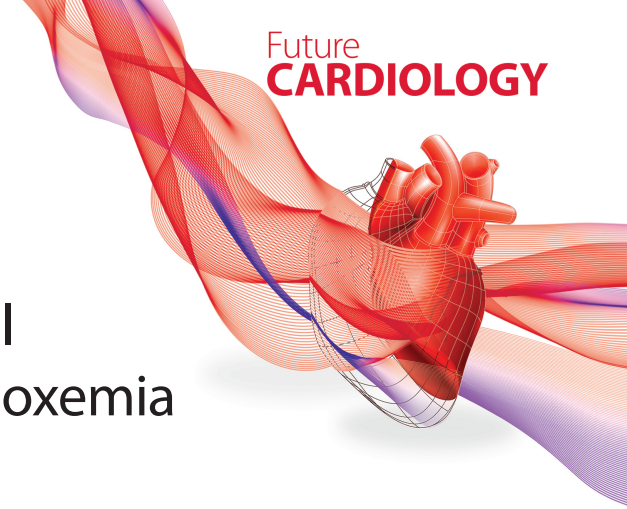
RESEARCH ARTICLE



Myocardial cardiotrophin-1 is differentially induced in congenital cardiac defects depending on hypoxemia

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ABSTRACT: **Aim:** Cardiotrophin-1 (CT-1) is upregulated by hypoxemia and hemodynamic overload and is characterized by potent hypertrophic and protective properties on cardiac cells. This study aimed to investigate whether CT-1 is differentially induced in the myocardium of infants with congenital cardiac defects depending on hypoxemia. **Methods & results:** Infants with Tetralogy of Fallot (n = 8) or with large nonrestrictive ventricular septal defect (n = 8) undergoing corrective surgery were investigated. Expression of CT-1 was assessed at mRNA and protein levels in the right atrial and ventricular myocardium. The activation of the STAT-3 and VEGF were measured. Degradation of cardiac troponin-I served as a marker of myocardial damage. CT-1 was detected in all patients with levels negatively correlating to the arterial oxygen saturation. Higher CT-1 expression in Tetralogy of Fallot patients was associated with activation of the JAK/STAT pathway and higher cardiac troponin-I degradation. **Conclusion:** CT-1 may mediate myocardial hypertrophy and dysfunction in infants with congenital cardiac defects, particularly in those with hypoxemia.

KEYWORDS
- congenital heart disease
- myocardial remodeling
- signal transduction

Cardiotrophin-1 (CT-1), a member of the IL-6 family, was originally discovered as a factor that can induce hypertrophy of cardiomyocytes, both *in vitro* and *in vivo* [1–3].

CT-1 has been shown to have a wide variety of growth and differentiation effects on different cell types, predominantly on the heart where it is synthesized [3–6]. CT-1 binds to a receptor system that consists of the IL-6 receptor and a common signal transducer, the glycoprotein 130/leukemia inhibitory factor complex. The signaling activity is characterized by activation of all three following distinct pathways: first, the JAK/STAT pathway; second, the MAPK, including extracellular SRK 1 and 2 signaling pathway; and third, the phosphatidylinositol 3-OH kinase pathway [1,3,7]. CT-1 most likely achieves its effects by a combination of these three pathways (Figure 1). Indeed, CT-1 induces myocyte hypertrophy and collagen synthesis, as well as prolonged survival of cardiomyocytes that are involved in ventricular remodeling [1,8]. CT-1 is also elevated in the serum of adult patients with hypertension and coronary artery disease. These levels correlate with disease severity in valve disease and heart failure [9–11]. Increased CT-1 secretion seems to be an early factor that occurs before the onset of left ventricular systolic dysfunction [12], and chronic CT-1 secretion impairs contractile function in reconstituted heart tissue [13]. The heart is a main source of circulating CT-1 in humans since CT-1 concentrations are significantly higher in the coronary sinus than in the aorta [14]. CT-1 synthesis is increased by hypoxia [15], mechanical stress [16] and proinflammatory cytokines such as IL-1β [15]. CT-1 may therefore take part in myocardial remodeling and failure in children with

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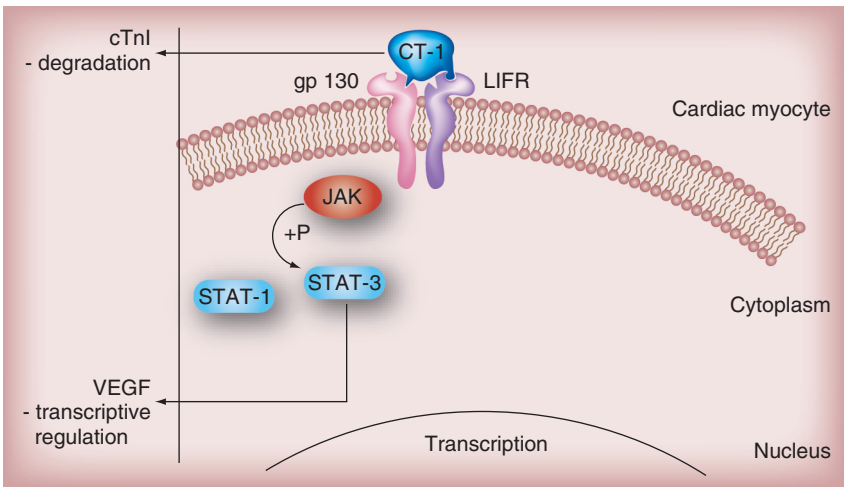

Figure 1. Cardiotrophin-1 and its interacting pathways. CT-I binds to a receptor system that consists of the IL-6 receptor and a common signal transducer, the gp 130/LIFR complex. The JAK/STAT pathway is activated.
+P: Phosphorylation; CT-1: Cardiotrophin-1; cTnI: Cardiac troponin-I; gp 130: Glycoprotein 130; LIFR: Leukemia inhibitory factor receptor.

congenital cardiac defects, but this has not been investigated thus far.

The aim of this study was, therefore, to test the hypothesis that CT-1 is differentially expressed in the myocardium of infants with congenital cardiac defects, depending on hypoxemia, and to investigate the signaling pathway related to its expression in this particular patient population.

Patients & methods

• Patients

After approval by the Human Ethical Committee of the Aachen University Hospital (Aachen, Germany) and informed consent of their parents, we prospectively investigated 16 infants aged 0.4–13.1 months undergoing primary corrective cardiac surgery. Eight patients had Tetralogy of Fallot and eight had a large nonrestrictive ventricular septal defect with pulmonary hypertension. All patients with Tetralogy of Fallot had a mild degree of hypoxemia (arterial oxygen saturation <90%). All patients had systemic levels of right ventricular pressure and right ventricular hypertrophy as shown by ECG and echocardiography, without clinical signs of right ventricular failure.

• Cardiac operation & sampling of myocardial biopsies

In all cases, drugs given for premedication and conventional general anesthesia consisted of midazolam, fentanyl sulfate and pancuronium bromide. Cefotiam hydrochloride was given for perioperative antibiotic prophylaxis. Dexamethasone (3 mg/m^2 body surface area) was given immediately prior to sternotomy. After institution of hypothermic cardiopulmonary bypass with a flow index of 2.7 l/min × m^2 body surface area for 15–20 min, the aorta was cross-clamped and cardiac arrest instituted by intra-aortal injection of a 4°C cold cardioplegic solution (30 ml/kg body weight), which was reaspirated in the right atrium. A biopsy was taken from the right atrial appendage before institution of cardiopulmonary bypass and from the right ventricular outflow tract immediately after aortic clamping, under the condition of deep hypothermia (22°C) and low flow bypass (0.65 l/min × m^2 body surface area), approximately 20 min after the start of cardiopulmonary bypass. Myocardial samples were taken from the infundibular portion of the right ventricular outflow tract. Samples obtained for reverse transcriptase PCR and western blotting were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis. Samples taken for immunocytochemistry were fixed in B*5 buffered solution (0.6% $ZnCl_2$ and 0.1% acid acetate), embedded in paraffin and cut into 3-µm thick sections.

• Reverse transcriptase PCR

Total RNA was extracted from the atrial and ventricular myocardium in all patients by using the RNeasy® kit (Qiagen Inc., Hilden, Germany). Total RNA (2 µg) was reverse transcribed to complementary DNA with random hexamers. Primers for human CT-1 (sense: 5′-AACTCTTGGACCCTCCTCGT-3′ and antisense: 5′-AGGAGGTGGGGATGGAAAAA-3′) and human 18S rRNA (sense: 5′-GTTGGTTTTCGCAACTGAGG-3′ and antisense: 5′-GGCCTCACTAAACCATCCAA-3′) were used to amplify complementary DNA products by PCR (35 cycles; 94°C for 30 s, 55°C for 30 s and 72°C for 2 min). PCR products were subjected to electrophoresis in a 1.8% agarose gel, stained with ethidium bromide and photographed. The predicted lengths of the amplification products for CT-1 and 18S rRNA were 152 and 834 base pairs, respectively.

• Western blotting

Total protein homogenates (100 µg) from atrial and ventricular myocardium were denatured and separated on 12 and 8% polyacrylamide gels by

SDS-PAGE. Primary antibodies in immunoblotting were monoclonal mouse antihuman CT-1 (Santa Cruz Biotechnology, Hamburg, Germany); polyclonal rabbit antihuman phospho-STAT-3 and STAT-3 (all from Cell Signaling Technology, Inc., Frankfurt am Main, Germany); polyclonal rabbit antihuman $VEGF_{165}$ (Santa Cruz Biotechnology); polyclonal goat antihuman troponin-I (Santa Cruz Biotechnology); and monoclonal mouse antihuman β-actin (Sigma, MO, USA). The bands were detected by a chemiluminescent system. Restaining with actin antibody ensured equal loading. Band intensities for CT-1, VEGF and cardiac troponin-I (intact: 32 kDa and degradation product: 26 kDa) were normalized for that of β-actin, and the band intensities of phospho-STAT-3 were normalized for that of STAT-3, respectively (Quantity One® Quantification software 4.1, Bio-Rad Laboratories BV, Veenendaal, The Netherlands). The density of the degradation products of cardiac troponin-I was expressed as a percentage of the total immunoreactivity **(Box 1)**.

• Electrophoretic mobility-shift assay

Nuclear extracts were prepared as previously described [17]. Protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad Laboratories BV). The electrophoretic mobility-shift assay was performed by using a double-stranded ^{32}P-labeled mutated sis-inducible element oligonucleotide from the *c-fos* promoter (m67 sis-inducible element: 5′-GAT CCGGGAGGGATTTACGGGGAAATGCTG-3′; MWG-Biotech AG, Ebersberg, Germany) [17]. For supershift assays, nuclear extracts were incubated with an antibody against STAT-3 (polyoclonal rabbit antihuman, Santa Cruz Biotechnology) overnight at 4°C before addition of the radiolabeled probe. The protein–DNA complexes were separated on a 6% polyacrylamide gel containing 7.5% glycerol in buffer (20 mM Tris, 20 mM boric acid and 0.5 mM EDTA) at 210 V. Gels were dried and autoradiographed.

• Immunocytochemistry

Immunostaining was performed using the immunohistochemistry mouse or rabbit kit (InnoGenex, Inc., CA, USA) to identify the cells producing CT-1 and STAT-3 in the ventricular myocardium. Briefly, sections were pretreated with a solution of peroxide block for 20 min to inhibit endogenous peroxidase activity and incubated with the power block reagent to block nonspecific protein binding. Subsequently, sections were incubated with the primary mouse antihuman CT-1 (1:100; Santa Cruz Biotechnology) or rabbit antihuman STAT-3 (1:100, Cell Signaling Technology, Inc.) for 4 h. After rinsing with phosphate-buffered saline solution plus 0.1% polysorbate, the slides were incubated for 30 min with the secondary antibodies. The slides were rinsed with phosphate-buffered saline and incubated for 20 min in a streptavidin–peroxidase conjugate. The color reaction was developed in aminoethyl carbazole substrate. Specimens were counterstained with hematoxylin. Negative controls were obtained by omitting the primary antibodies in the myocardial probes. Typical morphologic characteristics for cardiomyocytes, macrophages and endothelial cells were assessed by oil microscopy at 1000-fold magnification.

• Statistical analysis

Results are expressed by the mean value ± standard deviation. The Mann–Whitney U test was used to analyze differences between patients with cyanotic or acyanotic cardiac defects. Correlation of independent parameters was assessed by the Spearman rank correlation test. p-values <0.05 were considered significant and p-values <0.1 were considered to indicate a tendency towards significance. Data were analyzed with the Statistical Package for Social Sciences® (SPSS Software; IBM, Ehningen, Germany).

Results

• Clinical results

Patients with Tetralogy of Fallot and patients with ventricular septal defect showed similar parameters concerning age and weight. **Table 1** summarizes patient epidemiological data.

• Intramyocardial CT-1 synthesis

CT-1 was detected at mRNA and protein levels in the right atrial and ventricular myocardium of all patients. Concentrations of CT-1 were significantly higher in the right atrial and ventricular myocardium of patients with Tetralogy of Fallot compared with those with ventricular septal defect ($p < 0.05$, respectively; **Figure 2**).

Immunocytochemistry showed the presence of CT-1 in the cytoplasm of cardiomyocytes and

Box 1. Expression modus of degradation of cardiac troponin-I.

$$\text{Total immunoreactivity} = \left(\frac{\text{Density of the degradation products}}{\text{Density of intact band + intensity of degradation products}}\right) \times 100\%$$

Table 1. Patient data.

Parameter	TOF group (n = 8)	VSD group (n = 8)	p-value
Gender (female/male)	2/6	5/3	NS
Age at operation (months)†	3.8 ± 3.0	5.8 ± 4.3	NS
Weight (kg)†	7.5 ± 3.4	6.1 ± 2.7	NS
Preoperative SaO_2 (%)†	87.4 ± 3.6	97.7 ± 1.0	0.001

†Data are presented as the mean value ± standard deviation.
NS: Not significant; SaO_2: Transcutaneous arterial oxygen saturation; TOF: Tetralogy of Fallot; VSD: Ventricular septal defect.

of noncardiomyocytes, such as endothelial cells and fibroblasts (Figure 3).

Considering each of both patient groups, synthesis of CT-1 in the right atrium and ventricle negatively correlated with preoperative oxygen saturations (Spearman correlation coefficient: -0.48 [$p < 0.07$]; -0.60 [$p < 0.05$], respectively).

• Activation of STAT-3 in the myocardium

DNA-binding activity of STAT-3 was detected by performing an electrophoretic mobility-shift assay in the right ventricular myocardium of infants with Tetralogy of Fallot (n = 6) or with ventricular septal defect (n = 5), and was not different between both groups (Figure 4). Unlabeled oligonucleotide competition at a 100-fold concentration and supershift analysis confirmed the specificity of DNA-binding complexes.

Levels of phosphorylated STAT-3 measured by western blotting were not different between both groups in the atrium, but tended to be higher in the right ventricular myocardium of infants with Tetralogy of Fallot than in those with ventricular septal defect ($p < 0.1$; Figure 5). Immunocytochemistry showed the presence of STAT-3 in the nucleus of cardiomyocytes (Figure 3).

Intramyocardial synthesis of $VEGF_{165}$

$VEGF_{165}$ protein was detected in the right atrium and ventricle of all infants. Infants with Tetralogy of Fallot showed significantly higher levels of $VEGF_{165}$ in the right ventricle, but not in the right atrium compared with those with ventricular septal defect ($p < 0.05$; Figure 6).

• Degradation of cardiac troponin-I in the myocardium

Western blotting identified both intact cardiac troponin-I (32 kDa) and an additional, less

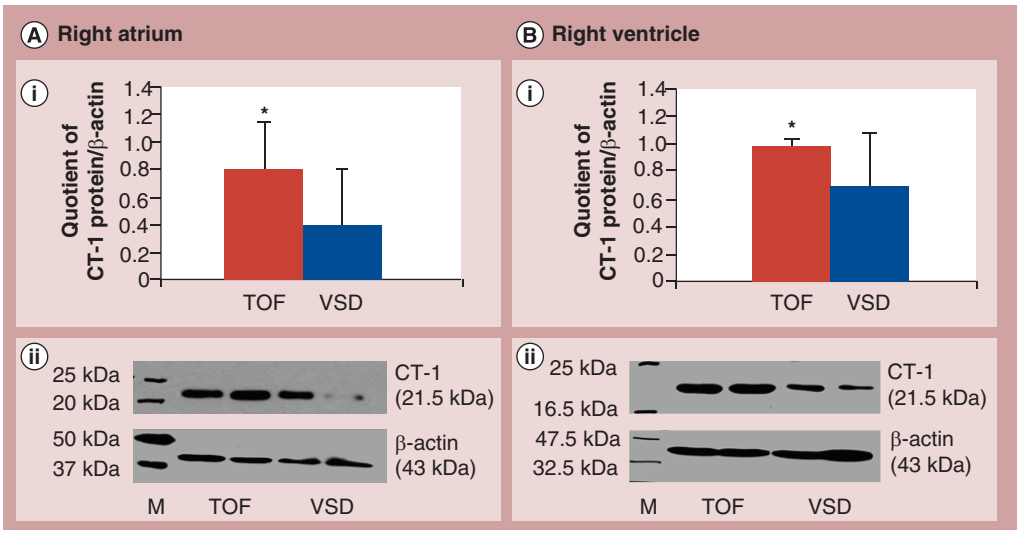

Figure 2. Myocardial expression of cardiotrophin-1. (i) Levels of CT-1 measured by western blotting in the **(A)** right atrium and **(B)** right ventricle of infants with TOF (n = 7) or with VSD (n = 8). Results are expressed as the mean value ± standard deviation. **(ii)** Exemplary gels obtained after western blotting of one representative experiment showing a higher synthesis of CT-1 in children with TOF.
*$p < 0.05$ between groups.
CT-1: Cardiotrophin-1; M: Marker; TOF: Tetralogy of Fallot; VSD: Ventricular septal defect.

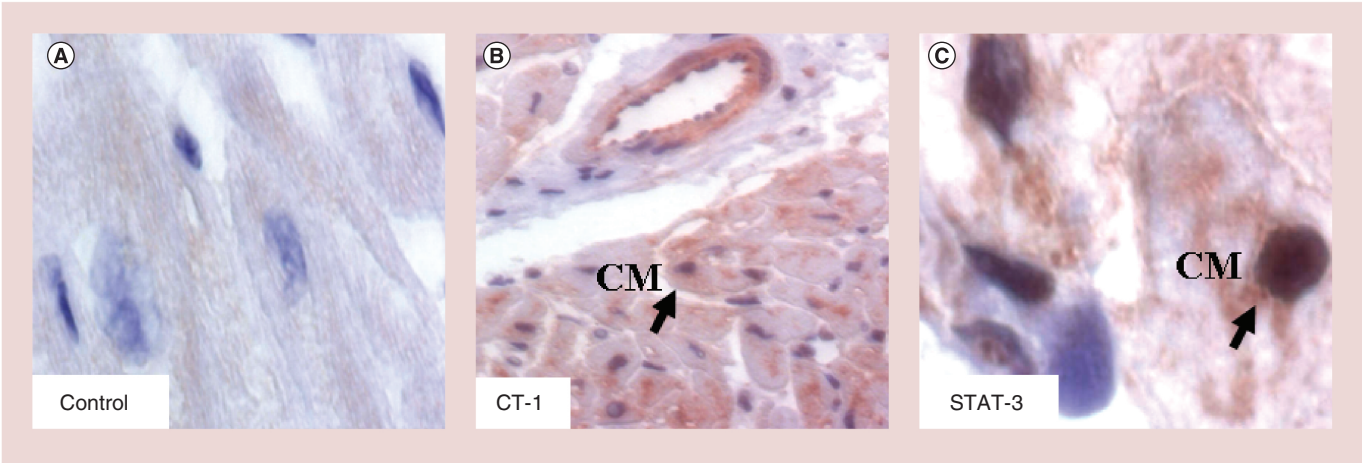

Figure 3. Presence of cardiotrophin-1 and STAT-3 shown by immunocytochemistry. (A) Control: negative control (no primary antibody). Immunocytochemistry of the right ventricular myocardium in one infant with Tetralogy of Fallot, showing **(B)** the presence of CT-1 in the CMs and **(C)** of STAT-3 in the nuclei of CMs. Original magnification ×400 (CT-1) and ×1000 (control and STAT-3).
CM: Cardiomyocyte; CT-1: Cardiotrophin-1.

intense band of 26 kDa in the right atrial and ventricular myocardium of all patients. The levels of intact (32 kDa) and total cardiac troponin-I (32 + 26 kDa) were significantly higher in the right ventricle, but not in the right atrial myocardium of infants with Tetralogy of Fallot compared with others (quotient of intact cardiac troponin-I: 1.09 ± 0.08 vs 0.87 ± 0.14; $p < 0.05$; total cardiac troponin-I: 1.62 ± 0.28 vs 1.09 ± 0.27; $p < 0.01$). Moreover, infants with Tetralogy of Fallot showed higher levels of 26-kDa cardiac troponin-I in the right atrium and ventricle than those with ventricular septal defect ($p = 0.1$ and $p < 0.05$, respectively; Figure 7).

Discussion

Our study demonstrates, for the first time, that CT-1 is expressed in the right atrial and ventricular myocardium of infants with congenital cardiac defects and that this is associated with the activation of the JAK/STAT-3 pathway and the degradation of cardiac troponin-I.

• Expression of CT-1 in the myocardium of infants with congenital cardiac defects

Although CT-1 is present in various organ tissues, including the developing and the adult heart [4], the expression of *CT-1* mRNA is markedly stimulated by hypoxia *in vitro* [15]. Furthermore, in an animal model of myocardial infarction, elevated CT-1 expression was observed in the infarcted zone at 24 h and persisted up to 8 weeks postinfarction [18]. Our results, which show a relationship between the degree of preoperative hypoxemia and CT-1 expression, suggest that hypoxemia stimulates CT-1 expression in infants. Besides hypoxemia, other stimuli are known to induce CT-1 expression. Hemodynamic overload [19], as well as proinflammatory cytokines [15], might have contributed to increased CT-1 expression in our patients [20].

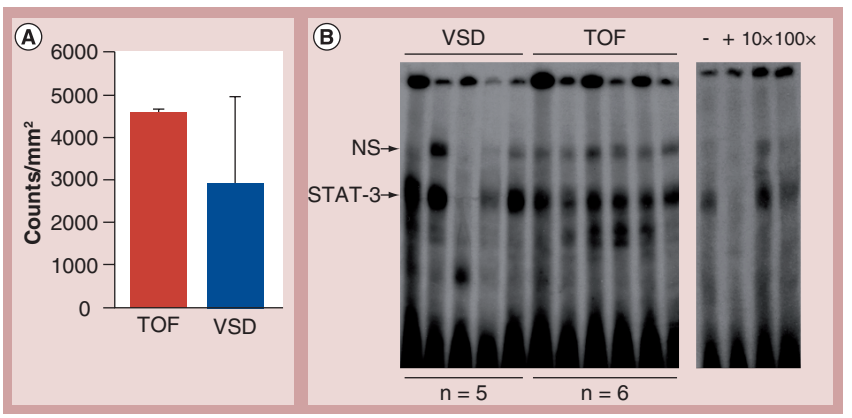

Figure 4. STAT-3 DNA-binding activity. (A) DNA-binding activity of STAT-3 measured by electrophoretic mobility shift assay and confirmed by supershift and unlabeled oligonucleotide competitors in the right ventricular myocardium of infants with TOF (n = 6) or with VSD (n = 5). Results are represented by counts/mm² and expressed as the mean value ± standard deviation. **(B)** Exemplary gel showing the effect of chronic hypoxemia on myocardial activation of STAT-3 as detected by electrophoretic mobility-shift assay and confirmed by supershift with anti-STAT-3 antibody (+) and competitors with tenfold (×10) and 100-fold (×100) unlabeled oligonucleotide in the right ventricular myocardium of infants with TOF or VSD. Results are shown as one representative experiment.
-: Nuclear extract without antibody and unlabeled oligonucleotide; NS: Nonspecific; TOF: Tetralogy of Fallot; VSD: Ventricular septal defect.

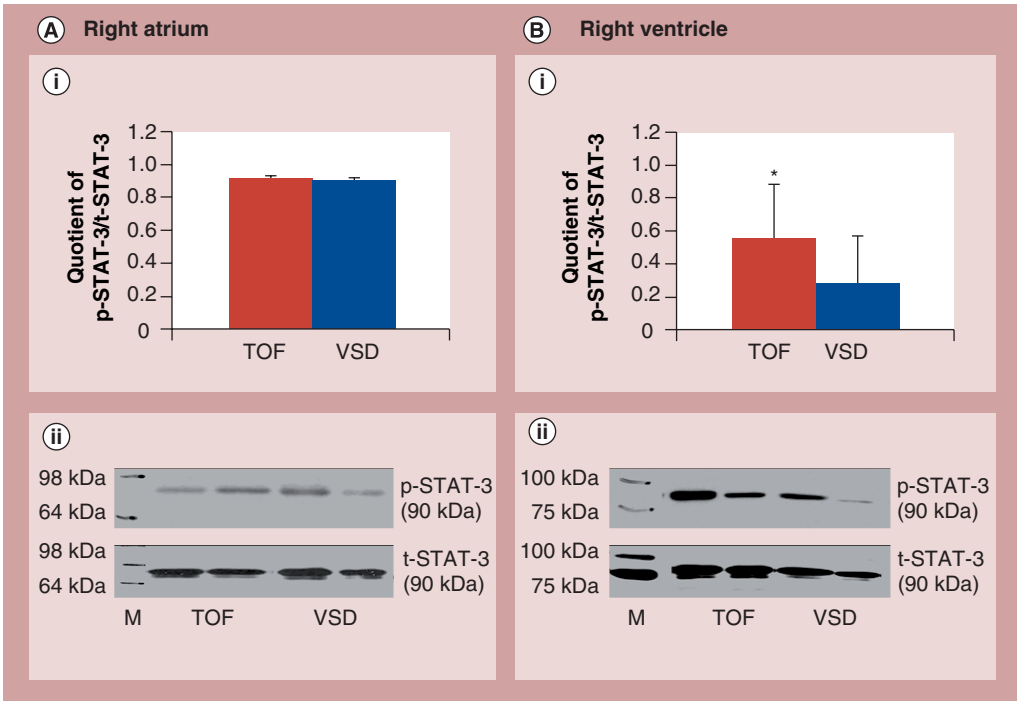

Figure 5. Expression of STAT-3. (i) Levels of p-STAT-3 measured by western blotting in the **(A)** right atrium and **(B)** right ventricle of infants with TOF (n = 7) or with VSD (n = 8). Results of p-STAT-3 are normalized for the bands of t-STAT-3 and are expressed as the mean value ± standard deviation. **(ii)** Exemplary gels obtained after western blotting of one representative experiment showing a higher activation of STAT-3 in children with TOF.
*p < 0.1 between groups.
M: Marker; p-STAT-3: Phospho-STAT-3; t-STAT-3: Total STAT-3; TOF: Tetralogy of Fallot; VSD: Ventricular septal defect.

• CT-1 induces myocardial hypertrophy in infants with congenital cardiac defects via the STAT-3 pathway

Infants with Tetralogy of Fallot or with a large ventricular septal defect show right ventricular hypertrophy, which is due to the severe pulmonary stenosis associated with the large ventricular septal defect in Tetralogy of Fallot and to high flow pulmonary hypertension in single ventricular septal defect. Therefore, both patient groups had pressure overload of the right ventricle at the systemic level and myocardial remodeling as previously reported [20,21]. In addition to right ventricular pressure overload, patients with ventricular septal defect also demonstrated volume overload of the left ventricle that may have enhanced CT-1 expression in response to inflammatory pathways, as a limit of this study and discussed above.

CT-1 binds to the leukemia inhibitory factor/glycoprotein 130 receptor, activating the JAK/STAT pathway, which is known to transduce hypertrophic signals in cardiac cells [3,22]. The JAK/STAT pathway is activated by hypoxia and mechanical stretch. This activation is mainly influenced by cytokines of the IL-6 family to which CT-1 belongs [6,15,16,23]. CT-1 induces phosphorylation of STAT-3 in a concentration-dependent manner and maintains its activation for 6 days in cultured neonatal cardiomyocytes [13], indicating its long-lasting effect. In the present study, intramyocardial phosphorylation and activity of STAT-3 suggest the activation of the JAK/STAT cascade by CT-1 in the myocardium of infants with congenital cardiac defects. Transgenic mice with cardiac-specific overexpression of STAT-3 manifest mild cardiac hypertrophy at the age of 12 weeks [24].

In addition, STAT-3 plays an important role in the transcriptional regulation of VEGF in cardiac myocytes: increased expression of VEGF was observed in cardiac myocytes after CT-1 stimulation, which was completely inhibited by dominant-negative STAT-3 *in vitro* and *in vivo* [25]. Phosphorylation of STAT-3 results in its nuclear translocation and DNA binding by which the target gene, VEGF, is regulated.

In our series, the overexpression of VEGF correlates with the levels of CT-1 and phospho-STAT-3 in the right ventricle. This suggests that CT-1 induces cardiomyocyte hypertrophy and angiogenesis during right ventricular remodeling in infants with congenital cardiac defects; the JAK/STAT signaling pathway seems to play a role.

• Effects of CT-1 on the myocardium

Furthermore, to induce cardiac hypertrophy via the JAK/STAT pathway, CT-1 expression was associated with cardiac troponin-I degradation. Cardiac troponin-I, an important component of the cardiac contractile apparatus, is degraded by Ca^{2+}-dependent proteases, which results in decreased maximal force and in relative insensitivity to calcium in myocardial ischemia and reperfusion injury [26,27]. The assumption that CT-1 mediates myocardial cell damage is supported by the fact that chronically increased synthesis of CT-1, as observed in human heart failure, might further accelerate contractile dysfunction and disease progression [9,10,12]. A recent study performed on reconstituted heart tissue demonstrated that chronic CT-1 stimulation impairs contractile function by downregulating expression of calsequestrin, a myocyte-specific protein involved in Ca^{2+}-handling [13]. CT-1 induces cardiac myocyte hypertrophy via the addition of sarcomeres in series rather than in parallel and via increased cardiac myocyte length, with little change in width [28]. This contributes to ineffective force generation. In addition, CT-1 increases the number of nonmyocyte cells including cardiac fibroblasts [29]. As we showed in this study by immunohistological staining, CT-1 is also present in nonmyocyte cells, suggesting that it might take part in the remodeling of the extracellular matrix of the infant myocardium. Indeed, CT-1 increases mature collagen synthesis [18,29,30].

This study shows, for the first time, differential expression of CT-1 in the myocardium of infants with congenital cardiac defects. Hypoxemia is likely to stimulate CT-1 expression in this age group. CT-1 activates the JAK/STAT pathway in these children, which mediates myocardial hypertrophy and leads to cardiac troponin-I degradation. The early myocardial remodeling with troponin degradation observed in our series points to the importance of early surgical management in patients with congenital heart defect and hypoxemia.

Future perspective

Hemodynamic overload and hypoxemia are known factors to upregulate intramyocardial

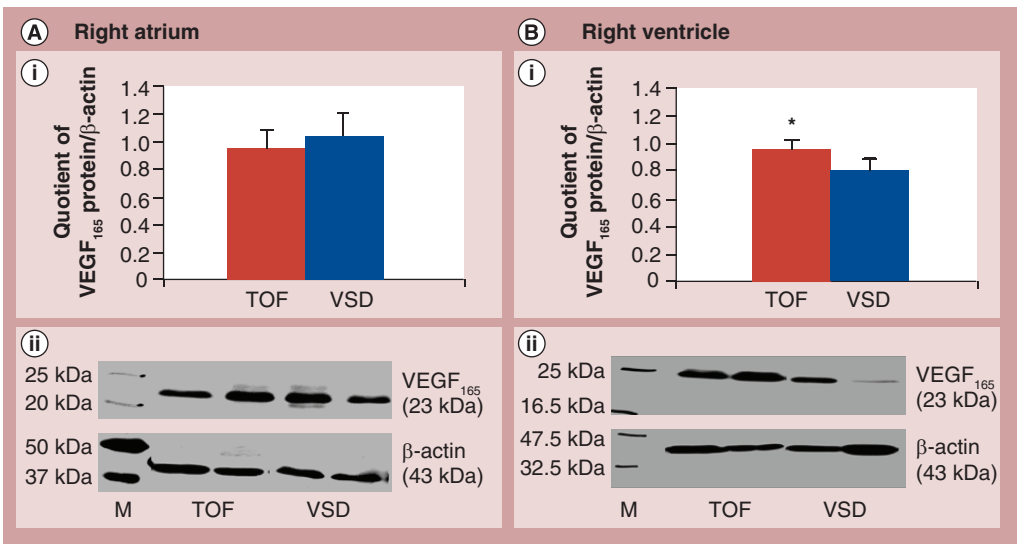

Figure 6. Expression of $VEGF_{165}$. (i) Levels of $VEGF_{165}$ measured by western blotting in the **(A)** right atrium and **(B)** right ventricle of infants with TOF (n = 7) or with VSD (n = 8). Results of $VEGF_{165}$ are normalized for the bands of β-actin and are expressed as the mean value ± standard deviation. **(ii)** Exemplary gels obtained after western blotting of one representative experiment. Children with TOF showed a higher synthesis of $VEGF_{165}$ in the right ventricle.
*$p < 0.05$ between groups.
M: Marker; TOF: Tetralogy of Fallot; VSD: Ventricular septal defect.

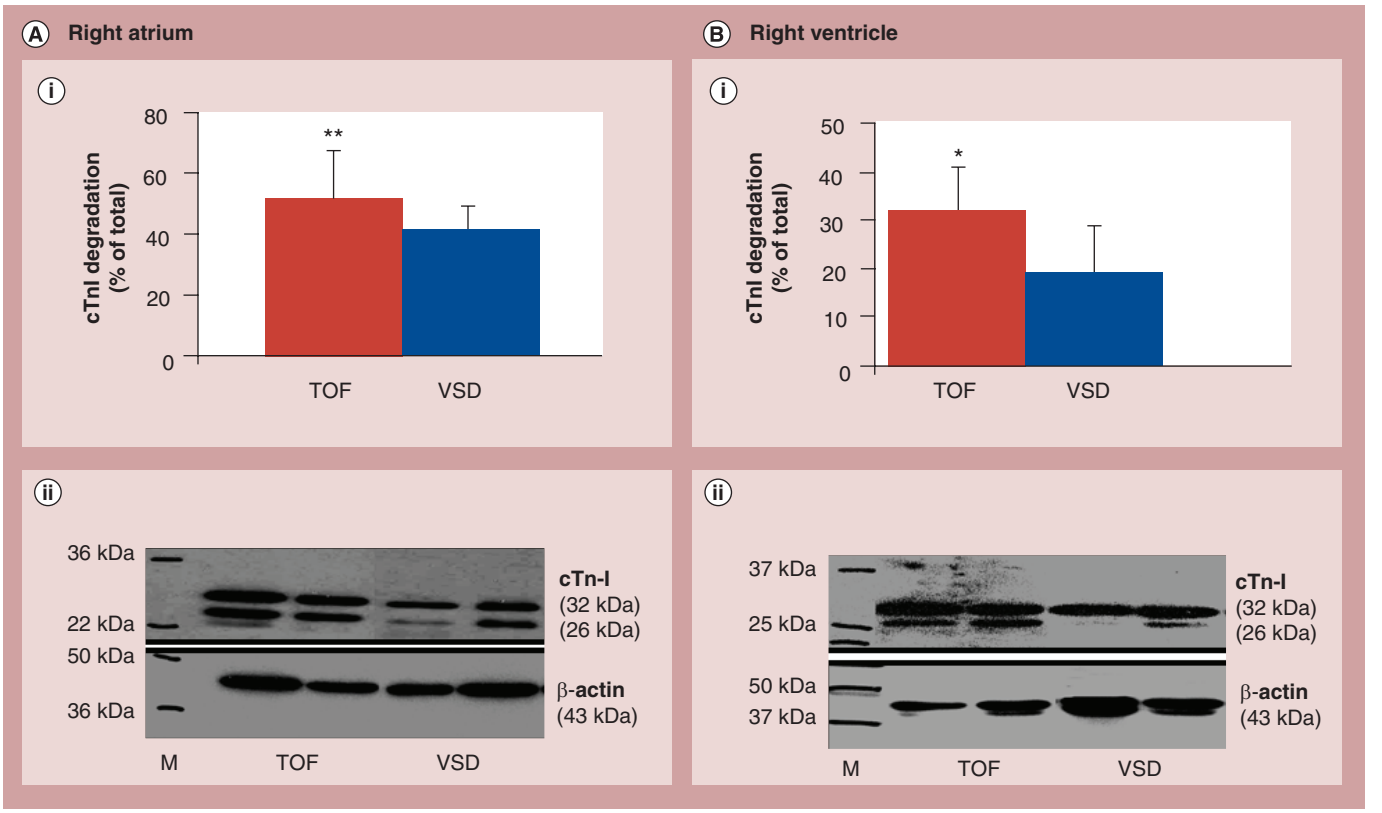

Figure 7. Expression of degradation products of cardiac troponin-I. (i) Degradation products (26 kDa) of cTnI measured by western blotting in the **(A)** right atrium and **(B)** right ventricle of infants with TOF (n = 8) or with VSD (n = 8). Degradation products (26 kDa) of cTnI are expressed as the percentage of total immunoreactivity (mean value ± standard deviation). **(ii)** Exemplary gels obtained after western blotting of one representative experiment showing higher degradation levels of cTnI in children with TOF.
*p = 0.1.
**p < 0.05 between groups.
cTnI: Cardiac troponin-I; M: Marker; TOF: Tetralogy of Fallot; VSD: Ventricular septal defect.

expression of CT-1, which is responsible for a variety of growth and differentiation effects, especially in cardiomyocytes. Children with Tetralogy of Fallot or a large ventricular septal defect are exposed to hemodynamic overload leading to myocardial changes. Investigation of the CT-1-induced pathways on a molecular level may unravel the underlying processes leading to myocardial transformation and clinical myocardial dysfunction. Therapy and management of children with congenital heart disease is determined by clinical requirements and the technical evolution of surgical techniques. Knowledge of ongoing intramyocardial processes in unoperated heart disease is expected to generate important additional information, which might have an impact on the future management of these patients. The influence of hypoxemia on intramyocardial processes might influence the optimal timing of surgical treatment in these children.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

- Cardiotrophin-1 (CT-1) is differentially expressed in the myocardium of infants with heart defects depending on hypoxemia.
- Higher CT-1 expression in patients with Tetralogy of Fallot is associated with activation of the JAK/STAT pathway and higher cardiac troponin-I degradation.
- CT-1 might influence myocardial hypertrophy and myocardial dysfunction in infants with congenital cardiac defects, particularly in those with hypoxemia.

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• **Implication of CT-1 in cardiac dysfunction and ischemia.**