

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

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33

34 **Abstract**

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

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

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

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

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

58

59 **1. Introduction**

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

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

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

88 At this stage, it seems clear that the mechanism of HIF-1 α activation is very complex and still needs
89 further investigation. Moreover, how this crucial transcription factor affects the heart is still unclear,
90 as it depends on many factors. Along this line, we recently discovered a novel pathway of HIF-1 α
91 activation in murine skeletal muscular myoblasts, which is different from the “canonical” inhibition
92 of prolyl hydroxylase 2 (PHD2) [10], and it is regulated by the membrane-bound sialidase NEU3
93 [11]. NEU3 is a glycosidase responsible for the removal of sialic acid from glycosphingolipids, and
94 it has been shown to possess trans-activity since it can also work on the gangliosides present on the
95 plasma membrane of adjacent cells [12-14]. These key features are likely instrumental for a crucial
96 role played by the enzyme in many cellular processes [15] including cell proliferation and
97 differentiation [16-18]. Specifically, we showed that sialidase NEU3 is transcriptionally activated
98 under hypoxia in skeletal muscle cells, and that activated NEU3 triggers a signaling cascade
99 mediated by ganglioside GM3, which activates the EGF receptor (EGFR) and its downstream anti-
100 apoptotic and pro-proliferative signaling pathways, ultimately leading to HIF-1 α activation.
101 Moreover, stable overexpression of sialidase NEU3 significantly enhances myoblasts resistance to
102 hypoxia, whereas stable silencing of the enzyme renders cells more susceptible to apoptosis [11].
103 These data support the working hypothesis of a physiological role played by NEU3 sialidase in
104 protecting cells from hypoxic stress.

105 Based on these premises, the main aim of this study was to test whether NEU3 plays a role in
106 cardiac muscle response to chronic hypoxia in congenital heart patients, ultimately triggering HIF-
107 1α signaling cascade.

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110 **2. Materials and methods**

111 **2.1. Patients**

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

118

119 **2.2. Tissue biopsies**

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

124

125 **2.3. mRNA isolation from cardiac tissue samples**

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

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

132

133 **2.4. mRNA purification and retro-transcription into the** 134 **corresponding cDNA**

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

139

140 **2.5. Gene expression analyses by Real-Time PCR**

141 For qPCR analyses, 10 ng of total RNA were used as a template with a StepOnePlus® Real-Time
142 PCR System (Thermo Fisher Scientific). The PCR mixture included also: 0.2 µM of primers
143 (Suppl. Table 1) and 10 µl of Power SYBR® Green Master Mix (Thermo Fisher Scientific), in a
144 final volume of 20 µl. Amplification and qPCR data acquisition were performed using the
145 following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles of 5 sec each at
146 95°C, 30 sec at 57°C and 30 sec at 72°C. A final elongation step was performed at 72°C for 2
147 minutes. **Relative quantification of target genes was performed in triplicate and was calculated by**
148 **the equation $2^{-\Delta\Delta Ct}$ using as housekeeper gene the ribosomal protein S14, which is a component of**
149 **the 40S subunit [19].** The accuracy was monitored by analysis of the PCR-product melting curve.

150

151 **2.6. Total protein extraction from Trizol-LS samples**

152 Proteins from the phenol/chloroform layer obtained during the RNA extraction with Trizol-LS,
153 were extracted by the following protocol: 1.2 mL of cold methanol were added to the
154 phenol/chloroform layer and vortexed, then samples were left for 10 min at 30°C and, after an
155 incubation on ice for 5 min, were centrifuged at 12,000 rcf for 10 min at 4°C to sediment the
156 proteins. The protein pellets were washed with 1 mL of cold methanol and centrifuged again at
157 12,000 rcf for 10 min at 4°C. After centrifugation, protein pellets were resuspended in 0.5 mL of
158 cold methanol and sonicated for 4 cycles of 5 sec each in ice using burst sonication to generate a
159 fine protein powder. Samples were then centrifuged for 1 min at 2,000 rcf and the methanol
160 evaporated at room temperature. Proteins were then resuspended with 200 µL of a 0.25%
161 Rapigest® solution in 50 mM ammonium bicarbonate and solubilized by alternate cycles of vortex
162 and heat at 60°C. The obtained proteins were stored at -80°C until used for Western Blot
163 experiments.

164

165 **2.7. Membrane and cytosolic protein separation**

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

177

178 **2.8. Protein expression analyses by Western Blot**

179 Protein concentrations were measured by the Pierce™ BCA Protein Assay Kit (Thermo Fisher
180 Scientific), following the Manufacturer's protocol. 30 µg of proteins were denatured by boiling for
181 5 min in sample buffer (6.5 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 5% 2-
182 mercaptoethanol (w/v), 0.01% bromophenol blue (w/v)) and separated on a 10% polyacrylamide
183 gel in denaturing conditions. Proteins were subsequently transferred into nitrocellulose membranes
184 by electroblotting using 100 Volt for 2 hours. Then, the membranes were incubated overnight in
185 Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl), 0.1% (v/v) Tween 20 (TBS-
186 Tween) containing 5% (w/v) dried milk or 5% (w/v) bovine serum albumin (BSA; Sigma) for the
187 blocking buffer. Blots were incubated with a primary antibody (Suppl. Table 2) in the appropriate
188 blocking solution for three hours at room temperature. Membranes were then washed four times for
189 10 min with TBS-Tween and then incubated with the appropriate secondary antibody horseradish
190 peroxidase (HRP) conjugated for 1 hour at room temperature. After four washes in TBS-Tween, the
191 protein bands were detected using an ECL detection kit (Thermo Fisher Scientific) as described by
192 the Manufacturer. Relative protein expression levels were calculated normalizing data on Calnexin
193 (Clx), Na⁺/K⁺ ATPase (membranes fraction) and EEA1 (cytosol fraction) **expression, which were**
194 **used as internal controls.**

195

196 **2.9. Statistical analysis**

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

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

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207 **3. Results**

208 **3.1. Characterization of patients**

209 Patients included in the study were divided in two groups based on the presence of cyanotic or
210 acyanotic congenital heart defects. The number of patients included in the acyanotic and cyanotic
211 groups was 17 and 18, respectively. Each group was composed by patients with different congenital
212 heart malformations, as shown in Table 1, to increase sample heterogeneity. Different clinical
213 parameters were measured in both cyanotic or acyanotic groups as shown in Table 2. BMI,
214 Creatinine, electrolytes (Na^+ ; K^+), PT, PTT and platelets count did not show any statistically
215 significant differences between the two populations. However, acyanotic and cyanotic patients were
216 statistically different for these clinical parameters: (a) SpO_2 : the level of oxygen saturation of
217 cyanotic patients was approximately 72.5% (SD: 15.38%) as compared to 98.9% (SD: 1.23%) for
218 control acyanotic patients; (b) glycaemia: the average levels of glycaemia measured in cyanotic
219 and acyanotic patients were 84.78 mg/dl (SD: 19.91 mg/dl) and 110.31 mg/dl (SD: 10.68 mg/dl),
220 respectively; (c) hemoglobin: the average value of hemoglobin in patients with cyanotic congenital
221 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
222 patients.

223

224 **3.2. HIF-1 α activation analysis in cyanotic patients**

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

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

249

250 **3.3. NEU3 activation in cyanotic patients**

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

262

263 **3.4. Analysis of EGFR signaling pathway in cyanotic patients**

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

273

274 **3.5. HIF-1 α and sialidase NEU3 correlation**

275 To test whether NEU3 activation under chronic hypoxia leads to an activation of HIF-1 α through
276 the EGFR signaling cascade, we analyzed the correlation between the mRNA expression levels of
277 NEU3 and HIF-1 α in acyanotic and cyanotic patients. Results showed that there was a statistically
278 significant positive correlation between NEU3 mRNA expression level and that of HIF-1 α in
279 acyanotic patients, as we expected from our previous results [11], with a Pearson's r coefficient of
280 0.6923 and a p-value of 0.0182. Moreover, a statistically positive correlation was found also
281 between NEU3 and HIF-1 α in cyanotic patients, with a Pearson's r coefficient of 0.7038, and a p-
282 value of 0.0231 (Fig. 5).

283

284 **4. Discussion**

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

300 supporting the hypothesis of a crucial role played by the enzyme in activating HIF-1 α under
301 hypoxia. Moreover, we found that SP1 and SP3, the two belonging to the Sp/KLF family of
302 transcription factors and known to bind to the NEU3 promoter region, were both up-regulated in
303 cyanotic patients, supporting the mechanism of activation of NEU3 under hypoxia that we
304 previously reported [11]. Then, we investigated the mechanism of NEU3-mediated HIF-1 α
305 activation. Similarly to what we reported in skeletal muscle [11], we found an increase of EGFR
306 protein expression and of its phosphorylated active form in cyanotic patients, as well as its
307 downstream targets ERK, AKT and p70S6K, also in their phosphorylated active forms. The direct
308 involvement of the EGFR/AKT/p70S6K pathway in the increased transcriptional and translational
309 expression of HIF-1 α was already described in smooth muscle cells and cancer. In particular, the
310 activation of AKT is able to induce the mRNA expression of HIF-1 α through the transcription
311 factor NF- κ B in pulmonary artery smooth muscle cells [22]. Moreover, it has been recently
312 demonstrated in ovarian cancer that the activation of AKT/ERK leads to the phosphorylation and
313 consequently to the activation of the ribosomal protein S6 kinase (p70S6K), which enhances HIF-
314 1 α mRNA translation resulting in HIF-1 α accumulation [23, 24]. Overall, all these evidences
315 support the hypothesis of a direct involvement of sialidase NEU3 in regulating HIF-1 α levels under
316 chronic hypoxia conditions in congenital heart patients. Thus, triggering HIF-1 α through the
317 activation of NEU3, or mimicking the sialidase effects by chemical inhibition of GM3 synthesis (as
318 we previously reported [25]), could be a novel tool to increase the physiological cell response to
319 chronic hypoxic stress. Moreover, **increasing the expression levels of active proteins** of pro-survival
320 pathways may represent a fundamental step to make cardiac cells and tissue more resistant to
321 reoxygenation injuries, thus possibly enhancing cyanotic cardiac patients' long-term outcome.
322 While sustained HIF-1 α activation may lead to cardiac fibrosis, as stated before, this should not be
323 viewed as detrimental, given that the process could be reversible and may represent a temporary
324 protective phenomenon during adverse conditions such as hypoxia. In fact, it has been shown that
325 HIF-1 α signaling has cardioprotective effects through reversible fibroblasts transformation of

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

331

332 **4.1. Conclusions**

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

339

340 **4.2. Study limitations**

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

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

352

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356

357 **Table 1. Acyanotic and Cyanotic patients group composition in terms of different congenital**
358 **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

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Table 2. Clinical characterization of acyanotic and cyanotic patients.

<i>Parameters</i>	<i>Acyanotic patients</i>	<i>Cyanotic patients</i>	<i>p-value</i>
	<i>Average</i>	<i>Average</i>	
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
SpO₂ (%)	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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473 **Figures Legend**

474 **Fig. 1. HIF-1 α activation in congenital cyanotic patients.**

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

483
484

485 **Fig. 2. PHD2 expression in congenital cyanotic patients.**

486 PHD2 mRNA expression was analyzed by Real-Time PCR and the results expressed as fold
487 changes as compared to the control group. Ribosomal protein S14 mRNA expression has been used
488 as internal control for Real-Time PCR experiments (A).

489 PHD2 protein expression was analyzed by Western Blot and the results expressed as arbitrary units
490 based on the densitometric analysis of the bands. Chaperone protein Calnexin (Clx) expression has
491 been used as internal control for Western Blot experiments (B). *p<0.05.

492

493 **Fig. 3. NEU3 activation in congenital cyanotic patients.**

494 The mRNA expression of the sialidase NEU3 (A) and of the transcription factors SP1/SP3 (B-C)
495 was analyzed by Real-Time PCR. Data of cyanotic patients are expressed as fold change as
496 compared to controls. Ribosomal protein S14 mRNA expression has been used as internal control
497 for Real-Time PCR experiments.

498 NEU3 protein expression in plasma membranes (D) and cytosol (E) fractions was measured by
499 Western Blot. The two subcellular fractions were characterized using the Na⁺/K⁺ ATPase as plasma
500 membranes marker and EEA1 as cytosol marker. Na⁺/K⁺ ATPase and EEA1 protein expression has
501 been used as internal control for membranes and cytosol fractions respectively. Results are
502 expressed in arbitrary unit. *p<0.05; **p<0.01.

503

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

505 EGFR signaling pathway activation was analyzed by Western Blot. Total proteins from cyanotic
506 and acyanotic patients were extracted and analyzed with anti-EGFR and anti-phospho-EGFR
507 antibodies (A); anti-ERK and anti-phospho-ERK antibodies (B); anti-AKT and anti-phospho-AKT
508 antibodies (C); anti-p70S6K and anti-phospho-p70S6K antibodies (D). Changes in protein content
509 were quantified by densitometry and data are reported in arbitrary units. **Chaperone protein**
510 **Calnexin expression has been used as internal control for Western Blot experiments.** *p<0.05;
511 **p<0.01.
512

513 **Fig. 5. HIF- α and NEU3 correlation.**

514 The correlation between HIF-1 α and sialidase NEU3 was analyzed by comparing mRNA
515 expression levels of both protein in cyanotic and acyanotic patients. The statistical significance of
516 the correlation was evaluated using the Pearson's r coefficient and the p-value. *p<0.05.
517

518 **Fig. 6. Representation of a novel HIF-1 α mechanism of activation in congenital cyanotic**
519 **patients mediated by NEU3.**

520 Pictures shows a schematic representation of: (A) HIF-1 α degradation by proteasome in normoxic
521 conditions; (B) HIF-1 α stabilization in hypoxic conditions mediated by the inhibition of PHD2
522 activity; (C) HIF-1 α up-regulation in hypoxic conditions by the sialidase NEU3 mediated activation
523 of the EGFR pathway.