Cardiac Transthyretin-derived Amyloidosis

Klaassen, Sebastiaan

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6

Wild-type transthyretin amyloidosis in Heart Failure patients with preserved or mildly reduced ejection fraction: Identification using serum proteins

S.H.C. Klaassen; J. Tromp; H.L.A. Nienhuis; M. Rienstra; J. Bijzet; R.H.J.A. Slart; B.P.C. Hazenberg; M.P. van den Berg; D.J. van Veldhuisen; P. van der Meer

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Abstract

Background:
Wild-type transthyretin amyloidosis (ATTRwt) is a cause of heart failure (HF) with preserved (HFpEF) or midrange (HFmrEF) ejection fraction. Diagnosis of ATTRwt is often missed. Therefore, a multi-biomarker profile was applied to identify ATTRwt in HFmrEF/HFpEF.

Methods:
We examined patients who were part of a prospective, investigator-initiated, study presenting with HFmrEF/HFpEF (n=101), in which we evaluated ATTRwt status. Serum biomarker profiles of ATTRwt-negative HF patients from this group ($HF^{ATTRwt-}$) were compared with those in a separate group (learning set) of cardiac amyloidosis patients (ATTRwt N=72; AL N=56), diagnosed at our national center of expertise for amyloidosis. The biomarker profiles found were validated in the ATTRwt-positive ($HF^{ATTRwt+}$)VIP-HF patients (validation set) diagnosed by bone scintigraphy and biopsy. All patients underwent extensive phenotyping and 364 biomarkers of different pathophysiological domains were measured using Olink CVD-II, CVD-III, Immune response and Oncology-II panels.

Results:
Out of 364 biomarkers, higher concentrations of three biomarkers — hepatocyte growth factor (HGF), S100 calcium-binding A4 (S100A4) and galactosidase beta-1 (GLB1) — distinguished cardiac amyloidosis from $HF^{ATTRwt-}$ in the learning set with an AUC of 0.98, $p<0.001$. Among the 101 patients, five patients (4.95 %; 95% confidence interval 1.63-11.2%) appeared positive for ATTRwt. The three biomarkers distinguished the five $HF^{ATTRwt+}$ from the $HF^{ATTRwt-}$ patients in the validation set (AUC 0.83).

Conclusion:
ATTRwt is relatively common in the male patient population with HFpEF/HFmrEF. Serum levels of the three biomarkers GLB1, S100A4, and HGF can identify ATTRwt patients within the HFpEF/HFmrEF patient population.

Key words

Cardiac amyloidosis – ATTR wild-type – HFpEF – Biomarker - OLINK

Introduction

Heart failure (HF) has a poor prognosis and is responsible for up to 1% of all acute hospitalizations.[1,2] Treatment options for patients with HF with preserved ejection fraction (HFpEF) are limited.[3] Cardiac amyloidosis, due to wild-type transthyretin-derived amyloidosis (ATTRwt) causes heart failure with preserved or midrange ejection fraction (HFmrEF/HFpEF) and is a potentially treatable disease.[4,5] The prevalence of ATTRwt has been estimated to be 3-14% of the patient population with HFmrEF/HFpEF. This is based on studies that are either post-mortem, single centre, or restricted to a selected population[4,5,6]

Tafamidis an oral benzoxazole derivative which stabilizes the transthyretin tetramer, reduces mortality and hospitalizations in patients with transthyretin amyloid cardiomyopathy and has shown to be most effective if initiated in early disease stages.[7] Several other treatments that are currently studied in clinical trials that can also be expected to be most beneficial when initiated at an early disease stage.[7,8,9]

Yet, diagnosis of ATTRwt is insidiously difficult and often missed or delayed. Both echocardiography and cardiac magnetic resonance imaging have limited discriminatory value in early stages of the disease and other diagnostic modalities such as cardiac biopsy or bone scintigraphy are often required, delaying diagnosis and ultimately treatment initiation.[10] There are no known biomarkers to reliably discriminate ATTRwt from other causes of HF. Few studies reported a single biomarker approach for the detection of amyloidosis, albeit with limited diagnostic value.[11]

Therefore, the aim of this study is to determine a multi-biomarker score to identify patients with ATTRwt, which may aid to diagnose this disease early in patients known to have HFpEF or HFmrEF.

Methods

Study design

We performed an analysis of the investigator-initiated, prospective, observational study, in the Ventricular tachyarrhythmia detection by implantable loop recording in patients with heart failure and preserved ejection fraction: the VIP-HF study.[12] In this population, bone scintigraphy was performed to screen for the presence of cardiac ATTR amyloidosis. All patients with a positive bone scintigraphy were further evaluated for confirmation and typing of the amyloid. In all patients a serum biomarker profile was established. In a second cohort of patients (learning cohort, see below) with cardiac amyloidosis (either ATTRwt or AL amyloidosis), a biomarker profile was determined as well. Firstly, the patients from the VIP-HF without ATTRwt ($HF^{ATTRwt-}$) were compared with patients with evident cardiac amyloidosis (CA), from the learning cohort,

to establish a distinct cardiac amyloidosis biomarker profile. Secondly, a selection of the top-3 biomarkers most differentiating between only ATTRwt patients, from the learning cohort, patients and the $HF^{ATTRwt-}$ patients was made. Lastly, the distinctiveness of the top three differentiating biomarkers was validated, using the patients from the VIP-HF study with ATTRwt ($HF^{ATTRwt+}$) compared with the $HF^{ATTRwt-}$ patients. As the biomarker profiles of the $HF^{ATTRwt+}$ group were not used earlier for the identification of the biomarker profile. A schematic representation of the study design is depicted in Supplementary Figure 1.

Study population

Prospective population. Patients presenting with HF with midrange (HFmrEF) or preserved (HFpEF) ejection fraction between January 2015 and December 2019 were included in the VIP-HF study and are fully described elsewhere.[12] In brief, patients presenting with HF [compatible with New York Heart Association (NYHA) functional class II-III] which required hospitalisation or had symptom relief with diuretics at the emergency room, were screened. Patients were included if the following inclusion criteria were present: left ventricular ejection fraction (LVEF) either had EF > 50% (HFpEF) or at least EF > 40% (HFmrEF), NT-proBNP levels of > 400 ng/L if sinus rhythm or > 1200 ng/L if atrial fibrillation (AF), and left ventricular hypertrophy (LVH) > 11 mm and/or left ventricular diastolic dysfunction (mean and septal and lateral e' <9 cm/s, or E/e' > 13), and/or left atrial dilatation (left atrial volume index > 34 mL/m2). Patients with pacemaker or ICD were excluded. All other inclusion and exclusion criteria are described previously.[12] All patients underwent a bone scintigraphy for screening for ATTR cardiomyopathy.[13,14] Patients with cardiac bone tracer uptake (Perugini score > 0) were further evaluated with blood tests to exclude AL-amyloidosis, subcutaneous abdominal adipose tissue aspirate for confirmation and typing of the amyloid and gene panel testing to exclude hereditary types of amyloidosis.

Retrospective population (learning set). This second cohort consisted of two different population: 1. patients with established ATTRwt amyloidosis, 2. Patients with AL amyloidosis and cardiac involvement. Patients were diagnosed with ATTRwt cardiomyopathy according to the diagnostic algorithm by *Gillmore et al.*[16] Patients with AL amyloidosis were diagnosed according to current guidelines and cardiac involvement was established using the International Society of Amyloidosis consensus criteria for organ involvement.[17,18] In addition, patients were included if the HF inclusion criteria of the prospective population (VIP-HF criteria) were met. Both AL and ATTRwt amyloidosis patients were treatment naïve.

Sample collection and processing

At baseline blood serum samples were collected in all patients in the prospective cohort. Blood serum samples of patients from the retrospective cohort had been collected prior to the start of the amyloidosis treatment and stored frozen at -80 °C until analysis. Data on

demographics, medical history, medication, clinical characteristics, echocardiography and laboratory measurements were collected at baseline of the study or at the time of cardiac amyloidosis diagnosis (retrospective cohort). All patients provided informed consent prior to the analyses of the serum samples. Directly after their collection, the samples were centrifuged at 2500 g for 15 minutes at 4 °C, stored at -20 °C for a standard period of two weeks, and subsequently stored at -80 °C until use. Samples were only once thawed before the analyses.

Biomarker analysis

In total 368 biomarkers from different pathophysiological domains using a high-throughput technique using the Olink Proseek CVD-II, CVD-III, Immune response and Oncology-II kits were determined by commercially available PCR methodology, of which assay characteristics are publicly available (https://www.olink.com/products/).

Statistical analyses

Descriptive statistics. Patient demographics, medical history and laboratory values were depicted as medians with interquartile ranges, means with standard deviations, or percentages depending on the variable. The student-t test, the $\chi 2$ test, the Kruskal-Wallis equality-of-populations rank test or the one-way analysis of variance (ANOVA) were used to test for significance where appropriate. A two-tailed P-value of <0.05 was considered to be statistically significant. These analyses were conducted using STATA version 16 (StataCorp LP, College station, Texas, USA).

Analytical statistics. An inductive approach to establish the optimal biomarker signature for ATTRwt consisted of three steps. Firstly, we performed a sparse partial-least-squares discriminant analysis (sPLS-DA) to identify a multi-biomarker signature of cardiac amyloidosis in general. For this analysis both the prospectively identified HFATTRwt-patients and the retrospectively selected cardiac amyloidosis population (AL & ATTRwt), as a "learning set", were used. We set a maximum of 5 components and used 5-fold cross validation repeated 10 times and depict the results for the optimal number of components achieved through cross validation. Secondly, to select individual biomarkers, we plotted the log2fold mean differences and -log10 (p-value) for difference of individual biomarkers in a volcano plot adjusting for possible confounders, in the CA-patients vs. $HF^{ATTRwt-}$ group. P-values were corrected for multiple testing using the Benjamini-Hochberg method. Thirdly, we selected the top three combination of biomarkers upregulated in the CA-patients group vs. $HF^{ATTRwt-}$ group starting with the biomarker with the greatest discriminatory value based on the smallest p-value in the volcano plot and calculated the change in AUC for adding each biomarker. Lastly, we conducted an internal validation and described if the suggested biomarker selection could diagnose the $HF^{ATTRwt+}$ patients from the prospectively included patients compared to $HF^{ATTRwt-}$ patients. These analyses were performed using R version 3.4.1 and all p-values were considered two-sided.

Results

Characteristics of the learning cohort and the VIP-HF ATTRwt-

The baseline characteristics of the retrospective cardiac amyloidosis (ATTRwt; N=72 & AL; N=56) cohort and the $HF^{ATTRwt-}$ patients (N=96) from the VIP study are depicted in *table 1*. The age did not significantly differ between the CA group and the HF ATTRwt-group. The CA group consisted of more men compared to the $HF^{ATTRwt-}$ group, 81% versus 49%, respectively, P<0.001). Both hypertension (79% vs. 15%, respectively) and type 2 diabetes (39.6% vs. 7.9%, respectively) were less prevalent in the CA group (P<0.001). The interventricular wall thickness (IVSD) was 16 mm (IQR: 13.0 – 19. 0) in the CA group and was 10 mm (IQR: 9.0-12) in the $HF^{ATTRwt-}$ group (P<0.001). The LVEF did not differ significantly between the groups, the LVEF in the CA group was 55% (IQR: 45-60) and in the $HF^{ATTRwt-}$ group 55% (IQR: 50-58), P=0.780. Serum concentrations of NT-proBNP were higher in the CA group 3127 ng/L (IQR: 1340 – 5695) than the $HF^{ATTRwt-}$ group 1490 ng/L (IQR: 687- 2491) (P<0.001).

Baseline characteristics of the VIP-HF cohort

Among the 101 patients with bone scintigraphy in the VIP-HF study, 5 (4.95%; 95% CI 1.63-11.2%) patients were diagnosed with ATTRwt ($HF^{ATTRwt+}$) and the $HF^{ATTRwt-}$ group consisted of 96 patients, baseline characteristics are depicted in *Table 2*. In 5 out of 7 patients with cardiac tracer uptake the diagnosis of ATTRwt cardiomyopathy was established and these patients were designated the $HF^{ATTRwt+}$ group. Amyloid cardiomyopathy could not be diagnosed in 2 of the 7 patients with grade 1 cardiac tracer uptake, these patients were excluded for further analysis. The remaining patients, with no cardiac tracer uptake, were designated the $HF^{ATTRwt-}$ group.[15] In all 5 patients, the presence of ATTRwt was histologically confirmed and AL amyloidosis was excluded. Mean age of the $HF^{ATTRwt+}$ was 73 $\pm$ 8 years and the age of $HF^{ATTRwt-}$ was 80 $\pm$ 6 years (P=0.028). The $HF^{ATTRwt+}$ group were predominantly men (80.0% vs. 49%, P=0.180). Hypertension was less prevalent in the $HF^{ATTRwt+}$ group (40.0% vs. 79.2%, P=0.042). IVSD was 13.0 mm (IQR: 12.0 – 21.0) in the $HF^{ATTRwt+}$ group and was 10 mm (IQR: 9.0-12) in the $HF^{ATTRwt-}$ group (P=0.033). The LVEF in the $HF^{ATTRwt+}$ group was 55% (IQR: 55-57) and 55% (IQR: 50-58) and did not differ significantly, P=0.500. Serum concentrations of NT-proBNP were 1275 ng/L (IQR: 1240 – 1312) in the $HF^{ATTRwt+}$ group and 1490 ng/L (IQR: 687- 2491) in the $HF^{ATTRwt-}$ group (P=0.770).

Table 1: Baseline characteristics testing cohort

	Cardiac amyloidosis (N=128)	HF$^{attrwt-}$ (N=96)	ATTRwt (N=72)	AL (N=56)	p-value*	p-value**
Age at diagnosis, years ± SD	71.3 ± 8.7	72.7 ± 7.8	74.3 ± 7.0	67.5 ± 9.2	.210	<.001
Men	80.5%	49.0%	93.1%	64.3%	<.001	<.001
Hypertension	15.1%	79.2%	13.9%	16.7%	<.001	<.001
Diabetes Mellitus type II	7.9%	39.6%	9.7%	5.6%	<.001	<.001
Myocardial Infarction	3.2%	21.9%	4.2%	1.9%	<.001	<.001
Medication						
Beta-blockers	60.5%	89.4%	71.4%	46.3%	<.001	<.001
ACE/ARB	64.5%	60.6%	74.3%	51.9%	.560	.032
Loop/thiazide diuretics	66.9%	91.5%	78.6%	51.9%	<.001	<.001
Rhythm					.052	<.001
Sinus rhythm	62.0%	62.5%	45.6%	80.4%		
Atrial fibrillation	30.6%	34.4%	42.1%	17.6%		
Atrial flutter	0.9%	3.1%	1.8%	0.0%		
Pacemaker	6.5%	0.0%	10.5%	2.0%		
Clinical						
Systolic blood pressure (mmHg)	124.0 (110.0, 140.0)	139.0 (125.0, 154.0)	126.0 (110.0, 140.0)	118.0 (103.0, 134.0)	<.001	<.001
Diastolic blood pressure (mmHg)	71.5 (60.0, 80.0)	73.0 (62.0, 82.0)	72.0 (64.0, 80.0)	71.0 (60.0, 80.0)	.990	.960
Echocardiography						
IVSD (mm)	16.0 (13.0, 19.0)	10.0 (9.0, 12.0)	18.0 (16.0, 21.0)	14.0 (12.0, 16.0)	<.001	<.001
LVPWD (mm)	14.0 (11.4, 18.0)	8.5 (8.0, 9.0)	17.0 (14.0, 19.7)	13.3 (10.6, 15.6)	<.001	<.001
LVMi	133.0 (108.0, 182.0)	95.0 (77.0, 121.0)	174.0 (133.5, 212.0)	112.0 (91.0, 142.0)	<.001	<.001
LVEF (%)	55.0 (45.0, 60.0)	55.0 (50.0, 58.0)	50.0 (40.0, 60.0)	60.0 (50.0, 60.0)	.780	.035
Laboratory						
NTproBNP (ng/L)	3126.5 (1340.0, 5695.0)	1490.0 (687.0, 2490.5)	3103.0 (1776.0, 5061.0)	3256.0 (619.0, 9570.0)	<.001	<.001
eGFR (ml/min * 1.73 m2)	59.0 (45.0, 74.0)	48.5 (37.0, 72.0)	60.0 (47.5, 71.5)	55.5 (43.0, 76.0)	.048	.130
Creatinine (µmol)	104.0 (86.0, 129.0)	106.0 (85.0, 143.0)	105.0 (90.0, 121.0)	101.5 (80.0, 135.0)	.540	.730
ALAT (IU/L)	23.0 (16.0, 34.0)	19.5 (14.0, 25.0)	23.0 (18.0, 35.0)	22.0 (15.0, 31.0)	.004	<.001

* P-value Cardiac amyloidosis vs. HFattrwt-; ** P-value HFattrwt- vs. ATTRwt vs. AL. Data are presented as medians with interquartile ranges, means with standard deviations, or percentages depending on the nature of the variable. IVSD = interventricular septal thickness at diastole; LVMi = left ventricular mass index; LVEF = left ventricular ejection fraction; eGFR = estimated glomerular filtration rate;

Table 2:baseline characteristics prospective cohort

	$HF^{ATTRwt-}$ (n=96)	$HF^{ATTRwt+}$ (n=5)	p-value
Age at diagnosis, years ± SD	72.7 ± 7.8	80.6 ± 5.6	.028
Men	49.0%	80.0%	.180
Hypertension	79.2%	40.0%	.042
Diabetes Mellitus type II	39.6%	20.0%	.380
Myocardial Infarction	21.9%	0.0%	.240
Rhythm			.900
Sinus rhythm	62.5%	60.0%	
Atrial fibrillation	34.4%	40.0%	
Atrial flutter	3.1%	0.0%	
Clinical			
Systolic blood pressure (mmHg)	139.0 (125.0, 154.0)	131.0 (129.0, 139.0)	.780
Diastolic blood pressure (mmHg)	73.0 (62.0, 82.0)	66.0 (65.0, 77.0)	.730
IVSD (mm)	10.0 (9.0, 12.0)	13.0 (12.0, 21.0)	.033
LVMi	95.0 (77.0, 121.0)	130.0 (108.0, 144.0)	.090
LVEF (%)	55.0 (50.0, 58.0)	55.0 (55.0, 57.0)	.500
Laboratory			
NTproBNP (ng/L)	1490.0 (687.0, 2490.5)	1275.0 (1240.0, 1312.0)	.770
eGFR (ml/min * 1.73 m2)	48.5 (37.0, 72.0)	52.0 (43.0, 56.0)	.880
Creatinine (μmol)	106.0 (85.0, 143.0)	116.0 (108.0, 118.0)	.570
ALAT (IU/L)	19.5 (14.0, 25.0)	22.0 (21.0, 29.0)	.320

Data are presented as medians with interquartile ranges, means with standard deviations, or percentages depending on the nature of the variable. IVSD = interventricular septal thickness at diastole; LVMi = left ventricular mass index; LVEF = left ventricular ejection fraction; eGFR = estimated glomerular filtration rate;

Serum markers profile analysis

The combination of the tested biomarkers resulted in an accumulative discriminatory value between the CA-patients and $HF^{ATTRwt-}$ patients, as is displayed by the first three components of the sPLS-DA with an AUC of 0.99 (*Figure 1A*). No significant difference of biomarkers profile within the CA group between ATTRwt and AL was present (P>0.05 for all). Secondly, in the CA-patients compared to the $HF^{ATTRwt-}$ group of the 368 individual biomarkers 205 biomarkers were significantly up or down regulated, after correcting for differences in sex, history of diabetes, hypertension and serum concentrations of NT-proBNP *(Figure 1B)*.

The top-3 biomarkers, irrespective if up or downregulated, most differentially expressed in patients with CA compared to the $HF^{ATTRwt-}$ patients were hepatocyte growth factor (HGF), S100 calcium-binding A4 (S100A4) and galactosidase beta-1 (GLB1). Combined,

HGF, S100A4 and GLB1 could distinguish ATTRwt from HF$^{ATTRwt-}$ patients with an area under the curve (AUC) of 0.98 (*Figure 2*).

Of the 205 significant differentially expressed circulating proteins 169 were upregulated in the ATTRwt group *(Supplementary Table 1)*. A pathway analyses between the top 30 biomarkers upregulated in the ATTRwt group is depicted in *Supplementary Figure 2;* all significant pathways (P<0.05) are depicted. Main significant pathways include: proteins related to cell surface interactions at the vascular wall, viral myocarditis, and proteins related to the extrinsic apoptotic signalling pathway, of which is the HGF protein. S100A4, GLB1 were not part of significant pathway.
The remaining 36 down-regulated proteins are shown in *Supplementary Table 2,* the subsequent pathway analysis is depicted in *Supplementary Figure 3*. The down-related proteins were associated with triglyceride catabolic processes, Asthma, and immunology related pathways. A pathway analysis of the remaining 36 down-regulated proteins, depicted in *Supplementary Figure 3*.

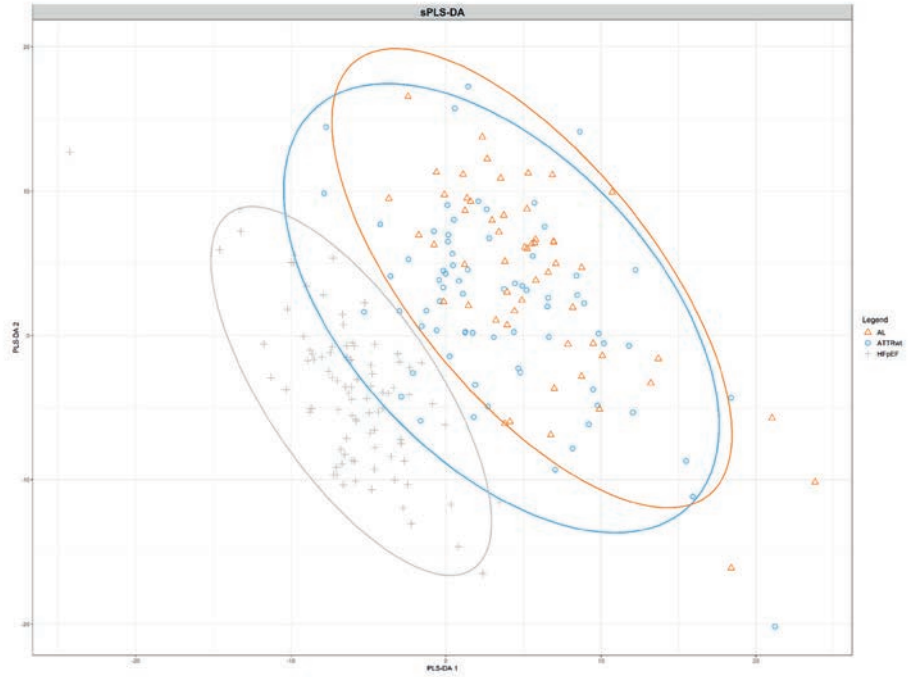

Figure 1: Panel A: Sparse partial least squares-discriminant analysis) (sPLS-DA). The grey cross is the HFATTRwt- group, the blue circle is the ATTRwt group and the orange rectangular is the AL group.

Figure 1: Panel B: Volcano plot of adjusted differential protein expression in the prospective cohort HFAT-TRwt- vs ATTRwt patients. Red dots are not significant. Blue dots are significant. The 15 biomarkers with the greatest p-value and greatest fold change are depicted. Corrected for differences in sex, history of diabetes, hypertension and serum concentrations of NT-proBNP

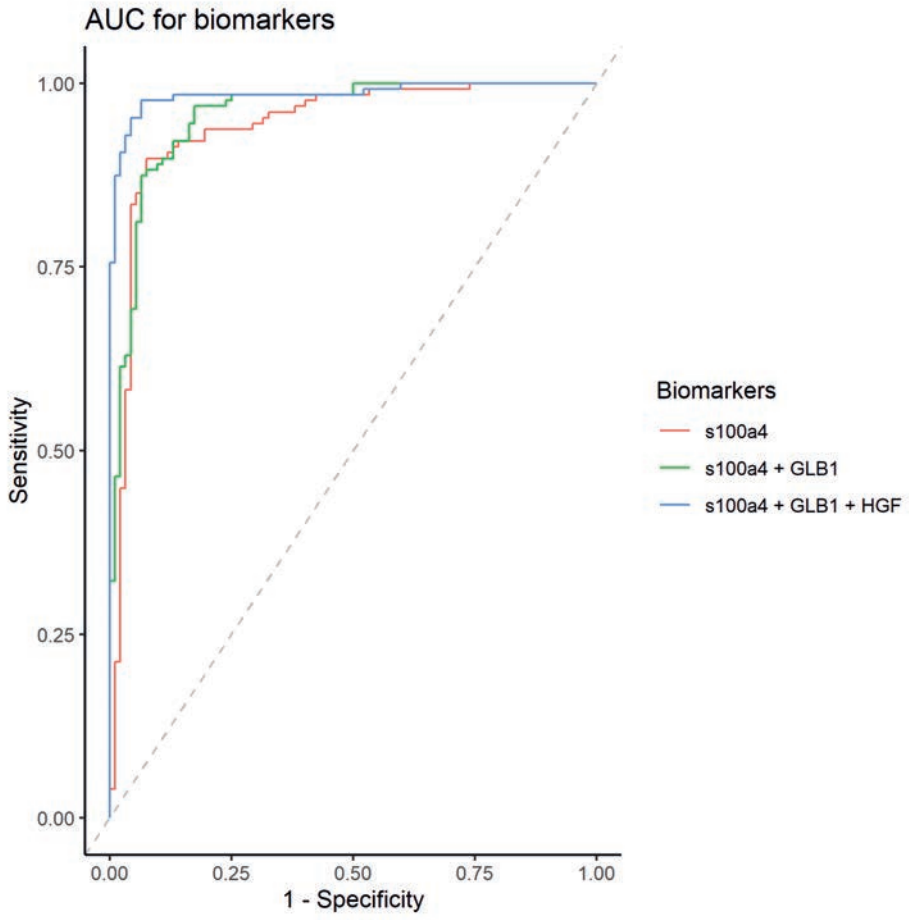

Figure 2: ROC curve of individual top 3 combined biomarkers (AUC 0.98).

Validation of the top three upregulated biomarker-selection

In *Figure 3 panel A-C*, the differences of normalized protein expression (NPX) for each of the biomarkers from the biomarker triplet between the prospective population groups with and without ATTRwt is shown. Panel A, patients with ATTRwt had a mean NPX of 10.6 and patients without ATTRwt had a NPX of 10.04, P=0.023. Panel B and C show the NPX results of S100A4, and GLB1, both biomarkers did not differ between patients with and without ATTRwt (P=0.222 and P=0.123, respectively). Lastly, to challenge our findings we determined whether the biomarker triplet was able to identify the patients with cardiac ATTRwt amyloidosis in our prospective cohort of patients with HFmREF and HFpEF. We found that the biomarker triplet could distinguish cardiac ATTRwt from patients without ATTRwt in the prospective cohort (AUC of 0.83).

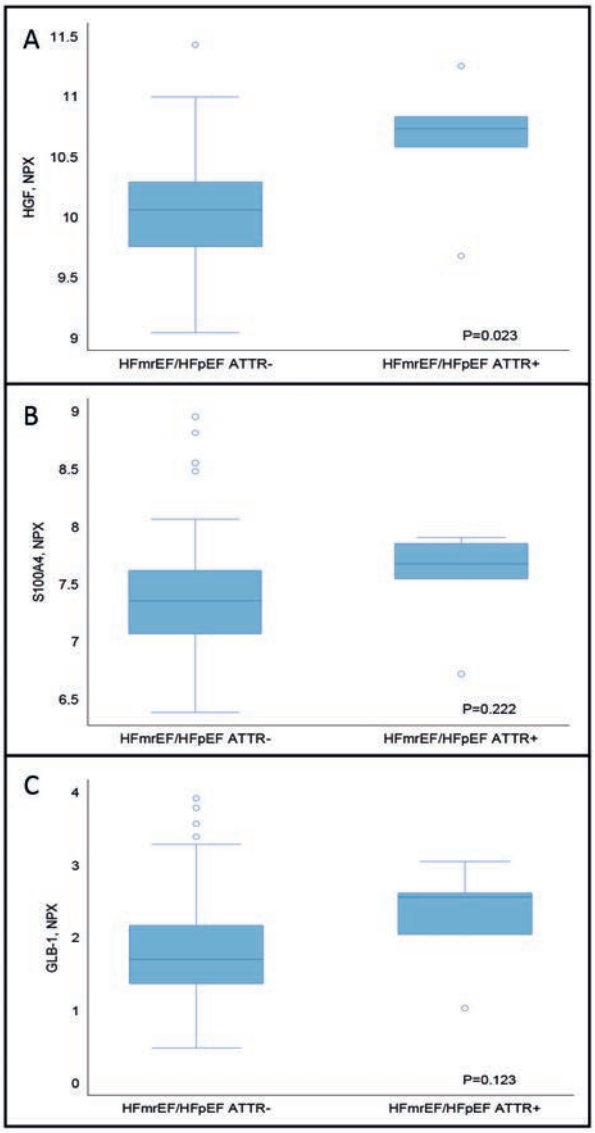

Figure 3: Panel A-C; top three biomarkers in the HFATTRwt- group vs. HFATTRwt+.

Discussion

This study shows that ATTRwt was present in 4.95% (95% confidence interval 1.63-11.2%) of patients with HFpEF or HFmrEF and biomarker profiles are significantly different between heart failure patients with versus without cardiac amyloidosis. Furthermore, we show that, three biomarkers—HGF, S100A4 and GLB1—can

distinguish patients with from patients without ATTRwt. Results of our study highlight the substantial prevalence of ATTRwt in patients with HFmrEF/HFpEF and that biomarkers have the potential to aid in the diagnosis of an often missed disease.

The prevalence of ATTRwt in HFpEF/HFmrEF is less than half of the prevalence reported by Gonzalez et al.[19] Gonzalez et al. reported a prevalence of 13% in HFpEF population, that showed some differences as compared to our population.[19] In contrast to Gonzalez et al. in our study patients with a midrange ejection fraction were included. Gonzalez et al. selected patients from 60 years of age and older, whereas our population consisted of patients older than 18 years. Because the incidence of HFpEF and ATTRwt amyloidosis increases with age,[5] we therefore believe that the difference of prevalence could firstly be explained by the age difference, as our population is significantly younger than the population of Gonzalez et al. Especially, because in our cohort patients with ATTRwt amyloidosis were all older than 75 years and had an LVEF of above 55%. Secondly, Gonzalez et al. included patient with severe conduction abnormalities requiring pacemaker implantation, whereas we did not. Lastly, *Abou Ezzeddine et al.* reported in a cohort of patients with heart failure and an LVEF of above 40% in combination with left ventricular wall thickness of 12mm or greater, an overall prevalence of ATTRwt to be 6.3%. However, prevalence did differ across multiple age categories.[20] Taken together we hypothesise that the "true" prevalence of ATTRwt in HFpEF is around 1 in 10 patients depending on patient selection.

Biomarker profiles are shown to differ between patients with AF and HFpEF or HFrEF, reflecting different pathophysiological mechanisms for AF in two HF subtypes.[20,21] In the present study we have demonstrated a distinctively different serum biomarker profile for patients with ATTRwt amyloidosis as compared to HFmrEF/HFpEF patients without ATTR amyloidosis. Although both groups are HF entities, the underlying pathophysiology is very different. We identified a distinctive triplet biomarker set consisting of HGF, S100A4 and GLB1.

Our results indicate that the biomarker triplet differentiates between the presence and absence of amyloid deposition in heart failure. However, we believe that the biomarker triplet has a strong predictive value for the presence of ATTRwt in HFmrEF/HFpEF. The first question is whether the triplet biomarker selection represents the activity of the amyloidogenic process or that it merely represents the response of body tissue to amyloid deposition.

Hepatocyte growth factor is a multi-functional cytokine and is a stimulator of mitogenesis, cell motility and matrix invasion.[22] In addition, HGF is known to be increased in chronic heart failure.[23] Therefore, it is possible that this elevated serum concentration of HGF is reflective of the pathophysiological mechanisms as a result of ATTRwt deposition in the body and does not reflect amyloidogenic processes. One other study reported the

diagnostic value of HGF in ATTRwt amyloidosis in heart failure.[11] Zhang et al. found that HGF was also elevated in ATTRwt cardiac amyloidosis as compared to all cause LVH and patients with heart failure with reduced ejection fraction (HFrEF). However, these last two entities do not represent the usual presentation of heart failure due to ATTRwt, which is predominantly HFpEF.[4]

S100 calcium binding protein A4 (S100A4) plays a role in cell cycle progression and differentiation and possibly in cancer metastasis, among other biological functions.[24] The third selected biomarker is GLB1 (Galactosidase Beta 1) and is involved in degeneration of glycolipids.[25] Both GLB1 and S100A4 appear not to play a role in amyloidogenesis. Furthermore, our initial sPLS-DA analysis showed no significant difference in biomarker profiles between AL and ATTRwt, although the etiology of these forms of amyloidosis are very different. This finding suggests that the amyloid specific biomarker pattern is mainly driven by the presence of amyloid fibril deposition in the myocardium than as a result of the pathogenesis of amyloidosis. Taken together to our opinion the elevated levels of the selected biomarker triplet may better reflect the response of body tissue to amyloid deposition than that they reflect the amyloidogenic process itself.

In addition, we sought to validate the biomarker triplet. The biomarker triplet discriminated the validation set of patients with ATTRwt, which were excluded from the analyses for the selection of the triplet, and the HFmrEF/HFpEF ATTRwt-negative patients. Despite the small size of the validation cohort, the AUC was high. This finding is of interest as these patients did not show the stigmata of more advanced ATTRwt, making this biomarker approach a potential tool for the early diagnosis of ATTRwt.

Limitations

Limitations of our study include the relative small samples size and the absence of a validation cohort. But well-defined HF cohorts in which bone scintigraphy is performed are scarce. In addition, in the VIP-HF cohort AL-amyloidosis was not a formal exclusion criterion. The reported biomarker concentrations are relative values and not interchangeable to other studies.

Conclusion

ATTRwt is prevalent (5%) in HFmrEF/HFpEF patients and a multi-biomarker approach can be used to detect the presence of cardiac amyloidosis in these patients. Such a serum-protein biomarker approach may lead to an earlier diagnosis of cardiac amyloidosis.

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Table 1: Up-regulated proteins in ATTRwt with a ratio >1.12

Uniprot	Assay	GenID	OLINKID	ATTRwt*	nonATTRwt*	Ratio ATTRwt/ NonATTRwt	pvalue
P16278	GLB1	2720	OID00937	3.0 (2.5, 3.7)	1.7 (1.4, 2.2)	1,765	<0.00001
P09874	PARP-1	142	OID00469	3.4 (2.8, 4.2)	2.1 (1.8, 2.6)	1,619	<0.00001
Q13158	FADD	8772	OID00691	2.1 (1.8, 2.5)	1.5 (1.3, 1.7)	1,400	<0.00001
P30048	PRDX3	10935	OID00953	-0.2 (-0.4, 0.2)	-0.5 (-0.6, -0.3)	1,400	<0.00001
Q9HCB6	SPON1	10418	OID00599	2.5 (2.2, 2.7)	1.9 (1.7, 2.1)	1,316	<0.00001
Q13421	MSLN	10232	OID00660	5.0 (4.4, 5.8)	3.8 (3.1, 4.4)	1,316	<0.00001
Q8N608	DPP10	57628	OID00956	2.1 (1.6, 3.2)	1.6 (1.4, 1.9)	1,313	<0.00001
P23229	ITGA6	3655	OID00959	1.3 (1.0, 1.6)	1.0 (0.7, 1.3)	1,300	0.00001
P19474	TRIM21	6737	OID00964	2.6 (2.1, 3.0)	2.0 (1.4, 2.3)	1,300	<0.00001
Q8WXI7	MUC-16	94025	OID00741	4.6 (3.9, 6.2)	3.6 (3.2, 4.2)	1,278	<0.00001
Q05516	ZBTB16	7704	OID00939	1.5 (1.2, 2.1)	1.2 (1.0, 1.6)	1,250	0.00169
Q9HCN6	GP6	51206	OID05026	3.5 (3.0, 4.1)	2.9 (2.7, 3.3)	1,207	<0.00001
O95786	DDX58	23586	OID01018	3.6 (3.2, 4.0)	3.0 (2.5, 3.2)	1,200	<0.00001
Q9BYF1	ACE2	59272	OID00457	4.9 (4.3, 5.5)	4.1 (3.7, 4.6)	1,195	<0.00001
Q9UHC6	CNTNAP2	26047	OID00943	2.5 (2.1, 2.7)	2.1 (2.0, 2.6)	1,190	0.00979
P78310	CXADR	1525	OID00992	3.2 (2.9, 3.9)	2.7 (2.4, 3.3)	1,185	<0.00001
Q14512	FGF-BP1	9982	OID00713	7.7 (7.3, 8.3)	6.5 (6.3, 6.8)	1,185	<0.00001
P04083	ANXA1	301	OID00745	5.2 (5.0, 5.9)	4.4 (4.0, 4.9)	1,182	<0.00001
P10747	CD28	940	OID00977	2.0 (1.8, 2.4)	1.7 (1.5, 2.0)	1,176	0.00003
P07585	DCN	1634	OID00444	6.1 (5.9, 6.4)	5.2 (5.0, 5.4)	1,173	<0.00001
Q12933	TRAF2	7186	OID00963	2.9 (2.5, 3.3)	2.5 (2.1, 2.9)	1,160	0.00105
Q9BXY4	RSPO3	84870	OID00737	7.3 (6.5, 7.9)	6.3 (5.6, 6.7)	1,159	<0.00001
P01241	GH	2688	OID00417	10.3 (9.0, 11.2)	8.9 (8.0, 10.6)	1,157	0.00031
Q9NQ25	SLAMF7	57823	OID00383	4.5 (3.9, 5.2)	3.9 (3.5, 4.3)	1,154	<0.00001
Q96PL1	SCGB3A2	117156	OID00636	3.8 (3.3, 4.2)	3.3 (2.8, 3.8)	1,152	0.00010
P20160	AZU1	566	OID00597	6.9 (5.8, 7.6)	6.0 (5.2, 6.7)	1,150	0.00002
P05164	MPO	4353	OID00600	4.6 (4.0, 5.1)	4.0 (3.7, 4.2)	1,150	<0.00001

Table 1: Up-regulated proteins in ATTRwt with a ratio >1.12

Uniprot	Assay	GenID	OLINKID	ATTRwt*	nonATTRwt*	Ratio ATTRwt/ NonATTRwt	pvalue
Q9C035	TRIM5	85363	OID00957	2.3 (2.0, 2.9)	2.0 (1.7, 2.3)	1,150	<0.00001
O14867	BACH1	571	OID00996	2.3 (2.0, 2.8)	2.0 (1.5, 2.6)	1,150	0.00741
P28845	HSD11B1	3290	OID00980	4.0 (3.7, 4.3)	3.5 (3.1, 3.7)	1,143	<0.00001
P48740	MASP1	5648	OID01014	2.4 (2.2, 2.7)	2.1 (1.8, 2.3)	1,143	<0.00001
Q96SB3	PPP1R9B	84687	OID00936	2.5 (2.2, 3.0)	2.2 (1.8, 2.6)	1,136	0.00026
P26447	S100A4	6275	OID00680	8.4 (8.1, 8.7)	7.4 (7.1, 7.6)	1,135	<0.00001
P12931	SRC	6714	OID00388	5.9 (5.2, 7.0)	5.2 (4.7, 5.9)	1,135	0.00012
Q04637	EIF4G1	1981	OID00976	3.4 (2.7, 4.2)	3.0 (2.5, 3.8)	1,133	0.03901
P16284	PECAM-1	5175	OID00652	5.2 (4.9, 5.5)	4.6 (4.3, 4.8)	1,130	<0.00001
NPPB	NT-proBNP	4879	OID00131	7.1 (6.0, 8.1)	6.3 (5.6, 7.2)	1,127	0.00123
Q13043	STK4	6789	OID00392	2.7 (2.1, 3.3)	2.4 (1.7, 2.9)	1,125	0.01973
P24158	PRTN3	5657	OID00618	6.4 (5.4, 7.2)	5.7 (5.2, 6.2)	1,123	0.00012
O14828	SCAMP3	10067	OID00668	4.6 (4.1, 5.2)	4.1 (3.8, 4.7)	1,122	0.00006
P08253	MMP-2	4313	OID00614	3.7 (3.4, 4.1)	3.3 (3.0, 3.6)	1,121	<0.00001
Q9Y624	JAM-A	50848	OID00625	6.5 (5.9, 7.1)	5.8 (5.4, 6.1)	1,121	<0.00001
P14210	HGF	3082	OID00706	11.3 (10.8, 11.8)	10.1 (9.8, 10.3)	1,120	<0.00001

*Normalized protein expression (IQR).

Table 2: All down-regulated proteins in ATTRwt

Uniprot	Assay	GenID	OLINKID	ATTRwt*	nonATTRwt*	p-value
Q9NQ76	MEPE	56955	OID00132	4.8 (4.2, 5.2)	5.0 (4.7, 5.4)	0.00772
P22004	BMP-6	654	OID00379	4.7 (3.5, 5.3)	5.0 (4.6, 5.4)	0.00758
P35318	ADM	133	OID00381	8.1 (5.1, 9.0)	9.5 (9.1, 9.8)	<0.00001
P29965	CD40-L	959	OID00382	6.4 (5.4, 7.3)	7.2 (6.6, 7.9)	0.00002
P35475	IDUA	3425	OID00393	4.4 (4.0, 4.7)	5.8 (5.4, 6.1)	<0.00001
P27352	GIF	2694	OID00407	7.6 (7.1, 8.4)	8.1 (7.4, 9.1)	0.00412
Q9NSA1	FGF-21	26291	OID00410	6.7 (5.9, 8.0)	8.3 (7.0, 9.4)	0.00002
Q96IQ7	VSIG2	23584	OID00429	5.0 (4.6, 5.6)	5.3 (4.8, 6.0)	0.01090
P47992	XCL1	6375	OID00433	5.5 (5.1, 5.9)	5.9 (5.6, 6.3)	<0.00001
P06858	LPL	4023	OID00446	10.4 (10.0, 10.8)	10.6 (10.3, 10.9)	0.01613
O00253	AGRP	181	OID00448	5.2 (4.5, 5.8)	5.4 (5.0, 5.9)	0.04397
P12104	FABP2	2169	OID00451	9.1 (8.6, 9.6)	9.6 (8.9, 10.0)	0.00290
P41159	LEP	3952	OID00463	5.8 (4.7, 6.8)	7.6 (6.7, 8.3)	<0.00001
Q99727	TIMP4	7079	OID00585	4.9 (4.6, 5.3)	5.2 (4.9, 5.6)	0.00136
P13686	TR-AP	54	OID00606	4.1 (3.6, 4.7)	4.9 (4.6, 5.2)	<0.00001
P56470	Gal-4	3960	OID00626	4.5 (4.1, 4.9)	4.9 (4.6, 5.3)	0.00007
P16870	CPE	1363	OID00657	4.8 (4.6, 5.3)	5.2 (4.9, 5.3)	0.00717
Q9UKR3	KLK13	26085	OID00658	2.7 (2.2, 3.1)	3.6 (3.2, 4.0)	<0.00001
O75888	TNFSF13	8741	OID00661	10.4 (10.1, 10.7)	10.6 (10.4, 10.9)	0.00426
P50591	TRAIL	8743	OID00672	7.0 (6.8, 7.3)	7.3 (7.1, 7.6)	<0.00001
O60259	hK8	11202	OID00676	7.0 (6.7, 7.3)	7.1 (6.8, 7.4)	0.04605
Q9P0G3	hK14	43847	OID00690	6.2 (5.4, 6.9)	6.7 (6.4, 7.2)	<0.00001
P09382	Gal-1	3956	OID00697	7.7 (7.4, 7.8)	7.9 (7.8, 8.1)	<0.00001
Q16674	MIA	8190	OID00701	11.0 (10.8, 11.2)	11.2 (11.0, 11.3)	0.00005
O95971	CD160	11126	OID00721	6.2 (5.7, 6.6)	6.5 (6.1, 7.1)	0.00234
Q99717	MAD homolog 5	4090	OID00734	4.3 (4.1, 4.4)	4.5 (4.4, 4.5)	<0.00001
Q8TE58	ADAM-TS 15	170689	OID00735	2.9 (2.5, 3.5)	4.1 (3.6, 4.4)	<0.00001
Q15661	TPSAB1	7177	OID00941	5.2 (4.9, 5.7)	5.5 (5.1, 5.9)	0.01668
Q8NHJ6	LILRB4	11006	OID00965	3.3 (2.9, 3.9)	3.8 (3.3, 4.2)	0.00015
P34130	NTF4	4909	OID00966	1.5 (1.3, 1.7)	1.8 (1.6, 2.0)	<0.00001
P08727	KRT19	3880	OID00967	2.4 (2.1, 3.1)	2.9 (2.4, 3.4)	0.00305
P51671	CCL11	6356	OID00970	8.2 (7.8, 8.5)	8.3 (8.0, 8.6)	0.03171
Q03431	PTH1R	5745	OID00978	4.0 (3.6, 4.3)	4.4 (4.2, 4.6)	<0.00001
P22301	IL10	3586	OID00993	3.7 (3.3, 4.1)	4.0 (3.8, 4.3)	0.00012
P52823	STC1	6781	OID00999	7.3 (7.0, 7.6)	7.5 (7.4, 7.8)	0.00003
P48061	CXCL12	6387	OID01008	1.2 (1.0, 1.5)	1.5 (1.3, 1.8)	<0.00001

*Normalized protein expression (IQR).

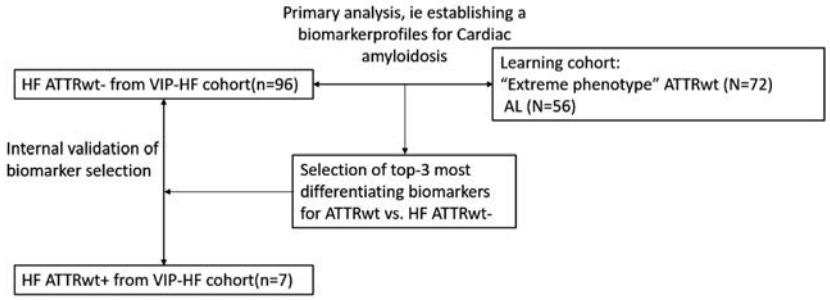

Figure 1: Schematic representation of study design
First analysis; a comparison between the patients form the VIP-HF without ATTRwt and learning cohort with cardiac amyloidosis (both ATTRwt and AL). Secondly, a top-3 biomarker selection most differentiating between HFATTRwt- and ATTRwt was used for the internal validation of the established biomarker profile within the VIP-HF cohort, between patients with and without ATTRwt.

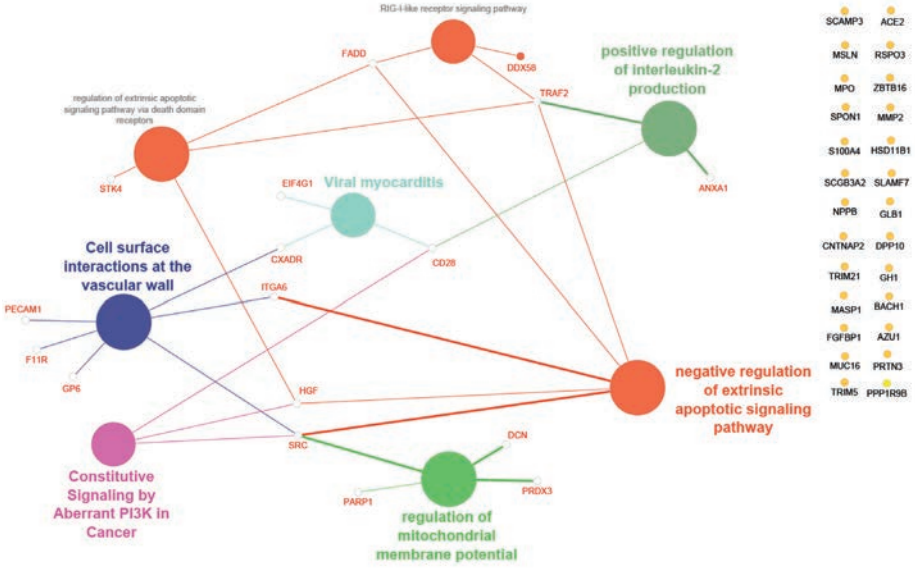

Figure 2: Pathway analysis of up-regulated proteins in ATTRwt

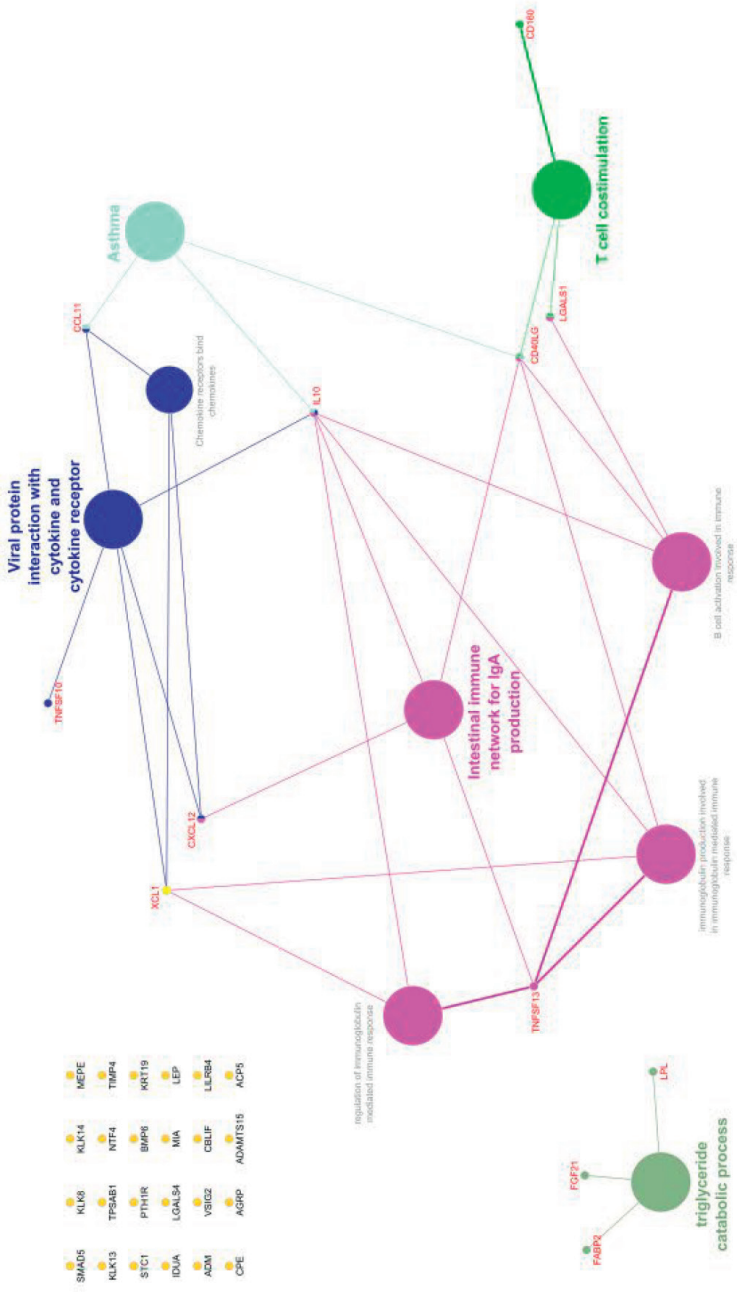

Figure 3: Pathway analysis down-regulated proteins in ATTRwt