005**
Nephrosclerosis		86 (24.2%)	40 (22.5%)	46 (25.8%)		67 (17.2%)	35 (17.9%)	32 (16.6%)			

Continued

Table 1 *Continued*

	Phase I—Derivation subcohort				Phase II—Replication subcohort				*P*-value		
	n	Global (*n* = 356)	Controls (*n* = 178)	Cases (*n* = 178)	*n*	Global (*n* = 389)	Controls (*n* = 196)	Cases (*n* = 193)	cases vs. controls Phase I	cases vs. controls Phase II	Phase I vs. Phase II
Glomerulonephritis or vasculitis		61 (17.1%)	26 (14.6%)	35 (19.7%)		71 (18.3%)	39 (19.9%)	32 (16.6%)			
Diabetes		46 (12.9%)	24 (13.5%)	22 (12.4%)		88 (22.6%)	35 (17.9%)	53 (27.5%)			
Tubulointerstitial disease		54 (15.2%)	18 (10.1%)	36 (20.2%)		63 (16.2%)	38 (19.4%)	25 (13.0%)			
Hereditary		48 (13.5%)	34 (19.1%)	14 (7.9%)		51 (13.1%)	29 (14.8%)	22 (11.4%)			
Other		61 (17.1%)	36 (20.2%)	25 (14.0%)		49 (12.6%)	20 (10.2%)	29 (15.0%)			
Diabetes	356	73 (20.5%)	35 (19.7%)	38 (21.3%)	389	117 (30.1%)	47 (24.0%)	70 (36.3%)	0.79	**0.011**	**0.003**
CV disease	356	177 (49.7%)	85 (47.8%)	92 (51.7%)	389	163 (41.9%)	64 (32.7%)	99 (51.3%)	0.52	**0.0002**	**0.033**
Myocardial infarction	356	57 (16.0%)	25 (14.0%)	32 (18.0%)	389	45 (11.6%)	18 (9.2%)	27 (14.0%)	0.39	0.16	0.088
Coronary revascularization	356	31 (8.7%)	11 (6.2%)	20 (11.2%)	389	24 (6.2%)	13 (6.6%)	11 (5.7%)	0.13	0.83	0.21
Peripheral vascular disease	356	59 (16.6%)	30 (16.9%)	29 (16.3%)	389	55 (14.1%)	16 (8.2%)	39 (20.2%)	1.00	**0.0007**	0.36
Congestive HF	356	27 (7.6%)	9 (5.1%)	18 (10.1%)	389	30 (7.7%)	14 (7.1%)	16 (8.3%)	0.11	**0.71**	1.00
Rosuvastatin	356	178 (50.0%)	92 (51.7%)	86 (48.3%)	389	196 (50.4%)	93 (47.4%)	103 (53.4%)	0.60	0.27	0.94
ACEI/ARB	356	112 (31.5%)	54 (30.3%)	58 (32.6%)	389	149 (38.3%)	67 (34.2%)	82 (42.5%)	0.73	0.096	0.055
Calcium-channel blocker	356	134 (37.6%)	58 (32.6%)	76 (42.7%)	389	136 (35.0%)	62 (31.6%)	74 (38.3%)	0.063	0.17	0.49
Beta-blocker	356	135 (37.9%)	73 (41.0%)	62 (34.8%)	389	143 (36.8%)	68 (34.7%)	75 (38.9%)	0.27	0.40	0.76
Diuretic	356	105 (29.5%)	46 (25.8%)	59 (33.1%)	389	128 (32.9%)	59 (30.1%)	69 (35.8%)	0.16	0.28	0.34
Platelet inhibitor	356	151 (42.4%)	71 (39.9%)	80 (44.9%)	389	179 (46.0%)	90 (45.9%)	89 (46.1%)	0.39	1.00	0.34
Vitamin D	356	173 (48.6%)	88 (49.4%)	85 (47.8%)	389	199 (51.2%)	96 (49.0%)	103 (53.4%)	0.83	0.42	0.51
Calcium substitution	356	265 (74.4%)	129 (72.5%)	136 (76.4%)	389	284 (73.0%)	148 (75.5%)	136 (70.5%)	0.47	0.30	0.68
Sevelamer	356	90 (25.3%)	44 (24.7%)	46 (25.8%)	389	104 (26.7%)	54 (27.6%)	50 (25.9%)	0.90	0.73	0.68
Erythropoietin	356	313 (87.9%)	149 (83.7%)	164 (92.1%)	389	343 (88.2%)	178 (90.8%)	165 (85.5%)	**0.022**	0.12	1.00

n: number of data available.
ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BP, blood pressure; CV, cardiovascular; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein.
Bold *P*-values denote statistical significance at the $P < 0.05$ level.

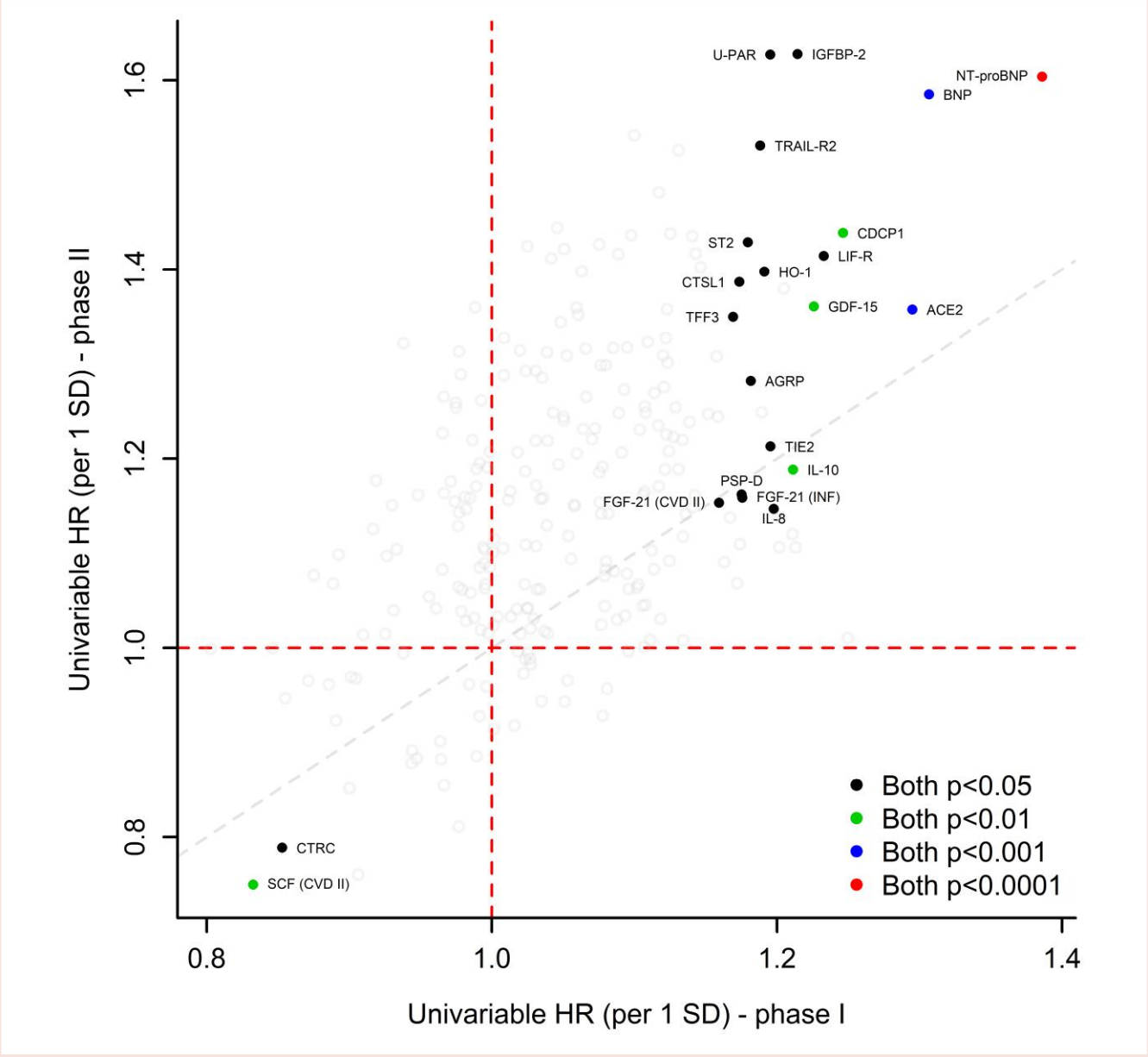


Figure 2 Comparison of univariable hazard ratios in Phase I and Phase II (AURORA).

The univariable clinical biological correlates of NT-proBNP and SCF are presented as online Supplementary material online, *Tables S4 and S5*.NT-proBNP was negatively correlated with higher BMI, albumin, and Hb, and was positively correlated with higher SBP and CRP in both phases. SCF was positively correlated with higher albumin in both phases.

External replication

The list of biomarkers measured in the AA cohort is available in Supplementary material online, *Table S6*. The global as well as subdivided baseline AA cohort characteristics according to the studied outcomes are presented in Supplementary material online, *Table S7*. In multivariable analyses (see Supplementary material online, *Table S8*), BNP was found significantly associated with MACE [sHR = 1.53 (1.26–1.86), $P < 0.0001$], while higher SCF was associated with less frequent CV deaths [sHR = 0.76 (0.60–0.96), $P = 0.022$].

Complex network approach

Based on existing knowledge on pathways and protein–protein interactions, the shortest paths between SCF and CV diseases on the one hand and kidney failure or chronic kidney failure on the other are presented in *Figure 3A*. Thirteen proteins acted as direct intermediates between SCF and kidney failure or chronic kidney failure, while 14 were found between SCF and CV diseases. Among these proteins, five were found to bridge SCF with both chronic kidney failure and CV diseases. Pathway enrichment analysis on this set of 22 intermediate proteins related to the SCF network revealed that most of these proteins were elements of signalling pathways (see Supplementary material online, *Table S9*).

Table 2 Multivariable Cox models using the variables identified in each phase

	Variables	Cox model in Phase I		Cox model in Phase II	
		sHR (95% CI)	*P*-value	sHR (95% CI)	*P*-value
Biomarkers identified in Phase I	SCF (CVD II)	0.79 (0.68–0.91)	**0.002**	0.82 (0.71–0.94)	**0.005**
	VSIG2	1.32 (1.13–1.54)	**0.0004**	0.88 (0.75–1.03)	0.11
	CCL22	0.78 (0.66–0.92)	**0.004**	1.03 (0.89–1.19)	0.73
	NT-proBNP	1.34 (1.15–1.56)	**0.0002**	1.61 (1.39–1.86)	**<0.0001**
	IL-10	1.20 (1.03–1.40)	**0.017**	1.21 (1.05–1.38)	**0.007**
Biomarkers identified in Phase II	SCF (CVD II)	0.85 (0.74–0.98)	**0.027**	0.83 (0.72–0.96)	**0.012**
	HSP 27	1.02 (0.87–1.19)	0.84	1.20 (1.02–1.41)	**0.024**
	OPN	1.05 (0.90–1.23)	0.54	1.34 (1.12–1.60)	**0.001**
	NT-proBNP	1.31 (1.11–1.54)	**0.002**	1.42 (1.22–1.64)	**<0.0001**
	LIF-R	1.13 (0.96–1.34)	0.14	1.25 (1.07–1.46)	**0.005**
	Age (years)	1.05 (0.90–1.23)	0.52	1.33 (1.14–1.55)	**0.0004**
	hsCRP (mg/L)	0.92 (0.79–1.08)	0.30	1.17 (1.03–1.34)	**0.020**
	Cardiovascular disease	0.99 (0.85–1.16)	0.92	1.18 (1.02–1.37)	**0.026**

sHR, standardized hazard ratio; CI, confidence interval.
CCL22, C-C motif chemokine 22; hsCRP, high-sensitivity C-reactive protein; HSP 27, heat shock 27 kDa protein; IL-10: Interleukin-10; LIF-R, leukaemia inhibitory factor receptor; NT-proBNP, *N*-terminal prohormone brain natriuretic peptide; OPN, Osteopontin; SCF, stem cell factor; VSIG2, V-set and immunoglobulin domain-containing protein 2.
Bold *P*-values denote statistical significance at the $P < 0.05$ level.

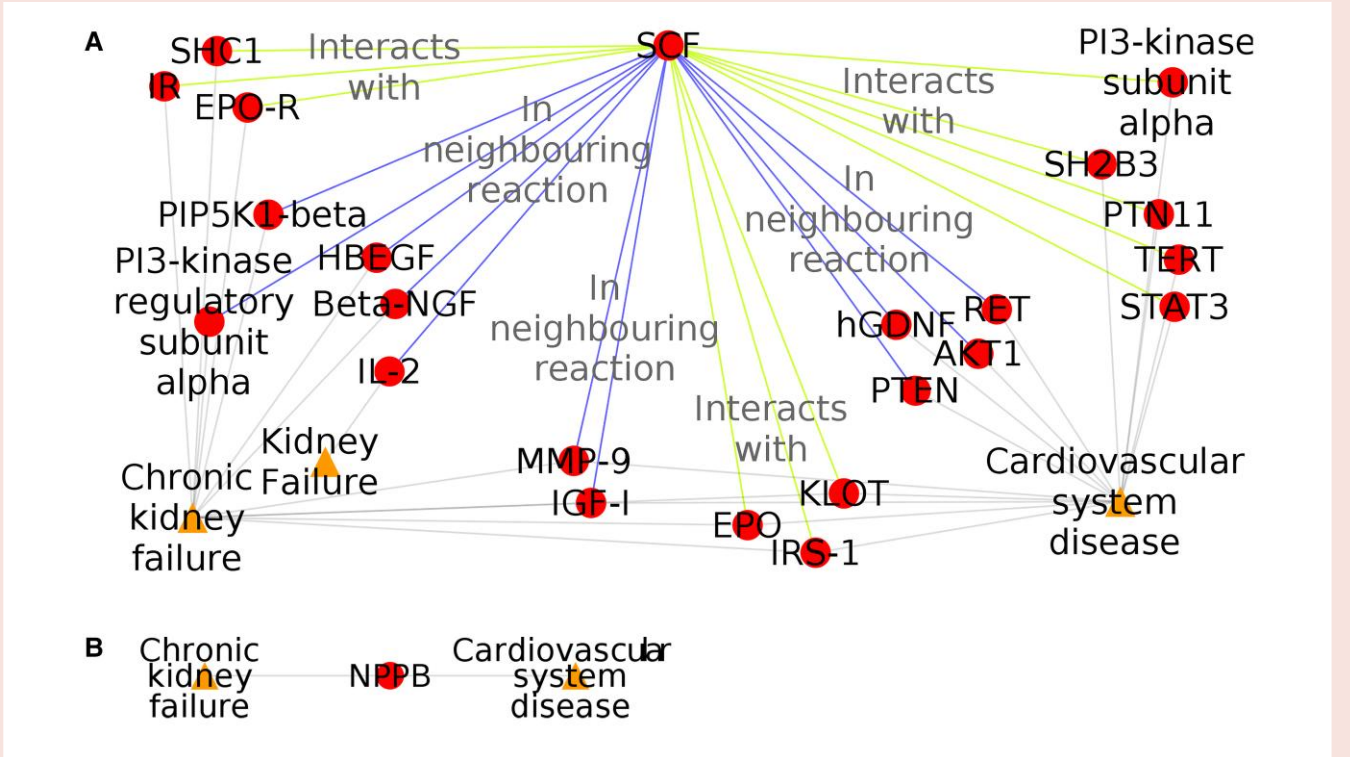


Figure 3 Visualization of the SCF—cardiovascular disease/kidney failure network. The shortest paths between SCF and cardiovascular diseases on the one hand and between SCF and kidney failure or chronic kidney failure on the other were retrieved from the FHF-GKBox). Triangles: disease nodes, circles: protein nodes, also representing the genes encoding the proteins. Disease–protein relationships are derived from disease-gene associations present in the DisGenet database. Relationships between red nodes represent protein–protein interactions derived from the STRING database (in green) or protein vicinity in signalling pathways derived from the Reactome database (in blue).

As a comparison, using the same methodological approach, the natriuretic peptide precursor (NPPB) was found directly associated with both kidney failure and CV diseases (*Figure 3B*).

Discussion

Of the 256 evaluable proteins (from the three Olink panels ontologically associated with CV diseases and/or inflammation), only two proteins were consistently found associated with the AURORA trial's composite primary endpoint of death from CV causes, nonfatal myocardial infarction or nonfatal stroke, independent from the clinical, and biological features at baseline. Increased NT-proBNP was associated with worse outcomes, while increased SCF was associated with better outcomes. These results obtained within a randomized clinical trial cohort population were replicated in a prospective real-life (AA) cohort of chronic HD patients. Indeed, in multivariable analyses, using the same statistical approach on a more limited set of biomarkers ($n = 92$) including NT-proBNP and SCF measured with the same methodology (Olink), BNP was found significantly associated with MACE while higher SCF was associated with less frequent CV deaths in these chronic HD patients. Importantly, we analysed the links between proteins using complex network. For SCF, this approach provided additional insights regarding the biological mechanisms underlying our results. But yet, no external biological nodes were identified using this refined approach for BNP.

In the present novel and innovative approach using proteomic profiling (256 proteins), elevated NT-proBNP was found to be the biomarker most associated with worse CV outcomes in a chronic HD population, and furthermore significantly improved the prognostic ability of the multivariable model, independently from clinical and biological baseline features.

The observation that NT-proBNP is a predictive factor for CV events in a chronic HD population was not unexpected. In our complex network analysis, based on available knowledge, a direct relationship between natriuretic peptides, CV diseases and kidney failure was shown. In a systematic review and metaanalysis of 23 studies ($n = 86\,915$ patients) including at least one of the following factors: age, sex, diabetes mellitus, BMI, previous CV disease, duration of HD, haemoglobin, albumin, white blood cells, C-reactive protein (CRP), parathyroid hormone (PTH), total iron binding capacity (TIBC), iron, ln ferritin, adiponectin, apolipoprotein A1 (ApoA1), ApoA2, ApoA3, HDL, total cholesterol (TC), haemoglobin A1c (HbA1c), serum phosphate, troponin T, and BNP, multiple markers and factors were found to be associated with the risk of mortality and cardiac death in patients undergoing HD. In particular, BNP was found to be associated with all-cause-mortality (RR: 1.99; 95% CI: 1.35–2.94; $P = 0.001$) where the association with cardiac death was not assessed.[29] More recently, a dedicated systematic review and a metanaalysis of 61 studies ($n = 19\,688$ people) identified ESRD-specific NT-proBNP and BNP level thresholds of elevation associated with increased risk for CV and all-cause mortality.[30] Our findings confirm the importance of (NT-pro) BNP and may have pathophysiological and potentially therapeutic implications. A minority of the AURORA population was enrolled with a history of congestive heart failure (7.6% in the derivation subcohort, 7.7% in the replication subcohort). Therefore, one may speculate whether the observed association was due to undiagnosed heart failure (a diagnosis that warrants, according to the Universal definition of Heart Failure,[31] the association of symptoms and/or signs of heart failure caused by a structural and or functional cardiac abnormality corroborated by at least one of the following: objective evidence of cardiogenic pulmonary or systemic congestion or elevated natriuretic peptides—while kidney disease is listed among the causes of elevated natriuretic peptide levels other than the primary diagnosis of heart failure) and/or to volume overload independent from heart failure. Importantly, no echocardiographic data were collected during the AURORA CV outcome trial. Volume overload is known to be associated with dismal outcomes in patients with ESRD receiving HD, as shown by the association between pulmonary congestion (as assessed by lung ultrasound) and death or cardiac events.[32] In a prospective cohort of 113 unselected chronic HD patients, an annual increase in BNP above 40% predicted all-cause and cardiac death in the subsequent year. In this latter survey, BNP increases did not reflect changes in markers of hypervolemia (e.g. body weight, interdialytic weight gain, and systolic blood pressure).[33] In contrast, in a retrospective cohort of 236 chronic HD patients, the observed BNP decrease in the first months of HD therapy was related to fluid excess correction.[34] More recently, Arrigo *et al.*[35] combined sCD146 (released from endothelial cells upon mechanical stress and considered as a biomarker of systemic congestion, independent from cardiac function) with BNP and echocardiographic data in chronic HD patients. The authors showed that over hydration (as determined by Body Composition Measurement by bioimpedance), systemic congestion and cardiac dysfunction did not necessarily coexist in a prospective cohort of 144 HD patients. Furthermore, cardiac systolic dysfunction and not systemic congestion *per se* was associated with high all-cause mortality.

It has been suggested that vascular progenitor cells may play a role in vascular repair and protection against CV diseases.[36] SCF is a dimeric molecule that exerts its biological functions by binding to the receptor tyrosine kinase c-Kit.[37] c-Kit has a number of functions in the CV system under normal and pathological conditions. It plays a role in vasculogenesis and may also be involved in atherosclerosis[37] and myocardial remodelling after myocardial infarction.[38] The local injection of SCF into the peri-infarct zone in mice directed significantly more c-Kit + stem cells of exogenous origin to the infarcted heart compared with control mice without SCF injection.[38] Mice with c-Kit dysfunction developed heart failure after myocardial infarction while bone marrow transplantation rescued the failing cardiac phenotype.[37,39] Neural stem/progenitor cells are known to migrate to sites of pathological insult such as various types of brain injury (i.e. ischaemia and blunt trauma) and tumours. Their migration towards damaged central nervous system tissue may represent an adaptive response for the purpose of limiting and/or repairing damage.[40] Interestingly, recombinant SCF has been reported to induce potent neural stem/progenitor cell migration both *in vitro* and *in vivo* through the activation of c-Kit in these cells.[40] SCF dose-dependently has also been found to promote survival, migration and capillary tube formation of human umbilical vein endothelial cells.[41] Furthermore, the transcription of c-Kit mRNA and the expression of c-Kit protein by vascular smooth muscle cells were found significantly upregulated in response to apoptotic stimulation. The increased c-Kit expression on VSMCs not only helped SCF exert its effects through protecting VSMCs from apoptosis and increasing VSMC proliferation but also facilitated the homing process of SCF-positive cells, which contributed to intimal hyperplasia after an experimental injury.[42]

A low SCF content in human carotid atherosclerotic plaques was found associated with less stable plaques, as determined by the amount of elastin and collagen.[36] The present finding of increased plasma concentrations of SCF being associated with better CV outcomes has never been previously reported in chronic HD patients, despite its biological plausibility strengthened by our complex network analysis, which identified biological several pathways potentially contributing to this observed association. That several pathways were engaged may have actually contributed to the lower added predictive ability of SCF (on top of the other retained biomarkers or conditions) compared with NT-ProBNP, which was found directly associated with both kidney failure and CV diseases by network analyses.

Of note, the complex analysis performed in this study did not reveal many novel markers that specifically characterize the ESRD patient with a particularly high risk. This might be taken as a possible indication for the fact that the condition 'ESRD' may not fundamentally and principally differ (in biological terms) from other advanced systemic diseases.

Our findings within the AURORA clinical population were confirmed in an independent 'real-life' setting of chronic HD patients. Importantly, within a Swedish population-based study, a prospective, nested case—

control study showed that a low SCF concentration was associated with increased carotid intima-media thickness, a surrogate marker of atherosclerosis, and a higher incidence of CV events.[36] From the same cohort, it was recently reported that patients with high plasma levels of SCF had a lower risk of development of both CV and all-cause mortality, as well as a lower risk of developing myocardial infarction, stroke, and heart failure. These associations (except for myocardial infarction) persisted after adjustment for age, sex, LDL and HDL cholesterol triglycerides, glucose, CRP, and systolic blood pressure.[43] In this latter population study, as in the present AURORA cohort, there was a negative association between SCF concentration and CRP concentrations, which may suggest that inflammation is a negative regulator of SCF.[43]

Limitations

We acknowledge that the present data are observational and do not establish a causal relationship, and that the analysis of biological biomarkers as continuous variables may have missed non-linear associations with clinical outcomes. We only had baseline protein measurement available within the AURORA trial, which prevented any analysis regarding longitudinal changes. In addition, it should be noted that the sample size of cohorts was relatively small for a proteomic discovery study. Furthermore, in this manuscript, we intended to identify biomarkers with risk-stratification properties. Other approaches, especially machine learning approaches, could provide additional results, targeting to a better biological understanding of underlying pathways involved in a given medical condition. Of note, clustering methods can identify phenotypes with homogeneous characteristics, including biological features. Such approaches have not been undertaken in the field of ESRD and should be targeted in future research projects. The latter should ideally encompass broader chronic kidney disease populations, in order to determine whether our findings are specific to ESRD. Finally, we used the primary endpoint of the AURORA trial as outcome and this did not include readmission for heart failure. Furthermore, heart failure was not specifically available as a cause of death diagnosis within the database.

Conclusion

However, our findings, supported by a robust design methodology (prospective cohorts, two-step derivation-replication, followed by external replication and complex network analysis to ascertain biological plausibility), robustly suggest that NT-pro-BNP and SCF may help identify ESRD patients with respectively high and low CV risk, beyond classical clinical predictors. These findings furthermore point to excess congestion/myocardial stretch and deficient stem cell factor as potential therapeutic targets in future ESRD CV prevention trials.

Lead author biography

Patrick Rossignol, MD, PhD, is a Nephrologist and Vascular medicine specialist, ESC/ESH certified hypertension specialist, Professor of Therapeutics at the University of Lorraine, France. He used to lead the Nancy University Hospital Inserm Plurithematic Clinical Investigation Centre. Since 2022 he is now heading the Medical specialties and Nephrology-hemodialysis department at the Princess Grace Hospital, and the Monaco Private hemodialysis Centre in Monaco. He is coordinating the French multidisciplinary network F-CRIN (French Clinical research Infrastructure Network): Cardiovascular and Renal Clinical Trialists (INI-CRCT) www.inicrct.fr. He is mainly involved in clinical trials, in heart failure, hypertension, and chronic kidney disease. Hyperkalemia is one of his main areas of interest (https://expertscape.com/ex/hyperkalemia). He has published more than 400 peer review publications and is the co-founder of the SME CardioRenal.

Data availability

Because of the sensitive nature of the data collected for this study, requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to the corresponding author.

Supplemental materials

Expanded methods Supplementary material online, Tables *S1–S9*.

Supplementary material

Supplementary material is available at *European Heart Journal Open* online.

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