Supplementary material

Urinary proteomic signature in acute decompensated heart failure: advances into molecular pathophysiology

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Supplementary Table S1: Clinical characteristics and risk factors of all patients.

	All patients N=67	NRF N=35	RD N=32	P-value
DEMOGRAPHIC CHARACTERISTICS				
Female/male, n	22/45	10/25	12/20	0.603
Age, years	71.0 [65.0-77.0]	69.0 [58.0-75.0]	74.0 [69.5-77.5]	**0.008**
Weight, kg	73.0 [61.6-86.8]	70 [61.2-86.6]	77.2 [62.0-88.6]	0.543
KIDNEY FUNCTION MARKERS				
Creatinine, μmol/L	105.0 [78.0-147.0]	78.0 [67.0-97.0]	147.0 [122.5-194.0]	**<0.001**
Glomerular filtration (MDRD-4)[a]	61.0 [40.9-83.3]	83.0 [68.9-99.0]	39.7 [31.3-45.0]	**<0.001**
Urea, mmol/L	10.2 [6.8-16.1]	7.0 [5.8-9.2]	16.5 [13.0-22.9]	**<0.001**
CARDIAC FUNCTION MARKERS				
NT-proBNP, ng/L	4.0 [2.3-8.6]	3.2 [1.9-6.4]	4.5 [2.8-14.7]	**0.026**
Left ventricular ejection fraction (LVEF), %	45.0 [33.0-58.0]	38.0 [33.0-57.0]	51.0 [35.5-59.5]	0.225
Preserved LVEF, N (%)	28 (42)	11 (31)	17 (53)	
Reduced LVEF, N (%)	27 (40)	18 (52)	9 (28)	0.125
Mid-range LVEF, N (%)	12 (18)	6 (17)	6 (19)	
Atrial fibrillation, N (%)	32 (48)	16 (47)	16 (50)	>0.999
Cardiovascular disease, N (%)	23 (34)	11 (31)	12 (38)	0.618
OTHER BIOCHEMICAL MARKERS				
Haemoglobin, g/L	122 [101-138]	128 [114-142]	110 [95-124]	**0.004**
RISK FACTORS; N (%)				
Active smoking	10 (15)	8 (23)	2 (6)	0.086
Hypertension	49 (74)	21 (62)	28 (88)	**0.024**
Pulmonary hypertension	16 (24)	8 (23)	8 (25)	>0.999
Diabetes mellitus type 2	30 (45)	11 (32)	19 (59)	**0.047**
Dyslipidaemia	46 (70)	20 (59)	26 (81)	0.063
BACKGROUND MEDICATION; N (%)				
Diuretics	49 (73)	21 (60)	28 (88)	**0.014**
Statins	43 (64)	18 (51)	25 (78)	**0.040**
Anticoagulants	27 (40)	14 (40)	13 (41)	>0.999
Antiplatelet agents	38 (57)	19 (54)	19 (59)	0.806
Beta-blockers	46 (69)	21 (60)	25 (78)	0.124
Antiarrhythmic agents	7 (10)	4 (11)	3 (9)	>0.999
Antidiabetics	27 (40)	8 (26)	19 (59)	**0.003**
Insulin	12 (18)	2 (6)	10 (31)	**0.010**
Oral antidiabetic agents	22 (33)	8 (23)	14 (44)	0.117
ACE inhibitor/ARB	43 (66)	26 (74)	17 (57)	0.189

[a]MDRD-4 levels expressed in mL/min/1.73m^2; Quantitative values were given in median [Q1-Q3]. P values of categorical variables were calculated with Fisher exact test, except for LVEF where χ^2 was used. P values of numerical data were calculated with Mann-Whitney, except for LVEF where Kruskall-Wallis was used. Diuretics: hydrochlorothiazide, furosemide, eplerenone, and spironolactone. Statins: atorvastatin, pravastatin, simvastatin, ezetimibe. Anticoagulants: warfarin, acenocumarol, bemiparin, heparin, dabigatran, rivaroxaban, edoxaban, and apixaban. Antiplatelet agents: acetylsalicylic acid and clopidogrel. Beta-blockers: bisoprolol and carvedilol. Antiarrhythmic agents: amiodarone. Oral antidiabetic agents: metformin and repaglinide. Angiotensin-converting enzyme inhibitors (ACEI) include: captopril, enalapril, and ramipril. Angiotensin receptor blockers (ARB): losartan, olmesartan, and valsartan.

Supplementary Table S2: Clinical characteristics and risk factors of discovery phase patients.

	2DE-MS group N=17	All patients N=67	P value
DEMOGRAPHIC CHARACTERISTICS			
Female/male, n	3/14	22/45	0.565
Age, years	72 [69-76]	71 [65-77]	0.624
Weight, kg	76 [66-87]	73 [62-89]	0.583
KIDNEY FUNCTION MARKERS			
Creatinine, μmol/L	101 [73-131]	105.0 [78.0-147.0]	0.693
Glomerular filtration (MDRD-4)[a]	68 [43.2-81.7]	61.0 [40.9-83.3]	0.476
Urea, mmol/L	9.0 [5.8-13.1]	10.2 [6.8-16.1]	0.367
CARDIAC FUNCTION MARKERS			
NT-proBNP, μg/L	2.4 [1.7-4.6]	4.0 [2.3-8.6]	0.160
Left ventricular ejection fraction (LVEF), %	48 [33-56]	45 [33-58]	0.738
Preserved LVEF >50%, N (%)	8 (47)	28 (42)	
Mildly reduced LVEF 40-49%, N (%)	3 (18)	12 (18)	0.916
Reduced LVEF <40%, N (%)	6 (35)	27 (40)	
OTHER BIOCHEMICAL MARKERS			
Haemoglobin, g/L	122 [106-139]	122 [101-138]	0.889
MEDICAL HISTORY; N (%)			
Atrial fibrillation	12 (71)	32 (48)	0.283
Cardiovascular disease	4 (24)	23 (34)	0.563
Active smoking	3 (5)	10 (15)	0.721
Hypertension	12 (71)	49 (74)	>0.999
Pulmonary hypertension	6 (35)	16 (24)	0.364
Diabetes mellitus type 2	6 (35)	30 (45)	0.586
Dyslipidaemia	11 (65)	46 (70)	>0.999
BACKGROUND MEDICATION; N (%)			
Diuretics	11 (65)	49 (73)	0.253
Statins	9 (53)	43 (64)	0.415
Anticoagulants	10 (59)	27 (40)	0.415
Antiplatelet agents	7 (41)	38 (57)	0.286
Beta-blockers	11 (65)	46 (69)	0.777
Antiarrhythmic agents	0 (0)	7 (10)	0.335
Antidiabetics	3 (24)	27 (40)	0.265
ACE inhibitor/ARB	13 (76)	43 (66)	0.562

[a]MDRD-4 levels expressed in mL/min/1.73m^2; Quantitative values were given in median [Q1-Q3]; 2DE-MS group refers to a subgroup of patients used for discovery phase of 2D electrophoresis coupled with mass spectrometry. P values of categorical were calculated with Fisher exact test, except for LVEF where χ^2 was used. P values of numerical data were calculated with Mann-Whitney, except for LVEF where Kruskall-Wallis was used. Diuretics: hydrochlorothiazide, furosemide, eplerenone, and spironolactone. Statins: atorvastatin, pravastatin, simvastatin, ezetimibe. Anticoagulants: warfarin, acenocumarol, bemiparin, heparin, dabigatran, rivaroxaban, edoxaban, and apixaban. Antiplatelet agents: acetylsalicylic acid and clopidogrel. Beta-blockers: bisoprolol and carvedilol. Antiarrhythmic agents: amiodarone. Oral antidiabetic agents: metformin and repaglinide. Angiotensin-converting enzyme (ACE) inhibitors include: captopril, enalapril, and ramipril. Angiotensin receptor blockers (ARB): losartan, olmesartan, and valsartan.

Supplementary Table S3: Extended data from table 1 regarding mass spectrometry characteristics of identified proteins in urine of ADHF patients.

Gel-ID	Protein name	Gene name	Swiss Prot number	Theoretical pI	Experimental pI	Theoretical MW (KDa)	Experimental MW (KDa)	MS or MS/MS	MASCOT Score	Coverage
1	Lysosomal acid phosphatase	*ACP2*	P11117	5.80	5.80	46.7	45.5	MS	63	7
2	Pancreatic α-amylase	*AMY2A*	P04746	6.45	6.45	57.7	51.6	MS/MS	61	-
3	Annexin A10	*ANXA10*	Q9UJ72	5.13	5.20	37.3	35.2	MS	60	19
4	Arylsulphatase A	*ARSA*	P15289	5.57	5.50	53.6	49.6	MS	67	10
5	Zinc-α-2-glycoprotein	*AZGP1*	P25311	5.58	4.8-5.1	34.3	41.6-43.5	MS	66	11
6	Complement C3	*C3*	P01024	6.00	6.75	187.1	54.8	MS	62	3
7	Carbonic anhydrase 1	*CA1*	P00915	6.63	6.70	28.9	30.1	MS/MS	71	-
8	Endosialin	*CD248*	Q9HCU0	5.14	4.70	80.9	44.6-45.6	MS/MS	54	
9	CD59 glycoprotein	*CD59*	P13987	5.18	4.90	14.2	22.7	MS/MS	82	-
10	Cathepsin D	*CTSD*	P07339	5.60	5.40	44.6	31.2	MS/MS	58	-
11	Fibrinogen β-chain	*FGB*	P02675	7.95	4.90	55.9	18.7-19.8	MS/MS	55	
12	Fibrinogen γ-chain	*FGG*	P02679	5.24	5.30-5.35	55.5	48.0-48.2	MS/MS	55	-
13	Vitamin D binding protein	*GC*	P02774	5.22	5.20	52.9	50.5	MS	94	17
14	Hemopexin	*HPX*	P02790	6.43	5.30-5.35	51.7	55.4	MS/MS	70	
15	Basement membrane-specific heparan sulphate proteoglycan core protein	*HSPG2*	P98160	6.03	5.40	468.8	24.9	MS/MS	102	-
16	Inter-α-trypsin inhibitor heavy chain H4	*ITIH4*	Q14624	6.00	4.9-5.1	103.4	36.6-37.2	MS	74	10
17	Kininogen-1	*KNG1*	P01042	6.23	4.7-4.9	72.0	50.5-53.3	MS	63	8
18	Vesicular integral-membrane protein VIP36	*LMAN2*	Q12907	6.06	5.20	40.2	35.2	MS/MS	60	-
19	Leucine-rich α-2-glycoprotein	*LRG1*	P02750	5.66	4.60	38.2	47.4	MS/MS	79	-
20	Retinol binding protein 4	*RBP4*	P02753	5.27	5.20	23.0	24.7-25.2	MS/MS	66	-
21	α-1-antitrypsin	*SERPINA1*	P01009	5.37	5.00-5.10	46.7	51.4-52.2	MS	130	19
22	Antithrombin III	*SERPINC1*	P01008	5.95	5.20	52.6	52.6	MS/MS	78	-
23	Serotransferrin	*TF*	P02787	6.70	6.00-6.40	77.1	56.5	MS	234	26
24	Trefoil factor 2	*TFF2*	Q03403	5.21	5.20	14.3	11.1	MS/MS	83	-
25	Transthyretin	*TTR*	P02766	5.31	5.30	15.9	15.9	MS/MS	61	-
26	Vitelline membrane outer layer protein 1 homolog	*VMO1*	Q7Z5L0	4.65	4.65	21.2	21.2	MS/MS	87	-

pI: isoelectric point; MW: molecular weight; MS: mass spectrometry.

Supplementary Table S4: Selected peptide information of proteins identified by MS/MS in urine of ADHF patients.

Gel-ID	Protein name	Gene name	Swiss Prot number	M/Z	Position	Sequence	Modification	Miss-cleavage
2	Pancreatic α-amylase	*AMY2A*	P04746	**1427.7**	307-318	ALVFVDNHDNQR	-	0
				1570.7	88-100	SGNEDEFRNMVTR	MSO: 97	1
7	Carbonic anhydrase 1	*CA1*	P00915	**985.4**	82-90	GGPFSDSYR	-	0
				970.6	161-169	VLDALQAIK	-	0
8	Endosialin	*CD248*	Q9HCU0	**1198.6**	93-101	QCQLQRPLR	Cys_CAM: 94	0
				1072.5	216-225	QPEGGVGWSR	-	0
9	CD59 glycoprotein	*CD59*	P13987	**1539.7**	67-78	FEHCNFNDVTTR	Cys_CAM: 70	0
10	Cathepsin D	*CTSD*	P07339	**1462.7**	393-403	YYTVFDRDNNR	-	1
11	Fibrinogen β-chain	*FGB*	P02675	**1032.6**	484-491	IRPFFPQQ	-	0
12	Fibrinogen γ-chain	*FGG*	P02679	**1034.5**	293-301	VGPEADKYR	-	1
				1194.5	32-40	DNCCILDER	Cys_CAM: 34, 35	0
				1545.8	418-432	LTIGEGQQHHLGGAK	-	0
14	Hemopexin	*HPX*	P02790	**1220.6**	92-102	NFPSPVDAAFR	-	0
				1268.7	209-219	FDPVRGEVPPR	-	1
				1684.9	209-222	FDPVRGEVPPRYPR	-	2
15	Basement membrane-specific heparan sulphate proteoglycan core protein	*HSPG2*	P98160	1666.8	4282-4295	LVSEDPINDGEWHR	-	0
				1601.9	4304-4318	RGSIQVDGEELVSGR	-	1
				2413.2	4358-4379	NLVLHSARPGAPPPQPLDLQ HR	-	0
18	Vesicular integral-membrane protein VIP36	*LMAN2*	Q12907	1343.7	208-218	NRDHDTFLAVR	-	1
				1073.5	210-218	DHDTFLAVR	-	0
19	Leucine-rich α-2-glycoprotein	*LRG1*	P02750	989.55	251-260	VAAGAFQGLR	-	0
				1152.6	165-175	ALGHLDLSGNR	-	0
20	Retinol binding protein 4	*RBP4*	P02753	**1106.5**	29-37	VKENFDKAR	-	2
				1302.7	172-181	QRQEELCLAR	Cys_CAM: 178	1
				1675.8	185-198	LIVHNGYCDGRSER	Cys_CAM: 192	1
22	Antithrombin III	*SERPINC1*	P01008	**1674.8**	202-215	LQPLDFKENAEQSR	-	1
24	Trefoil factor 2	*TFF2*	Q03403	**1696.7**	90-104	NCGYPGISPEECASR	Cys_CAM: 91, 101	0
25	Transthyretin	*TTR*	P02766	**1394.7**	56-68	AADDTWEPFASGK	-	0
26	Vitelline membrane outer layer protein 1 homolog	*VMO1*	Q7Z5L0	**1217.7**	185-196	GLGDDTALNDAR	-	0

M/Z: mass to charge ratio peaks, in bold those with highest MASCOT scores; position of amino acids corresponding to each peak and its sequence; variable modifications accepted: carbamidomethyl (CAM) and methionine sulfoxide (MSO); miss-cleavage: up to two trypsin miss-cleavages accepted.

Supplementary Table S5: Biological processes and molecular functions of differential urinary proteins.

SwissProt number	Gene	Biological processes				Molecular functions		
		Cell function	Haemostatic & complement system	Inflammation & immune response	Metabolic processes	Binding	Catalytic activity	Transport
P11117	*ACP2*				●		●	
P04746	*AMY2A*				●	●	●	
Q9UJ72	*ANXA10*				●	●		
P15289	*ARSA*				●	●		
P25311	*AZGP1*	●	●					●
P01024	*C3*		●	●	●	●	●	
P00915	*CA1*		●			●	●	
Q9HCU0	*CD248*	●				●		
P13987	*CD59*		●			●		
P07339	*CTSD*				●		●	
P02675	*FGB*	●	●	●		●		
P02679	*FGG*	●	●			●		
P02774	*GC*				●	●		●
P02790	*HPX*				●	●		●
P98160	*HSPG2*	●		●	●	●		
Q14624	*ITIH4*			●			●	
P01042	*KNG1*		●	●		●	●	
Q12907	*LMAN2*				●	●		●
P02750	*LRG1*	●		●		●		
P02753	*RBP4*		●		●	●		●
P01009	*SERPINA1*		●	●		●	●	
P01008	*SERPINC1*		●	●		●	●	
P02787	*TF*		●	●		●		●
Q03403	*TFF2*		●			●		
P02766	*TTR*				●	●		●
Q7Z5L0	*VMO1*					●	●	

Cell function includes: angiogenesis, cell adhesion, differentiation, and migration.

Supplementary table S6: Urinary transthyretin levels in relation to patient clinical characteristics.

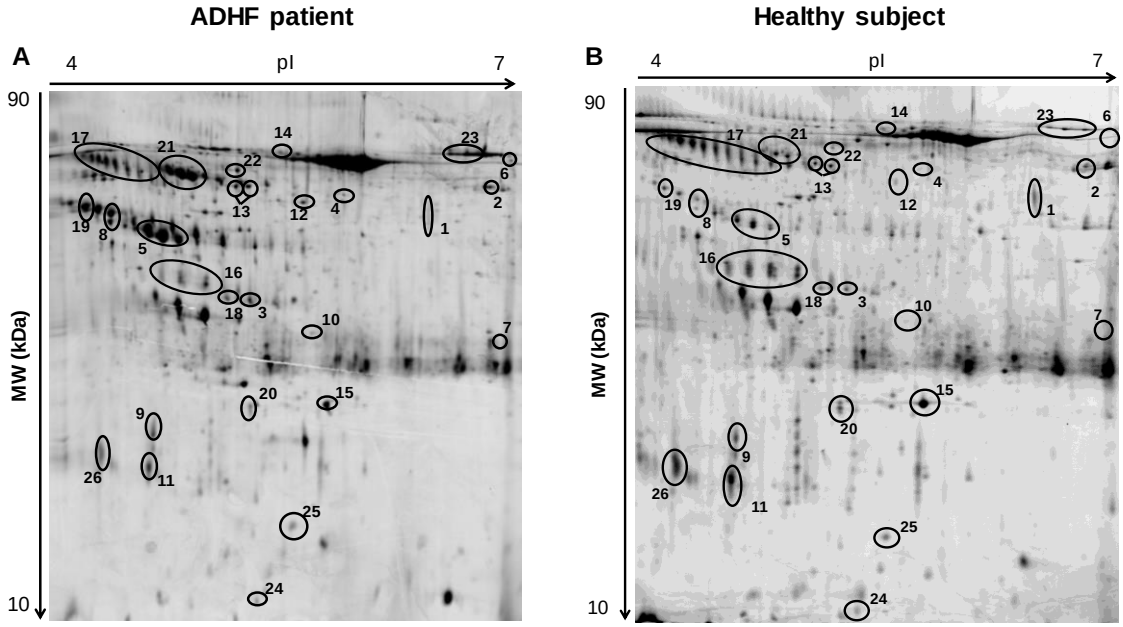
	ADHF patients		NRF		RD	
	N	Median [IQR]	N	Median [IQR]	N	Median [IQR]
LVEF						
Reduced (<40%)	27	12.3 [3.3-64.1]	18	12.3 [3.3-64.1][a]	9	12.0 [3.3-54.5][b]
Mildly reduced (40-49%)	12	15.0 [4.8-71.1]	6	9.7 [3.6-41.8]	6	19.6 [15.0-71.1]
Preserved (≥50%)	28	7.8 [3.7-64.4]	11	4.1 [1.6-5.0]	17	15.1 [7.2-70.7]
P value		*0.653*		*0.126*		*0.428*
Hypertension						
No	17	3.9 [2.8-64.1]	13	3.6 [2.4-64.1]	4	5.0 [3.3-70.7]
Yes	49	15.0 [5.0-64.4]	21	11.0 [4.2-43.0]	28	18.3 [7.6-71.1]
P value		*0.144*		*0.433*		*0.353*
Dyslipidaemia						
No	20	5.3 [3.3-54.5]	13	4.6 [3.0-40.5]	6	10.3 [5.0-54.5]
Yes	46	15.1 [4.3-67.1]	20	12.3 [2.2-44.2]	26	18.9 [7.6-72.3]
P value		*0.205*		*0.685*		*0.263*
Diabetes mellitus type II						
No	36	5.5 [3.3-44.2]	23	4.8 [3.3-41.8]	13	11.2 [5.0-54.5]
Yes	30	18.1 [7.6-71.1]	11	15.2 [1.6-45.0]	19	19.6 [7.6-85.2]
P value		*0.098*		*0.767*		*0.160*
Cardiovascular disease						
No	44	8.7 [3.4-43.4]	24	4.5 [2.4-35.1]	20	14.8 [5.0-64.4]
Yes	23	15.0 [5.3-97.3]	11	12.1 [2.8-64.1]	12	41.1 [7.2-128.3]
P value		*0.145*		*0.416*		*0.270*
Atrial fibrillation						
No	34	14.8 [4.8-64.4]	18	9.6 [3.3-64.1]	16	15.1 [7.2-64.4]
Yes	32	8.7 [3.3-54.5]	16	4.3 [2.2-41.8]	16	24.2 [3.7-84.8]
P value		*0.701*		*0.439*		*0.800*
Pulmonary hypertension						
No	51	11.2 [3.3-64.4]	27	5.2 [2.4-44.2]	24	18.3 [7.6-70.7]
Yes	16	12.3 [3.7-54.5]	8	8.2 [3.6-16.9]	8	15.0 [3.7-85.2]
P value		*0.893*		*0.962*		*0.811*

Urinary transthyretin levels in ng TTR/mg total protein. LVEF: left ventricular ejection fraction; NRF: ADHF patients with normal renal function at hospital admission; RD: ADHF patients with renal dysfunction at hospital admission; P values were calculated by Mann-Whitney test except for LVEF where Kruskall-Wallis test was used. Information of one patient was missing for hypertension, dyslipidaemia and diabetes mellitus type 2. [a,b]Comparison reduced vs preserved LVEF (Mann-Whitney), [a]P=0.049, [b]P=0.363.

Supplementary table S7: ROC (associated receiver operating characteristic) curve (AUC) analysis for determining power of urinary TTR and RBP4 levels in ADHF patients for GFR discrimination.

Protein	AUC Area	Lower limit	Upper limit	P-value
TTR	0.633	0.489	0.776	*0.070*
RBP4	0.742	0.614	0.870	*<0.001*
TTR + RBP4	0.826	0.705	0.947	*<0.0001*

TTR: transthyretin; RBP4: retinol binding protein 4, AUC: area under the curve.



Supplementary Figure S1. 2DE-PAGE gels of urinary samples from a representative ADHF patient (A) and a representative healthy subject (B) in a pI range of 4-7 and 12% SDS-PAGE gels.

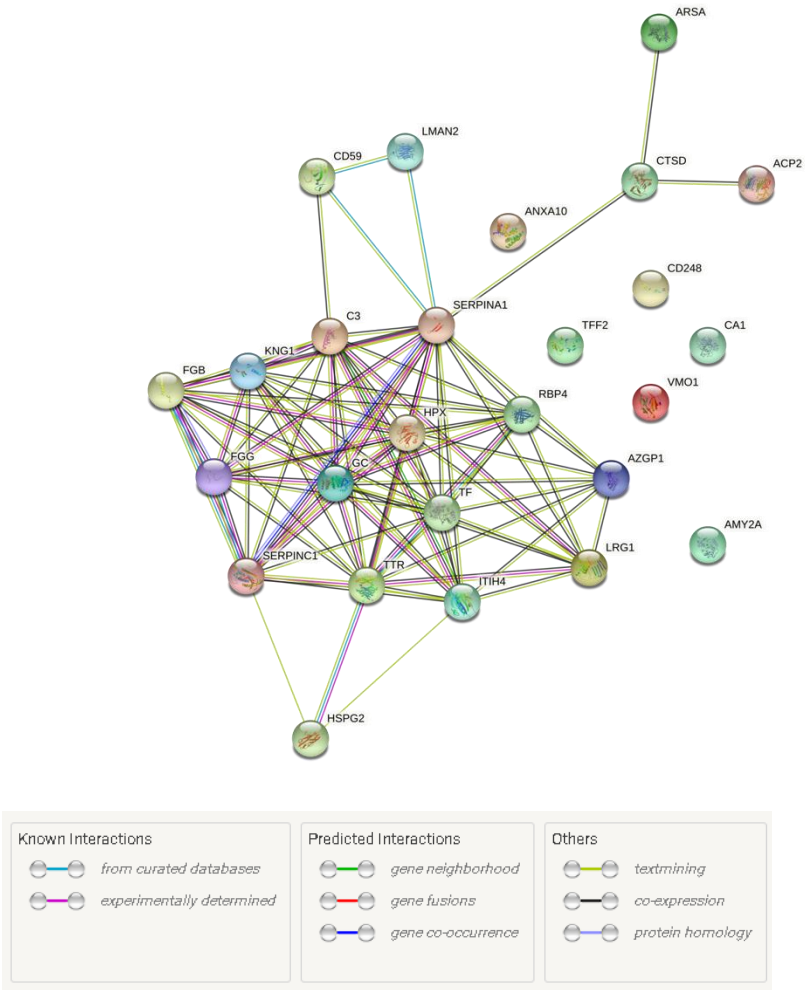
1	Lysosomal acid phosphatase
2	Pancreatic α-amylase
3	Annexin A10
4	Zinc α-2-glycoprotein
5	Arylsulphatase A
6	Complement C3
7	Carbonic anhydrase 1
8	Endosialin
9	CD59 glycoprotein
10	Cathepsin D
11	Fibrinogen β-chain
12	Fibrinogen γ-chain
13	Vitamin D binding protein
14	Hemopexin
15	Basement membrane-specific heparan sulphate proteoglycan core protein
16	Inter-α-trypsin inhibitor heavy chain 4
17	Kininogen 1
18	Vesicular integral-membrane protein VIP36
19	Leucine-rich α-2-glycoprotein
20	Retinol binding protein 4
21	α-1-antitrypsin
22	Antithrombin III
23	Serotransferrin
24	Trefoil factor 2
25	Transthyretin
26	Vitelline membrane outer layer protein 1 homolog

Protein	SwissProt number	Intensity change	MDRD-4			LVEF		
			T1	T2	T3	T1	T2	T3
FGB	P02675	down						
LMAN2	Q12907	down						
ANXA10	Q9UJ72	down						
RBP4	P02753	down						
HPX	P02790	up						
TTR	P02766	up						
GC	P02774	up						
ACP2	P11117	down						
AMY2A	P04746	down						
VMO1	Q7Z5L0	down						
ITIH4	Q14624	down						
SERPINA1	P01009	up						
KNG1	P01042	down						
FGG	P02679	up						
SERPINC1	P01008	up						
AZGP1	P25311	up						
HSPG2	P98160	down						
CD59	P13987	up						
C3	P01024	up						
CTSD	P07339	up						
ARSA	P15289	up						
TF	P02787	up						
CD248	Q9HCU0	up						
LRG1	P02750	up						
TFF2	Q03403	down						
CA1	P00915	up						

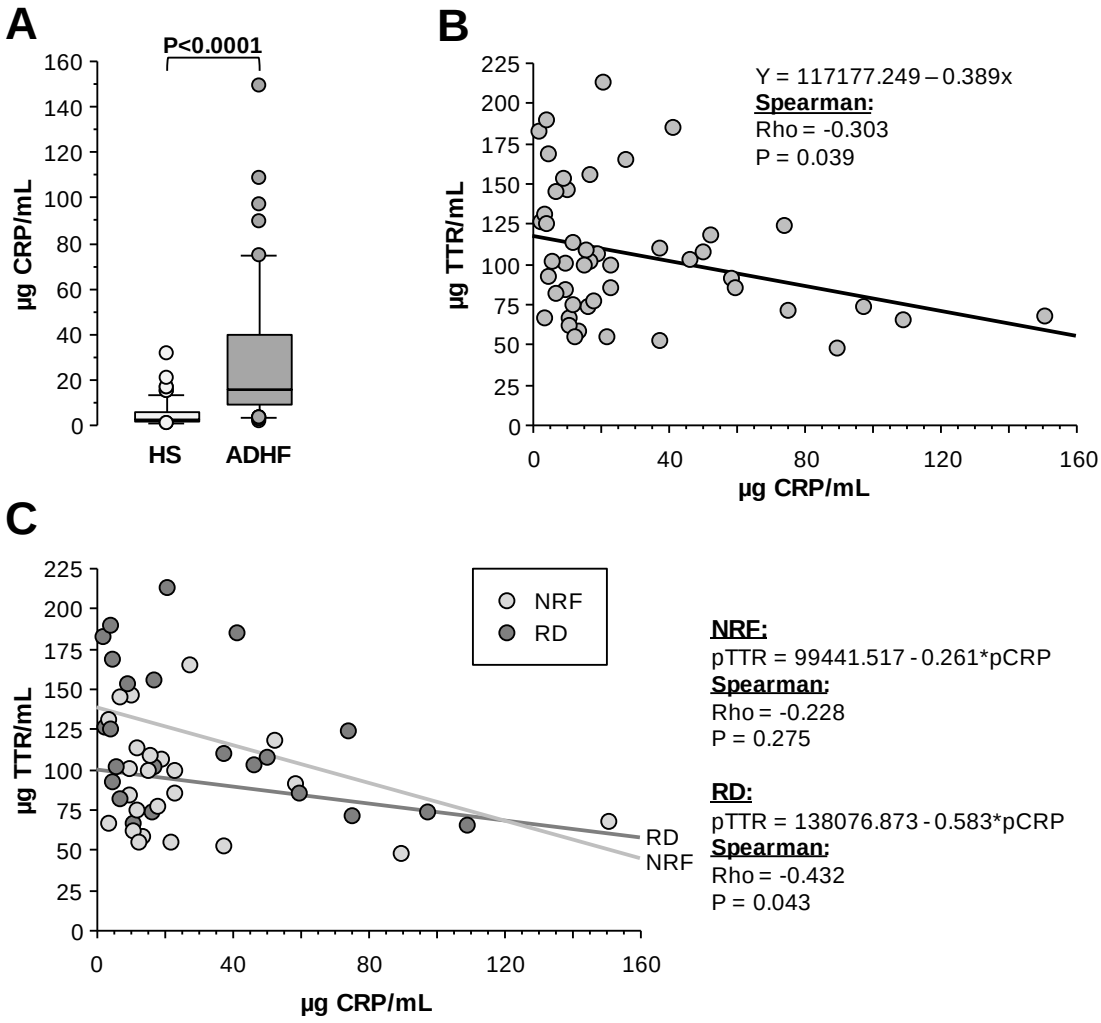
	Fold change
	1.5-2.0
	2.1-3.0
	3.1-5.0
	>5.1

	MDRD-4	LVEF
T1	33.7 [30.7-43.2]	33.0 [22.0-33.0]
T2	68.5 [62.5-71.7]	49.5 [47.0-55.0]
T3	97.8 [106.0-120.0]	60.0 [60.0-65.0]

Supplementary Figure S2. Fold change in urinary differential proteins according to renal function and left ventricular ejection fraction (LVEF) distributed by tertiles.



Supplementary Figure S3. Original STRING network showing the 26 differential proteins consistently detected in urine of ADHF patients and healthy subjects.



Supplementary Figure S4. Plasma C reactive protein (CRP) in ADHF patients at hospital admission. A) CRP levels of ADHF patients (N=67) and healthy subjects (HS). B) Significant correlation between CRP and TTR levels in ADHF patients. C) Regression lines of CRP and TTR correlations depending on kidney function, NRF in light grey and RD with dark grey.