NEU3 sialidase role in activating HIF-1α in response to chronic hypoxia in cyanotic congenital heart patients

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Abstract

Background: Hypoxia is a common feature of many congenital heart defects (CHDs) and significantly contributes to their pathophysiology. Thus, understanding the mechanism underlying cell response to hypoxia is vital for the development of novel therapeutic strategies. Certainly, the hypoxia inducible factor (HIF) has been extensively investigated and it is now recognized as the master regulator of cell defense machinery counteracting hypoxic stress. Along this line, we recently discovered and reported a novel mechanism of HIF activation, which is mediated by sialidase NEU3. Thus, aim of this study was to test whether NEU3 played any role in the cardiac cell response to chronic hypoxia in congenital cyanotic patients.

Methods: Right atrial appendage biopsies were obtained from pediatric patients with cyanotic/non-cyanotic CHDs and processed to obtain mRNA and proteins. Real-Time PCR and Western Blot were performed to analyze HIF-1α and its downstream targets expression, NEU3 expression, and the NEU3 mediated effects on the EGFR signaling cascade.

Results: Cyanotic patients showed increased levels of HIF-1α, NEU3, EGFR and their downstream targets, as compared to acyanotic controls. The same patients were also characterized by increased phosphorylation of the EGFR signaling cascade proteins. Moreover, we found that HIF-1α expression levels positively correlated with those recorded for NEU3 in both cyanotic and control patients.

Conclusions: Sialidase NEU3 plays a central role in activating cell response to chronic hypoxia inducing the up-regulation of HIF-1α, and this represent a possible novel tool to treat several CHD pathologies.

Keywords: HIF-1α, Sialidase NEU3, Congenital Heart Defects, Chronic Hypoxia, EGFR signaling pathway.

1. Introduction

Hypoxia significantly contributes to the pathophysiology of many common congenital heart defects, including the Tetralogy of Fallot (TOF) [1]. Before surgical correction, the heart, as well as the other organs and tissues of these patients, is exposed to chronic hypoxia, which is known to have lasting effects even after surgical repair [2]. Indeed, surgical repair usually consists in closing a ventricular septal defect and widening the right ventricular outflow tract to the pulmonary artery, which relives hypoxia and dramatically reduces the pressure. However, persistent pulmonary regurgitation after repair is common, and it contributes to progressive RV dilatation, often culminating in RV failure [3]. The rate at which these dysfunctions progress is variable among patients, suggesting that individual differences are crucial and should be elucidated for the development of novel therapeutic approaches [4]. Furthermore, the understanding of the biological pathways involved in RV remodeling becomes vital also for the development of new diagnostic tools for an early identification of high risk-patients, before significant RV remodeling has occurred. In particular, it is now clear that the hypoxia inducible factor-1α (HIF-1α), the master gene of cell response to hypoxia, plays a crucial role in cardioprotection [5]. In fact, when the oxygen level goes down, mammalian cells have developed an adaptive system to overcome conditions of moderate-severe hypoxia, mainly regulated by HIF-1α [6, 7], which eventually activates pro-survival signaling pathways.

There is now a general agreement that HIF-mediated cell response to hypoxia is different under acute or chronic conditions, and the effects of this transcription factor can be either beneficial or detrimental. In fact, it has been shown that while an increase in the level of HIF-1α is one of the first adaptations of the human heart to acute ischemic conditions, a sustained stabilization of this

factor, typical of chronic conditions, leads to dilated cardiomyopathy, with fibrosis, myocyte loss, lipid accumulation, and other detrimental effects [8]. However, surprisingly, HIF-induced fibrosis should not be always seen as detrimental, as it has been shown that patients with low expression of HIF-1α have less fibrosis at time of intervention, but enhanced adverse ventricular remodeling after surgical repair [9]. These results support the hypothesis that an activation of HIF-1α before surgical correction of the defects is desirable, as it promotes long-term beneficial effects in the patients. However, the mechanism behind this hypothesis has yet to be fully elucidated.

At this stage, it seems clear that the mechanism of HIF-1α activation is very complex and still needs further investigation. Moreover, how this crucial transcription factor affects the heart is still unclear, as it depends on many factors. Along this line, we recently discovered a novel pathway of HIF-1α activation in murine skeletal muscular myoblasts, which is different from the "canonical" inhibition of prolyl hydroxylase 2 (PHD2) [10], and it is regulated by the membrane-bound sialidase NEU3 [11]. NEU3 is a glycosidase responsible for the removal of sialic acid from glycosphingolipids, and it has been shown to possess trans-activity since it can also work on the gangliosides present on the plasma membrane of adjacent cells [12-14]. These key features are likely instrumental for a crucial role played by the enzyme in many cellular processes [15] including cell proliferation and differentiation [16-18]. Specifically, we showed that sialidase NEU3 is transcriptionally activated under hypoxia in skeletal muscle cells, and that activated NEU3 triggers a signaling cascade mediated by ganglioside GM3, which activates the EGF receptor (EGFR) and its downstream anti-apoptotic and pro-proliferative signaling pathways, ultimately leading to HIF-1α activation. Moreover, stable overexpression of sialidase NEU3 significantly enhances myoblasts resistance to hypoxia, whereas stable silencing of the enzyme renders cells more susceptible to apoptosis [11].

These data support the working hypothesis of a physiological role played by NEU3 sialidase in protecting cells from hypoxic stress.

Based on these premises, the main aim of this study was to test whether NEU3 plays a role in cardiac muscle response to chronic hypoxia in congenital heart patients, ultimately triggering HIF-1α signaling cascade.

2. Materials and methods

2.1. Patients

Pediatric patients with cyanotic and non-cyanotic congenital heart diseases, undergoing elective cardiac surgery correction with extracorporeal circulation at the Cardiac Surgery Division of San Donato Hospital, were enrolled for this study after understanding and signing an informed consent. Patients enrolled respected the inclusion and exclusion criteria of the study approved by the Hospital Ethics Committee and received an alpha-numeric code to be univocally identified. All data related to the patient disease and his cardiac surgery were recorded.

2.2. Tissue biopsies

Right atrial appendage biopsies were obtained from the point of atrial cannulation at the beginning of extracorporeal circulation. Tissue specimens weighted about 100 mg and were collected in cold phosphate buffer solution (PBS) at pH 7.4, kept in ice and processed within minutes after collection.

2.3. mRNA isolation from cardiac tissue samples

For mRNA extraction, tissue samples were retrieved from saline solution within 30 minutes, incubated with 1 ml of Trizol-LS reagent (Invitrogen, Carlsbad, CA, USA) and then homogenized using the Tissue homogenizer Lyser® (Qiagen). Each sample was processed with 2 cycles of 10

min each at 25 oscillations/sec, kept in ice for 30 min and finally centrifuged at 12000 rcf for 5 min at 4°C. After centrifugation, the supernatant of each sample was transferred in a new tube and total mRNA was extracted from Trizol-LS following the manufacture's protocol.

2.4. mRNA purification and retro-transcription into the corresponding cDNA

To eliminate possible genomic DNA contaminations in the extracted mRNA, samples were processed according to the "RNA Cleanup" protocol of RNeasy Mini Kit® (QIAGEN) supplied by the company. After isolation and purification, 1 µg of RNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the Manufacturer's instruction.

2.5. Gene expression analyses by Real-Time PCR

For qPCR analyses, 10 ng of total RNA were used as a template with a StepOnePlus® Real-Time PCR System (Thermo Fisher Scientific). The PCR mixture included also: 0.2 µM of primers (Suppl. Table 1) and 10 µl of Power SYBR® Green Master Mix (Thermo Fisher Scientific), in a final volume of 20 µl. Amplification and qPCR data acquisition were performed using the following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles of 5 sec each at 95°C, 30 sec at 57°C and 30 sec at 72°C. A final elongation step was performed at 72°C for 2 minutes. Relative quantification of target genes was performed in triplicate and was calculated by the equation $2^{-\Delta\Delta Ct}$ using as housekeeper gene the ribosomal protein S14, which is a component of the 40S subunit [19]. The accuracy was monitored by analysis of the PCR-product melting curve.

2.6. Total protein extraction from Trizol-LS samples

Proteins from the phenol/chloroform layer obtained during the RNA extraction with Trizol-LS, were extracted by the following protocol: 1.2 mL of cold methanol were added to the phenol/chloroform layer and vortexed, then samples were left for 10 min at 30°C and, after an incubation on ice for 5 min, were centrifuged at 12,000 rcf for 10 min at 4°C to sediment the proteins. The protein pellets were washed with 1 mL of cold methanol and centrifuged again at 12,000 rcf for 10 min at 4°C. After centrifugation, protein pellets were resuspended in 0.5 mL of cold methanol and sonicated for 4 cycles of 5 sec each in ice using burst sonication to generate a fine protein powder. Samples were then centrifuged for 1 min at 2,000 rcf and the methanol evaporated at room temperature. Proteins were then resuspended with 200 μL of a 0.25% Rapigest® solution in 50 mM ammonium bicarbonate and solubilized by alternate cycles of vortex and heat at 60°C. The obtained proteins were stored at -80°C until used for Western Blot experiments.

2.7. Membrane and cytosolic protein separation

To obtain membrane and cytosolic protein fractions, fragments of auricles were incubated with PBS containing 1% protease and phosphatase inhibitors (Sigma-Aldrich) and homogenized using the Tissue homogenizer Lyser® (Qiagen). Each sample was processed with 4-5 cycles of 10 min each at 25 oscillations/sec and the total tissue suspensions were lysed by sonication and then centrifuged at 800 rcf for 10 min to eliminate debris, unbroken cells and nuclear components. The obtained supernatants were subsequently centrifuged at 200,000 rcf at 4°C for 20 min on a TL100 Ultracentrifuge (Beckman) to obtain a soluble and a particulate fraction. The particulate fraction contains the plasmatic membranes, the lysosomal membranes, and the endoplasmic reticulum, while the supernatant contains the cytosol. Both fractions were used for NEU3 expression analyses by Western Blot. Relative NEU3 protein expression levels were calculated normalizing data using Na^+/K^+ ATPase as housekeeper for the membranes and EEA1 as housekeeper for the cytosol.

2.8. Protein expression analyses by Western Blot

Protein concentrations were measured by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), following the Manufacturer's protocol. 30 µg of proteins were denatured by boiling for 5 min in sample buffer (6.5 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 5% 2-mercaptoethanol (w/v), 0.01% bromophenol blue (w/v)) and separated on a 10% polyacrylamide gel in denaturing conditions. Proteins were subsequently transferred into nitrocellulose membranes by electroblotting using 100 Volt for 2 hours. Then, the membranes were incubated overnight in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl), 0.1% (v/v) Tween 20 (TBS-Tween) containing 5% (w/v) dried milk or 5% (w/v) bovine serum albumin (BSA; Sigma) for the blocking buffer. Blots were incubated with a primary antibody (Suppl. Table 2) in the appropriate blocking solution for three hours at room temperature. Membranes were then washed four times for 10 min with TBS-Tween and then incubated with the appropriate secondary antibody horseradish peroxidase (HRP) conjugated for 1 hour at room temperature. After four washes in TBS-Tween, the protein bands were detected using an ECL detection kit (Thermo Fisher Scientific) as described by the Manufacturer. Relative protein expression levels were calculated normalizing data on Calnexin (Clx), Na^+/K^+ ATPase (membranes fraction) and EEA1 (cytosol fraction) expression, which were used as internal controls.

2.9. Statistical analysis

Data were expressed as a percentage for nominal variables and mean with standard deviations for continuous variables using GraphPad Prism 6 (GraphPad Software Inc, California, USA). Statistical differences were determined by Unpaired T-test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

The correlation analysis was tested by Pearson method on Delta Ct values of gene expression of cyanotic and acyanotic patients using GraphPad Prism 6 (GraphPad Software Inc, California, USA).

3. Results

3.1. Characterization of patients

Patients included in the study were divided in two groups based on the presence of cyanotic or acyanotic congenital heart defects. The number of patients included in the acyanotic and cyanotic groups was 17 and 18, respectively. Each group was composed by patients with different congenital heart malformations, as shown in Table 1, to increase sample heterogeneity. Different clinical parameters were measured in both cyanotic or acyanotic groups as shown in Table 2. BMI, Creatinine, electrolytes (Na^+; K^+), PT, PTT and platelets count did not show any statistically significant differences between the two populations. However, acyanotic and cyanotic patients were statistically different for these clinical parameters: (a) SpO_2: the level of oxygen saturation of cyanotic patients was approximately 72.5% (SD: 15.38%**)** as compared to 98.9% (SD: 1.23%**)** for control acyanotic patients; (b) glycaemia: the average levels of glycaemia measured in cyanotic and acyanotic patients were 84.78 mg/dl (SD: 19.91 mg/dl) and 110.31 mg/dl (SD: 10.68 mg/dl), respectively; (c) hemoglobin: the average value of hemoglobin in patients with cyanotic congenital heart defects was 14.03 g/dl (SD: 3.05 g/dl) as compared to 11.98 g/dl (SD: 0.76 g/dl) of acyanotic patients.

3.2. HIF-1α activation analysis in cyanotic patients

Specimens of right auricle were isolated from patients with either cyanotic or acyanotic congenital heart diseases (hereafter simply *cyanotic* and *controls)*, and then they were processed for mRNA and protein extraction, as described in Materials and Methods. Analysis of HIF-1α expression by Real-Time PCR showed mRNA levels about 1.3 folds higher in cyanotic patients as compared to controls (Fig. 1A). HIF-1α protein expression by Western Blot was on average 3 folds higher in cyanotic patients than in controls (Fig. 1B). Moreover, the expression of VEGF, an important transcriptional target of HIF-1α and a key regulator in the formation of new blood vessels, was 1.2 folds higher in cyanotic patients as compared to controls by Real-Time PCR (Fig. 1C). Western Blot analysis of the VEGF protein expression showed a 3 fold increase in cyanotic patients as compared to control patients (Fig. 1D).

To further support the activation of the HIF-1α pathway in cyanotic congenital patients, the expression of several HIF-1α target genes involved in glucose metabolism was analyzed by Real-Time PCR and Western Blot. In particular, the ubiquitous glucose transporter 1 (GLUT1) expression was 3.1 folds higher in cyanotic patients as compared to controls (Fig. 1G). Furthermore, the shift from the oxidative to the glycolytic metabolism was confirmed by the upregulation of ALDOA and GAPDH, that resulted 1.6 and 3.2 folds higher in cyanotic patients than in controls by Real-Time PCR and Western Blot analyses, respectively (Figs 1E and 1H). Moreover, the pyruvate dehydrogenase kinase 1 (PDK1), the enzyme responsible for the inactivation of the pyruvate dehydrogenase complex, was found 1.4 fold higher in cyanotic patients as compared to controls (Fig. 1F). The expression of PHD2 was analyzed by Real-Time PCR and Western Blot in cyanotic and control samples. Although no significant differences were observed in PHD2 mRNA expression levels, PHD2 protein expression was found to be 2.5 folds higher in cyanotic patients than in acyanotic controls, using as internal control the chaperone protein Calnexin (Figs 2A and 2B).

3.3. NEU3 activation in cyanotic patients

To test whether chronic hypoxia affected sialidase NEU3 levels, its expression was tested by Real-Time PCR. Results showed that NEU3 mRNA levels were 1.7 folds higher in cyanotic patients as compared to controls (Fig. 3A). Moreover, the expression of SP1/SP3, transcription factors, which are known to be activated under hypoxia and to bind the NEU3 promoter region, both showed an increase of about 1.3 folds in cyanotic patients as compared to controls (Figs 3B and 3C). As the active form of the sialidase NEU3 has been recently shown to be present at the level of plasma membranes [20], the separation of cytosol and membranes protein fractions was performed on auricle samples from cyanotic and acyanotic patients. Analysis of NEU3 expression by Western Blot revealed a 1.7 fold increase of NEU3 protein level in the membranes of cyanotic samples, as compared to controls, whereas the cytosol fractions did not show any significant differences, as expected (Figs 3D and 3E).

3.4. Analysis of EGFR signaling pathway in cyanotic patients

The effects of chronic hypoxia on the EGFR signaling cascade were tested by evaluating its expression by Western Blot, as well as that of key proteins of the pathway in their inactive and/or active phosphorylated forms. Results revealed an increase of EGFR protein expression and of its phosphorylated active form, which were 6.3 and 4.2 folds higher, respectively, in cyanotic patients as compared to controls (Fig. 4A). Moreover, downstream targets of the EGFR-signaling cascade were also up-regulated in cyanotic patients. In particular, the expression of p42/p44 MAPK, AKT and p70S6K resulted 8, 2.5 and 2.2 folds higher, respectively, in cyanotic patients as compared to acyanotic controls. Similarly, the phosphorylated active forms of the same kinases were also significantly up-regulated in cyanotic patients, (Figs 4B, 4C and 4D).

3.5. HIF-1α and sialidase NEU3 correlation

To test whether NEU3 activation under chronic hypoxia leads to an activation of HIF-1α through the EGFR signaling cascade, we analyzed the correlation between the mRNA expression levels of NEU3 and HIF-1α in acyanotic and cyanotic patients. Results showed that there was a statistically significant positive correlation between NEU3 mRNA expression level and that of HIF-1α in acyanotic patients, as we expected from our previous results [11], with a Pearson's r coefficient of 0.6923 and a p-value of 0.0182. Moreover, a statistically positive correlation was found also between NEU3 and HIF-1α in cyanotic patients, with a Pearson's r coefficient of 0.7038, and a p-value of 0.0231 (Fig. 5).

4. Discussion

Herein we reported a novel mechanism of activation of HIF-1α in the heart of congenital cyanotic patients suffering of chronic hypoxic conditions, which is mediated by sialidase NEU3 (Fig. 6). While HIF-1α is constitutively expressed and is normally regulated by proteolysis in the ubiquitin-proteasome pathway (upon its oxygen-dependent hydroxylation by the prolyl hydroxylases, PHDs, as described in the introduction), in this study we corroborate our previous findings in mouse skeletal muscle, where we first described that HIF-1α is up-regulated at a protein level by sialidase NEU3 via a signaling cascade involving EGFR [11]. For this purpose, we analyzed heart biopsies, from the right atrial appendage of either cyanotic or acyanotic patients undergoing surgeries for congenital heart diseases. We found that HIF-1α expression was significantly higher in cyanotic patients as compared to acyanotic controls. Moreover, HIF-1α downstream targets, including VEGF and several key genes involved in the glycolytic pathway, were also up-regulated in cyanotic patients, as already reported by Quing *et al* [21]. Moreover, we also found that sialidase NEU3 was up-regulated in the same cyanotic patients, in terms of mRNA and of protein levels on the membranes, as we expected from our previous mechanistic study [20]. As we anticipated, NEU3 expression positively correlates with HIF-1α expression levels in both cyanotic and control patients,

supporting the hypothesis of a crucial role played by the enzyme in activating HIF-1α under hypoxia. Moreover, we found that SP1 and SP3, the two belonging to the Sp/KLF family of transcription factors and known to bind to the NEU3 promoter region, were both up-regulated in cyanotic patients, supporting the mechanism of activation of NEU3 under hypoxia that we previously reported [11]. Then, we investigated the mechanism of NEU3-mediated HIF-1α activation. Similarly to what we reported in skeletal muscle [11], we found an increase of EGFR protein expression and of its phosphorylated active form in cyanotic patients, as well as its downstream targets ERK, AKT and p70S6K, also in their phosphorylated active forms. The direct involvement of the EGFR/AKT/p70S6K pathway in the increased transcriptional and translational expression of HIF-1α was already described in smooth muscle cells and cancer. In particular, the activation of AKT is able to induce the mRNA expression of HIF-1α through the transcription factor NF-kB in pulmonary artery smooth muscle cells [22]. Moreover, it has been recently demonstrated in ovarian cancer that the activation of AKT/ERK leads to the phosphorylation and consequently to the activation of the ribosomal protein S6 kinase (p70S6K), which enhances HIF-1α mRNA translation resulting in HIF-1α accumulation [23, 24]. Overall, all these evidences support the hypothesis of a direct involvement of sialidase NEU3 in regulating HIF-1α levels under chronic hypoxia conditions in congenital heart patients. Thus, triggering HIF-1α through the activation of NEU3, or mimicking the sialidase effects by chemical inhibition of GM3 synthesis (as we previously reported [25]), could be a novel tool to increase the physiological cell response to chronic hypoxic stress. Moreover, increasing the expression levels of active proteins of pro-survival pathways may represent a fundamental step to make cardiac cells and tissue more resistant to reoxygenation injuries, thus possibly enhancing cyanotic cardiac patients' long-term outcome. While sustained HIF-1α activation may lead to cardiac fibrosis, as stated before, this should not be viewed as detrimental, given that the process could be reversible and may represent a temporary protective phenomenon during adverse conditions such as hypoxia. In fact, it has been shown that HIF-1α signaling has cardioprotective effects through reversible fibroblasts transformation of

stressed cells during chronic hypoxia in infancy, which results in better long-term myocardial adaptation to hemodynamic load [9]. In particular, this study has shown that patients with more HIF-1α-functioning alleles, i.e. more hypoxic response and RV fibrosis at time of TOF repair, had less progression of pulmonary insufficiency, less progression of RV dilation and dysfunction, and greater freedom from RV intervention and repair.

4.1. Conclusions

Our results further support that a better comprehension of the molecular mechanisms that affect cyanotic CHDs represents a mandatory step for the creation of personalized and more specific therapeutic approaches, with the possibility to ameliorate the short- and long-term outcomes of these patients. In this direction, the discovery that the modulation of sialidase NEU3 is crucial in HIF-1α activation provides a possible novel target for the development of more efficient therapeutic strategies for CHDs patients.

4.2. Study limitations

All the experiments were performed on right atrial tissue samples, which were the only heart specimens discarded during these interventions. However, it is known that is the ventricle, not the atrium, which is mostly sensitive to alterations and remodeling processes in response to chronic hypoxic conditions. As a consequence, we cannot exclude that our results, obtained using right auricle samples, do not precisely reflect what is happening at the level of the right ventricle. Anyhow, many studies have been conducted on NEU3 in other tissues, including tumors [26], and the results strongly support a general role played by sialidase NEU3 in regulating cellular responses to stress conditions, in particular activating pro-survival pathways, opposing cell death and increasing resistance to hypoxia [11, 16, 27]. Thus, based on these results, it is plausible to

speculate that the effects observed in the right atrium of cyanotic patients, which are analogous to those previously observed in other tissues, might be active also in right ventricle.

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Table 1. Acyanotic and Cyanotic patients group composition in terms of different congenital heart malformations.

Acyanotic Congenital Heart Defects	Number
Atrial Septal Defect (ASD)	7
Ventricular Septal Defect (VSD)	5
VSD (Ventricular Septal Defect) + ASD (Atrial Septal Defect)	1
Aortic Valve Stenosis	1
Partial Atrioventricular Canal Defect	1
Truncus Arteriosus Type 2	1
Tetralogy of Fallot (TOF)	1
Total number of patients	**17**

Cyanotic Congenital Heart Defects	Number
Tetralogy of Fallot (TOF)	8
Double Outlet Right Ventricle (DORV)	3
Transposition of the Great Arteries (TGA)	1
TGA + DORV	1
Complete Atrioventricular Canal Defect + DORV	3
Truncus Arteriosus Type 1	1

Pulmonary Atresia + VSD	1
Total number of patients	**18**

Table 2. Clinical characterization of acyanotic and cyanotic patients.

Parameters	**Acyanotic patients** *Average*	**Cyanotic patients** *Average*	*p-value*
Age (months)	**41.17 ± 23.8**	**19.32 ± 27.15**	0.0165; *
Body Mass index (BMI) (kg/m^2)	16 ± 2.5	16.35 ± 1.76	0.5321; ns
SpO_2 (%)	**98.94 ± 1.23**	**72.5 ± 15.38**	< 0.0001; ****
Glycemia (mg/dl)	**110.31 ± 10.68**	**84.78 ± 19.91**	< 0.0001; ****
Creatinine (mg/dl)	0.37 ± 0.09	0.34 ± 0.17	0.5223; ns
Na+ (mEq/L)	138.94 ± 1.44	138.22 ± 2.88	0.3608; ns
K+ (mEq/L)	4.65 ± 0.6	4.79 ± 0.65	0.5132; ns
Prothrombin Time (PT) (%)	96.125 ± 13.6	92.5 ± 11.44	0.3986; ns
Partial Thromboplastin Time (PTT) (sec)	30.16 ± 2.65	30.89 ± 5.48	0.6226; ns
Hemoglobin (Hb) (g/dl)	**11.98 ± 0.76**	**14.03 ± 3.05**	0.0111; *
Platelets (x 1000/μl)	285.31 ± 109.23	302.81 ± 114.69	0.6473; ns

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Figures Legend

Fig. 1. HIF-1α activation in congenital cyanotic patients.
HIF-1α expression was analyzed by Real-Time PCR (**A**) and Western Blot (**B**). The activation of HIF-1α pathway was analyzed measuring the expression of several HIF-1α target genes by Real-Time PCR as VEGF (**C**), ALDOA (**E**), PDK1 (**F**), GLUT1 (**G**) and by Western Blot as VEGF (**D**) and GAPDH (**H**). Real-Time PCR data of cyanotic patients are expressed as fold changes as compared to controls. Ribosomal protein S14 mRNA expression has been used as internal control for Real-Time PCR experiments. Western Blot data were measured by a densitometric analysis of the protein bands and expressed as arbitrary units. Chaperone protein Calnexin (Clx) expression has been used as internal control for Western Blot experiments. *p<0.05; **p<0.01; ***p<0.001.

Fig. 2. PHD2 expression in congenital cyanotic patients.
PHD2 mRNA expression was analyzed by Real-Time PCR and the results expressed as fold changes as compared to the control group. Ribosomal protein S14 mRNA expression has been used as internal control for Real-Time PCR experiments (**A**).
PHD2 protein expression was analyzed by Western Blot and the results expressed as arbitrary units based on the densitometric analysis of the bands. Chaperone protein Calnexin (Clx) expression has been used as internal control for Western Blot experiments (**B**). *p<0.05.

Fig. 3. NEU3 activation in congenital cyanotic patients.
The mRNA expression of the sialidase NEU3 (**A**) and of the transcription factors SP1/SP3 (**B-C**) was analyzed by Real-Time PCR. Data of cyanotic patients are expressed as fold change as compared to controls. Ribosomal protein S14 mRNA expression has been used as internal control for Real-Time PCR experiments.
NEU3 protein expression in plasma membranes (**D**) and cytosol (**E**) fractions was measured by Western Blot. The two subcellular fractions were characterized using the Na^+/K^+ ATPase as plasma membranes marker and EEA1 as cytosol marker. Na^+/K^+ ATPase and EEA1 protein expression has been used as internal control for membranes and cytosol fractions respectively. Results are expressed in arbitrary unit. *p<0.05; **p<0.01.

Fig. 4. EGFR signaling pathway activation in congenital cyanotic patients.

EGFR signaling pathway activation was analyzed by Western Blot. Total proteins from cyanotic and acyanotic patients were extracted and analyzed with anti-EGFR and anti-phospho-EGFR antibodies (**A**); anti-ERK and anti-phospho-ERK antibodies (**B**); anti-AKT and anti-phospho-AKT antibodies (**C**); anti-p70S6K and anti-phospho-p70S6K antibodies (**D**). Changes in protein content were quantified by densitometry and data are reported in arbitrary units. Chaperone protein Calnexin expression has been used as internal control for Western Blot experiments. *p<0.05; **p<0.01.

Fig. 5. HIF-α and NEU3 correlation.
The correlation between HIF-1α and sialidase NEU3 was analyzed by comparing mRNA expression levels of both protein in cyanotic and acyanotic patients. The statistical significance of the correlation was evaluated using the Pearson's r coefficient and the p-value. *p<0.05.

Fig. 6. Representation of a novel HIF-1α mechanism of activation in congenital cyanotic patients mediated by NEU3.
Pictures shows a schematic representation of: (**A**) HIF-1α degradation by proteasome in normoxic conditions; (**B**) HIF-1α stabilization in hypoxic conditions mediated by the inhibition of PHD2 activity; (**C**) HIF-1α up-regulation in hypoxic conditions by the sialidase NEU3 mediated activation of the EGFR pathway.