SAPIENZA
Università di Roma
Facoltà di Farmacia e Medicina

PhD in
MORPHOGENESIS AND TISSUE ENGINEERING

Ciclo XXXIII

(A.A.2019/2020)

Phosphodiesterases expression during murine cardiac development and Role of phosphodiesterase 5 in neonatal cardiomyopathy induced by gestational diabetes

PhD Student
Thays Maria da Conceição Silva Carvalho

Tutor:
Prof. Mauro Giorgi

Coordinator
Prof. Antonio Musarò

1.**The Thesis Explained**

Heart development is a complex process that requires an interplay of different transcription factors, signaling pathways, and morphological modifications. The cardiac formation is tightly controlled by a setup of signal transduction pathways, including the intracellular second messenger cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The intracellular levels of cyclic nucleotides are regulated by the balance between their synthesis and degradation. Despite the fact, the cAMP-cGMP/PDE pathway plays a critical role in cardiac processes like contraction/relaxation, cell differentiation, proliferation, and survival, very few data are available about PDE expression in cardiac development.

The first part of my PhD focuses on the characterization of the expression and enzymatic activity of the PDEs at different stages of murine heart formation. Obtained data by qRT-PCR, Western Blot, and Enzymatic activity demonstrated a modulation in the expression of some PDEs at the stages of heart development evaluated. PDE5 is one of the isoforms showing modulation of both expression and activity.

This PDE was expressed at a low level in prenatal hearts, confirmed by the low specific enzymatic activity. Over time PDE5 has been a target of pharmacological inhibition as treatment of some cardiac pathologies in patients and in the mouse. PDE5 inhibition has been associated with a cardioprotective effect. These lead us to hypothesize that PDE5 resembles fetal genes, which could be involved in congenital heart defects in response to a pathological stimulus.

Due to it, the second part of my thesis had a focus on verifying if PDE5 could play a role in neonatal cardiomyopathy induced by gestational diabetes. To accomplish this goal, I took advantage of a genetically modified animal to Pde5. Heterozygous Pde5 mice were mated and induced maternal

diabetes. Histological analysis from neonatal hearts of *Pde5*$^{+/+}$; *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ shows that reduced levels of *Pde5* decreased the incidence of congenital cardiomyopathy in newborns from mothers with maternal hyperglycemia, suggesting that PDE5 may exert a role also in the induction of neonatal heart diseases.

Part I:

Phosphodiesterases expression during murine cardiac development

1. Introduction

1.1 Murine heart development

The heart is one of the first organs to form and function during embryonic development (Sun & Kontadiris, 2018). Heart development is a complex process that requires an interplay of different transcription factors, signaling pathways, and morphological modifications (Boer et al, 2009).

In mice, the heart formation begins at embryonic day 7.5 (E7.5) when a group of mesodermal cells migrates to the midline forming a bilateral structure called a cardiac crescent (cc) which is comprised of the first and second heart fields (FHF & SHF) (Boer et al, 2009; Ivanovitch et al, 2017).

Further, at E8.5 these fields, through a complex morphogenetic process, change their configuration giving rise to the beating linear heart tube (HT). At this stage, the contractions are irregular becoming a regular beating heart at E9.5 (Savolainen et al, 2009).

At E9.5-E10.5 the cardiac loop begins, which presents undivided right and left ventricles. Meanwhile, the process of atrial septation occurs (Krishnan et al, 2014). At E11.5 the separation of the ventricles and the process of intraventricular septation begin (Krishnan et al, 2014).

At E12.5 cardiac trabeculation begins forming a network of projections called trabeculae that are responsible for an increase in cardiac output and for the nutrition and oxygen uptake of embryonic myocardium (Samsa et al, 2013). All these processes

end at E13.5, and at E14.5 the *four-chambered heart is formed* (Boer et al, 2009).

At E15.5 and E16.5, the atrioventricular valve leaflets and the coronary arteries start modifying, achieving definitive conformation, and at E18.5 the final external prenatal heart configuration is completed (Savolainen et al, 2009).

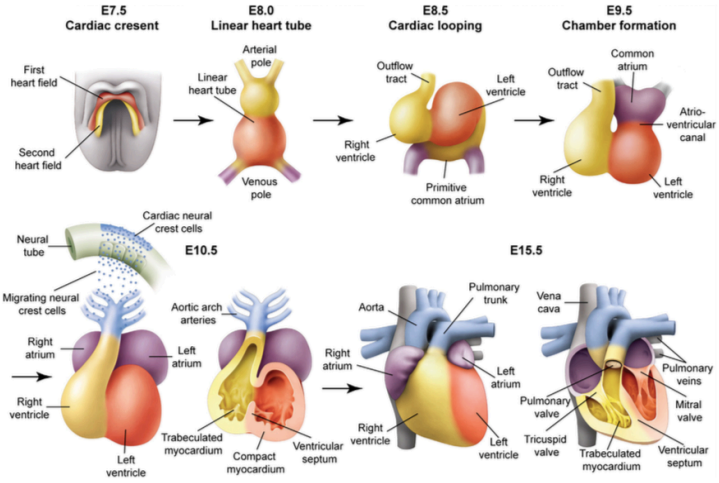


Figure 1. Schematic representation of heart development in human and mouse. (Adapted from: Epstein et al: Semaphorin signaling in cardiovascular development. Cell metabolis, 2015)

1.2 Signaling pathways

The process of heart formation is tightly controlled by a setup of signal transduction pathways including seconder messenger cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Roche et al, 2013).

cAMP and cGMP play a fundamental role in signal transduction that regulate some physiological and pathological

processes which include cellular growth, differentiation, proliferation, Ca^{2+} dependent signaling, gene transcription and cardiac function (Azevedo et al, 2013; Zaccolo, 2009).

In response to hormone and extracellular stimulation activating the G protein coupled receptors, into the heart, cAMP is the main second messenger involved in regulation of the heart rate and strength of contraction via β-adrenergic signaling (Chao et al, 2019; Boularou & Gales, 2015). Yet, cGMP acts as an antagonist of cAMP in the regulation of heart contractility (Drummond & Severson, 1979). cGMP is responsible for a decrease in contractility force and heart rate (Drummond & Severson, 1979).

Intracellular levels of cyclic nucleotides are regulated by the balance between their synthesis and degradation. cAMP is synthesized by adenylyl cyclases through agonist binding to G protein coupled receptors whereas cGMP is produced by a soluble guanylyl cyclase activated by nitric oxide and a particulate guanylyl cyclase activated by natriuretic peptides (Maurice et al, 2014). Both cyclic nucleotides are specifically hydrolyzed by 3'5'cyclic nucleotides phosphodiesterases (PDEs) into their inactive forms 5'-AMP and 5'-GMP.

1.3. 3'5' Cyclic nucleotide phosphodiesterase (PDE)

The 3'5' cyclic nucleotide phosphodiesterases are a superfamily of enzymes comprised of eleven genes (Bobin et al, 2016). PDEs are the unique enzymes that regulate the signal-transduction generated by cAMP and cGMP hydrolyzing both cyclic nucleotides in their inactive forms (cAMP-5'AMP; cGMP-5' GMP) (Bobin et al, 2016). PDEs vary in cellular function, catalytic properties, protein structure and affinity for cAMP and cGMP (Knight & Yan, 2012). PDE1, PDE2, PDE3, PDE10 and PDE11 hydrolyze both cAMP and cGMP; PDE4, PDE7 and PDE8 specifically hydrolyze cAMP, PDE5, PDE6

and PDE9 specifically hydrolyze cGMP (Figure 2) (Zaccolo & Movsesian, 2007).

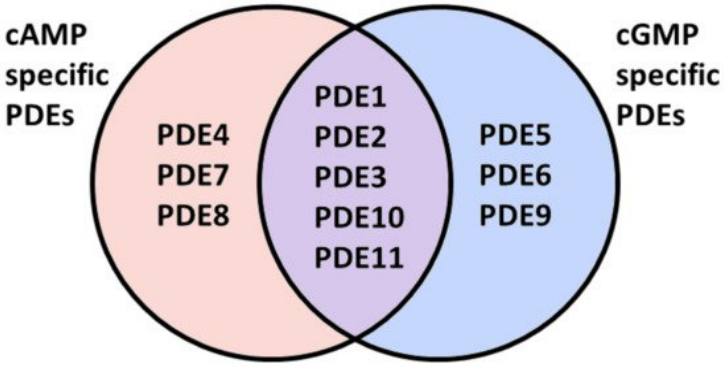


Figure 2: Substrate specificity of individual phosphodiesterases (PDEs) families. (Adapated from: Ercu & Klussmann; Roles of A-Kinase anchoring proteins and phosphodiesterases in the cardiovascular system. Journal of cardiovascular development and disease, 2018).

PDEs are widely expressed in different body tissues such as skeletal muscle, oocytes, the brain, smooth muscle, the immune system and in the heart (Omori & Kotera, 2007). In the cardiovascular system, PDEs exert different functions involved in the vascular smooth muscle contraction; phosphorylation of calcium channels, vasodilation and cardiac contractility (Azevedo et al, 2014). Seven PDEs are expressed in the adult heart: PDE1, PDE2, PDE3, PDE4, PDE5, PDE8 and PDE9 (Figure 3) (Zaccolo & Movsesian, 2007).

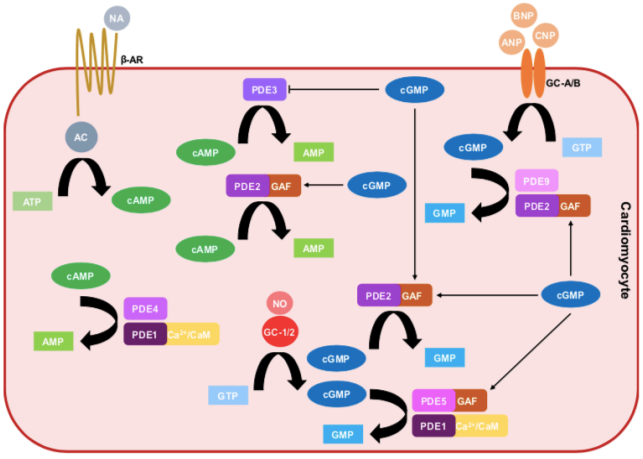


Figure 3: Phosphodiesterase regulation of cyclic nucleotides within cardiomyocytes. NP- natriuretic peptides; NO- nitric oxide; AC- adenylyl cyclase; ANP- atrial natriuretic peptide; ATP- adenosine 5' triphosphate; B-AR beta adrenergic receptor; BNP- brain natriuretic peptide; CNP- C-type natriuretic peptide; GC- guanylyl cyclase; GTP- guanosine 5' triphosphate, NA- noradrenalin. (Adapted from: Preedy, M.E.J. Cardiac cyclic nucleotide phosphodiesterases: roles and therapeutical potential in heart failure. Cardiovascular drugs and therapy, 2020).

Phosphodiesterase 1 (PDE1)

The PDE1 family is a Ca^{2+}/Calmodulin (CaM) stimulated enzymes comprised of 3 genes, PDE1A, 1B and 1C. All isoforms contain CaM binding domains which might increase in approximately 10-fold their hydrolytic capacity in response to Ca^{2+}/CaM binding (Knight & Yan, 2012). This group of PDEs present dual capacity to hydrolyze both cAMP and cGMP. PDE1A and 1B display the highest affinity in hydrolyzing cGMP, while PDE1C has comparably affinity in hydrolyzing cAMP and cGMP (Knight & Yan, 2012).

Phosphodiesterase 2 (PDE2)

PDE2, also called cGMP stimulated PDE, is a dual phosphodiesterase that can hydrolyze cAMP and cGMP with high affinity (Tsai & Kass, 2009). PDE2 presents a single gene, PDE2A, that is expressed in the cardiac tissue and in isolated cardiomyocyte being associated with the plasma membrane, Golgi complex, sarcoplasmic reticulum and the nuclear envelope (Knight & Yan, 2012). In the heart, PDE2 hydrolytic activity to cAMP increases through cGMP stimulation (Omori & Kotera, 2007).

Phosphodiesterase 3 (PDE3)

PDE3 family is formed by two genes, PDE3A and PDE3B. Both PDE3 isoforms are expressed in cardiac myocytes, however, 3A is more abundant than 3B (Movsesian et al, 2018). PDE3 is also a dual esterase capable of hydrolyze cAMP and cGMP (RAO & XI, 2009). It is known that PDE3A is mainly involved in the regulation of cardiac contractility, while PDE3B is correlated to metabolism regulation (Movsesian et al, 2018).

Phosphodiesterase 4 (PDE4)

Differently from the other PDEs described above, PDE4 is specific in hydrolyzing cAMP (Fertig & Baillie, 2018). This group of PDE is comprised of four genes, PDE4A, 4B, 4C, and 4D, which are expressed as multiple variants (Richter et al, 2011). In mice heart, a specific variant PDE4D3 was described and correlated with the regulation of Ryanodine receptor 2 phosphorylation and diastolic sarcoplasmic reticulum Ca^{2+} leak (Mika et al, 2013).

Phosphodiesterase 5 (PDE5)

PDE5 was the first specific cGMP phosphodiesterase described with a fundamental role in erectile and pulmonary vasomotor control (Lee et al, 2015). PDE5 is mainly involved in the hydrolysis of cGMP produced by the nitric oxide and is encoded from one gene with three spicing variants (PDE5A1, 5A2, and 5A3) (Lee and Kass, 2012). It is recognized that there is no–functional difference among the PDE5 variants, however, they share similar hydrolytic activity to cGMP (Korkneoz-Icoz et al, 2018). In mouse, PDE5 expression was found in platelets, brain, lung, and in the heart (Kotera et al, 2000).

Phosphodiesterase 8 (PDE8)

PDE8 belongs to the group of PDEs are specific in the hydrolysis of cAMP. This group of enzymes is formed by 2 genes 8A and 8B which are expressed in different tissues. PDE8A is widely expressed in testis and was detected in heart tissue in humans and mice (Patrucco et al, 2010). The PDE8B expression is detected in the adipose tissues, liver, kidney, and heart (Horvath et al, 2008).

Phosphodiesterase 9 (PDE9)

PDE9, as PDE5, is specific in the hydrolysis of cGMP (Lee et al, 2015), but differently from PDE5, it is responsible for the hydrolysis of cGMP generated through natriuretic peptides (NP) (Priksz et al, 2018). PDE9 was recently discovered and its expression was detected in testes, brain, skeletal muscle, and heart (Zhang & Kass, 2011).

2. AIM I

Although the cAMP-cGMP/PDE pathway plays a fundamental role in cardiac processes such as contraction/relaxation, cell differentiation, proliferation, and survival, very little data is available on PDE's expression in cardiac development. Previous studies reported that intracellular levels of cAMP and cGMP change over the course of heart formation in chicken embryos, which could be correlated with a major degradation of cyclic nucleotides by PDEs (Thakkar & Sperelakis, 1987).

In a previous study from our group, by a semiquantitave RT-PCR was verified that seven PDEs are expressed in murine hearts at different stages of development (Isidori et al, 2015). More importantly, we demonstrated that PDE2A plays a critical role in heart development because its genetic ablation induces congenital heart defects (Assenza et al, 2018).

Considering these observations, the present study was carried out to characterize the pattern of phosphodiesterase expression in cardiac development at mRNA, protein and activity levels. This information will provide a useful tool to clarify the role of PDEs in the framework of cardiac development and their potential role in cardiac disease.

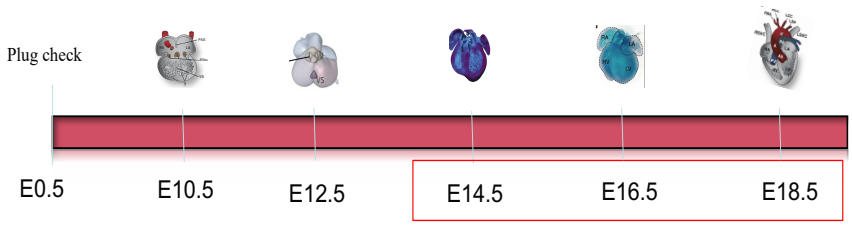
3. Materials and methods

3.1 Ethical statement

All the animal procedures were conforming to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were conducted with the approval of the Sapienza University's Animal Use for Research Ethic Committee and by the Italian Ministry of Health with protocol number DGSAF 24675-A.

3.2 Mouse husbandry and embryos collection

Male and female C57BL/6 wild type from eight to twelve weeks of age were mated to generate embryos and fetus mice. The day of vaginal plug was considered as embryonic day 0.5 (E0.5). The animals had access to food and drinking water ad libitum and maintained in a light-dark cyclic of 12-12 hours and temperature at 20-25°C. At 14.5, 16.5, 18.5 days of heart formation embryos and fetus were dissected from the yolk sac after cervical dislocation of pregnant mouse. The embryonic and fetal hearts were collected in phosphate buffer saline (PBS) and stored at -80º C to future analysis.



3.3 Real time PCR

Total RNA was isolated from five samples of embryonic heart tissue using total RNA isolation micropep kit- BioRad following the manufacturer's instructions. The RNA was treated with DNase I Zymol and after reversing transcribed to synthesize cDNA through Maxima H minus reverse transcriptase (Thermo Fischer Scientific).

For real time PCR, samples were amplified in the reaction mixture with PowerUp SYBR green Master Mix (Thermo Fisher Scientific) and specific primers for phosphodiesterases in Thermo Fisher Scientific 7500 Real-Time PCR instrument. For quantification analysis, the comparative threshold cycle (Ct) method was used. The Ct values of each gene were normalized to the Ct value of *Gapdh* in the same RNA sample. The gene expression levels were evaluated by the fold change using the equation 2^{-ddCt}. The primers used in the present study were listed below.

qRT-PCR primers sequence

Pde1a FW 5'AGGTATCATGCACTGGCTCA 3'
Pde1a RV 5'GAGCGGTCGTTGTACAGAAT 3'
Pde1c FW 5' ATGGGGATGATGCTTAGGAG 3'
Pde1c RV 5' CAATGCTTCGATTACAGCCG 3'
Pde2a FW 5' ACCGAAAGATCCTGCAACTG 3'
Pde2a RV 5' TTCTCCCAGCACTTTGTCTC 3'
Pde3a FW 5' AGAATCCATGCCACCGATGT 3'
Pde3a RV 5' CCCATGTGTCCGTGTGTAAA 3'
Pde4d FW 5' GCCTCTGACTGTTATCATGCAC 3'
Pde4d RV 5' GCAGCATGGATGTTGTTGTG 3'
Pde4a FW 5' TGCTGCAAGAGAACTGC 3'
Pde4a RV 5' AGGGTCATGTGCTTGGACAT 3'
Pde4b FW 5' AGGATCATCTAGCCAAGG 3'

Pde4b RV 5' GTAAGGGGCCGATTATGTGA3'
Pde5a FW 5' ATCCATGGACTCATCTCTGC 3'
Pde5a RV 5' GCTTCCTCCAATGTTGAACC 3'
Pde8a FW 5'TCAGAGTGTGCAATGGCAAC 3'
Pde8a RV 5'GTCCATCGAATGTTTCCTCC 3'
Pde9a FW 5'CTACGAGGAGCTGAAGCAGC 3'
Pde9a RV 5' AGTTTGGAGGAGAATGGCCT 3'
Gapdh FW 5'GTGAAGGTCGGTGTGAACG 3'
Gapdh RV 5'ATTTGATGTTAGTGGGGTCTCG3'

3.4 Western Blot

To evaluate the protein expression of PDEs during the cardiac development a western blotting analysis was performed. Protein samples were extracted from embryonic heart and lysed in RIPA lysis buffer 1x (EMD Milipore Corp, 20-188) supplemented with protease and phosphatase inhibitors.

The protein concentration was measured by Bradford method. Further, an amount of 30µg of protein was electrophoresed in 8-10% SDS-page gels and transferred onto nitrocellulose membrane.

Further, the membrane was immersed in 5% blocking milk (TBST + 5% non-fat milk) for 1 hour at room temperature and the incubated in primary antibodies (dilution 1:500 in TBST+ 5% BSA- bovine serum albumin- buffer) for phosphodiesterase detection (PDE1A FabGennix #PDE1A-101AP; PDE1C FabGennix #PD1C301AP; PDE2- Abcam #14672#; PDE3A Santa Cruz #sc-20792; PDE4D Invitrogen #PAS-79795# and PDE5A Santa Cruz #32884#) overnight at 4°C.

After the membranes were washed in TBST and incubated in appropriated horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000 dilution in TBST) for 1 hour at room temperature. Chemiluminescent images of immunodetected bands were recorded with the Syngene G-box

system (Syngene Bioimaging) and immunoblot intensities were quantitatively analyzed using ImageJ Software (NIH, Bethesda, MD).

3.5 PDEs activity assay

Embryonic hearts were homogenized using a glass homogenizer (15 strokes, 4°C) in 20mM Tris-HCl buffer pH 7.2 containing 0.2mM EGTA, 5mM β-mercaptoethanol, 2% v/v antiprotease cocktail (Sigma–Aldrich), 1mM PMSF, 5mM MgCl2, 0.2% v/v Triton X-100. The homogenates were centrifuged at 14,000g for 30 min at 4°C.

PDE activity was measured on the supernatant with the method described by Thompson and Appleman [1971] in 60mM Hepes pH 7.2, 0.1mM EGTA, 5mM MgCl2, 0.5mg/ml bovine serum albumin (BSA), and 30 mg/ml soybean trypsin inhibitor, in a final volume of 0.15ml. Ca^{2+}-calmodulin stimulation was determined in the presence of 1 mM $CaCl_2$ and 3 μg/ml calmodulin. The reaction was started by adding tritiated substrates at a final concentration of 1 μM [^{3}H] cAMP or [^{3}H] cGMP, and 0.1 μM [^{3}H] cAMP for PDE1C detection. The reaction was stopped by adding 50 μl of 0.1 N HCl and then neutralized with 50 μl of 0.1 N NaOH in 0.1M Tris–HCl pH 8.0.

Subsequently, 25 μl of 2mg/ml of 5'-nucleotidase (snake venom from Crotalus atrox; Sigma-Aldrich) in 0.1M Tris–HCl pH 8.0 were added. Samples were gently mixed and incubated at 30°C for 30 min to allow complete conversion of 5'-nucleotide to its corresponding nucleoside. Unhydrolyzed cyclic nucleotide and the corresponding nucleoside were separated by DEAE-Sephadex A-25 columns. The eluate was mixed with ULTIMA GOLD scintillation liquid (PerkinElmer) and counted on a Tri-Carb 2100TR Liquid Scintillation Counter (2000CA; Packard Instruments).

To evaluate the enzymatic specific activity of each PDEs, the specific inhibitors were added to the reaction mix at the following concentration: 10 μM milrinone (PDE3 inhibitor); 0.1 μM BAY 60-7550 (PDE2 inhibitor); 30 μM rolipram (PDE4 inhibitor); 0.1 μM sildenafil (PDE5 inhibitor); 1 μM PF04449613 (PDE9 inhibitor).

The percentage of each PDEs was calculated comparing the difference between the total PDE activity and the residual PDE activity assayed in the presence of the specific inhibitor with respect to the total PDE activity fixed as 100%.

3.6 Statistics analysis

The data obtained were statistically analyzed using the GraphPad Prism 5.0 (GraphPad software). Comparison among groups was performed with the One-way ANOVA or two-way ANOVA and Multiple comparisons by Tukey test. A p value of $p < 0.05$ was considered statistically significant; all data were presented as mean ± SD.

4. Results

4.1 PDEs mRNA expression variates between embryonal and fetal cardiac development

It was previously shown by semiquantitative RT-PCR that mRNAs of *Pde1, Pde2*, *Pde3, Pde4, Pde5, Pde8,* and *Pde9* were present in the heart of embryonic and fetal mice (Isidori et al, 2015).

To detect PDE's expression changes in developing heart, the mRNA level of selected PDEs was measured by qRT-PCR at E14.5, E16.5, and E18.5 (Figure 4).

Quantitative expression analysis showed that mRNA of *Pde1a* isoform was present at a similar level at E14.5 and E16.5, its level decreased at E18.5 of heart development without reaching a statistical significance. *Pde1c* mRNA was low expressed at E14.5, it increased its expression at E16.5 reaching the statistical significance at E18.5 ($p<0.05$) (Figure 4).

As mentioned before *Pde2a* expression is required for normal heart development (Assenza et al, 2018). *PDE2a* mRNA is low expressed at E14.5 and its level increases with time showing a significant modulation expression at E18.5 ($p<0.01$).

Pde3a mRNA is low expressed at E14.5, it increases at E16.5 and its level remains stable until E18.5 of cardiac formation.

The main *Pde4* isoforms expressed in the heart were analyzed: *Pde4a* mRNA shows a slight, but not significant, increase between E14.5 and 18.5; *Pde4b* mRNA shows a progressive significant decrease in expression at E16.5 ($p<0.0001$) and at E18.5 ($p<0.0001$); *Pde4d* mRNA is more expressed at E14.5 with a trend of reduced expression at E16.5 and E18.5 (Figure 4).

Pde5a mRNA is more expressed at E14.5 and it shows a slight and not significant decrease at E16.5, returning to increase at E18.5 (Figure 4).

Pde8 mRNA showed a decreased expression from E14.5 to E16.5, a small increase of expression occurred between E16.5 and E18.5 (Figure 4).

Pde9 mRNA shows a similar expression trend of *Pde4d,* showing a higher, but not significant, expression level at E14.5 compared to E16.5 and E18.5 (Figure 4).

These data show that mRNA expression of PDEs isoforms can be grouped into isoforms which are present at approximately the same level at any stage (*Pde1a*, *Pde3a*, *Pde5a* and *Pde8*), isoforms which tend to decrease with time (*Pde4b*, *Pde4d* and *Pde9*) and isoforms which are clearly upregulated with time (*Pde1c*, *Pde2a and Pde4a*).

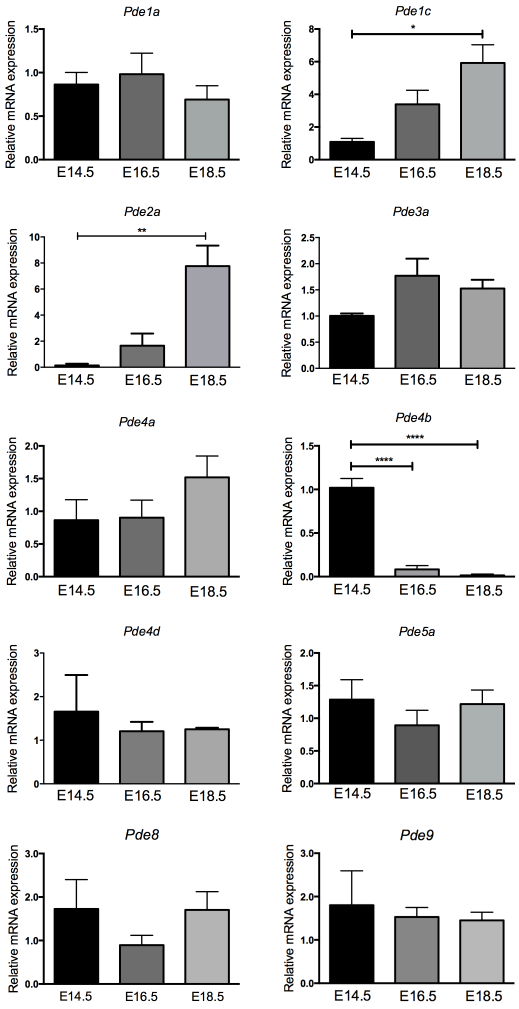


Figure 4. Phosphodiesterases gene expression during heart formation. qReal-time PCR analysis of *Pde1a*, *Pde1c*, *Pde2a*, *Pde3a*, *Pde4a, Pde4b, Pde4d*, *Pde5a*, *Pde8* and *Pde9* mRNA expression in E14.5, E16.5 and E18.5 mouse hearts. The relative expression was normalised versus *Gapdh* gene. Data represent the mean ± SD (n= 5 hearts for each stage). One-way ANOVA was used for statistical analysis. *p<0.05, **p<0.01, ****p<0.0001.

4.2 Variation of PDEs expression occurs at protein levels in embryonic and fetal heart

Since no data are available regarding the traslation of mRNA PDEs isoforms into protein in the prenatal heart, western blot analysis was performed to assess the presence of PDE isoforms immunoreactivity at the same days in which mRNA epression was evaluted (Figure 5).

Among the analyzed PDEs, PDE1A shows an increased protein expression at E16.5 compared to its level recorded at E14.5 and at E18.5. The PDE1C protein level was lowest at E14.5, it increased at E16.5 to reach the highest expression at E18.5 ($p<0.05$) (Figure 5).

PDE2A shows a pattern of expression similar to PDE1C: it is almost undetectable at E14.5 and it increases at E16.5 and E18.5 ($p<0.05$) (Figure 5).

PDE3A showed similar protein expression pattern at all stages evaluated. PDE4D expression was not modified at E14.5 and at E16.5, but a significant reduction of expression was detected at E18.5 ($p<0.05$).

Protein expression of PDE5A significantly decreased from E14.5 to E16.5 ($p<0.01$) reaching the lowest expression level at E18.5 ($p<0.01$) (Figure 5).

PDE8 and PDE9 proteins were below the limit of quantification of employed methods (data not shown).

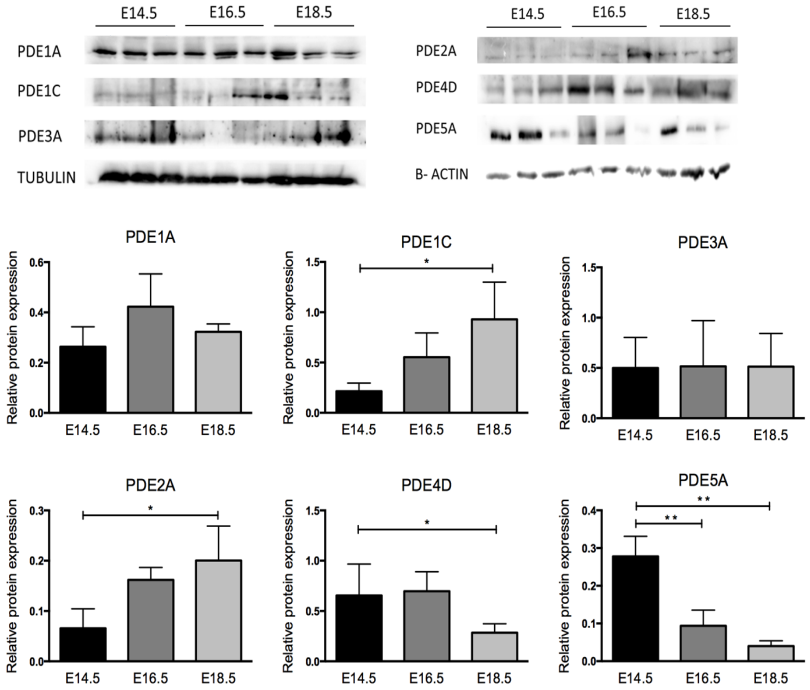


Figure 5. Phosphodiesterases protein expression during heart formation. Rapresentative image of western blot analysis and relative densitometric analysis of PDE1A, PDE1C, PDE3A, PDE2A, PDE4D and PDE5A proteins normalized with respect to Tubulin or β-Actin protein levels in E14.5, E16.5 and E18.5 mouse hearts. Data represent the mean ± SD of 3 heart for each embryonic stage. One-way ANOVA was used for statistical analysis, *p<0.05, **p<0.01.

4.3 The PDEs presenting enzymatic activity modulation at embryonic and fetal life

In the prenatal heart there was a substantial stability in the levels of cAMP and cGMP hydrolytic activities independently of the developmental time (Figure 6).

Taking advantage of availability of specific inhibitors for some PDE isoforms, it was possible to measure the enzymatic activity of PDE 2, 3, 4, 5 and 9 at different stages of the heart development. cAMP-PDE activity assays were performed in cardiac homogenate in presence of 10 μM of the specific PDE3 inhibitor milrinone and 30 μM of the specific PDE4 inhibitor rolipram. The obtained results show that almost 75% of the total cAMP hydrolytic activity in the prenatal heart is due to the PDE4 and the remaining 25% to PDE3 activity (Figure 6).

PDE3 activity decreased with time reaching the statistical significance at E18.5 ($p<0.05$). The enzymatic activity of PDE4 decreased more extensively compared to the PDE3 activity. PDE4 activity decreased of approximately 15% ($p<0.01$) from E14.5 to E16.5, whereas the reduction was of 20% from E14.5 to E18.5 ($p<0.01$).

cGMP-PDE activity assays were performed in cardiac homogenate in presence of 0.1 μM of the specific PDE2 inhibitor BAY 60-7550, 0.1 μM of the specific PDE5 inhibitor sildenafil or 1 μM of the specific PDE9 inhibitor PF04449613.

The obtained results show that in the prenatal heart at E14.5 almost 80% of the total cGMP hydrolytic activity is divided at the same extent between PDE2 and PDE5 and the remaining 10% of enzymatic activity is due to PDE9 (Figure 6).

PDE2-dependent cGMP hydrolytic activity increased from 40% of the total activity at E14.5 to 60% at E16.5 and E18.5. Concomitantly there was a significant decrease of PDE5 specific activity which passed from 40% of the total activity at E14.5 to

less than 17% at E18.5. PDE9 activity was about 15% of total cGMP activity at any evaluated stage.

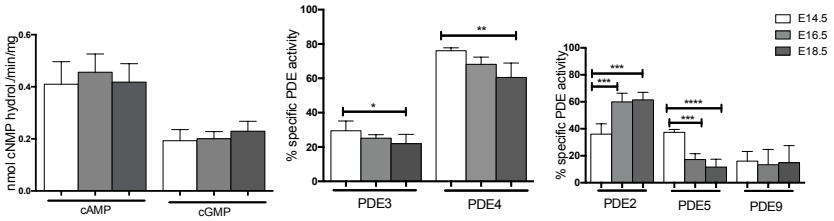


Figure 6. Total and specific PDEs enzymatic activity during the heart formation. Total cAMP and cGMP hydrolyzing activity from heart extracts at E14.5 (white columns), E16.5 (light grey columns) and E18.5 (dark grey columns). PDE3 and PDE4 specific activities are reported as percentage of total cAMP hydrolyzing activity evaluated after adding a fixed dose of the specific inhibitor milrinone and rolipram respectively (see Materials and Methods). PDE2, PDE5 and PDE9 specific activities as a percentage of the total cGMP hydrolyzing activity evaluated after adding a fixed dose of the specific inhibitor BAY 60-7550, sildenafil and PF04449613 respectively (see Materials and Methods). Data represent the mean of 3 independent experiments ± SD. Each experiment was performed with a pool of 5 hearts per stage. One-way ANOVA was used for statistical analysis. *$p<0.05$; **$p<0.01$; *** $p<0.001$, ****$p<0.0001$.

The activity of PDE1 family was evaluated taking advantage of their unique property to be stimulated by Ca^{2+}/calmodulin binding. Stimulated activity was first assessed with cGMP as the substrate to measure the total amount of all PDE1 isoforms. As shown in Figure 7, cGMP hydrolyzing activity is stimulated of about two-fold in the presence of calcium and calmodulin independently of the developmental stage.

Considering the lack of available specific inhibitors to PDE1 isoforms, the PDE1C enzymatic activity in hydrolyze cAMP was evaluated in presence of 0,1μM substrate concentration (Figure 7). This approach was performed because it was reported that the 1C isoform shows major affinity in hydrolyzing the cAMP than

cGMP (Omori & Kotera, 2007). The obtained results show a similar Ca^{2+}/calmodulin stimulation in both E14.5 and E16.5 prenatal hearts, and a significant increase of stimulated activity at E18.5 stage.

These results show that PDE4 is the main isoform responsible of cAMP hydrolyzing activity in the prenatal heart and that the constant cAMP hydrolysis during heart development can be explained by the increase of PDE2 enzymatic activity that parallels the slight decrease of PDE3 and PDE4 activity.

Similarly, the constant cGMP hydrolyzing activity present in the homogenate of the prenatal heart can be assigned to the clear shift between PDE2 and PDE5 basal activity along heart maturation.

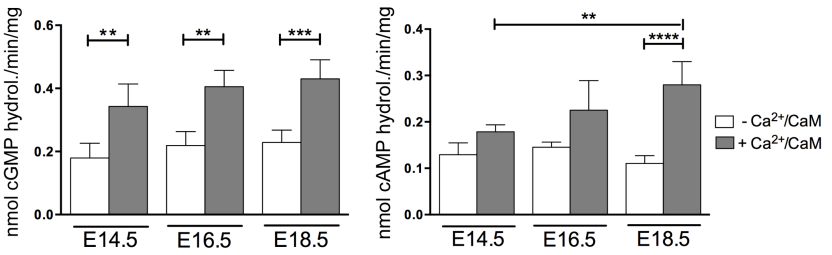


Figure 7. PDE1 enzymatic activity during the heart formation. Total PDE1 activity (left side) evaluated with 1μM cGMP as substrate in presence of calcium and calmodulin. PDE1C activity (right side) assessed with 0.1μM cAMP as substrate in presence of calcium and calmodulin. Data represent the mean of 3 independent experiments ± SD. Each experiment was performed with a pool of 5 hearts per stage. Two-way ANOVA were used for statistical analysis. **p<0.01, ****p<0.0001.

5. Discussion

The present study aimed to elucidate the developmental pattern of PDEs' expression and enzymatic activity at different stages of embryonic and fetal murine heart.

The 3'-5' cyclic nucleotides phosphodiesterases are the unique enzymes which hydrolyze the cyclic nucleotides cAMP/cGMP regulating the intracellular signal transduction of many hormones and growth factors involved in cardiac biology (Maurice et al, 2003).

Very few information is available about the levels of cyclic nucleotides in the developing heart. Thakkar and Sperelakis assessed the cAMP and cGMP content at basal level during chicken heart development (Thakkar & Sperelakis, 1987). Their data show that the cAMP content is highest at early stages of heart development and it decreases with time. On the contrary the basal cGMP content is lowest at the early stages and it increases with time. The authors hypothesized that the modifications of basal level content of cAMP and cGMP could be due either to a decrease of adenylyl and guanylyl cyclase activity either to an increase in the phosphodiesterase activity (Thakkar & Sperelakis, 1987).

The cAMP level reduction seems to continue even after birth in the chicken heart and it was proposed that this phenomenon could be related to changes of PDE activity (Hosey & Green, 1977). Our data show that basal levels of the cAMP and cGMP hydrolyzing activity does not change in mouse developing heart at different stages ruling out the possibility that any difference of cyclic nucleotide basal level could be due to a modification of the whole capacity of the cells to degrade them.

The emerging evidences suggesting a role of PDEs in heart development makes necessary to further investigate the pattern of PDEs' expression in embryonic and fetal murine heart. Previous data obtained by semi-quantitative PCR demonstrated

that nine PDEs are expressed in mouse heart at diverse stages of development (Isidori et al, 2015). These results were confirmed and extended by qRT-PCR showing that among the evaluated PDEs family *Pde1C* and *Pde2A* presents a significant increase in the mRNA expression passing from embryonic (E14.5) to fetal (E18.5) murine heart.

PDE1 is a Ca^{2+}/calmodulin stimulated phosphodiesterase comprised of 3 genes, PDE1A, PDE1B and PDE1C able to hydrolyze both cAMP and cGMP (Chen et al, 2018). The PDE1A and 1C were reported expressed in myocytes and in prenatal mouse heart (Wang et al, 2017; Isidori et al, 2015). Recently Wang *et al* (2017) reported that genetic ablation of *Pde1a* could induce an altered cardiac phenotype in mice since these animals have a lowest blood pressure, increased heart rate and elevated ejection fraction suggesting that this isoform could play a role in the cardiac function. We found that *Pde1a* expression was not changed at mRNA level, but it was mainly expressed at protein levels in fetal mouse heart at E16.5 suggesting that it could play a major role at this stage of heart development.

About *Pde1c* it was verified an increased mRNA expression which reached the highest level at E18.5. The protein expression followed the same pattern of mRNA with statistical significance at E18.5. The three PDE1 isoforms (A, B and C) show very similar affinity for cGMP hydrolysis (Bender & Beavo, 2006). The activity assay with cGMP 1μM as the substrate, in the presence of Ca^{2+}/CaM, shows the total activity of all three PDE1 isoforms, and reveals a constant basal activity throughout all heart development. PDE1C has a higher affinity for cAMP with respect to the other two isoforms which have a highest Km for this nucleotide (Bender & Beavo, 2006).

Taking advantage of this characteristic, it was possible to measure PDE1C specific activity with respect to the other two isoforms using cAMP substrate at low concentration upon stimulation with Ca^{2+}/CaM. The enzymatic activity of the

PDE1C isoform followed the same increase observed at mRNA and protein levels reaching the highest level at E18.5. Taken together these data suggest that PDE1 may play a major role in the fetal heart than in embryonic heart. *Pde1c* mutant mice do not present any cardiac alterations in physiological conditions. The genetic ablation of its isoform was associated with cardio protection since the PDE1C inhibition or deficiency attenuated myocyte apoptosis, cardiomyocyte hypertrophy, cardiac fibrosis and loss of contractile function induced by transverse aortic constriction in mice (Knight et al, 2016).

Pde2a gene gives rise to three isoforms localized in different cellular compartments which hydrolyze cAMP and cGMP at a similar rate (Pavlaki & Nikolaev, 2018). *Pde2a* expression markedly increased at mRNA and protein levels a E18.5 accounting for the 60% of the total cGMP hydrolyzing activity of fetal heart. The progressive increase in PDE2A expression over stages of heart formation was also demonstrated by Assenza et al (2018) using embryonic and fetal wild type heart and a genetically modified *Pde2a* knockout mouse. The importance of PDE2A expression is enlightened by the presence of cardiac alterations such as the incomplete intraventricular septum, ventricular enlargement, ventricular hypertrabeculation and, no compactation of the myocardium which are evident in the heart of *Pde2a* knockout embryo before the death at E15.5 (Assenza et al, 2018). Despite other phosphodiesterases being expressed in the heart, none of them are able to compensate the role of Pde2a during heart formation.

PDE3 is encoded by 2 genes (3A and 3B) differentially expressed in cardiomyocytes with a more abundant expression of the isoform 3A (Pavlaki & Nikolaev, 2018). Oikawa et al (2014) described that the inhibition and knockdown of *Pde3a* is associated with apoptosis in neonatal myocytes, suggesting a role of this PDE in the maintenance of normal heart function (Oikawa et al, 2014). *Pde3a* mRNA and protein expression were not significantly modified during the stages of heart formation.

Its enzymatic activity accounted of almost 20% of total cAMP hydrolyzing activity and it slightly decreased along time.

Among the PDEs which specifically hydrolyses cAMP, PDE4 provides the highest hydrolytic activity in the heart (Conti, 2017). The importance of PDE4 in developing heart is suggested by the fact that it is responsible for almost 75% of the total cAMP hydrolyzing activity in prenatal heart with a slight but consistent trend to decrease. PDE4 presents the highest percentage of cAMP hydrolytic activity compared to PDE3 in embryonic and fetal heart as reported by Ritcher et al (2008) in neonatal cardiomyocytes. Together these data suggest that the PDE4 is the main responsible to cAMP hydrolysis in embryonal, fetal and post-natal heart. PDE4 is a large family of enzyme with four genes (PDE4A, 4B, 4C and 4D) and it was shown that A, B and D are expressed in the developing mouse heart (Isidori et al, 2015). Despite this three PDE4 isoforms are expressed in comparable levels in murine cardiac tissue (Ritcher et al, 2008), the pharmacological inhibition and the genetic ablation of the *Pde4a* isoform in mice did not induce any cardiac alterations in animals at 6 months of age (Conti, 2017).

The different phenotypes induced by *Pde4* isoforms deletion are probably due to the different subcellular localization and macromolecular complex interaction. Indeed, Leroy et al (2011) using *Pde4b* knockout mice observed that this isoform, differently to *Pde4a*, is a critical regulator of L-type Ca^{2+} current ($I_{ca,L)}$ during the β-AR stimulation with intensified β-AR response of $I_{ca,L}$, Ca^{2+} transients and myocytes contraction followed by increased propensity to arrhythmias.

Pde4d knockout mice showed a progressive cardiomyopathy with a loss of β-adrenoceptor signaling in cardiac myocytes and heart failure after myocardial infarction (Lehnart et al, 2005; Xiang et al, 2005). In developing mouse heart, the *Pde4d* mRNA expression was not modified over the time. At protein level, PDE4D kept similar levels at E14.5 and E16.5 with a significant decreased expression at E18.5. The decrease in the cAMP-

hydrolytic activity of PDE4 that we observed in E18 hearts is in agreement with the decrease in the levels of 4B and 4D isoforms, while the slight decrease of 4A mRNA is probably not enough to parallel the increase of the other two isoforms.

In cardiomyocytes, PDE2A and PDE5A are both involved in the degradation of the cGMP synthesized by the soluble guanylate cyclase, whereas the cGMP synthesized by the particulate guanylate cyclase is mainly hydrolyzed by PDE2A (Pavlaki & Nikolaev, 2018). *Pde5a* mRNA level was stable during cardiac development but its protein level was highest at E14.5 to further reduce its expression reaching the lowest levels at E18.5. Concomitantly with the reduction of protein level there was a reduction of PDE5A specific activity which was compensated by an increased level of PDE2 activity. Our data show that the PDE2A and PDE5A might shift of activity in order to regulate the cGMP levels during the heart formation.

The potential role of PDE5 during heart development remains to be elucidated. Most information provided in the literature about PDE5 describe their role in different types of cardiomyopathies in human and adult mouse. In addition, their pharmacological inhibition has been reported to exert a cardioprotective effect in pathological condition (Das et al, 2015).

Pde9 mRNA level did not significantly change during heart formation and the protein level of PDE9 was not detectable by western blot analysis. The PDE9 enzymatic activity was lowest at all the evaluated stages accounting of 15% of cGMP hydrolysis activity. PDE9 is specific in hydrolyzing cGMP and its upregulation is correlated with cardiac hypertrophy and heart failure (Lee et al, 2015). The *Pde9* genetic ablation as well its pharmacological inhibition was reported to be cardio protective in response to a pathological stress; knockout mice submitted to a sustained pressure overload developed less heart dysfunction and myocyte hypertrophy compared with the wild type littermates (Lee et al, 2015). The low enzymatic activity of

PDE9 in prenatal heart and the absence of a cardiac phenotype in the *Pde9* knockout mice lead us to hypothesize that this phosphodiesterase could have small impact in the cardiac function in physiological condition but possibly playing fundamental role in the induction of cardiac diseases. The substrate specificity as well as the role of each PDEs are summarized in table 1.

In conclusion, it was demonstrated that there is a modulation of the PDEs expression at different stages of heart formation which tends to keep constant the total hydrolyzing activity of the heart. Further studies are necessary to verify if dysregulation of this pattern of expression could be involved in the cardiac malformations during the intrauterine life.

Table 1. Overview of PDEs.

PDE family	Substrate /specificity	Gene	Transgenic mice
PDE1	cAMP/ cGMP Ca^{2+}/CaM stimulated	3 genes 1A, 1B, 1C	Pde1a knockout: low blood pressure, elevated injection fraction and increased heart rate [38]. Pde1c knockout: cardioprotected against cardiac hypertrophy induced by TAC [40].
PDE2	cAMP / cGMP	1 gene 2A	Pde2 knockout: cardiac defects and embryonal death at E15.5. [33].
PDE3	cAMP /GMP cGMP-inhibited	2 genes 2A and 2B	PDE3A Knockout: Decreased left ventricular pressure and increased heart rate [48]. PDE3A overexpression: decreased heart rate anti-apoptotic effect in adult cardiomyocytes [42].
PDE4	cAMP	4 genes 4A, 4B, 4C, 4D	Pde4a knockout: No cardiac phenotype [43]. Pde4b knockout: intensified β-AR response of $I_{ca,L}$, Ca^{2+} transients and myocytes contraction followed by increased propensity to arrhythmias [45]. Pde4d knockout: progressive cardiomyopathy with a loss of β-adrenoceptor signaling in cardiac myocytes and heart failure after myocardial infarction [46;47].
PDE5	cGMP	1 gene 5A	Pde5 overexpression: adverse cardiac remodeling after myocardial infarction [49].
PDE9	cGMP	1 gene 9A	Pde9 knockout: cardioprotection in response to a pathological stress. Pde9 Knockout develop less heart dysfunction and myocyte hypertrophy when submitted to a sustained pressure overload [26].

6. References

1.Sun, C; Kontadiris, MI. Physiology of cardiac development: from genetics to signaling to therapeutic strategies. Curr Opin Physiol, 2018; 1: 123-139.

2. Boer, AB; Berg, GVD; Boer, PAJ et al. Growth of the developing mouse heart: an interactive qualitative and quantitative 3D atlas. Develop Biol, 2012; 368: 203-213.

3. Ivanovitch, K; Temino, S; Torres, M. Live imaging of heart tube development in mouse reveals alternating phases of cardiac differentitation and morphogenesis. Elife, 2017; 5;6: e30668

4. Savolainen, SM; Foley, JF; Elmore, SA. Histology atlas of the developing mouse heart with emphasis on E11.5 to E18.5. Toxicol Pathol, 2009; 37: 395-414.

5. Krishnan, A; Samtani, R; Dhanantwari, P et al. A detailed comparison of mouse and human cardiac development. Pediatr Res 2014; 6: 500-507.

6. Samsa, LA; Yang B; Liu, J. Embryonic cardiac chamber maturation: trabeculation, conduction and cardiomyocytes proliferation. Am J Med Genet C Semin Med. 2013; 3: 157-168.

7. Epstein, JA; Aghajanian, H; Singh, MK. Semaphorin signaling in cardiovascular development. Cell Metab, 2015; 2: 163-173.

8. Roche, P; Czubryt, MP; Wigle, JT. Molecular mechanisms of cardiac development- (Cardiac adaptations- chapter 2) *Springer*. In: Ostadal B, Dhalla, N. (eds) Cardiac Adaptations. Advances in Biochemistry in health and disease, vol 4. *Springer*, New York, NY. 2013.

9. Azevedo, MF; Faucz, FR; Bimpaki, E et al. Clinical and molecular genetics of the phosphodiesterases (PDEs). Endocr Rev, 2014; 2:195:233.

10. Zaccolo, M. cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. Br J Pharmacol, 2009; 1: 50-60.

11. Chao, YC; Surdo, NC; Pantano, S et al. Imaging cAMP nanodomains in the heart. Biochem Soc Trans, 2019; 47: 1383-1392.

12. Boularan, C; Gales, C. Cardiac cAMP: production, hydrolysis, modulation and detection. Front Pharmacol, 2015; 6: 203.

13. Drummond, GI; Severson, DL. Cyclic nucleotides and cardiac function. Circ Res, 1979; 2: 145-153.

14. Maurice, DH; Ke, H; Ahmad, F et al. Advances targeting cyclic nucleotide phosphodiesterases. Nat Rev Drug Discov, 2014; 13: 290-314.

15. Bobin, P; Belacel-Ouari, M; Bedioune, I et al. Cyclic nucleotide phosphodiesterases in the heart and vessels: a therapeutic perspective. Arch Cardiovasc Dis, 2016; 6-7: 431-443.

16. Knight, WE; Yan, C. Cardiac cyclic nucleotide phosphodiesterase: function, regulation and therapeutic prospects. Horm Metab Res, 2012; 44: 766-775.

17. Zaccolo, M; Movsesian, MA. cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. Circ Res, 2007; 11: 1569-1578.

18. Omori, K; Kotera, J. Overview of PDEs and their regulation. Circ Res, 2007; 3:309-327.

19. Lee, DI; Kass, DA. Phosphodiesterases and cyclic cGMP regulation in heart muscle. Physiology (Bethesda), 2012; 4: 248-258.

20. Tsai, EJ; Kass, DA. Cyclic cGMP signaling in cardiovascular pathophysiology and therapeutics. Pharmacol Ther, 2009; 3:216-238.

21. Movsesian, M; Ahmad, F; Hirsch, E. Functions of PDE3 isoforms in cardiac muscle. J Cardiovasc Dev Dis, 2018; 1:10.

22. RAO, YJ; XI, L. Pivotal effects of phosphodiesterase inhibitiors on myocyte contractility and viability in normal and ischemic hearts. Acta Pharmacol Sin, 2009; 1: 1-24.

23. Fertig, B; Baillie, GS. PDE4 mediated cAMP signaling. J Cardiovasc Dev Dis, 2018; 1:8.

24. Richter, W; Xie, M; Scheitrum, G et al. Conserved expression and functions of PDE4 in rodent and human heart. Basic Res Cardiol, 2011; 106:249-262.

25. Mika, D et al. Differential regulation of cardiac excitation-contraction coupling by cAMP phosphodiesterases subtypes. Cardiovasc Res, 2013; 2: 336-346.

26. Lee, DI; Zhu, G; Sasaki, T et al. Phosphodiesterase 9A controls nitric oxide independent cGMP and hypertrophic heart disease. Nature, 2015; 7544: 472-476.

27. Korkneoz-Icoz, S; Rodovits, T; Szabó, G. Targeting phosphodiesterase 5 as therapeutic option against myocardial ischemia/reperfusion injury and for treating heart failure. Br J Pharmacol, 2018; 175: 223-231.

28. Kotera, J; Fujishige, K; Omori, K. Immunohistochemical localization of cGMP binging cGMP- specific phosphodiesterase (PDE5) in rat tissues. J Histochem cytochem, 2000; 5: 685-693.

29. Patrucco, E; Albergine, MS; Santana, LF et al. Phosphodiesterase 8A (PDE8A) regulates excitation-contraction coupling in ventricular myocytes. J Mol Cell Cardiol, 2010; 2: 330-333.

30. Horvath, A; Giatzakis, C; Tsang, K et al. cAMP specific phosphodiesterase (PDE8B) that is mutated in adrenal hyperplasia is expressed widely in human and mouse tissues: a novel PDE8B isoform in human adrenal cortex. Eur J Hum Genet, 2008;10: 1245-1253.

31. Priksz, D; Bombicz, M; Varga, B et al. Upregulation of myocardial and vascular phosphodiesterase 9A in a model of atherosclerotic cardiovascular disease. Int J Mol Sci, 2018; 10: 2882.

32.Isidori, AM; Cornacchione, M; Barbagallo, F et al. Inhibition of type 5 phosphodiesterase counteracts β2-adrenergic signaling in beating cardiomyocytes. Circ Res. 2015, 106: 408-420.

33.Assenza, MR; Barbagallo, F; Barios, F et al. Critical role of phosphodiesterase 2A in mouse congenital heart defects. Cardiovasc Res, 2018; 114: 830-845.

34. Maurice, D.H.; Palmer, D.; Tilley, D.G.; Dunkerley, H.A.; Netherton, S.J.; Raymond, D.R. et al. Cyclic nucleotide phosphodiesterase activity, expression and targeting in cells of the cardiovascular systems. Mol pharmacol. 2003, 64: 533-546.

35. Thakkar, JK; Sperelakis, N. Changes in cyclic nucleotide levels during embryonic development in chick hearts. J dev physiol. 1987, 9: 497-505.

36. Hosey, MM; Green, RD. Effects of isoproterenol on cyclic AMP and cyclic AMP dependent protein kinase in developing chick myocardium. Biochim Biophys Acta. 1977; 500: 152-161.

37. Chen, S; knight, WE.; Yan, C. Roles of PDE1 in Pathological cardiac remodeling and dysfunction. J cardiovasc dev dis. 2018, 5 (2):22.

38. Wang, X; Yamada, S; LaRiviere, WB et al. Generation and phenotypic characterization of PDE1a mutant mice. PLoS One. 2017, 12 (7): e0181087.

39. Bender, AT; Beavo, JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev. 2006, 58(3):488-520.

40. Knight, WE; Chen, S; Zhang, Y et al. PDE1C deficiency antagonizes pathological cardiac remodeling and dysfunction. PNAS. 2016, 113: 7116-7125.

41. Pavlaki, N; Nikolaev, VO. Imaging of PDE2 and PDE3 mediated cGMP-to cAMP cross-talk in cardiomyocytes. J Cardiovasc dev dis. 2018, 5 (1): 4.

42. Oikawa, M; Wu, M; Lim, S et al. Cyclic nucleotide phosphodiesterase 3A1 protects the heart against ischemia reperfusion injury. J Mol Cell Cardiol. 2014, 64: 11-19.

43. Conti, M. Subcellular targeting of PDE4 in cardiac myocytes and generation of signaling compartments. In: Nikolaev, V.; Zaccolo, M. (eds) Microdomains in the cardiovascular system. Cardiac and Vascular Biology. Springer. 2017.

44. Richter, W; Day, P; Agrawal, R et al. Signaling from B1 and B2 adrenergic receptors is defined by differential interactions with PDE4. EMBO J. 2008; 27: 383-393.

45. Leroy, J; Richter, W; Mika, D et al. Phosphodiesterase 4B in the cardiac L-type Ca2+ channel complex regulates Ca2+ current and protects against ventricular arrhythmias in mice. J Clin Invest, 2011; 121: 2651- 2661.

46. Lehnart, SE; Wehrens, XHT; Reiken, S et al. Phosphodiesterase 4d deficiency in the Ryanodine-Receptor complex promotes heart failure and arrhythmias. Cell, 2005; 123: 25-35.

47. Xiang, Y; Naro, F; Zoudilova, M et al. Phosphodiesterase 4D is required for B2 adrenoreceptor subtype-specific signaling in cardiac myocytes. PNAS. 2005, 102: 909-914.

48. Sun, B; Li, H; Shakur, Y et al. Role of phosphodiesterase type 3A and 3B in regulating platelet and cardiac function using subtype selective knockout mice. Cell signaling. 2007, 19: 1765-1771.

49. Pokreisz, P.; Vandenwijngaert, S.; Bito, V et al. Ventricular phosphodiesterase-5 expression is increased in patients with advanced heart failure and contributes to adverse ventricular remodeling after myocardial infarction in mice. *Circulation*. 2009, 119(3):408-16.

50. Das, A; Durrant, D; Salloum, FN et al. PDE5 inhibitors as therapeutics for heart disease, diabetes and cancer. Pharmacol Ther, 2015; 147:12-21.

Part II:

Role of phosphodiesterase 5 in neonatal cardiomyopathy induced by gestational diabetes

7. Introduction

7.1 Congenital heart defects

Congenital heart defects (CHD) are the most common and serious birth defects worldwide (Engineer et al, 2019). It is estimated that CHD affects approximately 1-5% of live births and are one of the major causes of birth death (Wu et al, 2020; Bruneau, 2002).

CHD can be defined as any alteration in the structure and heart function caused by disturbances in the cardiac embryogenesis process (Zaid & Brueckner, 2017).

Cardiac defects are not manifested due to a single developmental aberration but owing to an etiologically multifactorial collection of alterations correlated with genetic disorders and environmental factors which exert teratogenic effects on embryos (Moran & Robin, 2013).

CHD resulting from genetic disorders are due to mutations in the expression and function of transcription factors and proteins recognized to play a fundamental regulatory role in cardiogenesis (Figure 8) (Marín-Garcia, 2009).

Regarding the nongenetic factors, which induce environmental insults during pregnancy, there are nicotine exposure, rubella infections, preeclampsia, maternal obesity, and gestational diabetes (Figure 8) (Engineer et al, 2019).

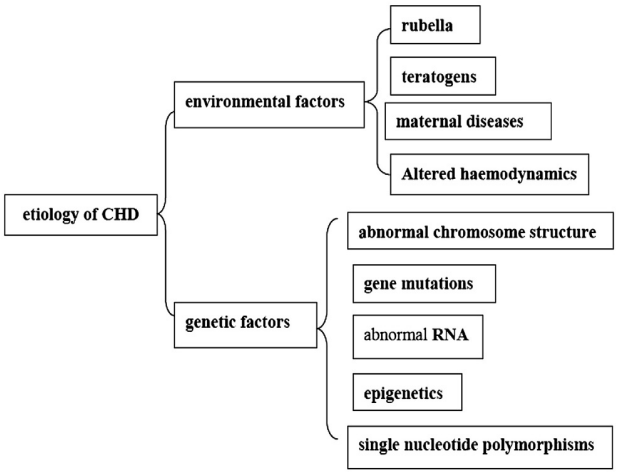


Figure 8: Etiology of congenital heart defects (CHD). (Adapted from: Jing-Bin et al. Molecular mechanisms of congenital heart disease. Cardiovascular pathology, 2010).

7.2 Gestational diabetes mellitus (GDM) definition and pathophysiology

GDM is a metabolic disorder of pregnancy characterized by impairment of glucose uptake inducing maternal hyperglycemia (Finer, S et al, 2015).

During normal pregnancy, a series of physiological modifications to supply the fetal demands are required (Plows et al, 2018). One of these, is a gradual alteration in glucose tolerance followed by insulin sensitivity (Plows et al, 2018).

This insulin sensitivity caused by pregnancy allows a reduction in the insulin dependent glucose uptake in tissues like skeletal muscle and fat serving as an adaptation to save carbohydrate to nourish the fetus (Egan et al, 2020).

Metabolic alterations in a normal pregnancy generally decrease quickly in the second and third trimester of pregnancy.

However, pregnancies diagnosed with GDM present an impaired ability of the beta pancreatic cells to respond to the increased insulin requirement over the course of gestation (Johns et al, 2018).

The inadequate insulin response results in many degrees of hyperglycemia in women without diabetes before the pregnancy (Egan et al, 2020). GDM may lead to some maternal outcomes, such as elevated risk to develop preeclampsia and type 2 diabetes mellitus after delivery (Baz et al, 2016).

7.3 Offspring outcome in response to GDM

Beyond maternal complications, it is recognized that maternal hyperglycemia may exert teratogenic effects on the fetus (Corrigan et al, 2009).

Offspring from mothers with GDM present an increased risk to develop congenital anomalies, such as macrosomia, central nervous system malformation and CHD (Ornoy et al, 2015).

Among the CHD found out in the offspring of diabetic mothers are transposition of great arteries, hypoplastic right and/or left ventricles, interrupted aortic arch, intraventricular septal defects, septum and ventricular hypertrophy and ventricular dilation (Han et al, 2015; Zhao, 2010).

These malformations in the heart occur as a result of abnormal early cardiac development (Sun & Kontaridis, 2018). Abnormalities in the processes of heart formation are closely related to alterations in expression of fetal genes and transcription factors over stages of cardiogenesis (Lister et al, 2019).

Other impacts of maternal hyperglycemia in the induction of CHD are through epigenetic modifications which regulate gene expression and signaling pathways involved in cellular processes of differentiation and development (Lister et al, 2019). However, a specific mechanism that could explain how GDM might induce heart malformations is still unclear.

7.4 Transcription factors and fetal genes involved in the heart development and CHD

The etiology of non-syndromic CHD, as cardiac malformations caused by GDM, is encoded by disruptions in the expression and function of transcription factors, such as *Tbx5*, *Tbx20*, and fetal genes such as *Gata4*, *Mef2c* and *Nkx2.5*, structural proteins (MYH6 and MYH7) and in epigenetic factors such as *Jarid2* (Figure 9) (Basu & Garg, 2018).

T-box genes are a family of transcription factors (TFs) characterized by conserved DNA binding region (Greulich et al, 2011). These TFs mediate transcriptional activation and/or repression of target genes (Greulich et al, 2011).

Six members of the T-box family (*Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, *Tbx18* and *Tbx20*) are expressed in different heart compartments with different regulatory functions during embryonic and post-natal life (McCulley & Black, 2012; Plageman & Youtzey, 2004). Together, these TFs likely contribute to chamber and non-chamber differentiation, valve formation and regulation of conduction system (Plageman & Youtzey, 2004).

Tbx5 and *Tbx20* are the key genes involved in the regulation of chamber differentiation. In non-chamber specification, the key gene is *Tbx2*, which is mainly involved in the atrioventricular septum and outflow tract formation (Plageman & Youtzey, 2004).

Gata4 is a member of the GATA family zinc finger that play a fundamental role in early cardiac development regulating cell lineage differentiation, cell survival and proliferation (Nemer & Nemer, 2010). In addition, *Gata4* is expressed in fetal and post-natal cardiomyocytes being required for the maintenance of heart function and regulation of gene expression (Mikhailov & Torrado, 2016).

Nkx2-5 is a member of the Nk family, consisting of homeodomains proteins which, through interaction with the

myocyte enhancer factor 2 (*Mef2*), have been implicated in the control of normal cardiac growth, development and regulation of the normal cardiac function (Azahi, A et al, 2006). Alterations in the expression of these transcription factors and fetal genes are associated with different types of CHD, such as cardiac conduction defects, septal defects, cardiac hypertrophy and ventricular dilation (Oka et al, 2007; McCulley & Black, 2012).

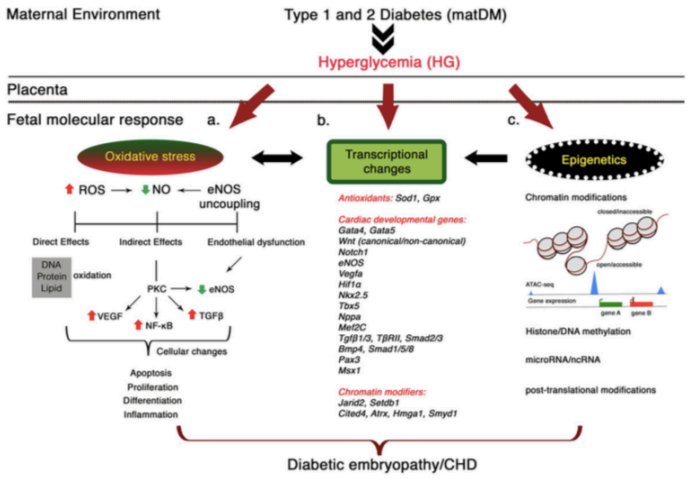


Figure 9: Fetal molecular responses to maternal hyperglycemia. (Adapted from: Basu, M.& Garg, V. Maternal hyperglycemia and fetal cardiac development: clinical impact and underlying mechanisms. Birth defects research, 2018).

7.5 PDE5 and cardiomyopathy

Most information regarding the role of PDE5 in the cardiomyopathies is provided through human patients and adult mice models (Vandenwijngart et al, 2013).

Metabolic diseases, such as Diabetes mellitus (DM), have been correlated with cardiac stress leading to diabetic cardiomyopathy (Rodovits et al, 2009). Several mechanisms are

involved in diabetic cardiomyopathy, among them, inflammatory processes, myocardial fibrosis, oxidative stress and endothelial dysfunction (Rodovits et al, 2009).

Some studies have reported that hyperglycemia reduces the bioavailability of Nitric Oxide (NO) responsible for producing cGMP due to increased levels of reactive oxygen species (Pandolfi &Filippis, 2007; Varma et al, 2012; Tsai & Kass, 2009). Together, these works demonstrated that the NO/cGMP/PDE5 signaling pathway is involved in cardiomyopathy caused by hyperglycemia.

Data from our group demonstrated that chronic inhibition of PDE5 by Sildenafil was able to improve diabetic cardiomyopathy in patients (Giannetta et al, 2012). Despite a great deal of information provided in the literature elucidating the role of Pde5 in adult cardiomyopathy and the cardioprotective effects generated through chronic inhibition of PDE, it remains unclear whether PDE5 also could play a role in CHD induced by maternal hyperglycemia

8. Aim II

Several studies have been developed with the goal to highlight the potential role of phosphodiesterase 5 in different types of cardiomyopathies. The NO/cGMP/PDE5 signaling pathway has been associated with diabetic cardiomyopathy and the inhibition of PDE5 was reported to exert a cardioprotective effect in diabetic mice and in patients.

Though the role of PDE5 in adult cardiomyopathy is widely explored remains to elucidate whether this PDE also could be associated with CHD. Disruptions in the signaling pathways involved in heart development and alteration in the expression of fetal genes and transcription factors are the principal causes of congenital heart malformation. One factor that may induce these disruptions is maternal hyperglycemia.

Gestational diabetes is associated with an increased risk of CHD in newborns. However, to our knowledge, there aren't data about the PDE5 and cardiomyopathy due to maternal hyperglycemia. Thus, the second part of my PhD was direct to investigate the potential role of PDE5 in neonatal cardiomyopathy induced by gestational diabetes.

9. Material and Methods

9.1 Experimental animals

Male and female *Pde5* heterozygous mice aged 8-12 weeks were mated to generate postnatal *Pde5*$^{+/+}$, *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ mice. The day of the vaginal plug was considered as E0.5. Diabetes was induced in pregnant *Pde5*$^{+/-}$ mouse, by intraperitoneal injections of streptozotocin for three consecutive days (75mg/kg, Sigma *aldrich*, dissolved in sodium citrate buffer 0,1M, pH4.5). Blood glucose levels were measured using a glucometer. Mice were considered diabetic with blood glucose levels >200mg/dL (11mmol/L). On the day of the birth the newborns were collected and sacrificed by decapitation. The newborns were genotyped by PCR.

9.2 Histological analysis

Neonate hearts were extracted and fixed in 4% paraformaldehyde overnight, dehydrated in ethanol and embedded in paraffin. Sections thick 5µm were stained in haematoxylin & eosin. The contra- lateral axes, the proximo distal axes length and the intraventricular septum thickness were measured on at least 20 sections for each newborn and the mean value was plotted.

9.3 qReal- time PCR

Total RNA was isolated from three samples of embryonic heart tissue using total RNA isolation micropep kit- BioRad following the manufacturer's instructions. The RNA was treated with DNase I Zymol and after reversing transcribed to synthesize cDNA through Maxima H minus reverse transcriptase (Thermo Fischer Scientific).

For real time PCR, samples were amplified in the reaction mixture with PowerUp SYBR green Master Mix (Thermo Fisher

Scientific) and specific primers for *Anp*, *Myh7*, *Gata4*, *Mef2c*, *Nkx2.5*, *Tbx2*, *Tbx5*, *Tbx20*, *Jarid2* and *Gapdh* in Thermo Fisher Scientific 7500 Real-Time PCR instrument. For quantification analysis, the comparative threshold cycle (Ct) method was used. The Ct values of each gene were normalized to the Ct value of Gapdh in the same RNA sample. The gene expression levels were evaluated by the fold change using the equation 2^{-ddCt}. The primers used in the present study were listed below.

qRT-PCR primers sequence

Anp Fw 5' AAGCTGCTGGAGCTGATAAGA 3'
Anp Rv 5' GATCCGATCCGGTCTATCTT 3'
Myh7 Fw 5' AACTGGATGGTGACACGCAT 3'
Myh7 Rv 5' TGTGGTGGTTGAAGAACTGC 3'
Gata4 Fw 5' TGACTTCTCAGAAGGCAGAG 3'
Gata4 Rv 5' TAGTCTGGCAGTTGGCACA 3'
Nkx2.5 Fw 5' CAATGCCTATGGCTACAACG 3'
Nkx2.5 Rv 5' GCCAAAGTTCACGAAGTTGC
Mef2c Fw 5' CGATGCAGACGATTCAGTAG 3'
Mef2c Rv 5' ACTGTTATGGCTGGACACTG 3'
Tbx20 Fw 5' GGCCTCCTTGCTCAATCTGAA 3'
Tbx20 Rv 5' CCACACTCTCCCTCTCAATGT 3'
Tbx5 Fw 5' CAAGGCAGGGAGGAGAATGT 3'
Tbx5 Rv 5' AGGCTCTGCTTTGCCAGTTA 3'
Tbx2 Fw 5' CGATGACTG CCGCTATAAGT 3'
Tbx2 Rv 5' GCATGGAGTTCAGGATGGTA 3'
Jarid2 Fw 5' CTGGCCTTCACTCTTCTGCA 3'
Jarid2 Rv 5' GCAGATCTGGCACCTCCTTT 3'
Gapdh Fw 5'GTGAAGGTCGGTGTGAACG 3'
Gapdh Rv 5'ATTTGATGTTAGTGGGGTCTCG3'

9.4 Neonatal cardiomyocytes culture and western blot

Neonatal mice were sacrificed by decapitation. Ventricles from newborns (P0) were digested 3 times by sequential incubation at 37°C for 3 minutes with collagenase type II pancreatin in ADS buffer (116 mmol/L NaCl, 5.4 mmol/L KCl, 5.5 mmol/L glucose, 18 mmol/L Hepes pH 7.3, 0.9 mmol/L NaH_2PO_4, 0.4 mmol/L $MgSO_4$), as previously described (Isidori et al, 2015). The enzymatic digestion was blocked by adding horse serum (HS- Gibco-Life technology, CA, USA).

Cells were harvested, resuspended in Dulbecco's modified Eagle's medium containing 10% Fetal bovine serum (FBS, Gibco-Life technology, CA, USA) penicilin and gentamicin (Sigma, CA, USA) and plated for 1 hour at 37°C to remove the contaminating fibroblasts.

Cardiomyocytes-enriched cells were re-suspended in the same medium and plated onto 35-mm collagen-coated culture for 24 hours. After 24 hours the Culture medium was changed and cardiomyocytes were maintained in DMEM low glucose that was supplemented with 1% fetal bovine serum and exposed to various concentrations of glucose and treated with Sildenafil citrate a specific inhibitor of phosphodiesterase 5 (5.5 mmol/l glucose, 5.5 mmol/l glucose + 1μM sildenafil, 33mmol/l glucose ,33mmol/l glucose + 1μM sildenafil) for 48 hours. 5.5 mmol/l glucose was used as a control.

After 48 hours, the treatment was stopped and the protein was extracted using RIPA lysis buffer 10x (EMD Milipore Corp, 20-188) supplemented with protease and phosphatase inhibitors.

The protein concentration was measured by Bradford method. Further, an amount of 40μg of protein was electrophoresed in 8-10% SDS-page gels and transferred onto nitrocellulose membrane. Further, the membrane was immersed in 5% blocking milk (TBST + 5% non-fat milk) for 1 hour at room temperature and the incubated in primary antibodies (dilution 1:500 in TBST+ 5% BSA- bovine serum albumin- buffer) for

tbx detection (Tbx2 abcam #ab33298# and Tbx20 Aviva #OAAB12587#) overnight at 4°C. After the membranes were washed in TBST and incubated in appropriated secondary antibodie (1:10000 dilution in TBST) for 1 hour at room temperature. At the end of the time, all the membranes were washed again in TBST and visualized. The relative densitometry was performed using ImageJ software.

9.5 Statistical Analysis

The data obtained were statistically analysed using the GraphPad Prism 5.0 (GraphPad software). The student t test and One way ANOVA analysis of variance with Turkey post-hoc correction was used to comparisons among the groups. A *$p \leq 0.05$ was considered to be significant; all data were presented as mean ± SEM.

10. Results

10.1 *Frequency of congenital heart malformations in offspring from mothers with gestational diabetes*

To assess the frequency and the type of congenital heart malformation, histological analysis was performed of newborns hearts from diabetic and no diabetic mothers.

The *Pde5*$^{+/+}$ newborns from diabetic mothers showed the highest heart malformation incidence since 57,2% of the animals were affected. The percentage of heart malformation was 40% and 37,5% in the *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$newborns respectively.

These data suggest that *Pde5*$^{+/+}$ newborns presented a higher susceptibility to cardiomyopathy in response to GDM compared to *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ newborns. Reduced levels of *Pde5* decrease the incidence of cardiac malformation in offspring exposed to GDM according with the genotype.

The majority of offspring exposed to hyperglycemic environment demonstrated features of ventricular dilatation. Moreover, intraventricular septum malformations were identified in two animals.

To quantify the type of congenital heart malformation in the newborns from mother with GDM the contra lateral axis, proximo-distal axis and the intraventricular septum (IVS) thickness were measured and compared to the values obtained in the heart of newborns of non-diabetic mothers.

The contra- lateral axis length of the heart of *Pde5*$^{+/+}$ neonates born from mothers with GDM was significantly increased compared to the length of heart of *Pde5*$^{+/+}$ neonates born from no diabetic mother ($p<0.05$). No difference was observed in length of the contra-lateral axis of hearts of *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ neonates from diabetic and no diabetic mothers (Figure 10).

The proximo- distal axis was measured to determine the presence of septal defects and the IVS thickness was measured

to detect significant septal hypertrophy. No difference of the length of the proximo distal axis and the IVS thickness was found among the newborns from diabetic and no diabetic mother (Figure 10). Just in one *Pde5*$^{+/+}$ and one *Pde5*$^{+/-}$ newborn feature of IVS hypertrophy was observed.

These results show that GDM affects the neonatal heart morphology inducing ventricular dilation that could be correlated with PDE5.

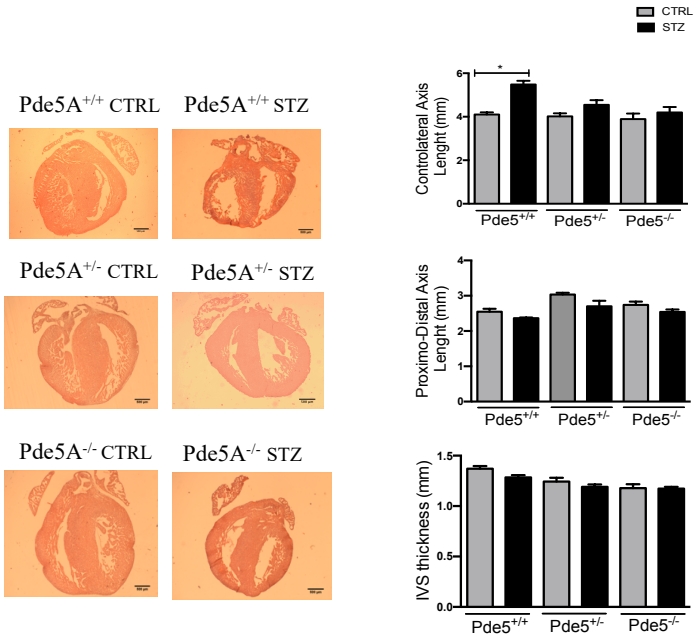


Figure 10. Representative histological staining of cardiac section with hematoxylin & eosin. The graphics represent the measure of the contra-lateral axis length, proximo-distal axis length and intraventricular septum thickness (IVS) of neonatal hearts from mothers with GD (STZ) and no GD (CTRL). (n= 4 pde5$^{+/+}$; 7 pde5$^{+/-}$; 3 pde5$^{-/-}$ neonatal heart from ctrl mothers and n= 7 pde5$^{+/+}$; 12 pde5$^{+/-}$ and 8 pde5$^{-/-}$ neonatal heart from STZ treated mothers). *Student t test* was used for statistical analysis. *p≤ 0.05. Scale bar 500µm.

10.2 *Maternal hyperglycemia increased expression of cardiac hypertrophic markers in neonatal hearts*

To quantify at molecular level the presence of cardiomyopathy, the mRNA expression of selected hypertrophic markers such as the atrial natriuretic peptide (Anp) and the myosin heavy chain 7 (Myh7) were evaluated.

Our findings show an increased *Anp* mRNA expression in *Pde5*$^{+/+}$ newborns heart from diabetic mother compared with the normal *Pde5*$^{+/+}$ newborns ($p<0.05$). Besides that, *Anp* mRNA expression significantly decreased according to genotyping. No difference in the *Anp* expression was detected in *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ newborns heart exposed to maternal hyperglycemia (Figure 11).

About *Myh7* mRNA a significant increased expression was identified in the hearts of *Pde5*$^{+/+}$ newborns exposed to maternal hyperglycemia when compared with the normal *Pde5*$^{+/+}$ newborns ($p<0.05$), but no significant alteration was observed in the *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ newborn hearts exposed to the maternal hyperglycemia (Figure 11).

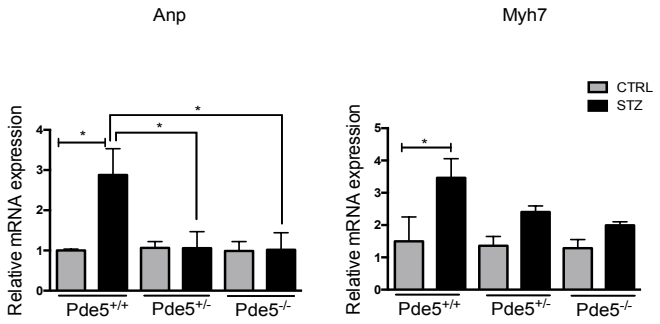


Figure 11. Maternal hyperglycemia increased mRNA expression of hypertrophic markers. qReal time PCR analysis of *Anp* and *Myh7* mRNA expression. The relative expression was normalized versus *Gapdh* gene. Data were reported as mean ± SEM from neonatal heart of offspring from mother with GDM (STZ) and no- GDM (CTRL) (n= 3 hearts for each genotype). One-way ANOVA was used for statistical analysis. *$p \leq 0.05$.

10.3 Reduced levels of PDE5 attenuates the upregulation of GATA4 and MEF2C mRNA expression in newborns heart exposed to GDM

Another characteristic of the congenital heart malformation is the altered expression of fetal genes, such as, *Gata4*, *Mef2c* and *Nkx2.5* (McCulley & Black, 2012).

To evaluate whether GDM could affect the mRNA expression of these fetal genes in newborns heart, a qRT-PCR was performed.

The *Gata4* mRNA was strongly increased ($p<0.01$) in $Pde5^{+/+}$ newborns hearts from mothers with GDM compared with the normal $Pde5^{+/+}$ newborns hearts. *Gata4* mRNA expression was not modified in the $Pde5^{+/-}$ and $Pde5^{-/-}$ newborn hearts in response to the maternal hyperglycemia. Considering the genotype, was observed that the mRNA levels of this gene decreased significantly in PDE5 dose dependent manner (Figure 12).

Mef2c mRNA expression was upregulated only in the $Pde5^{+/+}$ newborn hearts from mothers with GDM compared with the $Pde5^{+/+}$ normal mice ($p<0.05$) (Figure 12).

No statistical variation to *Nkx2.5* mRNA expression was detected in newborns hearts exposed to GDM (Figure 12).

Our data shown that maternal hyperglycemia induces upregulation of fetal genes in newborns heart. The mRNA levels of *Gata4* and *Mef2c* seem to be attenuated in relationship with reduced levels of *Pde5* in newborns heart.

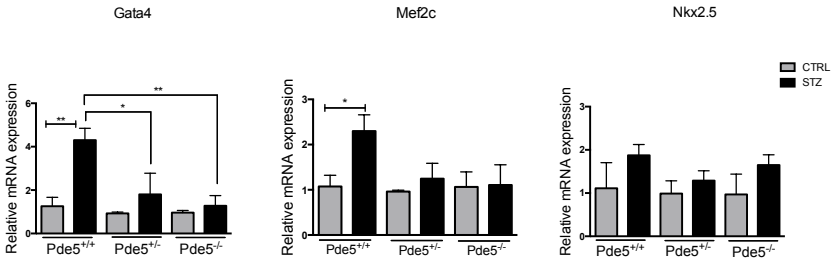


Figure 12. Maternal hyperglycemia alters the mRNA expression of fetal genes. *Gata4*, *Mef2c* and *Nkx2.5* mRNA expression was evaluated by qRT-PCR. *Gapdh* was used as housekeeping for normalization. Data were reported as mean ± SEM from neonatal heart of offspring from mother with GDM (STZ) and no- GDM (CTRL) (n= 3 hearts for each genotype). One-way ANOVA was used for statistical analysi. *p≤ 0.05; ** p≤ 0.01.

10.4 *Tbx mRNA expression decreases with the reduction of PDE5 levels in neonatal heart*

To elucidate the molecular mechanism which could be associated with the enlargement of the ventricles, it was evaluated the expression of *Tbx* genes implicated into cardiac and non- cardiac chambers formation (Singh et al, 2016; Horb & Thomsen, 1999).

In $Pde5^{+/+}$ newborn hearts *Tbx2* and *Tbx5* expression were strongly increased due to maternal hyperglycemia when compared with the expression in the normal $Pde5^{+/+}$ newborns heart (p≤ 0.001 and p≤0.05, respectively) (Figure 13). Also, a small but no significant increased expression of *Tbx5* mRNA was observed in the $Pde5^{+/-}$ and $Pde5^{-/-}$ newborn hearts in response to the maternal hyperglycemia. *Tbx20* mRNA expression shown no significant increase in the newborn's hearts exposed to GDM (Figure 13).

These data show that maternal hyperglycemia affect the *Tbx* mRNA expression in newborns heart. The disruption in the Tbx2 and Tbx5 expression might be responsible to the cardiac

malformation observed in newborns heart from mothers with GDM.

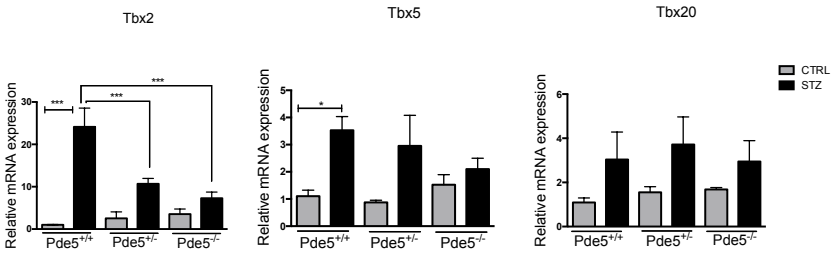


Figure 13. mRNA expression of Tbx transcription factors in neonatal heart exposed to maternal hyperglycemia. *Tbx2, Tbx5 and Tbx20* mRNA expression was evaluated by qRT-PCR. *Gapdh* was used as housekeeping for normalization. Data were reported as mean ± SEM from neonatal heart of offspring from mother with GDM (STZ) and no- GDM (CTRL) (n= 3 hearts for each genotype). One-way ANOVA was used for statistical analysis. *p≤ 0.05; ** p≤ 0.01; *** p≤ 0.001

10.5 *Inhibition of PDE5 activity might restores the protein expression of Tbx in isolated neonatal cardiomyocytes*

To verify the association of PDE5 enzymatic activity and the expression of Tbx transcription factors in hyperglycemic condition, neonatal cardiomyocytes were isolated and cultured under normal and high levels of glucose (5mM; 33mM respectively) and treated by sildenafil a specific inhibitor of PDE5 (5mM + sild; 33 mM + sild).

Analysis obtained by western blot shown that hyperglycemia increases the protein expression of Tbx2 in neonatal cardiomyocytes ($p<0.05$). On the contrary, no difference was found to Tbx20 protein expression (Figure 14).

However, a significant reduction in the Tbx2 protein expression was detected in neonatal cardiomyocytes exposed to hyperglycemia and treated with sildenafil ($p<0.05$) (Figure 14).

These data reinforce the hypothesis that PDE5 play a role in the neonatal cardiomyopathy induced by maternal

hyperglycemia altering the expression of transcription factors involved in the heart development.

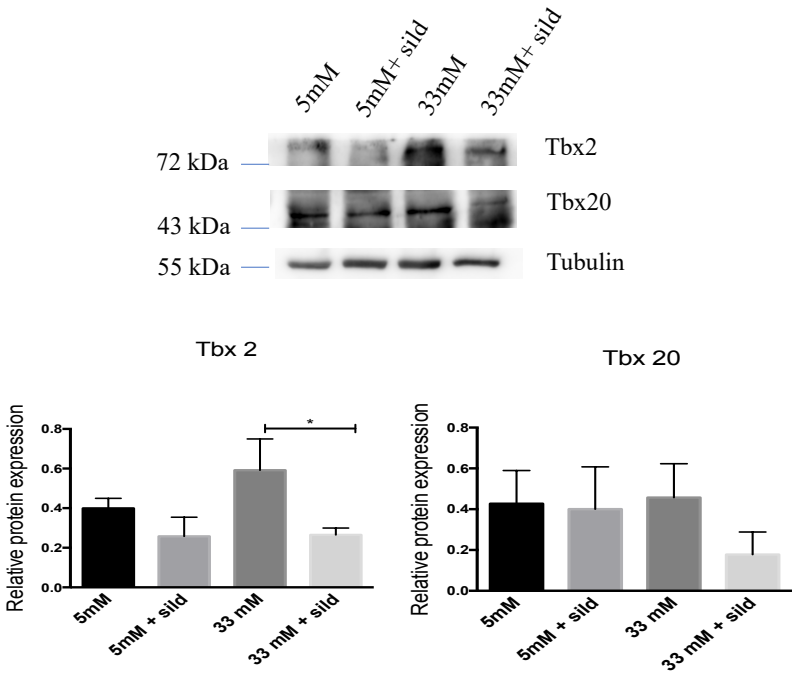


Figure 14. Tbx protein expression in isolated neonatal cardiomyocytes exposed to hyperglycemia and treated with sildenafil. Representative image of western blot analysis and relative densitometric analysis of Tbx2 and Tbx20 proteins with Tubulin level. Data represented by mean ± SEM of 3 independent experiments. One-way Anova was used for statistical analysis. *p≤ 0.05

10.6 *The Jarid2 epigenetic factor is upregulated in neonatal hearts exposed to maternal hyperglycemia*

Nitric oxide (NO) is a soluble molecule that is able to induce the production of cyclic guanosine monophosphate (cGMP) that is directly hydrolyzed by PDE5.

It has been reported that maternal hyperglycemia decreases the bioavailability of the NO (Basu et al, 2018), which has been associated with modulation of gene expression, such as *Jarid2* expression (Basu et al, 2017).

Consequently, we evaluated whether maternal hyperglycemia alters *Jarid2* expression in neonatal hearts. Our data demonstrated that mRNA expression of *Jarid2* was increased in $Pde5^{+/+}$ newborn hearts due to maternal hyperglycemia when compared with normal $Pde5^{+/+}$ newborn hearts ($p<0.05$). However, no statistical difference was found in $Pde5^{+/-}$ and $Pde5^{-/-}$ newborn hearts (Figure 15).

Our data suggests that NO/PDE5 signaling pathways could be involved in the disruption of normal expression of *Jarid2.*

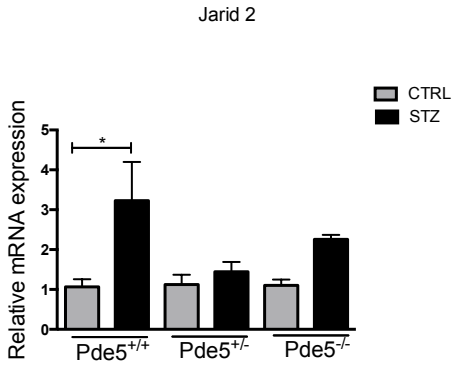


Figure 15. Maternal hyperglycemia affects the mRNA expression of *Jarid2*. The relative mRNA expression of *Jarid2* was evaluated by qRT-PCR. *Gapdh* was used as housekeeping for normalization. Data were reported as mean ± SEM from neonatal heart of offspring from mother with GDM (STZ) and no-GDM (CTRL) (n= 3 hearts for each genotype). One-way ANOVA was used for statistical analysis. *$p \leq 0.05$.

11. Discussion

This experimental study aimed to elucidate the potential role of PDE5 in neonatal cardiomyopathy induced by gestational diabetes mellitus.

GDM exerts teratogenic effects in offspring increasing approximately 5-fold the risk of congenital heart anomalies (Ma et al, 2015). Morphological analysis reveals that maternal hyperglycemia induces visible ventricular dilation in neonatal hearts. *Pde5*$^{+/+}$ newborns were more affected by the maternal hyperglycemia, with the highest percentage displaying ventricular dilation, confirmed by measuring the contra-lateral axis. The percentage of heart malformation was reduced in *Pde5*$^{+/-}$ and in *Pde5*$^{-/-}$ newborns. Similar cardiac malformation was reported by Gutierrez et al (2007). The authors demonstrated that a fetus from diabetic dams developed an obvious ventricular dilation.

Conversely, Gordon et al (2015) induced maternal hyperglycemia in the late period of gestation in rats and reported that fetuses exposed to hyperglycemia developed septal overgrowth. Results from echocardiography demonstrated that these animals had septal thickness greater than the fetus not exposed to hyperglycemia. However, in our model features of IVS hypertrophy were identified in just two animals.

Han et al (2015) evaluated the presence of cardiac hypertrophy in human fetuses as well as in mouse embryos and fetuses. Analysis from color ultrasound showed that human fetuses from mothers with diabetes presented a significant average of the intraventricular septum thickness compared to the control group. In addition, the average thickness of the posterior wall of the left ventricle (LVPW) in fetuses from diabetic mothers was greater than in the control group, demonstrating that fetuses exposed to maternal diabetes develop cardiac hypertrophy. In line with the data from human fetuses, the

authors reported similar results with mouse embryos and fetuses exposed to maternal hyperglycemia induced by streptozotocin. Fetuses at 18.5 days of development exposed to maternal hyperglycemia present significantly increased thickness of the right ventricle anterior wall (RVAW) compared to the control group; besides the IVS and the LVPW were greater than in the control group, which characterize a type of cardiac hypertrophy in fetuses from hyperglycemic mothers.

Another type of CHD in response to maternal hyperglycemia was reported by Bohuslavova et al (2013) who demonstrated that mouse embryos at 14.5 days developed incomplete interventricular septum. However, no feature of septum defect was observed in our animals.

All these results support the concept that maternal diabetes exerts teratogenic effects in a multifactorial manner that might explain the diversity in the types of CHD found in the offspring. In addition, differences in the time of exposition of offspring to uterine hyperglycemic environment could be a factor that might aggravate the morphological alterations of the heart.

Another factor that could be responsible for neonatal cardiomyopathy is PDE5. As observed, the reduction of the PDE5 levels had a potential cardioprotective effect on *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ newborns exposed to maternal hyperglycemia.

Studies in humans and in experimental mouse models have shown that PDE5/cGMP/NO signaling pathways are correlated with hypertrophic cardiomyopathy (Kass, 2012). In addition, PDE5 also has been correlated with diabetic cardiomyopathy (West et al, 2019). Inhibition of PDE5 enzymatic activity has been associated with attenuation of dilated cardiomyopathy (Lawless et al, 2019). These findings support results obtained in our experimental animal model using *Pde5*$^{+/-}$ mice.

Pde5$^{+/-}$ and *Pde5*$^{-/-}$ newborns presented less incidence in developing ventricular dilation in response to the hyperglycemia. This data points out the potential role of PDE5 in congenital heart defects.

One of the characteristics of ventricular dilation is an increased expression of certain molecular markers which are used as references for cardiac diseases. Among them, Anp and Myh7. The expression of Anp is downregulated after birth and becomes strongly expressed in adult mammalian hypertrophied hearts (Sergeeva & Christoffels, 2013). Myh7 encodes a sarcomere thick filament protein, which is more expressed during embryonic and fetal heart development, being switched with Myh6 after birth (Dirkx et al, 2013). Evaluating the mRNA expression of both markers was detected an upregulation of *Anp* and *Myh7* levels in $Pde5^{+/+}$ newborn hearts exposed to maternal hyperglycemia while no significant alteration was detected in the $Pde5^{+/-}$ and $Pde5^{-/-}$ newborns heart exposed to the same hyperglycemic environment. A recent study developed by Miyoshi et al (2019) using a knockout model of fetal heart failure demonstrated that maternal administration of tadalafil, a specific inhibitor of PDE5 enzymatic activity, was able to improve the left ventricular systolic function in fetuses with ventricular dilation. In addition, the inhibition of PDE5 activity also decreased the mRNA expression of *Anp* in these fetal hearts.

These results suggest that PDE5 might be involved in the regulation of *Anp* and *Myh7* in cardiac pathology.

It is well recognized that the cardiac defects observed in offspring exposed to GDM are linked with disruption of molecular signaling pathways that regulate the expression of fetal genes and transcription factors (Basu, 2018). Many studies have attempted to elucidate the potential mechanism involved and how maternal diabetes could affect these pathways (Kumar et al, 2007; Reinking et al, 2009; Basu et al, 2017).

Embryos exposed to maternal hyperglycemia have a significantly altered gene expression profile, especially the expression of key transcription factors (Ma et al, 2015), such as *Gata4* and *Mef2c* and *Nkx2.5*. Our results show that maternal hyperglycemia alter the mRNA expression of *Gata4* and *Mef2c*

but not *Nkx2.5* in *Pde5*$^{+/+}$ newborn hearts exposed to maternal hyperglycemia. However, Saiyin et al (2019) reported downregulation of *Gata4* mRNA expression in embryos mice exposed to pre-maternal diabetes. Meanwhile, no difference was found regarding *Nkx2.5* expression in embryo hearts. This data is in agreement with our results in newborn hearts. Maternal hyperglycemia was not able to alter the expression of this transcription factor. Regarding *Mef2c*, Bohuslavova et al (2013) found no alteration in the mRNA expression of *Mef2c* in wild type embryo hearts exposed to maternal hyperglycemia. However, the authors observed increased expression of *Mef2c* in heterozygous HIF-1 embryo hearts exposed to hyperglycemia. In our animal model, *Mef2c* was not modified in *Pde5*$^{+/-}$ and *Pde5*$^{-/-}$ newborns due to GDM.

The mRNA expression of *Tbx2*, *Tbx5,* and *Tbx20* which play an essential role during heart development and maturation were analysed. Our data shows that *Pde5*$^{+/+}$ newborns hearts exposed to hyperglycaemia presented an increased expression of *Tbx2* and *Tbx5* but no significant difference was found with *Tbx20*. Similar results were demonstrated by Liang et al (2010) in zebrafish. The authors exposed zebrafish embryos in different stages of development to high concentrations of glucose and observed by *In situ hybridization* a strong expression of *Tbx5* in the atrium and in the ventricles of wild type embryos exposed to hyperglycemia. In contrast, the expression of *Tbx20* was lower throughout the heart. Mikhailov & Torrado (2016) described that both over-and underexpression of *Tbx5* can exert equally deleterious effects on the heart. Alterations in *Tbx5* expression have been correlated with different kinds of CHD such as impaired ventricular trabeculation and ventricular abnormalities (Liberatore et al, 2000; Miyao et al, 2020). However, this is the first evidence regarding the effect of maternal hyperglycaemia in the expression of *Tbx2*.

Hyperglycaemia is also associated with increased cardiac expression of *Jarid2* (Lin et al, 2018). *Jarid2* regulates histone

methyltransferase complexes which play a critical role during heart development, as a transcriptional repressor (Basu et al, 2017). An increased expression of *Jarid2* is present in embryo hearts at 13.5 days of cardiac formation exposed to maternal hyperglycaemia (Basu et al, 2017). Similar increased expression of *Jarid2* was found in $Pde5^{+/+}$ neonatal hearts exposed to GDM but no significant difference of *Jarid2* expression was found between $Pde5^{+/-}$ and $Pde5^{-/-}$ neonatal hearts. This result suggests that the PDE5/cGMP signaling could be involved in the regulation of an epigenetic factor during heart development.

In the present study, it was demonstrated that GDM induces CHD in newborns mice. The cardiac malformation observed in the offspring might be correlated with disturbances in the expression of fetal genes and transcription factors. The incidence of CHD and alterations in the gene expression were attenuated in newborns hearts with reduced levels of PDE5. Together these results provide evidence that PDE5 might play a role in the induction of congenital heart malformations in response to the teratogenic effects caused by maternal hyperglycaemia.

13. Reference

1.Engineer, A; Saiyin, T; Greco, ER. Say NO to ROS: Their roles in embryonic heart development and pathogenesis of congenital heart defects in maternal diabetes. Antioxidants, 2019; 10: 436.

2. Wu, W; He, J; Shao, X. Incidence and mortality trend of congenital heart disease at the global, regional, and national level, 1990-2017. Medicine, 2020; 99:23.

3. Bruneau, BG. Mouse models of cardiac chamber formation and congenital heart disease. Trends Genet, 2002; 6: PS15-S20.

4. Moran, R; Robin, NH. Congenital heart defects. In: Emery and Rimoin's principles and practice of medical genetics, 2013; 1-51.

5. Zaidi, S; Brueckner, M. Genetics and genomics of congenital heart disease. Circ Res, 2017; 6: 923-940.

6. Marin-Garcia, J. Advances in Molecular genetics of congenital heart disease. Rev Esp Cardiol. 2009; 3: 242-245.

7. Finer, S; Mathews, C; Lowe, R et al. Maternal gestational diabetes is associated with genome-wide DNA methylation variation in placenta and cord blood of exposed offspring. Hum Mol Genet, 2015; 11:3021-3029.

8.Plows, JF; Stanley, JL; Baker, PN et al. The pathophysiology of gestational diabetes mellitus. Int J Mol Sci, 2018; 11:3342.

9. Egan, AM; Dow, ML; Vella, A. A review of the pathophysiology and management of diabetes in pregnancy. Mayo clin proc, 2020; 1-13.

10. Johns, E; Denison, FC; Norman, J et al. Gestational diabetes mellitus: mechanisms, treatment, and complications. Trends Endocrinol Metab, 2018; 11: 743-754.

11. Baz, B; Rivelline, JP; Gautier, JF. Gestational diabetes mellitus: definition, aetiological and clinical aspects. Eur J Endocrinol. 2016; 2:43–51.

12. Corrigan, N; Brazil, DP; McAuliffe, F. Fetal cardiac effects of maternal hyperglycemia during pregnancy. Birth Defects Res A Clin Mol Teratol, 2009; 6: 523-530.

13. Ornoy, A; Reece, EA; Pavlinkova, G et al. Effect of maternal diabetes on the embryo, fetus, and children: Congenital anomalies, genetic and epigenetic changes and developmental outcomes. Birth Defects Res, 2015; 105 81), 53-72.

14. Han, SS; Wang, G; Jin, Y et al. Investigating the mechanism of hyperglycemia induced fetal cardiac hypertrophy, PLoS One 2015; 9: e0139141.

15. Zhao, Z. Cardiac malformations and alteration of TGFB signaling system in diabetic embryopathy. Birth Defects Res, 2010; 89: 97-105.

16. Sun, C; Kontadiris, MI. Physiology of cardiac development: from genetics to signaling to therapeutic strategies. Curr Opin Physiol, 2018;1:123-139.

17. Lister, R; Chamberlain, A; Einstein, F et al. Intrauterine programming of diabetes induced cardiac embryopathy. Diabetes Obes Int J, 2019, 3:202.

18. Basu, M.& Garg, V. Maternal hyperglycemia and fetal cardiac development: clinical impact and underlying mechanisms. Birth Defects Res, 2018; 20:1504-1516.

19. Greulich, F; Rudat, C; Kispert, A. Mechanism of T-box gene function in the developing heart. Cardiovasc Res, 2011; 2:212-222.

20. McCulley, DJ; Black, BL. Transcription factor pathways and congenital heart disease. Curr Top Dev Biol, 2012; 100: 253-277.

21. Plageman, TF; Yutzey, KE. T-box genes and heart development: Putting the T in heart. Dev Dyn, 2005; 1:11-20.

22. Nemer, G; Nemer, M. Gata4 in heart development and disease. Heart Dev Regen, 2010; 599-616.

23. Mikhailov, AT; Torrado, M. Myocardial transcription factors in diastolic dysfunction: clues for model systems and disease. Heart Fail Rev, 2016; 6: 783-794.

24. Azahie, A; Fineman, JR; He,Y. Myocardial transcription factors are modulated during pathologic cardiac hypertrophy in vivo. J Thorac Cardiovasc Surg, 2006; 6:1262-1271.

25. Vandenwijngart, S; Pokreisz, P; Hermans, H et al. Increased cardiac myocyte PDE5 levels in human and murine pressure overload hypertrophy contribute to adverse cardiac remodeling. PLosOne, 2013; 8 (3): e58841.

26. Rodovits, T; Bomicke, T; Kokeny, G et al. The phosphodiesterase- 5 inhibitor vardenafil improves cardiovascular dysfunction in experimental diabetes mellitus. Br J Pharmacol, 2009; 6: 909-919.

27. Pandolfi, A, Filippis, EA. Chronic hyperglycemia and nitric oxide biovailability play a pivotal role in pro-atherogenic vascular modifications. Genes Nutr, 2007; 2:195-208.

28. Varma, A; Das, Anindita, Hoke, NN et al. Anti-inflammatory and cardioprotective effects of tadalafil in diabetic mice. PlosOne, 2012; 9: e45243.

29. Tsai, EJ; Kass, DA. Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. Pharmacol Ther, 2009; 122: 216-238.

30. Giannetta, E; Isidori, AM; Galea, N et al. Chronic inhibition of cGMP phosphodiesterase 5A improves diabetic cardiomyopathy. Circulation. 2012; 19: 2323-2333.

31. Singh, MK; Christoffels, VM; Dias, JM et al. Tbx20 is essential for cardiac chamber differentitation and repression of tbx2. Mol Cell Biochem, 2016; 414:129-136.

32. Horb, ME & Thomsem, GH. Tbx5 is essential for heart development. Development, 199; 126: 1739-1751.

33. Isidori, AM; Cornacchione, M; Barbagallo, F et al. Inhibition of type 5 phosphodiesterase counteracts β2-adrenergic signaling in beating cardiomyocytes. Cardiovasc Res. 2015; 3: 408-420.

34. Ma, RCW; Tutino, GE; Lillycrop, KA et al. Maternal diabetes, gestational diabetes and the role of epigenetics in their long-term effects on offspring. Prog Biophys Mol Biol, 2015; 1-2: 55-68.

35. Gutierrez, JC; Hrubec, TC; Prater, MR et al. Aortic and ventricular dilation and myocardial reduction in gestation day 17 ICR mouse fetuses of diabetic mothers. Birth Defects Res, 2007;6:459-464.

36. Gordon, EE; Reiking, BE; Hu, S et al. Maternal hyperglycemia directly and rapidly induces cardiac septal overgrowth in fetal rats. J Diabetes Res, 2015: 479565.

37. Bohuslavova, R; Skvorova, L; Sedmera, D et al. Increased susceptibility of HIF-1 heterozygous- null mice to cardiovascular malformations associated with maternal diabetes. J Mol Cell Cardiol, 2013; 60:129-141.

38. West, TM; Wang, Q; Deng, B et al. Phosphodiesterase 5 associates with β2 Adrenergic Receptor to Modulate Cardiac Function in Type 2 Diabetic Hearts. J Am Heart Assoc, 2019;15:e012273.

39. Lawless, M; Caldwell, JL; Radcliffe, EJ et al. Phosphodiesterase 5 inhibition improves contractile function and restores transverse tubule loss and catecholamine responsiveness in heart failure. Sci Rep, 2009; 9:6801.

40. Segeeva, IA; Christoffles, VM. Regulation of expression of atrial and brain natriuretic peptide, biomarkers for heart development and disease. Biochim Biophys Acta, 2013; 12: 2403–2413.

41. Dirkx, E; Martins, PAC; Windt, LJ. Regulation of fetal gene expression in heart failure. Biochim Biophys Acta, 2013; 2414-2424.

42. Miyoshi, T; Hisamitsu, T; Ishibashi-Ueda, H et al. Maternal administration of tadalafil improves fetal ventricular systolic function in a *Hey2* knockout mouse model of fetal heart failure. Int J Cardiol, 2020; 302:110-116.

43. Reinking, BE; Wedemeyer, EW; Weiss, RM et al. Cardiomyopathy in offspring of diabetic rats is associated with activation of the MAPK and apoptotic pathways. Cardiovasc Diabetol, 2009; 8:43.

44. Basu, M; Zhu, JY; LaHaye, S et al. epigenetic mechanisms underlying maternal diabetes-associated risk of congenital heart disease. JCI insight, 2017; 20: e95085.

45. Lin, N; Cai, Y; Zhang, L et al. Identification of key genes associated with congenital heart defects in embryos of diabetic mice. Mol med rep, 2017; 17:3697-3707.

46. Saiyin, T; Engineer, A; Greco, ER et al. Maternal voluntary exercise mitigates oxidative stress and incidence of congenital heart defects in pre-gestational diabetes. J Cell Mol Med 2019;8: 5553–5565.

47. Liang, J; Gui, Y; Wang, W et al. Elevated glucose induces congenital heart defects by altering the expression of tbx5, tbx20 and has 2 in developing zebrafish embryos. Birth Defects Res, 2010; 480-486.

48. Liberatore, CM; Searcy-Schrick, RD; Yutzey, KE. Ventricular expression of tbx5 inhibits normal heart chamber development. Develop Bio, 2000; 169-180.

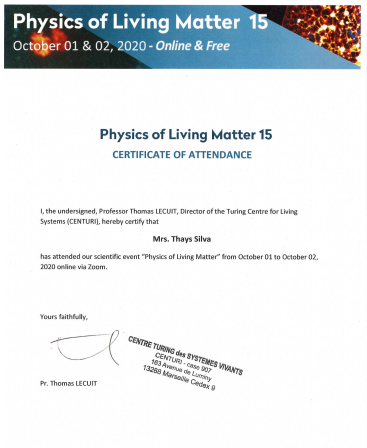
49. Miyao, N; Hata, Y; Izumi, H et al. TBX5 R264K acts as a modifier to develop dilated cardiomyopathy in mice independently of T-box pathway. PlosOne, 2020; 4: e0227393.

14. List of Congress, Symposium, Meetings and Publications

1. 2 Symposium “Fragment Screening in Drug Discovery”, as part of the 49th Annual SBBq Virtual Meeting activities, October 1, 2020.

2. Physics of living matter 15, Virtual Meeting October 01 & 02,

3. La percezione pubblica della scienza: I giovani ricercatore di fronti a temi scientifici di forte interesse pubblico, politico e mediatico. 27 e 28 aprile 2020. Online.



Activities developed in the third year of PhD

In the third year of PhD, I focused on developing the last experiments to answer and achieve the goals proposed in my research.

As an experimental approach, I evaluated the enzymatic activity, the protein, and mRNA expression of phosphodiesterases which were not evaluated in the first and second year. Besides that, I also performed a molecular analysis of mice neonatal hearts exposed to maternal hyperglycemia. In addition, in vitro experiments with isolated neonatal cardiomyocytes under hyperglycemic condition was performed.

As activities involved in my formation, I followed the cycle of seminaries proposed by the PhD school. Besides, I followed the symposium online as formative activities that could improve my formation as PhD student.

PhD Seminars

12-02-20 Dissecting the interplay between endothelin receptor and B1 integrin in invadopodia function and metastatic process of ovarian cancer. Phd Ilinia Massi.
12-02-20 Modellizzazione neurofisiologica dello stato cognitive di lavoratori europei in età avanzata. Phd Vincenzo Ronca
19-02-20 Ruolo degli Rna non codificanti nel controllo post-trascrizionale dell'espressione genica nel tumore alla mammella triplo negativo. PhD Gabriella Esposito
19-02-20 Dissecting the role of novel onco miRs and their molecular targets in the evolution of resistance to target therapies in melanoma. PhD Vittorio Castaldo

26-02-20 Role of N6-methyladenosine in skeletal muscle regeneration PhD Francesco Millozzi
03-04-20 Retinoic acid, proteasome inhibition and oxidative stress as a new combined strategy to induce myeloid leukemic progenitor cell death PhD Francesca Liccardo
03-04-20 Role of circRNAs-m6A dependent during stress response in myeloid leukemic cells
28-04-20 The effects of extracellular vesicles on ALS ex vivo and in vivo models: development of innovative functional measurement technique and testing tools PhD Flavia Forconi
06-05-20 The cellular mechanotransduction: from mechanical stimuli to cellular Young's modulus measurement PhD Serrena Carraro
06-05-20 Gene therapy for ataxia-telangiectasia syndrome PhD Bruna Sabino
13-05-20 Control of macrophage programming in the tumor microenvironment: interplay between TPC2/ calcium signaling and autophagy PhD Samantha Barbonari
13-05-20 Toxicogenomic effects of benzo(a)pyrene on human adult testis PhD Irene Tartarelli
22-05-20 Non- invasive analysis of the embryonic genome for the development of improved preimplantation genetic testing (PGT) protocols PhD Antonio Capalbo
03-06-20 Kinematic study on ALS Phd Marco Ceccanti
03-06-20 The activation of miR-125a-5p/IP6K1 axis upon myo-inositol administration: a potential novel target for breast cancer therapy PhD Mirko Minini
10-06-20 A novel 3D culture system as an in vitro model to study muscle biology and disease PhD Mariana Consentino
10-06-20 Hormonal involvement and new drugs delivery system against melanoma disease PhD Giada Pontecorvi
17-06-20 Role of DACH1 in prostate cancer and its involvement in radioresistance PhD Ilinia Giordani
17-06-20 Molecular and cellular networks driving neurogenic muscle atrophy PhD Daisy Proiety

24-06-20 Anti- tumor effect of oleic acid in hepatocellular carcinoma cell lines via autophagy reduction PhD Federico Giulitti
24-06-20 Role of muscarinic receptors in ephitelial ovarian carcinoma (EOC) PhD Marilena Taggi
01-07-20 Molecular characterization of ALS pathogenic mechanisms PhD Elisa Lepore
08-07-20 Role of STAT3 signaling in control of muscle regeneration, growth and autophagy during age-related degenerative neuromuscular diseases PhD Giorgia Catarinella
08-07-20 Oxytocin as a physiological anti-cachectic agent PhD Alexandra Benoni
15-07-20 The interplay between metabolic alterations and circadian clock PhD Irene Casola
22-05-20 Charaterization of non-human primate spermatogonal compartment PhD Chiara Caponi
22-05-20 Analysis of radioresistance mechanisms in a model of bone metastasis from prostate cancer PhD Silvia Sideri
29-07-20 C-met- activated signalling pathway promotes malignant behaviour of NT2D1 non-seminoma cell line PhD Luisa Gesualdi
15-09-20 Brain K tomography PhD Marco Bertiol
15-09-20 Brain segmentation in preclinical and clinical MRI PhD Ricardo di Feo
23-09-20 Targeting PKC theta to ameliorate dystrophic heart fibrosis in a novel model mdx of accelerated pathology PhD Jacopo Morroni
23-09-20 Epigenetic role of nuclear microRNAs in normal and neoplastic hematopoiesis PhD Martina Gentile
25-09-20 Role of phosphodiesterase type 5 in the induction of cardiac hypertrophy PhD Ana Gabriela Rego
07-10-20 Neurophysiological models to predict the mental state of users in industrial context PhD Alessa Vozzi

CONFIDENTIALITY NOTICE

Reviewers and PhD committee members are obliged to keep the files confidential and to delete all records after completing the review process.

Il ricevimento degli elaborati scientifici, per l'ottenimento del titolo di Dottore di Ricerca, in qualità di Membro del Collegio dei Docenti del Dottorato in Morfogenesi ed Ingegneria Tissutale richiede di osservare le seguenti normative:

i. considerare le Informazioni confidenziali e riservate come strettamente private e ad adottare tutte le ragionevoli misure finalizzate a mantenerle tali;

ii. utilizzare le Informazioni confidenziali e riservate unicamente allo scopo per le quali sono state fornite o rese note, impegnandosi a non divulgarle a soggetti terzi le informazioni contenute negli elaborati ricevuti;

iii. a garantire la massima riservatezza, anche in osservanza alla vigente normativa in materia di marchi, di copyright e di brevetti per invenzioni industriali e in base alla normativa sulla privacy, ai sensi del D.Lgs. 196/2003, riguardo il know-how e tutte le informazioni acquisite, che non potranno in alcun modo, in alcun caso e per alcuna ragione essere utilizzate a proprio o altrui profitto e/o essere divulgate e/o riprodotte o comunque rese note a soggetti terzi.

Reviewer: Meacci Elisabetta

To whom it may concern I evaluated the thesis entitled "Phosphodiesterases expression during murine cardiac development and Role of phosphodiesterase 5 in neonatal cardiomyopathy induced by gestational diabetes" of THAYS MARIA SILVA CARVALHO, PhD student of the Doctoral Course in MORFOGENESI E INGEGNERIA TISSUTALE at Sapienza University of Rome. Following are my comments to the PhD study The thesis project is based on the knowledge of the fundamental role played by the signals triggered by cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in cardiac functions. In particular, since few data are available on phosphodiesterase expression in cardiac development, the study, focusing on the characterization of the expression pattern of PDEs during heart formation, leads to interesting conclusions on the different role of PDE2A and PDE5A expression/activity that shift in order to regulate the cGMP levels. In the second part of the thesis, the potential involvement of the PDE5 isoform in the cardiomyopathy due to maternal hyperglycemia has been investigated. The reported data provide convincing evidence that PDE5 might mediate the appearance of congenital heart malformations in response to the teratogenic effects caused by the maternal hyperglycemia. Each part of the thesis, divided in paragraphs state of art, methodology, results, discussion is well written and the conclusions supported by the data, which are discussed taking into consideration previous studies in the lab and the literature. Even if the methodological approaches are not very innovative, multidisciplinary experimental techniques have been used, data are well described, allowing to have a wide vision of the potential role of PDEs in murine cardiac development. The current version of the thesis is well presented and adequate for admission of the candidate to the defense of the work in front of a national evaluation board. However, this referee suggest minor

revisions to the candidate in order to improve the document before the final submission.

Reviewer comment 1. The comparison between the biochemical properties of the different isoforms in terms of substrate specificity and regulation is quite complicated. Therefore, this referee suggests summarizing this information in a table in order to better describe the relevance of each isoform in heart development.

PhD student: First of all, I am grateful for the positive and constructive comments from the review process.
As required by the reviewer the PDEs information was summarized in a table. Table 1 was added in the final of the discussion regarding part I of the present thesis. Besides, was added two new references.
References 48 and 49.

Reviewer comment 2. The expression profile (either at mRNA and protein level) of the known PDE isoforms at different stages of cardiac development (from 14.5 to 18.5 days) shows a potential role of PDE1C, PDE2 and PDE5 in heart formation. This approach is appropriate but it should be better discussed.

PhD student: The discussion developed about the role of Pde1c, Pde2a, and Pde5a during heart formation was developed based on data reported in the present thesis and taking into consideration the information provided in the literature. However, to achieve a better discussion as highlighted by the reviewer some additional details were written about the PDEs described above.

Reviewer comment 3. In the second part of the thesis, the comparison between transgenic (PDE5+/- , +/+ and -/-) pregnant mice injected with streptozotocin to promote diabetes

convince the reviewer that the reduction of the PDE5 levels had a potential cardioprotective effect on Pde5+/- and Pde5-/- newborns exposed to maternal hyperglycemia. Unfortunately, the effect of specific PDE5 inhibitor in primary culture cell shas not been evaluated on other genes othen than Tbx.

PhD student: I agree with the reviewer regarding the fact that just the Tbx protein expression was evaluated under the sildenafil treatment. This point is one limitation of the present study. This limitation will be raised with future investigations using the sildenafil treatment in isolated neonatal cardiomyocytes.

Reviewer comment 4. This reviewer suggest to update the literature and to revise the text in order to eliminate word repetitions (i.e AIM II) and spelling mistakes. Please re-formulate the title of paragraphs in order to better specify the content (i.e the 10.3 and 10.4 taking into consideration the effects of PDE5 expression on fetal genes and Tbx).

PhD student: The text was revised and some of the literature references were updated as required, and the spelling mistakes were corrected.

References added.

Ref. number 2. Wu, W; He, J; Shao, X. Incidence and mortality trend of congenital heart disease at the global, regional, and national level, 1990-2017. Medicine, 2020; 99:23.

Ref. number 14. Ornoy, A; Reece, EA; Pavlinkova, G et al. Effect of maternal diabetes on the embryo, fetus, and children: Congenital anomalies, genetic and epigenetic changes and developmental outcomes. Birth Defects Res, 2015; 105 81), 53-72.

The title of paragraphs 10.3 and 10.4 were re-formulated according to the suggestion made by the reviewer.

10.3 *Maternal hyperglycemia induced re-expression of cardiac fetal genes in neonatal hearts*

Re-formulated:
10.3 Reduced levels of PDE5 attenuates the upregulation of GATA4 and MEF2C mRNA expression in newborns heart exposed to GDM

10.4 *Tbx mRNA expression was affected by maternal hyperglycemia*

Re-formulated:
10.4 Tbx mRNA expression decreases with the reduction of PDE5 levels in neonatal heart

Reviewer: Gomes Dayane Aparecida

Report The thesis presents clear objectives, with well-designed experiments. The results are promising using this knockout model for PDE5.

I am grateful for Prof. Dayane Gomes appreciating the PhD thesis.

Licenza