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ORIGINAL RESEARCH

Associations Between Blood Biomarkers, Cardiac Function, and Adverse Outcome in a Young Fontan Cohort

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BACKGROUND: Patients who have undergone the Fontan procedure are at high risk of circulatory failure. In an exploratory analysis we aimed to determine the prognostic value of blood biomarkers in a young cohort who have undergone the Fontan procedure.

METHODS AND RESULTS: In multicenter prospective studies patients who have undergone the Fontan procedure underwent blood sampling, cardiopulmonary exercise testing, and stress cardiac magnetic resonance imaging. Several biomarkers including NT-proBNP (N-terminal pro-B-type natriuretic peptide), GDF-15 (growth differentiation factor 15), Gal-3 (galectin-3), ST2 (suppression of tumorigenicity 2), DLK-1 (protein delta homolog 1), FABP-4 (fatty acid-binding protein 4), IGFBP-1 (insulin-like growth factor-binding protein 1), IGFBP-7, MMP-2 (matrix metalloproteinase 2), and vWF (von Willebrand factor) were assessed in blood at 9.6 (7.1–12.1) years after Fontan completion. After this baseline study measurement, follow-up information was collected on the incidence of adverse cardiac events, including cardiac death, out of hospital cardiac arrest, heart transplantation (listing), cardiac reintervention (severe events), hospitalization, and cardioversion/ablation for arrhythmias was collected and the relation with blood biomarkers was assessed by Cox proportional hazard analyses. The correlation between biomarkers and other clinical parameters was evaluated. We included 133 patients who have undergone the Fontan procedure, median age 13.2 (25th, 75th percentile 10.4–15.9) years, median age at Fontan 3.2 (2.5–3.9) years. After a median follow-up of 6.2 (4.9–6.9) years, 36 (27.1%) patients experienced an event of whom 13 (9.8%) had a severe event. NT-proBNP was associated with (all) events during follow-up and remained predictive after correction for age, sex, and dominant ventricle (hazard ratio, 1.89; CI, 1.32–2.68). The severe event-free survival was better in patients with low levels of GDF-15 (P=0.005) and vWF (P=0.008) and high levels of DLK-1 (P=0.041). There was a positive correlation (β=0.33, P=0.003) between DLK-1 and stress cardiac magnetic resonance imaging functional reserve.

CONCLUSIONS: NT-proBNP, GDF-15, vWF, DLK-1, ST-2 FABP-4, and IGFBP-7 levels relate to long-term outcome in young patients who have undergone the Fontan procedure.

Key Words: biomarker ■ congenital heart disease NT-proBNP ■ outcome ■ univentricular heart

Since the introduction of the contemporary modifications of the Fontan operation, which is the treatment of choice in most patients with a functional univentricular heart, long-term survival has improved, resulting in a rapidly increasing number of surviving patients who have undergone the Fontan procedure.[1–4]

However, long-term complications are common and include circulatory failure, thromboembolic

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CLINICAL PERSPECTIVE

What Is New?

- Blood biomarkers such as NT-proBNP (N-terminal pro-B-type natriuretic peptide), ST2 (suppression of tumorigenicity 2), GDF-15 (growth differentiation factor 15), DLK-1 (protein delta homolog 1), vWF (von Willebrand factor), FABP-4 (fatty acid-binding protein 4), and IGFBP-7 (insulin-like growth factor-binding protein 7) are associated with cardiac function and/or long-term outcome in patients who have had a Fontan procedure.
- NT-proBNP was associated with all events (severe adverse events and re-interventions) during the follow-up, and severe event-free survival was better in patients with low levels of GDF-15 and vWF and high levels of DLK-1.

What Are the Clinical Implications?

- These findings suggest a possible role of these blood biomarkers, especially NT-proBNP, in the clinical follow-up and risk stratification of young patients who have undergone the Fontan procedure.

Nonstandard Abbreviations and Acronyms

CPET	cardiopulmonary exercise testing
DLK-1	protein delta homolog 1
FABP4	fatty acid-binding protein 4
FR	functional reserve
Gal-3	galectin-3
GDF-15	growth differentiation factor 15
IGFBP-1	insulin-like growth factor-binding protein 1
IGFBP-7	insulin-like growth factor-binding protein 7
IGFBPs	insulin-like growth factor-binding proteins
MMP-2	matrix metalloproteinase 2
ST2	suppression of tumorigenicity 2
VO_2	oxygen uptake
vWF	von Willebrand factor

events, arrhythmias and death.[1,4,5] In general the incidence of heart failure in patients with congenital heart disease (CHD) is 1.2 per 1000 patients-years and increases with age.[6] One-year mortality after admission for heart failure in patients with CHD is 24%.[6] In patients after atriopulmonary Fontan (which was the original surgical procedure), 28-year freedom from death, heart transplantation or heart failure is only 45%.[2,7] In children and adolescents who have undergone contemporary modifications of the Fontan operation in a staged approach, the incidence of heart failure seems lower although exact data are lacking.[3,8] In patients who have undergone the Fontan procedure early identification and treatment of heart failure is important.[2]

Blood biomarkers are a new potential tool in risk stratification in patients with CHD. In recent years various pathways of myocardial stress, inflammation, fibrosis, remodeling and vascularization and related blood biomarkers have been discovered, mostly in adult patients with heart failure.[9–13] In previous studies biomarkers such as NT-proBNP (N-terminal pro-B-type natriuretic peptide), Gal-3 (galectin-3), ST2 (suppression of tumorigenicity 2), GDF-15 (growth differentiation factor 15), vWF (von Willebrand factor), and MMP-2 (matrix metalloproteinase 2) have been related to clinical condition, heart failure, or impaired cardiac function in groups of patients with CHD with mixed diagnoses.[9,12,14–17] However, in young patients who have undergone the Fontan procedure relatively few biomarkers have been studied. Therefore, the aim of this study was to explore the relationship between levels of multiple promising biomarkers (assessed from the literature) and clinical outcomes in a young contemporary cohort who have undergone the Fontan procedure.

METHODS

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

Patients and Methods

We included all patients who have undergone the Fontan procedure ≥8 years old from whom blood samples taken at a single moment in time were stored in the setting of 2 cross-sectional and prospective studies in 5 tertiary referral centers between 2009 and 2018.[18,19] The institutional review boards of participating centers approved the studies. Patients with contraindications for cardiac magnetic resonance imaging (CMR) were excluded. All participants, and if necessary their parents, gave written informed consent before inclusion in these studies. At the baseline study assessment all patients underwent blood sampling, CMR, and cardiopulmonary exercise testing (CPET) according to a standard protocol in all contributing centers. The patients were subsequently

followed in the setting of usual care, commonly 1 to 2 visits/year.

Blood Sample Analysis

Blood samples were taken from a peripheral vein and collected in EDTA tubes. Samples were stored at −80°C. The frozen samples were shipped to Olink Proteomics AB (Uppsala, Sweden) for analysis with the Olink Cardiovascular panel III. Using proximity extension assay technology, the levels of biomarkers were measured; this technique has been described extensively before.[20] All blood samples were coded; therefore, laboratory staff was blinded for the patients' clinical and study data. Likewise the physician did not know the outcomes of the biomarkers assessment. Biomarker levels were not used for clinical decision-making. The biomarker values are presented as normalized protein expression units on a Log2 scale.

For the aim of the current study, we examined 10 biomarkers that have been associated with CHD, the Fontan circulation, cardiac fibrosis, or heart failure in general.[11,16,17,21–24] These biomarkers were GDF-15, Gal-3, DLK-1 (protein delta homolog 1), FABP-4 (fatty acid-binding protein 4), IGFBP-1 (insulin-like growth factor-binding protein 1), IGFBP-7, NT-proBNP, MMP-2, ST2 and vWF. These biomarkers were selected from the literature before the data analysis.

Clinical Data

CMR Acquisition and Analysis

CMR imaging was performed with a dedicated phased-array cardiac surface coil. All images were acquired without sedation during free breathing. Scans were performed at rest and repeated during continuous low-dose (7.5 µg/kg per minute) dobutamine-hydrochloride infusion (Centrafarm Services, Etten-Leur, The Netherlands). Dobutamine infusion was decreased to 5.0 µg/kg per minute (or if necessary stopped) when the heart rate increased >50%, when the systolic and/or diastolic blood pressure increased >50% or decreased <20%, when rhythm disturbances were seen, or with complaints of the patient. Details on our dobutamine stress protocol have been published previously.[18,25]

Analyses were performed with the software packages MASS and FLOW (Medis Medical Imaging Systems, Leiden, The Netherlands). Contours were manually drawn in end-diastole and end-systole; papillary muscle and trabeculae were excluded from the blood pool. All CMRs were analyzed by one of the authors (E.v.d.B.) under supervision of one of the authors (W.H.) with long-standing experience in CMR. End-diastolic volume and end-systolic volume were obtained and used to calculate ejection fraction (EF). Ventricular volumes were defined as the sum of the volumes of the systemic ventricle and the hypoplastic chamber. All ventricular volumes were indexed for body surface area. Data on the reproducibility of the CMR analyses have previously been published by our group.[26]

Changes in CMR parameters during dobutamine stress were calculated as follows: parameter change (Δ)=$\text{parameter}_{\text{stress}}$−$\text{parameter}_{\text{rest}}$, functional reserve is described as the $\text{EF}_{\text{stress}}$−$\text{EF}_{\text{rest}}$.[5]

Cardiopulmonary Exercise Tests

CPETs were performed on a bicycle ergometer according to protocols used in previous studies by our group.[18,25] From these exercise tests the peak oxygen uptake (VO_2) was assessed and expressed as percentage of predicted values. Exercise tests with a peak respiratory exchange rate of ≥1.0 were included in the analysis.

Study End Point

After the baseline study assessment, patients received regular patient specific care. For the purpose of the current study the medical records of the latest outpatients visit were reviewed and all cardiac events during follow-up (since the baseline study measurement) were recorded until June 2018. The survival status of the patients was also checked in the Municipal Population Register.

Severe events were defined as death, out of hospital cardiac arrest, heart transplantation (listing), or cardiac reoperations. Overall events included the severe events as well as cardiac reintervention and hospitalization or cardioversion/ablation for arrhythmias.

Patients who experienced multiple events were considered to have reached the study end point at the time of the first event.

Statistical Analysis

Continuous baseline variables are summarized as mean value±SD, and as median value (25th–75th percentile). Differences between patients with and without events were analyzed using Mann-Whitney *U* tests. Categorical variables are presented as numbers and percentages, whereas between-group differences are evaluated by chi-square tests, or Fischer's exact tests (in case expected values <5).

Linear regression analysis is applied to study the relation between the selected biomarkers with CPET, CMR, and clinical parameters, while adjusting for age and sex.

We applied Cox proportional hazard regression analyses to explore the association between the selected biomarkers and the incidence of the specified study end points. Biomarkers were entered as

standardized continuous variables (*Z*-score). We report hazard ratios (HR) with corresponding 95% CI, which are estimated using Firth's method for bias reduction in small samples. For "overall" events we present regression results as (1) unadjusted HRs, (2) HRs that are adjusted for age and sex, and (3) HRs that are adjusted for age, sex, and dominant ventricle. For "severe" events we present only unadjusted HRs, because the number of such events was limited.

The Cox regression model provides a relative measure of association between the explanatory variable (ie, the biomarker) and the study end point. We produced Kaplan-Meier event-free survival curves in order to also provide an impression of the relation between biomarker levels and absolute incidences. For that particular purpose, patients were categorized by quartiles (quartile 1–2 versus quartile 3–4) of the corresponding biomarker, whereas differences between groups were evaluated using the log-rank test, in particular the permutation version (in view of the relative small number of events).

Analyses were performed using SPSS (version 24.0) and R (version 4.0.0; mainly the "coin" and "coxphf" packages) statistical software. Two-sided $P<0.05$ is considered statistically significant.

RESULTS

A total of 133 patients were included in this analysis at a median of 9.7 (7.1, 12.1) years after the Fontan operation. The median age at the baseline study assessment was 13.2 years (10.4–15.9). At baseline a CMR was performed in 119 patients and a successful CPET was performed in 103 patients. The available blood sample was successfully analyzed in all 133 patients. Table 1 shows patient characteristics in relation to study end point events.

Overall Events and Baseline Characteristics

During a median follow-up of 6.2 (4.9, 6.9) years since the baseline study assessment, 36 (27.1%) patients experienced an overall event (see Table 2). The main cause for overall events were cardiac catheter interventions. One patient experienced an out of hospital cardiac arrest and received an implantable cardioverter-defibrillator.

There was no difference in median age or other surgical or baseline characteristics between the patients who did and did not develop an event during follow-up. Patients with an event had a significantly diminished increase in EF during dobutamine stress CMR (FR), 5±6% versus 10±6%, $P=0.001$.[5]

Overall Events and Biomarkers

Results of Cox regression analyses relating the selected biomarkers "overall events" are given in Table 3. It appeared that only NT-proBNP was significantly associated with the incidence of an "overall event." After adjustment for age, sex, and dominant RV, the HR for a 1-SD difference was 1.88 (95% CI, 1.31–2.69; $P=0.001$). ST2 was also associated with "overall events," but statistical significance was not reached (adjusted HR for 1 SD difference 1.38; 95% CI, 0.98–1.89; $P=0.063$).

Severe Events and Biomarkers

A total of 13 (9.8%) patients experienced a severe event during follow-up, see Table 1. There were no statistically significant differences in median age or other surgical or baseline characteristics between the patients with and without a severe event. Patients with a severe event had a lower baseline EF, a higher ventricular mass, and a lower FR compared with patients without a severe event.[5]

The severe event-free survival was better in patients with lower levels of vWF ($P=0.008$). In addition patients with the highest DLK-1 levels ($P=0.041$) and lowest GDF-15 levels ($P=0.005$) experienced the best severe event-free survival (see Figure). In a univariable Cox regression model, higher levels of NT-proBNP, ST2, and vWF were associated with severe events during follow-up (Table 4).

Association of Biomarkers With Other Clinical Parameters

In Table 5 associations, corrected for age and sex, between the biomarkers and several baseline CMR and CPET parameters are shown. DLK-1 was associated with FR; for every percentage of increase in FR, DLK-1 increased with a factor $\beta=0.33$, $P=0.003$.

DISCUSSION

In this explorative, prospective multicenter study in young patients who have undergone the Fontan procedure we demonstrated an association of several blood biomarkers and ventricular FR with clinical condition and events during 6 years of follow-up. We observed that NT-proBNP, vWF, DLK-1, ST2, and GDF-15 were related with clinical events during follow-up. Other biomarkers such as FABP-4 and IGFBP-7 seemed associated with parameters of cardiac function. Although the observed relations resemble findings of previous studies (primarily in acquired heart disease) and can be understood from pathological point of view (as we discuss later), we still consider these as hypothesis generating, given the explorative nature of our study and the broad range of biomarkers studied.

Patients who have undergone the Fontan procedure are at high risk for late death, arrhythmias, and reinterventions.[1,2,4] Even in our young (median age 13.2 years)

Table 1. Baseline Patient Characteristics in Patients Who Reached Study End Points and Those Who Remained Event-Free

	Overall Event	No Overall Event	*P* Value	Severe Event*	No Severe Event	*P* Value
No. of patients	36	97		13	120	
Age at baseline, y	13.9±4.9	13.9±4.2	0.98	15.8±5.4	13.7±4.3	0.097
	12.9 (9.8–15.5)	13.2 (10.4–16.2)	0.67	14.8 (11.2–20.9)	12.9 (10.4–15.8)	0.16
Male, n (%)	20 (55.6)	55 (56.7)	1	10 (76.9)	65 (54.2)	0.15
Resting saturation (%)	94±5	95±3	0.23	95±3	95±3	0.94
	95 (93–97)	95 (94–97)	0.58	95 (94–98)	95 (94–97)	0.98
Length, cm	152±17	157±15	0.17	160±18	155±16	0.30
	151 (137–166)	156 (144–168)	0.16	160 (144–177)	155 (142–167)	0.37
Weight, kg	44±17	47±15	0.32	51±19	46±15	0.23
	38 (30–54)	46 (34–58)	0.19	48 (36–63)	43 (33–57)	0.35
Body surface area, m^2	1.35±0.33	1.42±0.29	0.25	1.50±0.35	1.39±0.30	0.25
	1.26 (1.09–1.57)	1.44 (1.09–1.64)	0.16	1.47 (1.20–1.75)	1.38 (1.13–1.63)	0.38
Dominant ventricle						
Left, n (%)	24 (66.7)	60 (61.9)	0.69	9 (69.2)	75 (62.5)	0.77
Right, n (%)	11 (30.6)	36 (37.1)	0.54	4 (30.8)	43 (35.8)	1.00
Indifferent, n (%)	1 (2.8)	1 (1.0)	0.47	0 (0.0)	2 (1.7)	1.00
Cardiac diagnosis						
Hypoplastic left heart syndrome, n (%)	8 (22.2)	12 (12.4)	0.18	3 (23.1)	17 (14.2)	0.41
Tricuspid atresia, n (%)	11 (30.6)	30 (30.9)	1.00	5 (38.5)	36 (30.0)	0.54
Pulmonary atresia, n (%)	4 (11.1)	11 (11.3)	1.00	1 (7.7)	14 (11.7)	1.00
Double inlet left ventricle, n (%)	6 (16.7)	13 (13.4)	0.59	2 (15.4)	17 (14.2)	1.00
Double outlet right ventricle, n (%)	3 (8.3)	18 (18.6)	0.19	1 (7.7)	20 (16.7)	0.69
Other, n (%)	4 (11.1)	13 (13.4)	1.00	1 (7.7)	16 (13.3)	1.00
Age at Fontan procedure y	3.2±1.1	3.5±1.3	0.31	3.5±1.5	3.4±1.3	0.88
	3.2 (2.4–3.9)	3.2 (2.6–3.9)	0.54	3.3 (2.4–4.3)	3.2 (2.5–3.9)	0.90
Type of Fontan						
Extra cardiac conduit, n (%)	20 (60.6)	56 (58.9)	0.85	6 (46.2)	70 (58.3)	0.56
Intra-atrial lateral tunnel, n (%)	13 (39.4)	39 (41.1)	0.70	6 (46.2)	46 (38.3)	0.77
Other, n (%)	3 (8.3)	2 (2.1)	0.12	1 (7.7)	4 (3.3)	0.41
Maximal Exercise Parameters	**n=24**	**n=79**		**n=11**	**n=92**	
Peak VO_2, mL/min per kg)	32.1±8.5	33.1±6.8	0.55	33.3±9.7	32.8±6.9	0.85
	33.5 (24.4–38.8)	32.4 (28.2–38.0)	0.59	36.1 (23.6–40.4)	32.2 (28.0–38.0)	0.81
Peak VO_2 (% of predicted)	78.3±18.1	82.1±15.9	0.32	77.9±22.1	81.6±15.7	0.48
	77.3 (64.4–92.7)	81.5 (69.5–92.6)	0.34	78.2 (55.0–93.8)	80.7 (69.7–92.6)	0.66
CMR	**n=32**	**n=87**		**n=11**	**n=108**	
EDV, mL/m^2	97±34	90±19	0.28	118±43	89±20	0.053
	85 (72–102)	87 (76–101)	0.75	99 (94–140)	86 (76–100)	0.006
ESV, mL/m^2	47±29	42±12	0.28	65±41	41±12	0.078
	40 (31–52)	40 (33–48)	0.71	57 (49–62)	39 (32–48)	0.002
SV, mL/m^2	48±14	48±11	0.67	53±13	48±12	0.25
	47 (41–54)	46 (42–58)	0.97	47 (45–65)	46 (51–54)	0.27
EF (%)	53±10	54±7	0.54	47±11	54±8	0.007
	53 (46–60)	54 (50–59)	0.64	47 (44–56)	55 (49–60)	0.023
Mass, g/m^2	61±19	56±15	0.10	67±19	56±16	0.037
	57 (46–71)	53 (44–65)	0.22	71 (52–76)	53 (45–65)	0.036
Mass/EDV ratio, g/mL	0.66±0.24	0.63±0.15	0.32	0.58±0.10	0.64±0.18	0.24
	0.62 (0.53–0.74)	0.61 (0.51–0.74)	0.73	0.61 (0.48–0.64)	0.62 (0.52–0.75)	0.23

(*Continued*)

Table 1. Continued

Δ Stress: Stress–Rest CMR	n=19	n=57		n=8	n=68	
EDV, mL/m^2	−15±13	−12±9	0.31	−12±11	−13±10	0.75
	−12 (−21–−6)	−10 (−18–−5)	0.51	−11 (−24–0)	−10 (−18–−5)	0.79
ESV, mL/m^2	−10±9	−13±7	0.13	−10±9	−13±7	0.37
	−9 (−14–−4)	−13 (−17–−9)	0.057	−6 (−21–−3)	−12 (−17–−9)	0.36
SV, mL/m^2	−4±8	2±7	0.005	−1±5	0±8	0.61
	−3 (−6–0)	2 (−2–6)	0.005	−3 (−4–3)	0 (−3–6)	0.28
EF (%)	5±6	10±6	0.001	5±4	10±7	0.033
	5 (1–9)	10 (6–15)	0.002	5 (1–7)	10 (5–14)	0.016

Continuous baseline variables are summarized as mean value± SD, and as median value (25th–75th percentile). Categorical variables are presented as numbers and percentages. CMR indicates cardiovascular magnetic resonance imaging; EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; SV, stroke volume; and VO_2 peak, maximum oxygen uptake.

*Definition severe event: death, out of hospital cardiac arrest, heart transplantation (listing), or cardiac reoperations

cohort of patients 27% experienced an event during midterm follow-up, and in 9.8% this was a severe event. These observations provide information hardly available so far on the level of events in this age cohort after Fontan operations with contemporary strategies and point toward the importance of risk-stratification even in relatively young patients who have undergone the Fontan procedure.

Potentially, assessment of blood biomarkers levels is a relatively simple and harmless method to monitor the clinical condition. Because there may be differences in pathways involved in heart/circulatory failure in children with CHD compared with adults with heart failure, including adults with CHD, assessment of markers in young patients is important.[12,13,27] As such, our study explored biological pathways that are involved in the maintenance of cardiac function and mid- to long-term outcome in a young cohort who have undergone the Fontan procedure.

Fontan failure is generally divided in ventricular failure, systemic venous failure, and pulmonary vascular failure.[2] Common pathways that are most likely involved in the development of heart failure in CHD relate to myocardial hypertrophy, inflammation, fibrosis, remodeling, vascularization, cardiac metabolism, and repair.[27] The biomarkers we found that were associated with an increased risk for poor clinical outcome have been associated with ventricular failure and fibrosis (NT-proBNP, GDF-15, DLK-1) or potential endothelial failure (vWF).[11,28–31] A potentially highly interesting finding in this setting is that of IGFBP-7, because this factor has been associated with cardiac regeneration in zebrafish and mice.[32]

We subsequently discuss the biological role of these blood biomarkers and associations with clinical outcomes in our study.[11,16,17,21–24]

Table 2. Clinical State at Latest Follow-Up

	Patients (n=133)
Median age at latest follow-up, y	18.3 (16.0–21.8)
Median time after blood sampling, y	6.2 (4.9–6.9)
First overall event, n (%)	36 (27.1)
Median time after study until first overall event, y	2.8 (1.1–4.7)
Median time after Fontan until first overall event, y	12.6 (9.7–17.1)
OHCA, survived, n (%)	1 (0.8)
Cardiac reoperation, n (%)	8 (6.0)
Cardiac catheter intervention, n (%)	13 (9.8)
Hospitalization/ablation for arrhythmias, n (%)	12 (9.0)
Implantation pacemaker, n (%)	2 (1.5)
Second overall event, n (%)	12 (9.0)
Severe event*, n (%)	13 (9.8)
Deceased, n (%)	1 (0.8)
OHCA, survived, n (%)	1 (0.8)
Heart transplantation listing, n (%)	1 (0.8)
Cardiac reoperation, n (%)	10 (7.5)
Extra cardiac conduit replacement, n (%)	3 (2.3)
Closure tunnel leakage, n (%)	2 (1.5)
Bentall procedure, n (%)	2 (1.5)
Mitral valve replacement, n (%)	1 (0.8)
Other, n (%)	2 (1.5)

OHCA indicates out of hospital cardiac arrest.

*Definition severe event: death, OHCA, heart transplantation (listing) or cardiac reoperations.

N-Terminal Pro-B-Type Natriuretic Peptide

NT-proBNP is secreted mainly by the ventricle as response to increased myocardial stress and ventricular volume and pressure overload.[28] It is a well-known biomarker in acquired heart failure and adult patients with CHD; elevated NT-proBNP levels are associated with mortality and adverse events.[12,24,28,33,34] In asymptomatic patients who have undergone the Fontan procedure NT-proBNP levels are often within the normal range,[34] but elevated NT-proBNP levels have been associated with an older surgical technique and impaired ventricular function.[24,35] Although associations between

Table 3. Cox-Regression Analyses for Biomarkers and the Overall Events

	Crude Univariable Model			Model Adjusted for Age and Sex			Clinical Model*		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Levels (per 1 SD difference)									
Protein delta homolog 1	0.85	0.62–1.18	0.335	0.86	0.61–1.20	0.369	0.86	0.61–1.21	0.382
Fatty acid-binding protein 4	1.28	0.92–1.77	0.141	1.28	0.91–1.77	0.148	1.29	0.93–1.78	0.129
Galectin 3	1.04	0.77–1.33	0.776	1.04	0.77–1.33	0.784	1.03	0.77–1.32	0.823
Growth differentiation factor 15	1.11	0.83–1.46	0.468	1.11	0.80–1.50	0.529	1.12	0.81–1.52	0.482
IFGBP-1	1.15	0.83–1.61	0.400	1.16	0.83–1.62	0.390	1.14	0.82–1.60	0.428
IFGBP-7	1.27	0.93–1.76	0.137	1.29	0.94–1.79	0.117	1.32	0.95–1.81	0.097
MMP-2	1.19	0.85–1.65	0.305	1.21	0.86–1.69	0.266	1.23	0.88–1.70	0.224
N-terminal pro-B-type natriuretic peptide	1.72	1.25–2.33	0.001	1.90	1.33–2.70	0.001	1.89	1.32–2.68	0.001
Suppression of tumorigenicity 2	1.35	0.98–1.80	0.065	1.38	0.99–1.89	0.060	1.38	0.98–1.89	0.063
von Willebrand factor	1.27	0.91–1.74	0.153	1.31	0.93–1.83	0.116	1.31	0.93–1.82	0.118

HR indicates hazard ratio; and IGFBP, insulin-like growth factor-binding protein.
*Clinical model: Cox model adjusted for age, sex, and single ventricle type.

elevated NT-pro(BNP) levels and adverse outcome have been observed,[28,34,36] monitoring of (NT-pro)BNP is not specifically mentioned in recent international guidelines for the follow-up of patients who have undergone the Fontan procedure.[37] Our results highlight the potential value of NT-proBNP in the routine follow-up of young patients who have undergone the Fontan procedure.

von Willebrand Factor

vWF is produced mainly in endothelial cells. In recent years it has emerged as a mediator of inflammation.[29,30] vWF levels have been associated with an increased risk of myocardial infarction, cerebral stroke, and coronary artery disease.[29,38] In patients with CHD and especially in patients who have undergone the Fontan procedure,

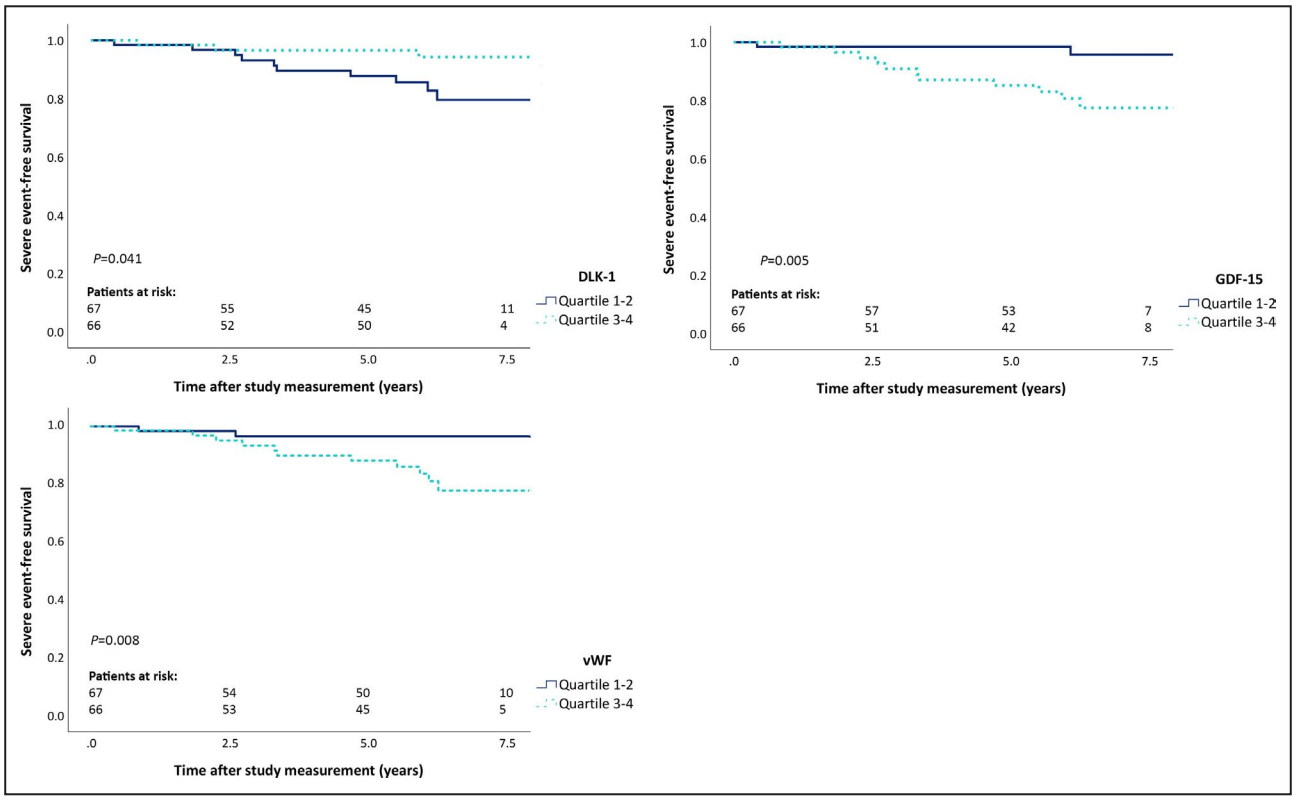

Figure 1. Kaplan-Meier curves for severe event-free survival for the lowest vs highest quartiles of DLK-1, GDF-15, and vWF.
DLK-1 indicates protein delta homolog 1; GDF-15, growth differentiation factor-15; and vWF, von Willebrand factor.

Table 4. Cox-Regression Analyses for Biomarkers and Severe Events

	Crude Univariable Model		
	HR	95% CI	*P* Value
Levels (per 1 SD difference)			
Protein delta homolog 1	0.62	0.37–1.06	0.084
Fatty acid-binding protein 4	1.70	0.99–2.85	0.053
Galectin 3	0.98	0.53–1.55	0.952
Growth differentiation factor 15	1.49	0.96–2.19	0.073
IFGBP-1	1.40	0.82–2.42	0.218
IFGBP-7	1.42	0.82–2.50	0.216
Matrix metalloproteinase 2	1.40	0.81–2.35	0.227
N-terminal pro-B-type natriuretic peptide	2.01	1.27–3.08	0.004
Suppression of tumorigenicity 2	1.67	1.02–2.54	0.040
von Willebrand factor	1.77	1.05–2.94	0.032

HR indicates hazard ratio; and IGFBP, insulin-like growth factor-binding protein.

the role of elevated vWF and an elevated risk of thrombosis has been evident.[30,39] Beyond thrombosis and hemostasis vWF has been associated with adverse events in CHD.[17] We noted that higher vWF levels are associated with a worse severe event-free survival. None of the events in our patients was thromboembolic.

Protein Delta Homolog 1

DLK-1 is a member of the epidermal growth factor-like family.[11] DLK-1 plays a role in angiogenesis, muscular differentiation, and fibrosis.[11,40] DLK-1 knockout mice display increased collagen deposition, left ventricle dilatation, and reduced myocardial contractility.[11] In human ischemic myocardial tissue DLK-1 mRNA expression was downregulated compared with healthy tissue.[11] In our study, patients with higher DLK-1 levels who have undergone the Fontan procedure have a better severe-event-free survival. Also, higher DLK-1 levels were associated with a higher FR during dobutamine stress CMR. We recently showed that higher FR is associated with a better event-free survival in young patients who have undergone the Fontan procedure in whom other known predictors did not differentiate between events.[5]

Our findings, combined with the existing literature, indicate a potential role of DLK-1 in the maintenance of cardiac function.

Suppression of Tumorigenicity 2

ST2 is a member of the interleukin-1 receptor family and can be expressed in a soluble form and a transmembrane form (ST2 ligand).[9,22] ST2 is upregulated in response to myocardial stress and is a marker for inflammation and remodeling, fibrosis, and apoptosis in the myocardium.[9,22,41] In acquired heart failure, higher ST2 levels have been associated with adverse outcomes.[41] In large cohorts (n=602 and n=169) of adult patients with CHD with mainly biventricular circulations, patients with complex CHD displayed higher soluble ST2 levels, which predicted all-cause mortality and events during follow-up.[9,22] Likewise in children with several types of CHD, elevated pre- and postoperative ST2 levels have been associated with 30-day re-admission rate and mortality.[42] In another pediatric CHD cohort (n=36), including a range of defects, a negative correlation between soluble ST2 levels and left ventricular EF was observed.[21]

In our cohort, higher ST2 levels at baseline were associated with severe events during follow-up. Indicating a possible role for ST2 in the clinical follow-up of young patients who have undergone the Fontan procedure.

Growth Differentiation Factor 15

GDF-15 is a member of the TGFβ (transforming growth factor beta) family and during ischemia, oxidative stress, or reperfusion it is expressed in the heart.[31] GDF-15 is also involved in several cancers and diabetes mellitus and may inhibit body-growth, potentially contributing to the "failure to thrive" mechanism.[31,43] In adults with CHD, higher GDF-15 levels correlate with poor functional status, cardiac dysfunction, lower VO_2 max, elevated pulmonary pressure, and adverse outcome.[15,24,31] A small (n=38) study in young (15.0 years) patients who have undergone the Fontan procedure observed that patients with an echocardiographic EF <50% had significantly higher GDF-15 levels compared with patients with preserved systolic function.[44] Higher GDF-15 levels were associated with reduced severe event-free survival in our cohort who have undergone the Fontan procedure, not with max VO_2.[31] However, max VO_2 values in children with CHD are often more preserved compared with adults with CHD.[4]

Insulin-Like Growth Factor-Binding Protein 7

IGFBPs are a family of proteins that regulate and modulate IGF activity and have indirect effects on growth hormone.[45] IGFBP-7 is highly expressed in endothelial cells and has been linked to collagen deposition.[46–48] Interestingly, IGFBP-7 has been linked to postinfarction myocardial repair.[32] In both mouse and zebrafish heart regeneration, infarct border zone cardiomyocytes seem to be the most prone to divide. IGFBP7 is upregulated in this border zone of the injured mouse and zebrafish heart, suggesting a role in cardiac regeneration.[32] IGFBP-7 has been identified as potential biomarker for the prediction of adverse outcome in patients with acquired heart failure[23] and is associated

Table 5. Association Between Study Parameters and Biomarker Levels, Corrected for Age and Sex

Dependent Variable		VO_2 Max (Per 1 mL/min per kg)	EF (Per 1%)	Functional Reserve (ΔEF) (Per 1%)
Protein delta homolog 1	β	−0.02	0.07	0.33
	95% CI	−0.02 to 0.02	−0.01 to 0.02	0.01 to 0.05
	P value	0.82	0.42	0.003
Fatty acid-binding protein 4	β	−0.38	0.17	−0.05
	95% CI	−0.05 to −0.01	−0.02 to 0.001	−0.02 to 0.02
	P value	<0.001	0.061	0.65
Galectin 3	β	−0.03	0.12	0.11
	95% CI	−0.01 to −0.01	−0.004 to 0.02	−0.01 to 0.02
	P value	0.77	0.23	0.36
Growth differentiation factor 15	β	−0.15	−0.15	−0.04
	95% CI	−0.03 to 0.004	−0.02 to 0.002	−0.02 to 0.02
	P value	0.14	0.090	0.75
IGFBP-1	β	−0.07	−0.05	0.14
	95% CI	−0.05 to −0.02	−0.03 to 0.02	−0.02 to 0.06
	P value	0.51	0.62	0.26
IGFBP-7	β	−0.27	−0.245	0.05
	95% CI	−0.03 to −0.004	−0.02 to −0.00	−0.01 to 0.02
	P value	0.013	0.009	0.71
Matrix metalloproteinase 2	β	−0.20	0.16	0.09
	95% CI	−0.02 to 0.001	−0.02 to 0.001	−0.01 to 0.02
	P value	0.072	0.094	0.46
N-terminal pro-B-type natriuretic peptide	β	−0.19	−0.04	−0.20
	95% CI	−0.07 to 0.003	−0.03 to 0.02	−0.08 to 0.003
	P value	0.069	0.66	0.069
Suppression of tumorigenicity 2	β	−0.07	−0.11	0.20
	95% CI	−0.02 to 0.01	−0.02 to 0.005	−0.002 to 0.04
	P value	0.49	0.23	0.075
von Willebrand factor	β	−0.04	0.002	0.02
	95% CI	−0.02 to 0.02	−0.02 to 0.02	−0.02 to 0.03
	P value	0.73	0.98	0.88

Interpretation: for every difference in mL/m^2 or %, the biomarker difference is a factor β. EF indicates ejection fraction; IGFBP, insulin-like growth factor-binding protein; and VO_2 max, maximum oxygen uptake.

with left ventricle diastolic dysfunction and lower VO_2 max.[47] In patients with CHD, the role of IGFPBs in cardiac function or prognosis is largely unexplored but has been linked to general growth, failure to thrive, and nutritional status.[49,50] Our study is, to our knowledge, the first study in patients with CHD who have undergone the Fontan procedure that observed an association between IGFBP-7 levels and cardiac function and VO_2 max.

Fatty Acid-Binding Protein 4

FABP-4 is highly expressed in adipocytes and elevated levels of FABP-4 are associated with adiposity, female sex, diabetes mellitus, and systemic hypertension.[23,51,52] FABP-4 displays some expression in macrophages. It is thought that in macrophages FABP-4 increases foam cell formation and induces an inflammatory response.[51,52]

FABP-4 levels have been associated with left ventricle hypertrophy and systolic and diastolic dysfunction.[51] In patients with chronic heart failure, higher FABP-4-levels were independently associated with adverse outcome during follow-up.[23] In patients with CHD little is known about FABP-4. Although in our study higher FABP-4 levels were not associated with events, higher FABP-4 levels were associated with lower peak VO_2. A diminished peak VO_2 is a known predictor for poor outcome in CHD.[53] FABP-4 may be a potential biomarker in CHD and therefore further research on

the role of FABP-4 levels in CHD is required to assess its value in clinical practice.

Limitations

We studied a total of 133 patients who have undergone the Fontan procedure, which can be considered a small sample. However, compared with the existing literature on biomarker assessment in patients who have undergone the Fontan procedure our sample is relatively large. The patients who have undergone the Fontan procedure in our study were relatively young and in good clinical condition; therefore, the number of hard end points during follow-up was limited. This is a known limitation in CHD research.[37] Because of the limited number of end points, especially severe events, it is possible that we have missed associations between biomarkers and end points in this study. For this reason, we also could not assess the additional value of combining different biomarkers to predict end points. At the other hand, we acknowledge that false positives are a competing explanation for some of the found associations, as we did not adjust for multiple testing. And finally, although we did adjust for age and sex in an additive model, we are aware of the possibility of residual confounding. We consider our explorative study mainly as hypothesis generating.

In our study we assessed some of the biomarkers of the Olink cardiovascular III panel to detect possible patterns between biomarkers and cardiac outcome. We did not assess all the measured biomarkers of the Olink panel. Detecting biomarker cutoff values for clinical use was not part of this study and further research is necessary to determine the possible role of the observed biomarkers in clinical practice.

Late gadolinium enhancement or T1 mapping, useful in detecting local or generalized fibrosis in the myocardium, was not performed in our imaging protocol owing to time constraints.[54] Therefore, we could not investigate associations between myocardial fibrosis with potential fibrosis blood biomarkers.

CONCLUSIONS

In this explorative, prospective multicenter study, we performed an analysis of blood biomarkers and their relation to cardiac function and subsequent outcome in a young and contemporary population who have undergone the Fontan procedure. We observed that in addition to NT-proBNP, ST2, and GDF-15, biomarkers such as DLK-1, vWF, FABP-4, and IGFBP-7 relate to cardiac function and long-term outcome, as did the ventricular response to dobutamine stress CMR.[5] These biomarkers, especially NT-proBNP, may have a role in the clinical follow-up and risk stratification of patients who have undergone the Fontan procedure.

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