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Gene Expression Analysis in Cardiac and Adipose Derived Fibroblasts: potential influence of origin and *in vitro* culture

Dissertation for a Master's Degree in Biomedical Engineering

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ABSTRACT

Cardiac fibroblasts represent one major cell population in the heart, and are responsible for many important cardiac functions. They also arise from different cardiac lineages, suggesting that they are primed to transdifferentiation, and may be directly involved in cardiogenesis.

The objective of this study was to achieve a detailed analysis of the transcriptional activity of human cardiac fibroblasts, derived from right and left atrium, assuming a possible contribution of the fibroblast population in cardiac development.

Cardiac samples were collected, both from adult patients, suffering from coronary and valvular heart disease, and from infant patients suffering from congenital heart disease. Control fibroblasts from adipose tissue, of the same patient, were cultured as a reference. Gene expression was evaluated by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), and subsequently subjected to data analysis. The gene expression was compared between cardiac, and adipose-derived fibroblasts. In addition, the results were analysed according to age, and different cell passages.

Gene expression comparison between atrial cardiac fibroblasts (CF) and their respective adipose tissue fibroblasts (AF), show that certain cardiac transcription factors, such as *GATA5,* are only expressed in CFs and absent in AFs. Only in children, the same occurs for *GATA4* and *WT1*. However, typical fibroblast markers like *CD90* or *DDR2*, seem to have a higher expression level in AFs. Age-dependent effects are small if any. Passage 0 (P0) fibroblasts, usually show higher gene expression levels than passage 2 (P2) fibroblasts.

Interestingly, *in vitro* cell culture appears to affect gene expression. Therefore, it is important to use unbiased approaches for gene expression analysis such as directly use *ex vivo* purified cardiac fibroblasts. Further gene expression studies, focused on diagnoses, may contribute to gene interference in cell culture differences. The eventual possibility of using fibroblasts in cardiac regeneration strongly encourages further research in this area.

Keywords

Fibroblasts; Cardiac; Transcription factors; Gene Expression; Heart Disease

RESUMO

Os fibroblastos cardíacos são a principal população de células no coração, e são responsáveis por muitas funções cardíacas importantes. Também são provenientes de linhagens cardíacas diferentes, sugerindo que estão predispostos para a transdiferenciação e podem estar diretamente envolvidos na cardiogénese.

O objectivo deste estudo foi realizar uma análise detalhada da actividade transcripcional dos fibroblastos cardíacos humanos, provenientes da aurícula direita e esquerda, assumindo uma possível contribuição da população de fibroblastos no desenvolvimento cardíaco.

Foram recolhidas amostras cardíacas, provenientes de doentes adultos, com patologia de doença coronária e valvular, e de crianças com patologias cardíacas congénitas. Tendo sido usados como referência, fibroblastos de controle de tecido adiposo do mesmo doente. Relativamente à metodologia usada, após os testes de reacção de transcriptase reversa RT-qPCR (*quantitative Reverse Transcriptase Polymerase Chain Reaction*), procedeu-se a uma análise de dados. Finalmente, foi efectuada uma comparação do padrão de expressão entre fibroblastos cardíacos (CF) e de tecido adiposo (AF). Os resultados foram comparados tendo em conta os grupos etários, e as diferentes passagens.

Em relação ao perfil de análise de expressão genética dos CF de crianças e adultos, em comparação com os respectivos AF, os resultados mostram que certos factores de transcrição, como o *GATA5,* é apenas expresso em CFs e não nos AFs. O mesmo acontece apenas em amostras de crianças para *GATA4* e *WT1*. No entanto, os marcadores de fibroblastos típicos, como *CD90* ou *DDR2*, parecem ter um nível maior expressão nos AFs. Os efeitos dependentes da idade são baixos, não sendo significativos. Os fibroblastos da passagem 0 (P0), mostram geralmente níveis de expressão mais elevadas do que os da passagem 2 (P2).

Curiosamente, a cultura *in vitro* parece afectar a expressão genética, pelo que, futuramente, a análise da expressão dos genes, deverá ser efectuada *ex vivo,* usando directamente, fibroblastos cardíacos purificados. Estudos futuros de expressão genética, focados nos diagnósticos, poderão contribuir para uma melhor compreensão da interferência genética na cultura de células. A eventual possibilidade do uso de fibroblastos na regeneração cardíaca, incita a uma maior investigação nesta área.

Palavras-Chave

Fibroblastos; Cardíaco; Factores de Transcrição; Expressão Genética; Doença Cardíaca

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LIST OF ABBREVIATIONS

ACVB	Aorto-coronary vein bypass
AF	adipose derived fibroblasts
aq. bidest	double distilled water
ASD	Atrial Septal Defect
ASDII	Atrial Septal Defect Secundum
AVR	Aortic valve replacement
CAVSD	Complete Atrium Ventricle Septum Defect
CD105	Cluster of Differentiation 105
CD90	Cluster of Differentiation 90
cDNA	complementary DNA
CF	cardiac fibroblasts
CHD	Congenital Heart Disease
CoA	Coarctation of the aorta
DDR2	Discoidin domain-containing receptor 2
DMEM	Dulbecco´s Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	deoxyribose nucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
EMT	Epithelial-to-mesenchymal transformation
EPDC	Epicardium-derived cell
FCS	Fetal Calf Serum
GATA4	GATA-binding protein 4
GATA5	GATA-binding protein 5
GATA6	GATA-binding protein 6
HBSS	Hank’s balanced saline solution

HKG	House Keeping Gene
HLHS	Hypoplastic left heart syndrome
ICC	Immunocytochemical staining
IHC	Immunohistochemical staining
M-MLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MVR	Mitral valve repair
P	Passage
PBS	Phosphate buffered saline
PDGRFA	Platelet Derived Growth Factor Alpha
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
POSTN	Periostin
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
TBE	Tris/Borate/EDTA
TBX18	T-box Transcription factor 18
TBX20	T-box Transcription factor 20
TBX5	T-box Transcription factor 5
TCF21	Transcription factor 21
VIM	Vimentin
WT1	Wilms' Tumor 1

1. INTRODUCTION

The cardiovascular system is one of the most important systems in the human body, as it is essential for the distribution of nutrients and oxygen to all cells of the body. During embryonic development, the heart is the first organ to develop. Cardiogenesis is a complex, well-orchestrated series of processes, including specification, proliferation, migration, and differentiation (1). Accordingly,, cardiac development is exquisitely sensitive, as any perturbation in the cells involved in this process leads to cardiac malformations. These frequently result in the death of the embryo (2). If, instead, the child is born, congenital abnormalities in cardiac growth, contractility and vascularity, will affect the child wellbeing, leading to an early life filled with medical interventions and a poor quality of life. If the malformations are too extreme death will occur (3).

1.1 Heart Development

Heart development has been considerably studied in mice, towards understandingt this process, to determine the biological pathways and responsible genes involved, as well as lineage tracing (1).

Cardiac development starts with the formation of the precardiac mesoderm. At embryonic day 6.5 (E6.5), gastrulation takes place and mesoderm cells migrate anteriolaterally to form bilaterally paired heart fields. At E7.75 the two fields meet, forming the cardiac crescent, also denominated as the first heart field (1). In this location, primary cardiac progenitor cells will contribute to left ventricle and atria (3). The second heart field is derived from the pharyngeal mesoderm, and contributes primarily to the right ventricle and outflow tract (4). They also contribute a majority of cells to the atria (5). Both halves of the cardiac crescent migrate to build the linear heart tube by E8. By E9.5, the heart looping occurs, in which the primitive heart tube loops asymmetrically. This rightward looping, and differential growth, brings the primitive heart chambers into alignment before their future integration. This process ends, when the main regional divisions of the mature heart and primordium of the great arterial trunks become established preceding septation, by E10.5 (1). The four-chambered heart starts to form, beginning with neural crest cells migrating into the heart. Resulting from a derivation of cells from cardiac muscle cells, the development of the conduction system follows. Cardiac chamber is formed (atria and ventricles), and septa construction occurs, a process in which atrial and ventricular septum are generated and organized, to separate these four enclosed cavities within the heart (6). Along with smooth muscle cells, the venous and arterial vasculature is modulated. Finally, valves and endocardium, originated by endothelial cells, are formed (3).

Examples of transcription factors, expressed by primary cardiac progenitor cells, are GATA Binding Protein 4/5/6 (GATA4/5/6), and T-box transcription factor 5/20 (TBX5/20) will be studied in this project in further detail.

The GATA family factors constitute a family of zinc finger DNA binding proteins that control the development of diverse tissue-specific profiles by activating or repressing transcription (7) (8). These transcriptional factors control gene expression and differentiation in a variety of cell types, along with cellular maturation; proliferation and survival (9) (10).

GATA4 encodes a transcriptional activator that plays a key role in cardiac development. It is expressed in adult heart and during embryonic development it is expressed from E7.5 (formation of first heart field) until E10.5 (when the four-chambered heart is formed) (11). *GATA5* is required during cardiovascular development and is known for playing an important role in smooth muscle cell diversity. Regarding *GATA6*, it is expressed during early embryogenesis and localizes to endo- and mesodermally derived cells during later embryogenesis, around E13.5 (12) (13) (14). Other than the heart it also plays an important role in gut and lung development. Highest levels of expression were seen in smooth muscle cells of the aorta and pulmonary artery, but also in atrial and ventricular myocardium (12).

TBX5 plays a role in heart development and specification of limb identity. It encodes a DNA-binding protein that regulates the transcription of several genes and is involved in heart development and limb pattern formation. *TBX5* is expressed in the embryonic heart from E8, when the cardiac crescent migrates to form the linear heart tube, to E10.5, when the four-chambered heart is formed (7). *TBX20* is present in the cardiac progenitor differentiation pathway and heart development. It plays a role in endoderm formation and branching involved in blood vessel morphogenesis (15) (16).

These five transcription factors act interactively and collaboratively in cardiac development. This regulatory network of transcription factors controls the responses to intrinsic, and extrinsic inductive factors, which establish patterns, and guide morphogenesis (17).

1.2 Fibroblasts

Fibroblasts are typically identified by their spindle-shaped flattened morphology and ability to adhere to culture plates, as seen in figure 1.1. Fibroblasts synthesize most of the extra cellular matrix (ECM) of connective tissue (fibrillar collagens and fibronectin). Their nuclei are large and euchromatic and, possess prominent nucleoli. Fibroblasts are characterized as being nonvascular, non-epithelial, and non-inflammatory cells (18). Other than their main function, structural, these cells are also known for their part in inflammation regulation, and wound healing (19) (20).

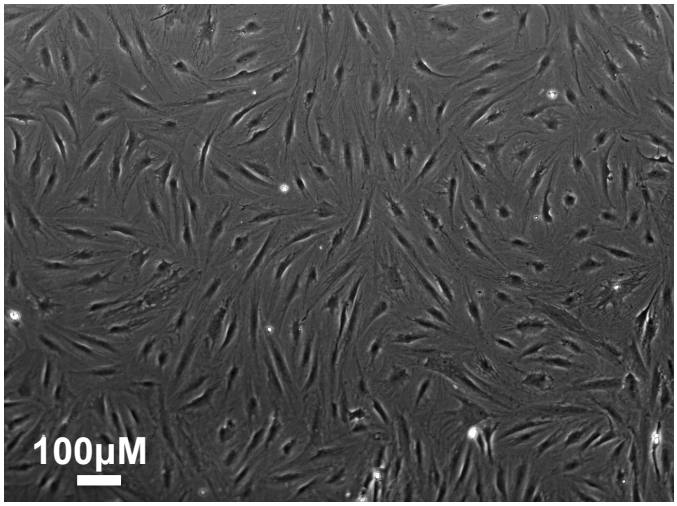

Figure 1.1 - Photograph of AF at P0 from a child after 4 days in culture, with 10x. The image was not edited.

As a heterogenous population, fibroblasts are widely distributed in numerous tissues of the vertebrate organism, being associated with various forms of connective tissue and repairing function. However, fibroblasts from differing anatomic sites have distinct transcriptional patterns and, may even exhibit different morphology. Lipid metabolism, cell signalling pathways that control proliferation, cell migration and cell fate determination are other functions that can vary.

1.2.1. Cardiac Fibroblasts

Fibroblasts, along with cardiomyocytes, smooth muscle cells and endothelial cells, are the resident cell populations in the adult heart. They form a major cell population in the heart (up to two-thirds in a normal adult heart), which is largely interspersed in the collagen network. (21).

Cardiac fibroblasts (CF) serve diverse functions in the heart providing structural, mechanical, electrical, and biochemical contributions (6). CF are responsible for synthesis and maintenance of a 3D scaffold for cardiomyocytes, which insures the functional integrity of the myocardium. In addition, CF also have the ability to sense mechanical stress through multiple pathways, including integrins, ion channels, and secondary messenger responders. Finally, mechanical stimulation may result in ECM gene expression, growth factor production, and collagenase activity (22).

During embryonic development, CF have further vital functions, such as synthesis of ECM and instructive epithelial differentiation. Different populations of fibroblasts originate from different sources: the epicardium; endocardium and from the neural crest, and co-exist within the heart (23) (24) (25). CF derive mainly from the epicardium (26) (27), thus, the epicardial markers T-box transcription factor 18 (*TBX18*); Wilms' tumor 1 (*WT1*), and Transcription factor 21 (*TCF21*) are important to analyse.

TBX18 gene codes for a member of a family of transcription factors that plays a crucial role in embryonic development (28). It is expressed in epicardium. In the heart, it contributes to formation of the sinus horn myocardium at the venous pole of the heart (29). In adults, TBX18 is found in differentiated smooth muscle of the coronary vessels; including the coronary arteries, and in some coronary vascular smooth muscle cells development (28).

The zinc finger transcription factor encoded by the *WT1* carries a mutation in Wilm's tumor patients, cancer of the kidneys that usually affects children and rarely adults. This gene is expressed in epicardium, among other locations in the heart and kidney (25).

Transcription factor 21 is a member of the class basic helix-loop-helix (bHLH) family of transcription factors that manage cell-fate specification. It promotes cardiac fibroblasts identity and persists in differentiated cardiac interstitial and adventitial fibroblasts in the postnatal and adult heart. *TCF21* is mainly required for epicardial development and fibroblast lineage. Besides the epicardium, it is expressed in kidney, lung and reproductive tract (27) (30).

As for the endothelial lineage; most mesenchymal cells are derived from endothelial-to-mesenchymal transition (EMT) of a subset of endocardial cells, making this a compelling candidate for the source for the endothelially derived fibroblasts in myocardium (23). Markers such as Cluster of Differentiation 105 (*CD105*) (an endothelial marker), Platelet-derived growth factor receptor α (*PDGRFA*) and Periostin (*POSTN*) (both mesenchymal markers) are important for endothelial/mesenchymal lineage.

Indeed, the Endoglin gene, *CD105*, encodes a homodimeric transmembrane glycoprotein of the vascular endothelium involved in angiogenesis and regulation of endothelial cell proliferation (31) (32). In an immunohistochemical study, *CD105* is expressed in the human foetal heart at the 9^{th} (in endocardium) and 10^{th} week (in epicardial and myocardial vessels), throughout gestation, and postnatally. This embryonic human heart development stage would correspond to E14.5 of the mouse (33). Mutation of *CD105* causes hereditary hemorrhagic telangiectasia, an autosomal dominant inherited vascular dysplasia (34).

Tyrosine kinase receptor *PDGRFA* is a mesenchymal marker involved in the arrangement and development of the cardiovascular system. The fact that fibroblast main function is structural, synthesising most of the extra cellular matrix of connective tissue, *PDGRFA* comprehensively marks fibroblasts generally, as it found to stimulate collagen synthesis (35). Other than cardiac fibroblasts, in normal heart, it also marks smooth muscle cells and cardiomyocytes. (23).

POSTN, codes a TGFβ superfamily-responsive matricellular protein, which is secreted throughout cardiovascular morphogenesis and postnatal cardiac homeostasis. It is required for maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart (36). It is expressed in the periosteum and periodontal ligament, injured vessels, metastatic cancer

cells, and in cells undergoing EMT. It is used to mark CF population but, since it is an extracellular protein, it has limitations in fibroblast labelling by means of antibodies (22). It is expressed in small part of adult heart (6).

1.2.2. Fibroblasts Markers

CF have been extensively studied in respect to their origin, to determine when and where distinct fibroblasts are generated, their lineage, and how they move to the final position in the heart. For this, it is crucial to adjust cell-staining techniques to identify fibroblasts. In the late 19th century, fibroblasts would be identified only by their structure. Nowadays immunostaining also allows identification of fibroblasts, and lineage tracing permits the divination of the path of origin of a specific fibroblast, thus permitting the understanding of CF lineages, above mentioned (37).

There are several fibroblast markers, however none is exclusive or expressed by all types of fibroblasts. The lack of specific fibroblasts markers is one of the main problems concerning the study and purification of these cells. Nevertheless, Cluster of Differentiation 90 (*CD90*), Discoidin domain receptor 2 (*DDR2*) and Vimentin (*VIM*) have been analysed.

CD90, or Thymocyte antigen 1 (*Thy-1*), codes a cell surface membrane glycoprotein. It is known to label subsets of fibroblasts as well as immune and endothelial lineages (6) (23). It is also involved in the biological process of angiogenesis, and expressed mainly in smooth muscle cells.

DDR2 encodes a tyrosine kinase receptor protein (38). Representing a cell surface receptor (39), it is used to mark fibroblasts; however, it only identifies subsets of fibroblasts (22). Nevertheless, *DDR2* is also expressed by smooth muscle cells, epithelial cells, and bone marrow-derived cells (39).

The intermediate filament protein coding gene, *VIM*, is part of the cytoskeleton. It is mostly used to label cardiac fibroblasts, but it also labels endothelial, immune system cells, and mesenchymal-derived cells (38). However, the staining is mostly comprehensive, i.e. all CF are marked positive (6).

1.3 Heart Disease

Cardiac disease is nowadays a worldwide public health problem. Therefore, the study of the heart and the mechanisms during development are very important to potentially develop treatment and prevention therapies. A major part of this condition comprises acquired heart disease, which develop during life and is manifested in adults. In contrast, precocious congenital heart disease affects the embryonic development, leading to major and severe

cardiac malformations. In many cases the aetiology of congenital heart disease remains largely unknown.

1.3.1 Congenital Heart Disease

The heart is the first functional organ during embryogenesis. Unfortunately, approximately 4 to 14 per 1.000 live births are diagnosed with congenital heart disease (CHD), a condition, in which malformations of the heart anatomy occur disturbing the circulatory system. The underlying malformations range from small atrial or ventricular septal defects to highly complex malformations, resulting in serious hemodynamic changes. Hypoplastic left heart syndrome (HLHS) and Tetralogy of Fallot are major and most severe clinical malformations (40).

CHD is the most common birth defect as it is estimated that 2% of live births have it (41). Furthermore, the incidence of this disease may be more than 10 times greater in nonviable embryos (i.e., spontaneously aborted foetuses or still-births) and these malformations contribute to advanced heart failure in the paediatric and adult population (1), (41) (42) (43).

Regarding aetiology and pathogenesis; 90% of cases are unknown and in the 10% of cases the environmental and genetic factors may lead to CHD. There are single gene mutations, chromosomal deletion or additions and single gene mutations that affect proteins of transcription factors.

The diagnosed CHDs in this project are presented in the following paragraphs.

HYPOPLASTIC LEFT HEART SYNDROME

HLHS results from a defective development by a stenotic or atretic aortic and/or mitral valve, a high-grade hypoplasia of the ascending aorta and a highly hypoplastic or even completely missing left ventricle. Because of the abnormal circulation, the ductus arteriosus and foramen ovale are patent and the right atrium, right ventricle, and pulmonary artery are enlarged. Until recently, HLHS was a uniformly fatal pathologic condition (40).

Severe atrial septal leads to obstruction to left ventricular outflow, causing hypoplasia of the left ventricle and ascending aorta.

HLHS can be caused by mutation in the Gap Junction Protein, Alpha-1 gene (*GJA1*) gene and in the NK2 Homeobox 5 gene (*NKX2-5*) gene. This last gene is essential in cardiac development and its mutation causes various congenital heart malformations. Cardiac expression of *NKX2-5* continues throughout development and into adult life (44). However, this gene interacts with the genes *GATA4*, *TBX5* and *TBX20* (45), (46), (47), (17).

CO-ARCTATION OF THE AORTA

The aortic co-arctation (CoA) is an obstructive congenital anomaly described by the narrowing of part of the aorta causing obstruction of blood flow to the body. The obstruction of the left ventricular outflow results in pressure hypertrophy of the left ventricle.

No particular gene has been directly associated with this clinical diagnosis.

ATRIAL SEPTAL DEFECT

ASD I (Atrial Septal Defect Primum), consists of a cardiac septal defect. It occurs less than 10% of ASD cases and is next to AV valves, mitral cleft. ASD has some variations, which can be distinguished by its clinical synopsis.

ASD II (Atrial Septal Defect secundum type), for instance is described clinically when the patient exhibits (beyond the atrial septal defect) pulmonary valve thickening or stenosis and, in some patients, even ventricular septal defects and atrioventricular septal defect. In comparison to ASDI it usually occurs 90% of ASD cases and is distinguished by a defective fossa ovalis. This variation is known to be caused by a mutation in the *GATA4* gene. (48) (49) (50) (51)

ASD has other variations (52), like ASD4 and ASD9 are caused by mutation in the *TBX20* (53) and *GATA6* gene (54), respectively. There is no description of clinical synopsis for ASD4, but characteristics of ASD9 consist of atrial septal defect, ostium secundum type and in some patients: tricuspid valve disease; pulmonary valve disease and pulmonary artery hypertension.

COMPLETE ATRIOVENTRICULAR SEPTAL DEFECT

The term 'atrioventricular septal defect' (AVSD) covers a spectrum of congenital heart malformations characterized by a common atrioventricular junction coexisting with deficient atrioventricular septation. In ostium primum atrial septal defect (ASD) there are separate atrioventricular valvar orifices despite a common junction, whereas in complete AVSD (CAVSD) the valve itself is also shared (55).

The 2 syndromes most frequently associated with AVSD are Down syndrome, in which AVSD is the most frequent congenital heart defect, and Ivemark syndrome (56). Other variations like AVSD4, which is caused by mutation in the *GATA4* gene (57) (58), and AVSD5 caused by mutation in the *GATA6* gene (12), are not rare.

1.3.2. Heart Disease in Adults

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels. CVDs are the number 1 cause of death globally: more people die annually from CVDs than from any other cause (59).

In adults, coronary heart disease is a type of cardiovascular disease that affects the blood vessels supplying the heart muscle, causing myocardial ischemia, due to reduction in the coronary blood flow, as a result of obstructive atherosclerosis in the coronary artery. Aorto-coronary Vein Bypass (ACVB) consists of the establishment of a bypass by using veins from the aorta to the coronary vessels (59).

Valvular heart disease consists of the malfunctioning of a valve in the heart. Aortic Valve Replacement (AVR), consists in the replacement of the aortic valve in case of stenosis or regurgitation. As for Mitral Valve Repair (MVR), it consists in the repair of the mitral valve also in case of stenosis or regurgitation. This clinical diagnosis, in the case of mitral prolapse, is associated with connective tissue disorders like Marfan syndrome (autosomal dominant) (59).

In adults, there is a weak contribution that genes may have in heart diseases, since a somatic cause is more expected. However, they may be used as an indicative. Age, the medication history, and clinical history (risk factors like smoking, obesity or lack of exercise, poor diet and excessive alcohol consumption) are the main responsible factors of these diseases.

1.4 Scope

The main objective of this project is to identify the expression patterns of primary CF from children and adults. Relative gene expression patterns are also compared between culture passages at time zero and after two passages with the purpose to see whether if culture conditions would change the gene expression profile.

Cardiac samples were collected, from adult patients, suffering from coronary and valvular heart disease, and from children suffering from congenital heart disease (Figure 1.2). Control fibroblasts, from adipose tissue of the same patient, were used as a reference. These cells were cultured for two passages. Gene expression of passage zero (P0) and passage two (P2) was evaluated by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and subsequently subjected to data analysis. The gene expression was compared between cardiac and adipose-derived fibroblasts. In addition, the results were analysed according to the age, different cell passages and diagnosis. Cultured fibroblasts of passage one (P1) were used for

extra staining assays, immunocytochemical (ICC) and immunohistochemical (IHC), to confirm the analysis of fibroblasts only and not of other cells.

Genes that are relevant during cardiac development were used in this work. Besides those genes, some fibroblast markers were also included.

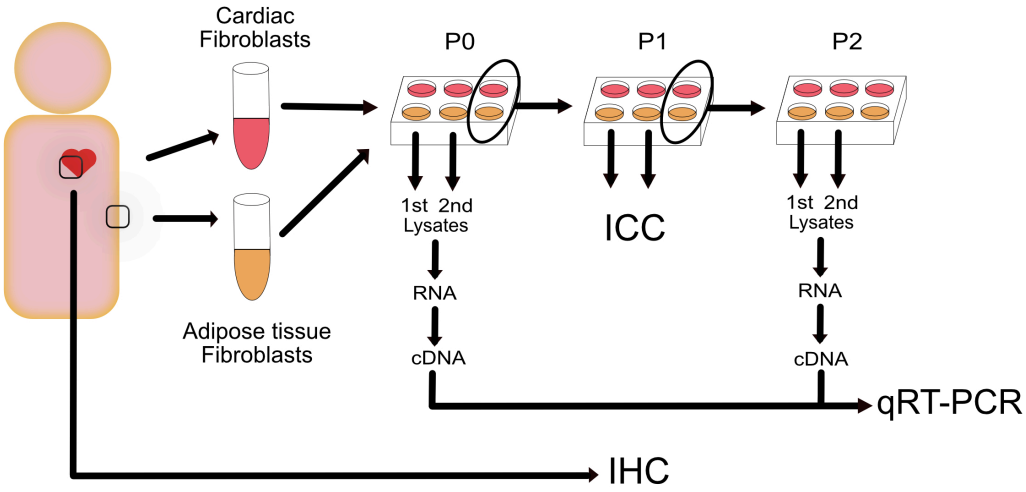

Figure 1.2 - Experimental procedure representation

Abbreviations: P0, passage zero; P1, passage one; P2, passage two; ICC, immunocytochemical staining; IHC, immunohistochemical staining; RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid; RT-qPCR, quantitative reverse transcriptase polymerase chain reaction.

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2. MATERIALS

A list of all the materials used in this work such as chemicals and reagents, equipment, kits, produced medium, produced solutions is presented in the next sections.

2.1. Chemicals and Reagents

The tables containing the consumables, primers and software, were displaced to Attachment A – “List of consumables, software and used primers”.

All chemicals and reagents used in this work are listed in table 2.1.

Table 2-1 - Chemicals and reagents

Designation	**Item number**	**Manufacturer**
Bovine Serum Albumin (BSA)	B14	Thermo Scientific
DMEM (4,5g/l Glucose, with L-Glu (stable))	FG-0435	Merck Millipore
EDTA (0,5M)	CN06.2	Carl Roth GmbH & Co. KG
Ethanol (>99,8%)	K928.1	Carl Roth GmbH & Co. KG
Fetal Bovin Serum (FCS) (ESC-qualified)	10270-106	Invitrogen
GeneRuler 50bp DNA Ladder (0,5µg/µl)	SM0371	Thermo Scientific
H_2O (double distilled)	R91051	H. Kerndl GmbH
PeqGold Universal Agarose Powder	35-1020	Peqlab
Trypsin/EDTA- solution (0,25%)	25200-056	Life Technologies
Dithiothreitol (DTT) (0,1M)	P/N Y00147	Invitrogen
DNA Loading buffer (6x)	R0611	Fermentas
dNTPs (10mM)	R0181	MBI Fermentas
First strand buffer (5x)	P/N Y02321	Invitrogen
Collagenase Type2	LS004176	Worthington
L-ascorbic acid -2 – phosphate (5g)	A8960	Sigma Aldricht
M-MLV Reverse Transcriptase (200U/µl)	28025-013	Invitrogen
Sodium pyruvate (100mM)	11360-039	Life Technologies
Random hexamer Primers (250ng/µl)	48190-011	Invitrogen
SybrGreen Quantitect	204145	Qiagen
Tris (99%)	T 6066	Sigma Aldrich

2.2. Equipment

Next, table 2.2 follows listing all the equipment used in the lab.

Table 2-2 - Equipment

Device	**Manufacturer**
Analytical Balance EW600-2M	Kern & Sohn GmbH
CO2-Incubator AutoFlow CO2 Water Jacket (37°C, 5% CO2)	NuAire
Battery powered pipette filler- Pipetus	Hirschmann
Fluorescencemicroscope Axiovert 200M	ZEISS
Electrophoresis Gel Device - Power Supply Gel	Bio-Rad
Gel Imaging System – ChemDoc XR	Bio-Rad
Gel Chambers (50ml, 150ml)	Bio-Rad
Fridge 4°C	Liebherr
Fridge -20°C	Liebherr
Fridge -80°C	Thermo Scientific
Hemocytometer	Carl Roth GmbH & Co. KG
Microwave MW 7849 900W	Severin
NanoDrop 2000 Spectrophotometer	Thermo Scientific
Pipetes (various)	Eppendorf Research
Safety workbench Class II	NuAire
Thermomixer comfort	Eppendorf
Thermocycler C1000	BIO-RAD
Table Centrifuge 5417R	Eppendorf
Vortex device	Scientific Industries
Water bath (37°C)	Memmert
Medium bench centrifuge – Heraeus Megafuge 2.0R	Thermo
ABI PRISM 7000	Applied Biosystems
Cryostat Leica CM1850	Leica Biosystems
Precellys® 24	Peqlab

2.3. Kits

Table 2.3 lists the original kits used for RNA extraction, cDNA production and PCR.

Table 2-3 - Kits

Kit	Item number	Manufacturer
PeqGOLD Total RNA Kit	126834	Peqlab
PeqGOLD DNase I Digest	12-1091-02	Peqlab
High Pure PCR Product Purification Kit	11 732 668 001	Roche

2.4. Produced Media and Solutions

Produced media, used in cell culture, and solutions, used in digestion of samples, cell culture and electrophoresis are listed below in table 2.4

Table 2-4 - Produced Media and Solutions

Media and Solutions	Composition
MEF Medium	DMEM + 10% (v/v) FCS + 1%(v/v) Na-Pyruvate (100mM) + 1% (v/v) Pen (100U/ml) / Strep (100µg/ml)
TBE-Buffer (10x) Solution	55.64g boric acid + 109g Tris 9.3g EDTA; dissolve Disodium salt dihydrate in aq. bidest, set pH to 8.3 with 1M HCl; fill up to 1L with aq. Bidest
TBE-Buffer (1x) Solution	100ml TBE-Buffer (10x) + 900ml aq. bidest
Trypsin/EDTA-Solution (0.05%)	1ml Trypsin/EDTA-Solution (0.25%) + 4ml PBS
Collagenase Solution (0.2%)	100mg Collagenase Type 2 +10ml PBS, sterile filter and wrap falcon tube in aluminium foil to protect from light, stored at 4°C

2.5. Antibodies and Serum Used

A list of the serum and antibodies used in Immunocytochemistry (ICC) and Immunohistochemistry (IHC) staining assays are listed below in table 2.5. Dilution used is also presented as well as the fluorescence of the 2nd antibody, Alexa Fluor 555 and Alexa Fluor 488, corresponding to red and green respectively.

Table 2-5 - Antibodies and serum used

Staining		**Antibody**	**Manufacturer, number**	**Dilution**	**Fluorescence**
ICC	1st	Anti-Vimentin: rabbit polyclonal IgG	Abcam ab45939	1:500	-
	1st	Anti-DDR2: rabbit polyclonal IgG	LSBio, LS-C99151	1:10	-
	2nd	Goat anti rabbit IgG	Abcam ab150078	1:500	Alexa Fluor 555
IHC	1st	Anti-α-Actinin: mouse monoclonal sarcomere antibody	Abcam, ab9465	1:100	-
	2nd	Goat anti mouse IgG	Abcam, ab150114	1:500	Alexa Fluor 555
	1st	Anti-Vimentin: rabbit polyclonal IgG	Abcam ab45939	1:500	-
	1st	Anti-DDR2: rabbit polyclonal IgG	LSBio, LS-C99151	1:50	-
	2nd	Goat anti rabbit IgG	Abcam ab150078	1:500	Alexa Fluor 488
Staining		**Serum**		**Dilution**	**Fluorescence**
ICC/IHC	-	Goat Serum	Abcam	-	-

2.6. Biological material

All donors have signed an informed consent and the procedure was approved by the local ethical committee of the medical faculty of the Technical University of Munich.

3. METHODS

3.1. Establishment of primary cardiac and adipose fibroblast cultures

3.1.1. Cell Culture

Cell culture is the process, by which eukaryotic cells are grown under controlled conditions. There are two types of culture: cells in suspension and adherent cells. Epithelial and fibroblasts-like cells both attach to a substrate; the difference between them is the morphology: epithelial like-cells appear flattened and polygonal in shape, while fibroblasts appear elongated and bipolar. Lymphoblast-like cells do not attach to a substrate and remain in suspension with a spherical shape (60).

The aim of cell culture is to provide an environment to maintain the proliferation of a certain cell type which then allows the analysis of selected parameters (e.g. gene expression). Cell culture requires sterile conditions (avoid contamination by bacteria, etc); and defined parameters (e.g. temperature; humidity; CO_2, growth medium) and suitable surface for attachment (culture vessel) for adherent cells. Figure 3.1

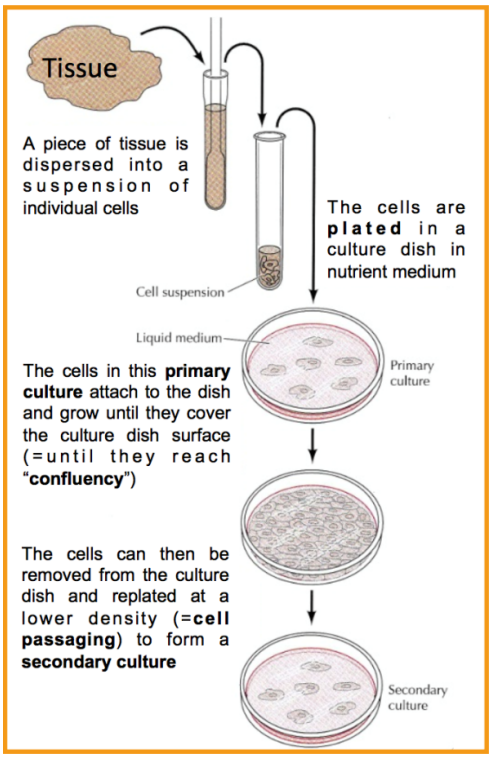

Figure 3.1 - Cell Culture Procedure
Source: (60)

Regarding the sterile conditions, all work is done in a laminar flow. Cultures are incubated (with defined temperature, gas mixture and humidity level, usually: 37ºC; 5% CO_2 in air and more than 95% humidity, with these last two helping stabilizing pH).

Cell culture media, chemically defined, have a neutral pH (7.2-7.4), contain essential amino acids, vitamins, inorganic salts, and glucose, among others. The most commonly used media are Dulbecco's Modified Eagle Media (DMEM) and Roswell Park Memorial Institute Medium (RPMI). Usually they are supplemented with antibiotics (penicillin, streptomycin, etc.) to inhibit the growth of contaminants. Media are sterile and stored at 4ºC.

Cell culture vessels used for fibroblasts culture (adherent cells) are T-flasks (T-25, T-75 - the number indicates cm^2 of surface area) and multiwell plates, which come in various sizes. The ones used in this project are 12-well plates (60).

Cell cultures from 5 children and 5 adults with congenital or acquired coronary and valvular heart disease were established. The surgical intervention or the type of diagnosis of each patient is listed in table 3.1. Right atrium (RA) was the type of cardiac tissue harvested for all patients, except for one adult patient with the MVR diagnosis, whose cardiac tissue harvested and analysed was left atrium (LA).

Table 3-1 - List of the diagnosis of the collected samples

Age Group	Patient	Intervention/Diagnosis	Cardiac Tissue
Children	1	Coarctation of Aorta (CoA)	RA
	2	Complete Atrium Ventricle Septum Defect (CAVSD)	RA
	3	Atrium Septum Defect (ASD)	RA
	4	Hypoplastic Left Heart Syndrome (HLHS)	RA
	5	Atrium Septum Defect Secundum (ASDII)	RA
Adults	6	Aortic Valve Replacement (AVR)	RA
	7	Aortic Valve Replacement (AVR)	RA
	8	Mitral Valve Repair (MVR)	LA
	9	Aorto-coronary Vein Bypass (ACVB)	RA
	10	Mitral Valve Repair (MVR)	RA

3.1.2. Protocol used

Human cardiac biopsies or subcutaneous adipose tissue are transferred on ice from the operating room to the lab in phosphate-buffered saline (PBS). The sample is cut into small fragments (approx. $1mm^3$) using scalpels. A small part of the sample is snap frozen in liquid nitrogen and kept at -80ºC. This will serve as a tissue control. The remaining tissue is digested with 0.2% collagenase II in PBS for approximately 1h with vigorous shaking at 37ºC.

Optionally, the digested tissues may be filtered through a 70µm filter to remove larger undigested pieces. Cells are centrifuged for 10min at 300xg and resuspended in MEF Medium (DMEM high glucose, 1% sodium-pyruvate, 10% FCS and 1% Pen/Strep). Depending on the size, the cells are distributed into a T25 flask or in 3 wells of a 12-well plate in the same medium. T-flasks and multiwell plates were labeled with project name (fibroblasts project), and patient number (differing samples from children and adults, and cardiac and adipose tissue). Date of seeding is also present, as well as passage number. Dates of medium changing and passaging were noted.

After overnight incubation, fibroblasts should have attached to the plastic surface. To get rid of cellular debris and non-attached cells the wells are then washed and receive fresh medium.

After 5 to 7 days, P0 cultures should present the typical fibroblast morphology and are ready for the next culture passages, namely the P1 to be used for downstream experiments and P2 for further procedures.

All cell culture work was done under sterile conditions in the laminar flow. Unsterile solutions were filtered; material and devices were sprayed with 70% (v/v) ethanol before use. Cells are incubated at 37°C and 5% (v/v) CO_2 in a atmosphere with *circa* 95% (v/v) of humidity.

3.1.3. Cell passaging

Cell lines cannot be kept in culture indefinitely due to the gradual rise in toxic metabolites, use of nutrients limitation and increase in cell number (avoid inhibition due to contact). The aim is to keep the cells actively and healthily growing and expand the number of cells, which leads to the necessity to subculture at regular intervals (cell passaging). For adherent cells, it involves breaking the bonds or cellular 'glue' that attaches the cells to the substrate and to each other. The use of proteolytic enzymes, like trypsin, breaks down proteins that facilitate adhesion.

When the fibroblasts reach 80% confluence (referring to the proportion of the surface of the flask, which is covered by cells, usually cells are cultured only until 80% confluence, and not 100%, to avoid contact inhibition), medium is aspirated and the plate wells are washed with sterile phosphate-buffered saline (PBS). This PBS pre-rinse step is made, due some confluent cultures being more difficult to detach. Thus, it will remove the serum, which neutralizes the action of the trypsin, from the well. Cells were detached from the plastic with Trypsin (1:4, Trypsin/EDTA: PBS), as it stripped proteins from the cell surface, after a 5-minute incubation at 37ºC (trypsin's operating temperature). Incubation time and concentration were chosen carefully, as incubating cells (with too high a trypsin concentration) for too long will damage cell membranes and kill the cells. After inspecting cells under the microscope, and making some they are already detached, trypsinized cells, with a round shape, as they now are suspended, were pipetted to a 15mL falcon tube. Trypsin strips proteins from the cell surface and will

continue to act as a proteinase until an inhibitor, such as serum, neutralizes it, thus MEF medium was added, inactivating trypsin, due to the FCS content. Afterwards cells were centrifuged for 5 min at 1500 rpm. The supernatant was aspirated and the cells were resuspended with MEF medium.

Cells were distributed in 3 new wells in a 12-well plate, designated passage one. These cells were again passaged and grown to 80% confluence (passage two, P2).

3.2. RNA Extraction

RNA extraction consists on the purification of RNA from biological samples, and is used to generate complementary DNA (cDNA), and afterwards amplify this cDNA with primers, specific for the gene of interest in quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR).

However, why chose this technique, when DNA can be purified and amplified with quantitative Polymerase Chain Reaction (qPCR), saving work? In order to distinguish a few particular genes of interest, among all other coding and non-coding sequences, which are sitting in the nucleus, the only one possible way is to follow the messenger RNA (mRNA), corresponding to the genes of interest. When extracting a particular mRNA, its corresponding gene is being expressed (or productive) in the moment of extraction. The coding DNA is present in all nucleated cells, whereas the coding mRNA is expressed only in those cells that are actively synthesizing the protein. Therefore, if interested only in the cells that are active, i.e. expressing the RNA, RNA extraction has to be accomplished.

The RNA extraction was performed with the peqGOLD Total RNA Kit (Peqlab). For the cultured fibroblast, medium was aspirated and 400µL of RNA Lysis buffer were added directly to the confluent cells. The culture surface was rinsed multiple times with the lysis buffer to capture as many cells as possible. The lysate was collected with a pipette and added to a 1.5mL Eppendorf tube. The lysates were promptly stored at -20°C until further use.

Regarding the tissue control, samples were retrieved from -80ºC on dry ice. Samples were transferred to 2 mL tubes with 1.4mm ceramic (zirconium oxide) beads, designed for soft tissue homogenization, which were put on ice. 400µL of the previously used RNA Lysis are buffer is added. Lysates placed in the Precellys® 24, piece of benchtop equipment dedicated to the grinding, lysis, and homogenization of biological samples. With 3D speed and motion, the beads crush the biological sample to release the desired RNA. The settings parameters were 6500rpm-2x20-005; (velocity - 6500rpm; number of digestion intervals -2; duration of digestion

intervals – 20s and the pauses between digestion intervals – 005s). After homogenization, the tubes were put on ice until RNA extraction.

RNA isolation from fibroblasts samples and tissue samples, was performed accordingly to the manufacturer's instructions, (PeqGOLD Total RNA Kit, #126834).

The lysate was transferred into a DNA removing column, placed in a 2ml tube. After 1min centrifugation at 10,700rpm at room temperature, it was transferred to a new 1.5ml tube. An equal volume (400µl) 70% Ethanol was added to the lysate and mixed by vortexing, preventing hydrolysis of the RNA. A RNA binding column was placed in a new 2ml tube. The lysate was added directly to the membrane of the column. It was centrifuged at 10,700rpm for 1min and the tube containing the flow-through liquid was discarded. In the first washing step, the RNA binding column was placed in a new 2ml tube. 500µl of RNA wash buffer I was added, and the lysate was centrifuged at 10,700rpm for 15sec.The flow-through liquid was discarded, but the tube was reused. DNase digestion step followed, to remove DNA. A 75µL reaction mix for each column was prepare, containing 73.5µL of DNase I Digestion Buffer and 1.5 µL of RNase-free DNase I (20 Kunitz units/µl). The DNase digestion reaction mix, was added to the column, directly onto the membrane. After a 15min incubation at room temperature, the columns were placed into a new 2ml tube. 400µl RNA Wash Buffer I was added and a 5min incubation at benchtop followed. After a 15sec centrifugation at 10,700rpm, the flow-through was discarded. In the second washing step, 600µl of completed RNA wash buffer II was added and the column was centrifuged for 15sec at 10,700rpm. The flow-through liquid was discarded and the step was repeated, discarding again the low-through liquid. In the drying step, the column was centrifuged at 10,700rpm for 2min. Finally, in the elution step, the last step, the column was placed into a new 1.5ml tube. 50µL of sterile RNase-free dH20 (DEPC-water) were added directly to the membrane of the column and it was centrifuged for 1min for 6,900rpm to elute RNA.

After RNA extraction, purified samples are frozen at -20°C until further use.

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3.3. cDNA synthesis

Complementary DNA (cDNA) is synthesized from purified RNA. It consists of a double-stranded DNA synthesized from a single stranded purified RNA (mRNA) template in a reaction catalysed by the enzyme reverse transcriptase.

Prior to reverse transcription, the RNA concentration must be measured at 260nm using the NanoDrop 2000 Spectrophotometer.

Firstly 1.5µL random hexamer primers (250ng/µL), 1.5µL of deoxyribonucleotide triphosphates (dNTPs) (10mM), 100ng RNA from sample are added to a tube which is filled up with RNase-free water to a final volume of 20.5µL. The mix is pipetted into a tube and briefly centrifuged. The mixture is incubated for 5 min at 65°C and chilled on ice.

In the second step 6µL of first strand buffer (5x) and 3µL of DTT (100mM) are added, gently mixed and incubated at 37°C for 2 min.

After cooling the mix down on ice for some minutes, 0.5 µL of the enzyme Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200 units) is added and mixed. The mixture is incubated (10 min at 25°C), followed by a 50-min incubation at 37°C. Finally, the enzyme is inactivated at 75°C for 10 min. The samples are frozen at -20°C until further use.

In table 3.2, a typical approach to produce cDNA is shown. The values for RNA and water are not fixed (depend on the RNA concentration value). Thus, being X represented in the volume of RNA per sample.

Table 3-2 - cDNA synthesis protocol.

Steps	**Reagents**	**Volume per sample [µL]**	**Conditions**	
			Duration	**Temperature**
1st step	RNA (100ng)	X	5min	65°C
	dNTPs (10mM)	1,5		
	Random Primers (250ng/µL)	1,5		
	DEPC Water	*		
2nd step	First Strand Buffer (5x)	6	2min	37°C
	DTT (100mM)	3		
3rd step	M-MLV RT (200U/µL)	1	10min	25°C
			50min	37°C
			15min	75°C
The volume of RNA per sample is denominated X as it depends on RNA concentration.				

The RNA concentration will determine the volume of RNA per sample, in equation 3.1, and the volume of diethyl pyrocarbonate (DEPC) water, used to inactivate RNase enzymes.

$$\frac{100}{RNA\ concentration\ value} = RNA\ volume\ per\ sample$$

Equation 3-1 - RNA Volume per sample

$$RNA\ volume\ per\ sample + DEPC\ Water\ volume\ per\ sample = 17.5\mu L$$

The sum of RNA and RNase-free water must be 17.5 µL. Thus, if the RNA concentration measured is too low, the maximum volume added is 17.5 µL and no water is added.

3.4. Polymerase Chain Reaction

With the help of Polymerase Chain Reaction (PCR) it is possible to amplify specific DNA-fragments. In this process, a DNA-polymerase synthesizes a new DNA strand complementary to a single-stranded nucleic acid template, starting in the sequences where the primers will specifically bind. Primers are oligonucleotides (small sequences of specific base-pairs) that serve as starter molecules, which are complementary to parts of the templates and defining the sections of the template that will be amplified. When they bind specifically to the original region that will be synthesized, they allow the DNA-polymerase to start amplification, following a sequence of three main steps: denaturation, annealing and polymerisation.

In denaturation, usually using high temperature, the separation of complementary molecules of the DNA chain occurs. The next step is annealing occurring at a low temperature and allowing the primers to bind to the specific regions that will give the order to start the following step, polymerisation. This last step occurs at a specific temperature in order to allow the polymers to make a copy of the template sequences (normal and complementary, 5' and 3'), from the first (forward) primer until the second (reverse) one.

This sequence of steps will be repeated several times, duplicating the material at the end of each cycle. This means that after the first cycle, there will be two copies of the template molecule, for the second cycles it will duplicate, making it four, for the third cycle it will make 8 and so on, progressing in an exponential manner (2^x), where x represents the number of cycles that will be performed. Each cycle will run for a specific time, accordingly to the protocol used. This is the crucial principle of PCR, where the cyclic repetition of the reaction steps allows the DNA-strand to be exponentially amplified.

3.5. Reverse Transcriptase - Quantitative Polymerase Chain Reaction (RT-qPCR) with SybrGreen

3.5.1. Principle of RT-qPCR

The expression of endogenous genes is verified and demonstrated through RT-qPCR using cDNA as a template.

RT-qPCR is based on the principle that after each amplification cycle the fluorescence of the sample is measured. The more cDNA is amplified, the stronger the fluorescence signal. The dye SYBR Green intercalates with the double stranded cDNA and fluoresces. The signal rises proportionally to the amount of cDNA in the sample.

Tissue samples were used as a control to differential gene expression. Cardiac, epicardial, fibroblast and other genes, such as *GATA4/5/6*; *TBX5/18/20*; *CD90/105*; *VIM*; *WT1*; *TCF21 DDR2*; *POSTN* and *PDGRFA* were tested. The whole heart control gene expression is presented in the results.

At the end of the program, quantification of the fluorescence signal is applied over the number of cycles. The threshold value defines the number of cycles, after which the fluorescent signal exceeds the background signal (i.e., the CT value). The threshold value was set to 0.2 in all evaluations.

Concerning the preparation of the plate for RT-qPCR, firstly a master mix is prepared, which contains all the reagents for a RT-qPCR run. The used amounts are demonstrated in table 3.3.

Table 3-3 - Mastermix composition for a RT-qPCR, for one sample.

Designation	Amount [µL]
Primer Forward (5µM)	1.2
Primer Reverse (5µM)	1.2
SybrGreen and enzyme mix	10.0
Aq. bidest.	6.6
Total	19.0

The master mix was vortexed and briefly centrifuged.

One µL of cDNA sample was pipetted in a 96 Multiwell Plates well followed by 19µL of the master mix. As a negative control, instead of the template, 1µL aq. bidest was used.

The plates were centrifuged and the RT-qPCR is performed on an ABI Prism 7000, with the conditions described in table 3.4. The 2-step program cycles between 95°C and 60°C with a total number of 40 cycles.

Table 3-4- RT-qPCR Operating conditions

Temperature	Duration
50°C	2min
95°C	10min
95°C	15sec
60°C	60sec

At the end of each run, after the amplification, a melting curve analysis is conducted, to verify if the product in the sample is clean or if eventually primer dimers or other products have accumulated.

3.5.2. Quantification of RT-qPCR samples by means of Standard Curves

Regarding quantification of relative gene expression, in the respective RT-qPCR sample, standard curves are generated for the target genes. Therefore, from a positive sample, which contains the target gene, a dilution series is accomplished. The value of undiluted samples is arbitrarily set to 10^6 arbitrary units (AU), the further log (10)-dilutions correspondingly less, as presented on table 3.5.

Table 3-5 - Dilution series for a standard curve

Sample	Dilution	Arbitrary Units (AU) per µL
1	Not diluted (1:1)	$1x10^6$
2	1:10	$1x10^5$
3	1:100	$1x10^4$
4	1:1.000	$1x10^3$
5	1:10.000	$1x10^2$
6	1:100.000	$1x10^1$
7	1:1.000.000	$1x10^0$

Each sample will be measured as a duplicate in the RT-qPCR. The standard curves result from the Ct-values of the measured samples plotted against the logarithm of the arbitrary units.

From the slope and the intersection with the y-axis of the standard curve the relative number of amplified PCR-products can be determined as indicated in equation 3.2.

$$AU = e^{\frac{Ct - Intersection\ with\ y-axis}{slope}}$$

Equation 3-2 - Amplified PCR-products

In attachment B, an example of a standard curve is exemplified for *CD90* gene.

3.5.3. Normalisation with housekeeping gene β-Actin

Gene expression is the process by which information from a gene used in synthesis of a functional gene product, often a protein. Some specific genes, for each cell type, have a constant expression, designated housekeeping genes. Others are up-regulated (i.e. become expressed, switched on, or productive) in particular cellular conditions - normal physiological (development, aging, fertilization) or pathological (apoptosis, inflammation, mutations, etc). Gene expression, of a gene of interest, is usually compared toward the expression (or production) of a housekeeping gene, which is accepted as a constant.

For each sample, a RT-qPCR run was performed with primers specific for the β-Actin gene. β-Actin is expressed more or less constantly to a certain level at any time in the cell; it is therefore referred to as a housekeeping gene.
This β-Actin specific PCR allows to determine whether the sample contains amplifiable cDNA and the values of β-Actin of the RT-qPCR run serve as reference to normalize the values of the target genes.

This allows to compare different independent samples and to determine the relative frequency of gene expression in the samples.

Subsequently, the relationship between the target genes and reference gene, and thus the relative gene expression can be determined with the following equation 3.3.

$$relative\ expression = \frac{AU\ (target\ gene)}{AU\ (reference\ gene)}$$

Equation 3-3 - Target Genes / Reference Genes

3.5.4. Agarose Gel Electrophoresis

The principle of gel electrophoresis consists in the migration of the DNA fragments through the pores of the gel towards the anode, due to its negative charge, upon application of a voltage. The size of the gel's pores varies depending on agarose concentration. For a 3% agarose gel, 1,5g of agarose powder were weighed in an Erlenmeyer flask and heated with 50ml TBE-buffer (1x) in the microwave, until dissolved completely.

Subsequently 10µL of ethidium bromide (EtBr) (500µg/ml) were added and mixed with the gel. EtBr intercalates between the bases of DNA, which becomes visible under UV-light.

The liquid gel was poured in the gel chamber and incubated for 30min at RT, until the gel was polymerized.

The samples applied to gel, were first mixed with 4µL Loading buffer (6x) and afterwards pipetted into the wells of the solidified gel. Additionally, a molecular weight marker, which consists of different DNA fragments of known size (50bp), was added to the gel.

The exact composition is listed in the table 3.6 below.

Table 3-6 - Composition List

Designation	**Volume [µL] per well**
DNA molecular weight-marker (0.5µg/µl)	5µL
Sample	20µL
Loading buffer	4µL

The DNA-fragment separation was done at 120V and 400mA for 35min. The gel is photographed under UV-light with the gel documentation device Molecular Imager Gel Doc XR System.

3.5.5. Data Analysis

Some outliers and extreme values were not used due to a defected β–actin value measurement, which ended up affecting all complementary lysates for that value. Regarding descriptive statistics, all the collected data, (relative gene expression in relation to age, passage and cell type) by means of an exploratory data analysis (determination of averages, standard deviations, medians, 95% confidence intervals, extreme values), was examined and assessed.

Three main analysis were made. The first only compares the mean results of children and adults, without any concern for the different pathologies (a heatmap is presented later in Figure 5.1). The second one takes into account the different diseases, being gene by gene analysis (regarding AF vs. CF and P0 vs. P2), considering each result by itself (heatmaps are presented in Figures 5.3, 5.4, 5.5 and 5.6) The third analysis does not consider any values. Instead, a ratio calculus was performed, in order to understand if there is an increase or decrease of expression, regarding AF vs. CF, P0 vs. P2 (for both tissues) and, children vs adults. Only the last analysis is considered for this project. The first one was ruled out due to the pathology (gene) influence, and the second one due to not being statistically viable.

3.6. Immunostaining

Immunostaining is used to detect the distribution and localization of specific proteins within individual cells or tissues, by the use of specific antibodies (AB) to detect a single target protein. Detection of antigens in cultured cells is referred to as immunocytochemistry (ICC), whereas their detection in paraffin tissue sections is generally referred to as immunohistochemistry (IHC). Both methods involve exposure of fixed cells or tissues to primary antibodies directed against one or more proteins of interest. Bound antibodies are then detected using commercially available secondary antibodies directed against the invariant heavy chain portion of the primary antibody. Immunostaining is used in cell biology to study differential protein expression, localization and distribution at the tissue, cellular, and subcellular level (41).

Antibodies are typically developed in mouse or rabbit and have high affinity for specific proteins. Only specific antibodies may be used to detect the antigenic protein in fixed samples of animal tissue and within fixed individual mammalian cells (61).

3.6.1. Immunocytochemistry

Cells were grown on gelatine-coated cover slips. They were fixed in ice-cold acetone at -20ºC, for 15min and washed with PBS afterwards. Permeabilisation follows by adding 0.1% Triton-X100 in PBS for 15min at RT. This step will open the pores of the cellular membrane allowing the inside of the cell to be stained, as Vimentin binds inside the cell and only DDR2 binds to the surface of the cell. Unspecific binding sites were blocked by incubation with 5% of goat serum for 1h at room temperature. After 1h incubation at 37ºC with the first antibody (Anti-Vimentin and Anti-DDR2) and a washing step, the secondary antibody (Goat anti rabbit) was added for 1h at room temperature in the dark. Thereafter, the cells were washed, air-dried and mounted in a medium containing DAPI to stain the nuclei followed by analysis under a fluorescence microscope.

3.6.2. Immunohistochemistry

Immunohistochemistry (IHC) is a common technique for morphological characterization. It separates itself from ICC by detecting and analysing protein expression while maintaining the composition, cellular characteristics and structure of native tissue. This process can be divided in four steps: fixation, cryo embedding, cutting and staining.

Firstly, human cardiac tissue was transferred on ice from the operating room to the lab in PBS.

Tissues were fixed using paraformaldehyde to prevent degradation while preserving the morphology of the specimen for 1h at RT by 4% PFA. After washing the sample 3 times with PBS, it stayed overnight at 4°C in 30% Sucrose: OCT (2:1).

The sample was carefully (avoiding the formation of bubbles) cryo-embedded into OCT in an embedding shape mould. This mould was on top of a metal tray, which had liquid nitrogen underneath. After the OCT had turned white, the sample was removed from the mould and placed on an appropriately labelled (sample number; date) Petri dish. The sample was stored rapidly at -80°C until further use.

The slicing step follows. Firstly, the temperature was set to -20°C in the Cryostat Leica CM1850 device, since each tissue has a preferred temperature for processing, according to the device's manual. Heart tissue may be sectioned between -15°C and -25°C. The cryostat is a device with a microtome, a slicer capable of slicing sections as thin as 1 µm, inside a freezer.

Cryocompound was applied to the specimen disc at room temperature and the sample was positioned thereon. The specimen disc was frozen and placed back onto the device.

A glass cover slip was positioned at the end of the specimen disc and a precooled blade was placed in the knife holder and clamped. The knife angle was adjusted as well as the thickness

setting (8µm). Unlocking the handwheel, allowed sectioning. The first cuts had the only purpose to adjust more accurately the settings.

A slice was picked upon the glass cover slip. Since these were warmer than the sample; the slice adhered to the glass. In some cases, the slice was prone to roll on itself and must be unrolled with tweezers (that were found inside the freeze chamber). Three slices per glass cover slip were checked under the microscope. The glass slides were labelled and immediately stored at -80°C.

Finally staining follows with a two-day protocol. Figure 3.2 represents the cover glass scheme with the antibodies used for each slice, along with the dilutions. On the right, the patient sample was identified as a human biopsy, indicating patient number, the number of the cover glass and the date the cuts were performed. On the left three divisions are displayed, the circles represent the slices and above the antibodies used for each slice, including dilutions and fluorescence.

On the first day, the previously chosen glass cover slip is retrieved from -80ºC and left 10min at RT to warm up. The sections were washed two times with PBS for 2min each time. The permeabilisation step follows as we leave for 10min the slices with 1% Goat Serum in PBS-T (0.4% TritonX in PBS) at RT.

Next, unspecific binding sites were blocked by incubation with 10% Goat serum + 0.1% BSA in PBS-T. After 1h at RT, the cuts were washed once with PBS-T for 2min. This was the blocking step. Before the 1st AB is used, with the help of a cotton swab the glass between the slices is dried and a thin layer of red varnish is applied. This prevents the different antibodies to stain other slices. The last step is achieved by applying to each slice the 1st AB, diluted in 1% Goat Serum in PBS-T.

Notice that 2 AB are added per slice: α-Actinin/DDR2 and α-Actinin/VIM, as displayed in figure 3.2. In the negative control slice, only PBS-T/1%goat is added. The glass cover slip stays overnight at 4ºC. On the second day, it is washed 3 times for 5min with 1% Goat Serum in PBS-T. The second AB, diluted in 1% Goat Serum in PBS-T, is added (1%Goat Serum + Goat and Mouse + Goat and Rabbit). The slices are left for incubation for 1h at RT in the dark.

<table>
<tr><td>α-Actinin/DDR2
1:100/1:50
Goat anti mouse IgG
1:500 (Alexa 555)
Goat anti rabbit IgG
1:500 (Alexa 488)</td><td>α-Actinin/VIM
1:100/1:500
Goat anti mouse IgG
1:500 (Alexa 555)
Goat anti rabbit IgG
1:500 (Alexa 488)</td><td>Neg. Ctrl
Goat anti mouse IgG
1:500 (Alexa 555)
Goat anti rabbit IgG
1:500 (Alexa 488)</td><td rowspan="2">huBiopsy
#patient nr
OT_17
(cut on: date)</td></tr>
<tr><td>O</td><td>O</td><td>O</td></tr>
</table>

Figure 3.2 - Diagram of cover glass with three slices of tissue and the stainings performed in IHC.

Afterwards they are washed again 3 times for 5min with 1% Goat Serum in PBS-T and one time rinsed with water. The excess of the washing reagent is carefully removed with a cotton swab. One to 3 drops of anti-fade mounting Medium with DAPI (previously retrieved from 4ºC and placed upside-down) are added to each slice. It was let absorb for 3min. A cover glass was placed thereon and the cuts are ready to visualize fluorescence under the microscope.

4. RESULTS

4.1. Primary cardiac and control fibroblast cultures

For this project, ten samples were collected: 5 from children and 5 from adults. During this work, more than 30 samples were digested and cultured. However, it was not always possible to obtain both adipose and cardiac samples from the OR, due to restrictions during the operation. In addition, not all samples from children biopsies grew in culture, probably, due to the limited size of samples.

In Figure 4.1 an example of a 100% confluent well culture of adipose tissue fibroblasts, at a 10x magnification, from a child is shown. The typical fibroblast morphology, as a branched cytoplasm surrounding an elliptical (22), is observed. Speckled nuclei, that typically have 1 or 2 nucleoli, are visible at a higher magnification.

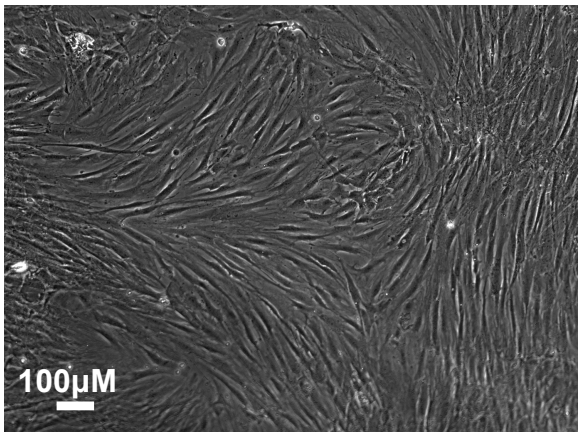

Figure 4.1 - Photograph of confluent AF at P0 after a week in culture from a child sample, with 10x. The image was not edited.

In Figure 4.2 a comparison of AF (Fig. 4.2 A) and CF (Fig. 4.2 B) from the same patient (child) is presented. While fibroblasts from different anatomical sites have similar morphology, they have their own gene-expression profile and characteristic phenotypes in different locations. Thus, they synthesize extracellular matrix proteins and cytokines in a site-specific manner (62). The main morphological difference observed between these two types of fibroblasts is that adipose tissue fibroblasts tend to be more elongated while cardiac ones appear shorter.

In comparison to Figure 4.1, an observation can be made, that the cells have not reached 100% confluence. However, AF appeared to reach confluence faster than CF, also noticeable in Figure 4.1. This comparison was easily made as both samples (from the same patient) would be cultured in the same day. This may be due to the fact that the received cardiac samples from

children were quite small in comparison with the fat samples, thus taking more time for cells to cover the well surface.

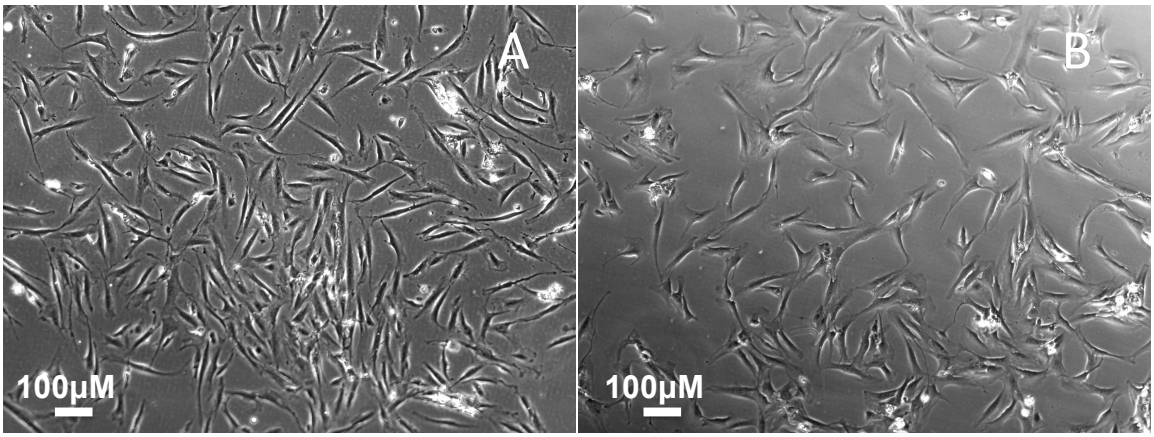

Figure 4.2- Photographs of AF (A) and CF (B, right atrium) at P0 after 3 days of culture from a child, with 10x. The image was not edited.

Another point to consider is that children's fibroblasts grow more rapidly than adult ones. Adult samples took approximately one month to grow while child samples took almost half of that time. During P0 cells from children would take a week to grow. After passaging, it only took one day for the cells to reach confluency for P1. The same is true for P2.

4.2. Immunostaining

After analysing fibroblasts morphology in culture, both ICC and IHC assays were performed to prove that the cells undergoing analysis are fibroblasts.

Regarding ICC, Figure 4.3 provides representative immunofluorescence data to show the expression of *VIM* and *DDR2*. Vimentin is expressed in both cultivated CF (figure 4.3 A) and AF (figure 4.3 B). *DDR2* is also expressed in both cell types, represented in figures 4.3 C and D, respectively.

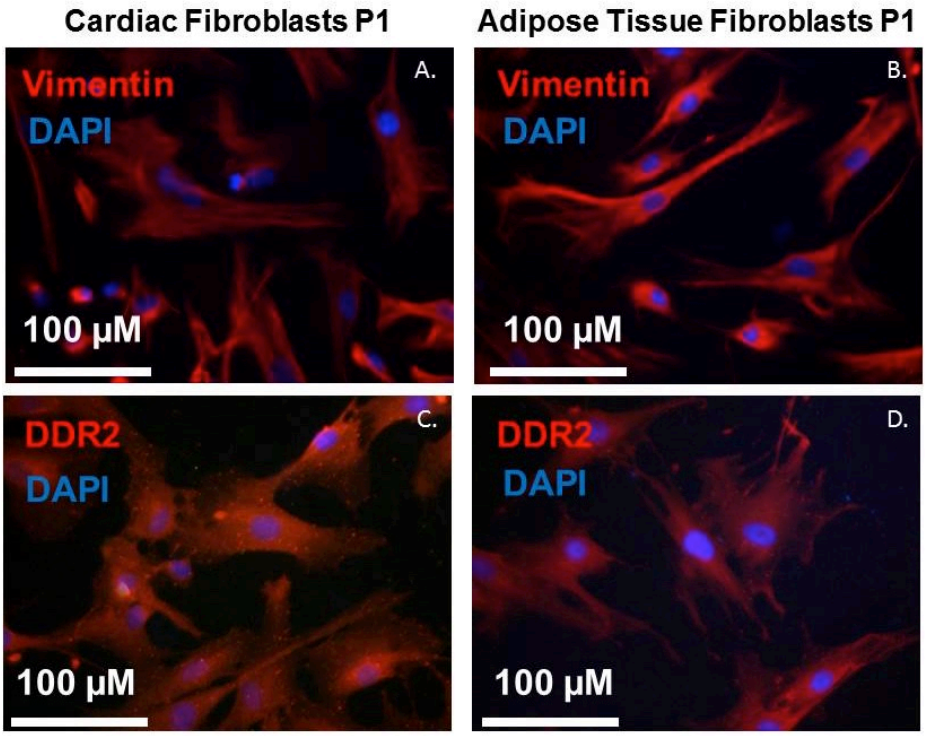

Figure 4.3 A. Right atrium CF from P1, from an adult sample stained with *VIM* (1:500), Alexa Fluor 555, DAPI, with 40x. **B.** AF from P1, from an adult sample, stained with *VIM* (1:500), Alexa Fluor 555, DAPI, with 40x.**C.** Right Atrium CF from P1, from adult sample, stained with *DDR2* (1:10), Alexa Fluor 555, DAPI, with 40x. **D.** AF from P1, from adult sample, stained with *DDR2* (1:10), Alexa Fluor 555, DAPI, with 40x. No images were edited.

ICC was performed to show where the tested proteins are expressed: within the cell, like the nucleus, or on the extracellular matrix or even on the surface of the cell. In this case, *VIM* and *DDR2* markers were chosen because they are established indicators of the fibroblast phenotype. These two markers (red) are expressed in the fibroblasts. As described in the introduction, the *VIM* gene is a structural constituent of cytoskeleton, thus the *VIM* gene marker marks structural filaments of the fibroblasts, which are visible in figures 4.3 A and B. *DDR2*, on the other hand, functions as cell surface receptor for fibrillar collagen, one of the main components of the fibroblast, thus being the filaments not marked, as shown in figures 4.3 C and D.

It is also important to mention that at this magnification, fibroblast morphology is more distinguishable than in figures 4.1 and 4.2, since the nuclei (counterstained with DAPI - blue), are now visible.

Regarding IHC, a histological analysis of cardiac tissue (right atrium) from adult was performed, to show the location of the fibroblasts in the heart. Both fibroblast markers *DDR2* and *VIM* were

tested along with α-Actinin and DAPI. The results are presented in figures 4.4 A and B respectively.

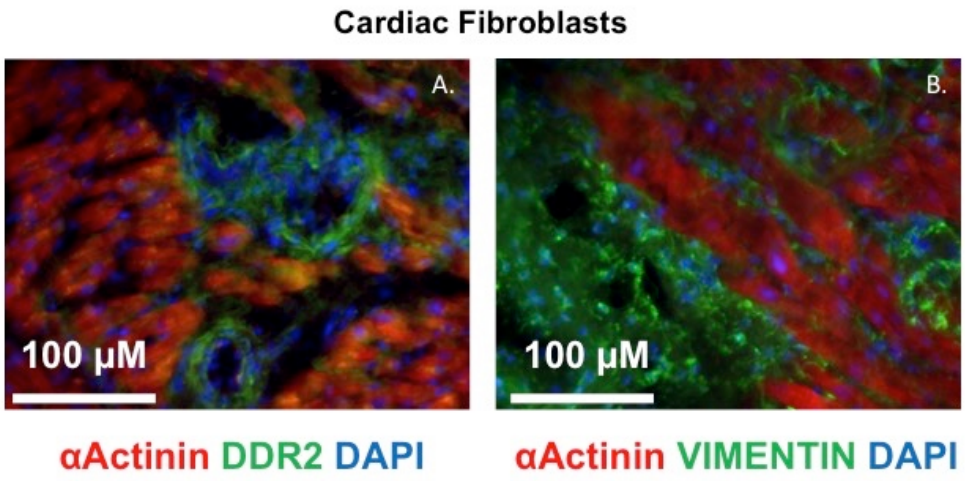

Figure 4.4 A. Immunohistochemistry results. Right atrium histological cut, from an adult sample stained with *DDR2* (1:50) and Alexa Fluor 488, α-Actinin (1:100) and Alexa Fluor 555 and DAPI, with 40x. **B.** Right atrium histological cut, from an adult sample, stained with *VIM* (1:500) and Alexa Fluor 488, α-Actinin (1:200) and Alexa Fluor 555 and DAPI, with 40x. No images were edited.

The presence of the fibroblasts markers *DDR2* is shown in figure 4.4A and *VIM* in figure 4.4B.

In figure 4.4 A and B, α-Actinin (red) labels cardiomyocytes, which clearly stand out with their distinct morphology. These cells consist of a basic contractile unit (the sarcomere), a group of actin-, and myosin-filaments (myofibrils). Myofibrils are seen as transverse-tubules (t-tubules), which run on the transverse axis, and are aligned normally along the radial axis of the cardiomyocytes (63).

This assay not only validates the sample being cardiac for the existence of cardiomyocytes but also allows the distinction of cardiac fibroblasts from other cell types in the heart (stained with DDR2 and VIM, already stained in CF in ICC).

Despite the accuracy and reliability of the IHC, results depend on the specificity and sensitivity of the antibodies used. Combined with ICC, IHC is a powerful approach for evaluating cell populations within a tissue. In this case, fibroblasts (previously stained by ICC) can be distinguished from cardiomyocytes.

4.3. Gene expression

After lysis, RNA extraction and cDNA production, a RT-qPCR was performed in order to identify potentially unique genes expressed in cardiac and adipose tissue fibroblasts. Normalization to the expression values of β-Actin, a house-keeping gene, was performed. This is explained in detail in methods 3.5.3.

Low values

In most cases the obtained values were used to analyse the expressiveness of genes. But gene expression is not comparable between genes, because the level of expression is different in different types of tissue and also during stages of development, i.e. during embryonic development these genes may be highly expressed in cardiac tissue and have a residual (or very low) expression in a formed individual, an adult. In this sense, there are genes that, besides of exhibiting values very close to zero, still are considered.

From another point of view, the gene expression of cultured adipose and cardiac fibroblasts is compared with whole heart tissue. The results show that, for instance for some cardiac genes, expression is absent in adipose tissue but present in both cardiac fibroblasts and heart tissue, as seen in attachment F. Other populations of cardiac cells, like smooth muscle cells or cardiomyocytes, that may express the same genes, exist in the heart tissue. Thus, what is expressed by fibroblasts may only be a part of what is expressed in the heart tissue. Therefore, even though the gene expression values in fibroblasts are low, they cannot be disregarded.

Also, RNA corresponds to what is expressed in the cell. As, when extracting a particular mRNA, its corresponding gene is being expressed (or productive) in the moment of extraction, actively synthesizing a protein, for instance. Since cDNA was produced from the extracted RNA, if there is gene material it will be measure in the RT-qPCR. Hence low values could be considered.

Another point to take into consideration concerns to the gene functions in the cell, i.e. a functional gene product, often a protein, is produced. However, genes could up-regulated (i.e. become expressed, switched on, or productive) in particular cellular conditions. These conditions are related or influenced by other gene's expression, and when the cell expresses the gene, depending on its necessity, it may have a higher or lower expression, thus having more or less mRNA available. Is the expression always low, or is it derived from the diagnostic? This angle allows the possible explanation for the low gene expression.

Control

Concerning the topic of relative gene expression, it would suggest that, for it to be relative gene expression of the patients, it would have to be compared to gene expression of people with no

heart conditions. However, there is no possibility to obtain human cardiac samples from healthy people. Therefore, for standard curves, cDNA of patients is used. This cDNA was previously checked for every gene in each tissue, and both passages, in electrophoresis gels, in order to make sure that gene expression values are obtained. This allows observation of different expression in different cell populations. cDNA is quantifiable and through dilutions and application of formulas (explained in methods 3.3), standard curves were obtained for each gene. This technique was performed following laboratory protocols, where this study was conducted.

Missing values

There are cases in which expression is detected in P2, but not P0. A hypothesis is that the gene is expressed in P0 but the limited sensitivity of the equipment does not allow it to be detected. In P2, since the sample has been in culture for a longer period of time and more genetic material is available, the equipment is able to detect the expression.

Analysis

Regarding the analysis, since variations with genetic cause are presented, even within each age group, the average results may not be conclusive. Therefore, no average results were analysed. An individual analysis of each patient is also not viable. Thus, a qualitative analysis of gene expression increase or decrease was calculated, as explained in methods 3.5.5. and discussed in the discussion.

Results

The results of relative gene expression of P0 and P2 for both AF and CF for the 14 studied genes and each age group, are presented in attachment C, separated in 4 tables dividing the genes in four groups (cardiac, epicardial, fibroblasts marker and others).

In order to facilitate the analysis three relative comparisons were calculated: for P0 the ratio between CF and AF; for AF, the ratio between P0 and P2 and the same for CF, shown in tables, 4.1; 4.2; 4.3 and 4.4. Ratios were calculated dividing relative gene expression values (these results are presented in Attachment C). This indicates, in which tissue and passage, there appears to be a higher expression. The red arrow indicates increased expression and the blue arrow decreased expression. The white boxes marked with '-', mean that it was not possible to calculate the ratio, since there was no expression detected for AF. The values are presented by patient, indicating their diagnosis, and age. The results are presented in 4 tables, with the tested genes: cardiac transcription factors (*GATA4/5/6* and *TBX5/20*) (Table 4.1); epicardial factors (*TBX18*, *WT1* and *TCF21*) (Table 4.2); fibroblasts markers (*CD90*, *DDR2* and *VIM*) (Table 4.3) and endothelial and mesenchymal markers (*CD105*, and *PDGRFA* and *POSTN*) (Table 4.4).

Table 4.1 - Gene expression AF/CF in P0 and P0/P2 in both tissues comparison ratio for cardiac genes in children and adults.

Table 4-1 - Results of ratio analysis between tissue and passages, for cardiac genes.

				P0	AF	CF	P0	AF	CF
Gene	Age Group	Diagnosis	Age	CF/AF	P0/P2	P0/P2	CF/AF	P0/P2	P0/P2
GATA4	Children	1 - CoA	6d	-	-	0.28	-	-	↓
		2 - CAVSD	7d	-	-	1.10	-	-	↑
		3 - ASD	33m	-	-	0.28	-	-	↓
		4 - HLHS	54m	-	-	0.58	-	-	↓
		5 - ASD2	8y	-	-	2.17	-	-	↑
	Adults	6 - AVR	60y	144.61	11.06	0.12	↑	↑	↓
		7 - AVR	60y	116.61	0.48	0.31	↑	↓	↓
		8 - MVR	66y	-	-	3.22	-	-	↑
		9 - ACVB	69y	4.00	4.32	0.46	↑	↑	↓
		10 - MVR	73y	58.84	0.63	0.95	↑	↓	↓
GATA5	Children	1 - CoA	6d	-	-	0.12	-	-	↓
		2 - CAVSD	7d	-	-	1.13	-	-	↑
		3 - ASD	33m	-	-	0.47	-	-	↓
		4 - HLHS	54m	-	-	0.78	-	-	↓
		5 - ASD2	8y	-	-	1.39	-	-	↑
	Adults	6 - AVR	60y	-	-	-	-	-	-
		7 - AVR	60y	-	-	1.28	-	-	↑
		8 - MVR	66y	-	-	-	-	-	-
		9 - ACVB	69y	-	-	1.55	-	-	↑
		10 - MVR	73y	-	-	1.38	-	-	↑
GATA6	Children	1 - CoA	6d	4.20	0.46	0.26	↑	↓	↓
		2 - CAVSD	7d	2.75	0.55	1.56	↑	↓	↑
		3 - ASD	33m	3.56	0.82	0.74	↑	↓	↓
		4 - HLHS	54m	0.48	17.81	0.84	↓	↑	↓
		5 - ASD2	8y	1.12	3.97	1.39	↑	↑	↑
	Adults	6 - AVR	60y	0.68	2.38	0.49	↓	↑	↓
		7 - AVR	60y	0.94	0.60	0.61	↓	↓	↓
		8 - MVR	66y	2.99	1.60	0.83	↑	↑	↓
		9 - ACVB	69y	0.72	1.69	0.79	↓	↑	↓
		10 - MVR	73y	1.34	0.44	0.86	↑	↓	↓
TBX5	Children	1 - CoA	6d	0.41	0.59	0.44	↓	↓	↓
		2 - CAVSD	7d	0.54	0.92	0.69	↓	↓	↓
		3 - ASD	33m	0.22	1.11	0.39	↓	↑	↓
		4 - HLHS	54m	0.14	13.84	2.14	↓	↑	↑
		5 - ASD2	8y	0.20	4.94	2.50	↓	↑	↑
	Adults	6 - AVR	60y	0.60	10.50	0.46	↓	↑	↓
		7 - AVR	60y	0.14	1.68	0.43	↓	↑	↓
		8 - MVR	66y	0.34	3.19	1.00	↓	↑	↓
		9 - ACVB	69y	0.18	1.30	1.19	↓	↑	↑
		10 - MVR	73y	14.70	0.14	1.07	↑	↓	↑
TBX20	Children	1 - CoA	6d	535.28	-	0.38	↑	-	↓
		2 - CAVSD	7d	29162.82	0.00	1.60	↑	↓	↑
		3 - ASD	33m	-	0.00	0.44	-	↓	↓
		4 - HLHS	54m	-	0.00	0.99	-	↓	↓
		5 - ASD2	8y	549.07	0.28	3.66	↑	↓	↑
	Adults	6 - AVR	60y	313.93	0.19	0.21	↑	↓	↓
		7 - AVR	60y	570.88	-	0.55	↑	-	↓
		8 - MVR	66y	-	-	1.52	-	-	↑
		9 - ACVB	69y	2.20	2.14	0.74	↑	↑	↓
		10 - MVR	73y	27.71	0.87	1.15	↑	↓	↑

Table 4.2 - Gene expression AF/CF in P0 and P0/P2 in both tissues comparison ratio for epicardial genes in children and adults.

Table 4-2 - Results of ratio analysis between tissue and passages, for epicardial genes.

				P0	AF	CF	P0	AF	CF
Gene	Age Group	Diagnosis	Age	CF/AF	P0/P2	P0/P2	CF/AF	P0/P2	P0/P2
TBX18	Children	1 - CoA	6d	1.43	0.51	0.29	↑	↓	↓
		2 - CAVSD	7d	1.21	0.93	0.86	↑	↓	↓
		3 - ASD	33m	0.49	3.27	0.60	↓	↑	↓
		4 - HLHS	54m	-	0.00	0.37	-	↓	↓
		5 - ASD2	8y	0.87	3.78	2.31	↓	↑	↑
	Adults	6 - AVR	60y	1.54	0.48	16.56	↑	↓	↑
		7 - AVR	60y	0.89	1.13	0.46	↓	↑	↓
		8 - MVR	66y	0.31	0.85	0.30	↓	↓	↓
		9 - ACVB	69y	0.28	1.36	0.77	↓	↑	↓
		10 - MVR	73y	0.77	1.14	0.59	↓	↑	↓
WT1	Children	1 - CoA	6d	-	-	0.78	-	-	↓
		2 - CAVSD	7d	-	-	0.92	-	-	↓
		3 - ASD	33m	-	-	0.62	-	-	↓
		4 - HLHS	54m	-	-	0.54	-	-	↓
		5 - ASD2	8y	-	-	1.90	-	-	↑
	Adults	6 - AVR	60y	-	0.00	-	-	↓	-
		7 - AVR	60y	0.97	0.73	0.38	↓	↓	↓
		8 - MVR	66y	-	-	1.04	-	-	↑
		9 - ACVB	69y	0.52	1.91	1.38	↓	↑	↑
		10 - MVR	73y	2.70	0.89	1.05	↑	↓	↑
TCF21	Children	1 - CoA	6d	27.15	2.81	0.27	↑	↑	↓
		2 - CAVSD	7d	-	0.00	5.44	-	↓	↑
		3 - ASD	33m	92.44	1.33	2.50	↑	↑	↑
		4 - HLHS	54m	0.82	-	1.59	↓	-	↑
		5 - ASD2	8y	17.17	2.48	3.67	↑	↑	↑
	Adults	6 - AVR	60y	0.15	4.37	0.44	↓	↑	↓
		7 - AVR	60y	0.74	0.69	0.90	↓	↓	↓
		8 - MVR	66y	0.94	8.18	0.70	↓	↑	↓
		9 - ACVB	69y	1.93	3.66	0.91	↑	↑	↓
		10 - MVR	73y	5.07	0.63	1.90	↑	↓	↑

Table 4.3 - Gene expression AF/CF in P0 and P0/P2 in both tissues comparison ratio for fibroblasts markers in children and adults.

Table 4-3 - Results of ratio analysis between tissue and passages, for fibroblasts markers

				P0	AF	CF	P0	AF	CF
Gene	Age Group	Diagnosis	Age	CF/AF	P0/P2	P0/P2	CF/AF	P0/P2	P0/P2
CD90	Children	1 - CoA	6d	0.53	0.66	0.33	↓	↓	↓
		2 - CAVSD	7d	0.50	1.62	0.81	↓	↑	↓
		3 - ASD	33m	0.30	2.03	0.59	↓	↑	↓
		4 - HLHS	54m	0.09	14.82	0.79	↓	↑	↓
		5 - ASD2	8y	0.35	4.57	1.74	↓	↑	↑
	Adults	6 - AVR	60y	0.32	3.30	0.33	↓	↑	↓
		7 - AVR	60y	0.39	1.31	0.94	↓	↑	↓
		8 - MVR	66y	0.43	4.23	0.53	↓	↑	↓
		9 - ACVB	69y	0.11	6.01	0.80	↓	↑	↓
		10 - MVR	73y	0.69	0.67	1.10	↓	↓	↑
DDR2	Children	1 - CoA	6d	0.60	0.65	0.32	↓	↓	↓
		2 - CAVSD	7d	0.79	0.73	0.85	↓	↓	↓
		3 - ASD	33m	0.58	1.54	0.78	↓	↑	↓
		4 - HLHS	54m	0.12	16.52	1.47	↓	↑	↑
		5 - ASD2	8y	0.46	5.14	4.02	↓	↑	↑
	Adults	6 - AVR	60y	0.58	0.90	0.25	↓	↓	↓
		7 - AVR	60y	0.32	1.19	1.31	↓	↑	↑
		8 - MVR	66y	0.69	2.36	0.56	↓	↑	↓
		9 - ACVB	69y	0.07	3.69	1.35	↓	↑	↑
		10 - MVR	73y	1.21	0.55	1.43	↑	↓	↑
VIM	Children	1 - CoA	6d	0.93	0.29	0.24	↓	↓	↓
		2 - CAVSD	7d	0.69	1.04	0.62	↓	↑	↓
		3 - ASD	33m	0.65	0.98	0.49	↓	↓	↓
		4 - HLHS	54m	0.08	8.58	0.78	↓	↑	↓
		5 - ASD2	8y	0.43	3.42	1.44	↓	↑	↑
	Adults	6 - AVR	60y	0.90	1.15	2.55	↓	↑	↑
		7 - AVR	60y	1.26	0.31	0.63	↑	↓	↓
		8 - MVR	66y	0.41	3.21	0.67	↓	↑	↓
		9 - ACVB	69y	0.22	1.68	0.59	↓	↑	↓
		10 - MVR	73y	1.16	0.92	0.69	↑	↓	↓

Table 4.4 - Gene expression AF/CF in P0 and P0/P2 in both tissues comparison ratio for endothelial and mesenchymal markers in children and adults.

Table 4-4 - Results of ratio analysis between tissue and passages, for other markers.

				P0	AF	CF	P0	AF	CF
Gene	Age Group	Diagnosis	Age	CF/AF	P0/P2	P0/P2	CF/AF	P0/P2	P0/P2
CD105	Children	1 - CoA	6d	1.30	0.59	0.55	↑	↓	↓
		2 - CAVSD	7d	0.35	1.92	0.51	↓	↑	↓
		3 - ASD	33m	0.41	1.34	0.41	↓	↑	↓
		4 - HLHS	54m	1.36	0.86	2.62	↑	↓	↑
		5 - ASD2	8y	1.12	1.40	0.54	↑	↑	↓
	Adults	6 - AVR	60y	0.83	0.43	2.17	↓	↓	↑
		7 - AVR	60y	0.09	3.11	0.28	↓	↑	↓
		8 - MVR	66y	0.66	0.91	0.70	↓	↓	↓
		9 - ACVB	69y	0.09	2.15	1.10	↓	↑	↑
		10 - MVR	73y	1.13	0.61	1.10	↑	↓	↑
PDGRFA	Children	1 - CoA	6d	0.63	0.42	0.17	↓	↓	↓
		2 - CAVSD	7d	0.35	2.34	1.09	↓	↑	↑
		3 - ASD	33m	0.20	3.94	0.39	↓	↑	↓
		4 - HLHS	54m	0.11	18.57	1.00	↓	↑	↑
		5 - ASD2	8y	0.59	5.77	2.93	↓	↑	↑
	Adults	6 - AVR	60y	0.90	0.49	2.46	↓	↓	↑
		7 - AVR	60y	0.29	0.60	0.30	↓	↓	↓
		8 - MVR	66y	1.02	2.07	1.35	↑	↑	↑
		9 - ACVB	69y	0.06	1.76	0.54	↓	↑	↓
		10 - MVR	73y	0.96	0.74	0.49	↓	↓	↓
POSTN	Children	1 - CoA	6d	0.15	0.55	0.14	↓	↓	↓
		2 - CAVSD	7d	0.11	2.00	0.22	↓	↑	↓
		3 - ASD	33m	0.31	0.72	0.57	↓	↓	↓
		4 - HLHS	54m	0.04	17.75	0.77	↓	↑	↓
		5 - ASD2	8y	0.28	2.53	1.39	↓	↑	↑
	Adults	6 - AVR	60y	0.42	1.24	1.75	↓	↑	↑
		7 - AVR	60y	0.63	6.19	1.09	↓	↑	↑
		8 - MVR	66y	0.70	1.81	0.43	↓	↑	↓
		9 - ACVB	69y	0.42	4.08	1.11	↓	↑	↑
		10 - MVR	73y	0.18	0.55	0.89	↓	↓	↓

4.3.1. Comparison between AF and CF

In order to facilitate visualisation, a graphic representation of the CF/AF ratio, for P0, was made. Like tables, the red colour represents ratios where the expression in CF is higher than AF, and the colour blue indicates the opposite.

Comparison between AF and CF is analysed, only for P0, in further detail.

All the cardiac genes are expressed in CF, as shown in table 4.1. *GATA4* is not expressed in AF except for four patients, however the expression levels are quite low, as CF/AF ratio values are very high, shown in both table 4.1 and graph A of figure 4.5. Expression of *GATA5* is absent in AF, thus there is no value for CF/AF ratio. In one sample, GATA5 is also not found in CF, in both passages (patient 8) and as well as in passage. *GATA6* is found in both cell populations. *TBX5* is also expressed in both tissues, being more expressed in AF than CF for most samples. Regarding *TBX20*, extremely low expression is found in AF, thus the extremely high values of CF/AF ratio. Three patients, showed no expression in AF. In the other patients, expression in CF is distinctly higher.

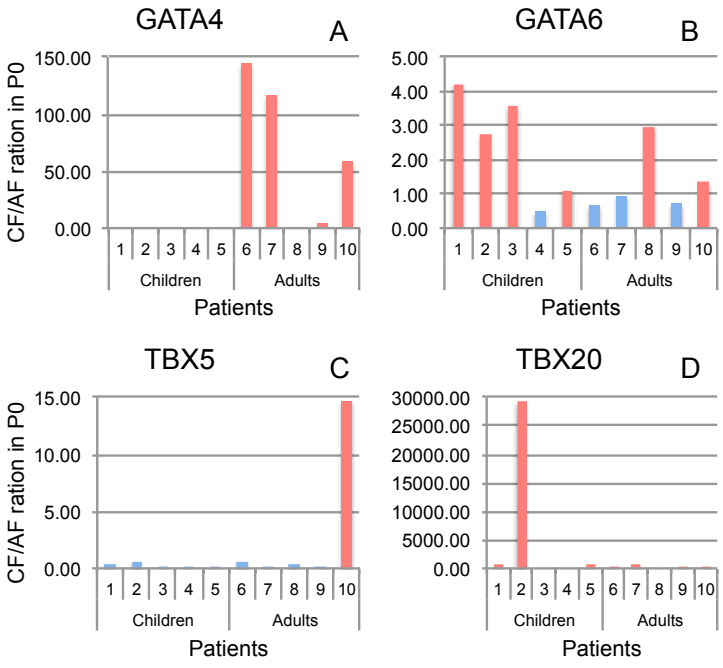

Figure 4.5 CF/AF ratio analysis of relative gene expression of the following cardiac genes *GATA4* (A), *GATA6* (B), *TBX5* (C), and *TBX20* (D) for P0. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero.

Concerning the gene expression of epicardial genes, the results may be seen in table 4.2. They are expressed in both cell populations. *TBX18*, represented in graph A of figure 4.6, is more highly expressed in AF than CF; however, there is a patient that has no expression detected in AF. As for *WT1* it is mostly absent in AF, as seen in graph B of figure 4.6, with the exception of three patients, who show expression. Expression of *TCF21* is present in both cell populations with the exception of one patient, where it is absent. Most of the samples show a higher expression in CF than in AF, as shown in graph C of figure 4.6.

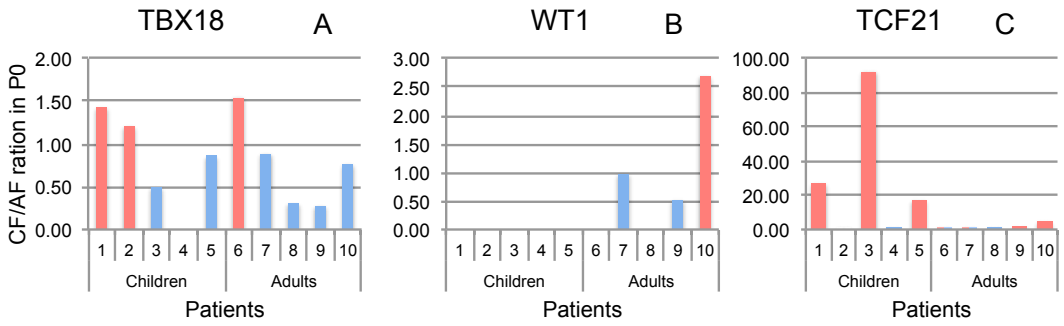

Figure 4.6 - CF/AF ratio analysis of relative gene expression of the following epicardial genes *TBX18* (A), *WT1* (B), and *TCF21* (C), for P0. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero.

Moving on to fibroblasts markers, *CD90*, *DDR2* and *VIM*, shown in table 4.3, they are all expressed in both cell populations but more highly expressed in AF, as seen in Figure 4.7.

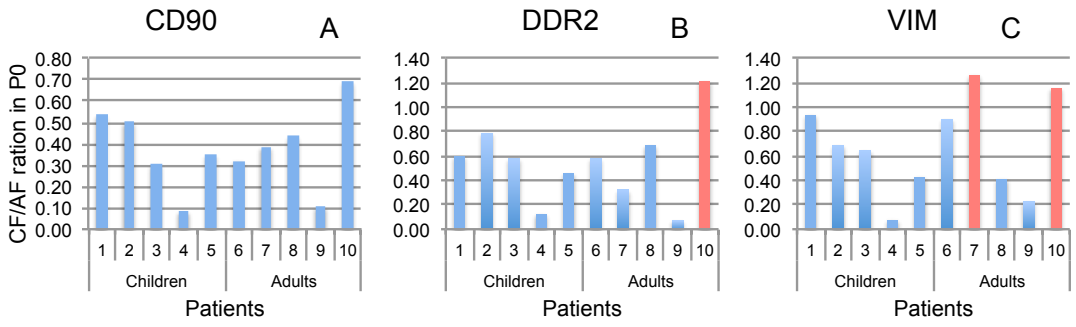

Figure 4.7 - CF/AF ratio analysis of relative gene expression of the following fibroblasts markers *CD90* (A), *DDR2* (B), and *VIM* (C), for P0. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent

children, and 6 to 10, adults. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero.

Finally, consulting table 4.4 and figure 4.8., regarding the results of gene expression of both endothelial (*CD105*) and mesenchymal (*PDGRFA* and *POSTN*) markers, it may be concluded that, similarly to the previous group of markers, they are all expressed in both cell populations and to a higher extent in AF.

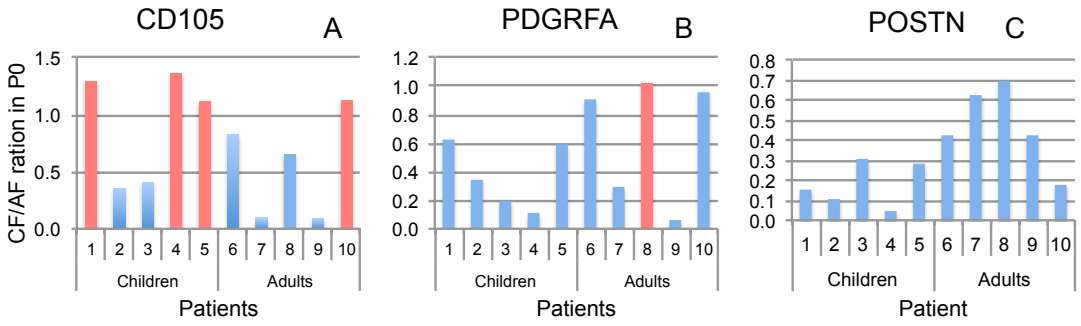

Figure 4.8 - CF/AF ratio analysis of relative gene expression of the following endothelial and mesenchymal markers *CD90* (A), *DDR2* (B), and *VIM* (C), for P0. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero.

4.3.2. Comparison between Passages zero and two

In order to facilitate visualisation, a graphic representation of the P0/P2 ratio, for AF and CF, was made. Like tables, the red colour represents ratios where the expression in CF is higher than AF, and the colour blue indicates the opposite.

Starting with the cardiac gene *GATA4*, since there is no expression in AF there is no difference in expression in the two passages, as it is shown in table 4.1, and graph A of figure 4.9. As for CF, expression diminishes with culture in most of the patients, as shown in graph A of figure 4.10. *GATA5* is absent in all AF, thus no difference in expression in the two passages is shown in table 4.1. *GATA5* is not found in both passages of CF of patient 8 (MVR) and in P0 of patient 6 (AVR). Considering CF, expression of *GATA5* tends to decrease with culture, as shown in graph B of figure 4.10. Concerning the expression of *GATA6*, in AF half of the samples increase, and the other half decreases, as shown in graph B of figure 4.9, thus no conclusions were made. There is an overall increase of expression in CF, for *GATA6*, as seen in graph C of figure 4.10. As for *TBX5*, a decrease of expression is mostly detected in AF (graph C figure 4.9), and the opposite in CF (graph D figure 4.10). As for *TBX20*, it was absent in AF samples of

two children and one adult, thus there is not a significant difference in expression in the two passages, as it is shown in table 4.1 and graph D of figure 4.9. The expression of the remaining genes, mostly rises with culture in AF, and in most of CF samples as well (graph E of figure 4.10).

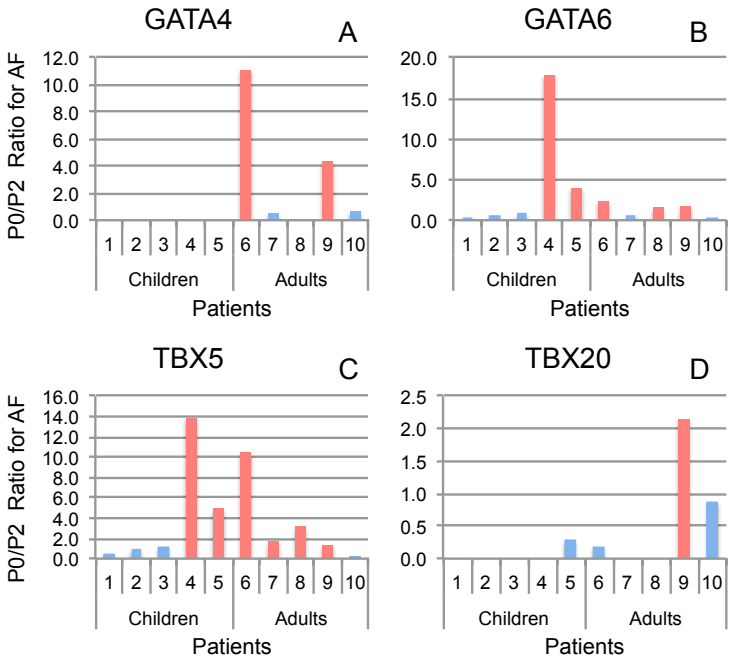

Figure 4.9 – P0/P2 ratio analysis of relative gene expression of the following cardiac genes *GATA4* (A), *GATA6* (B), *TBX5* (C), and *TBX20* (D), for AF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; AF, adipose derived fibroblasts.

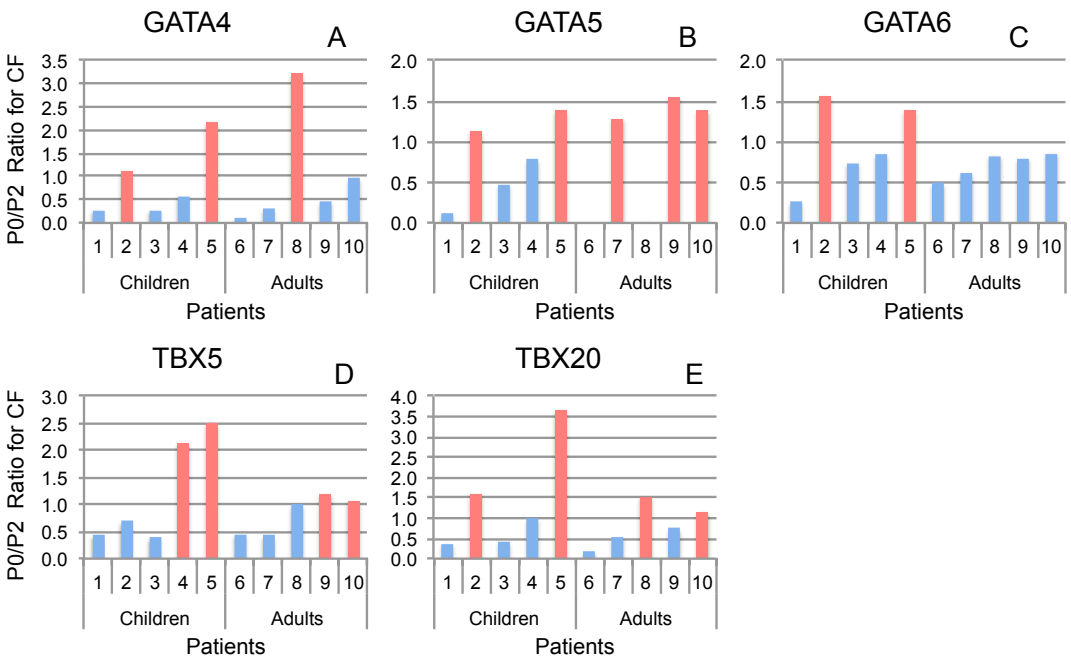

Figure 4.10 - P0/P2 ratio analysis of relative gene expression of the following cardiac genes *GATA4* (A), *GATA5* (B), *GATA6* (C), *TBX5* (D), and *TBX20* (E), for CF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; CF, cardiac fibroblasts.

Taking a closer look at the expression change, in culture, of epicardial genes, presented in table 4.2, concerning *TBX18*, there is an overall rise of expression in both cell populations, AF and CF, as seen in both graphs A of figures 4.11 and 4.12. There is no expression of *WT1* in more than half of the samples, thus not exhibiting a difference between the two passages in AF, shown in graph B of figure 4.11. In CF, graph B of figure 4.12, expression tends to rise in more than half of the samples with culture. *TCF21* presents a higher expression in P0 in most samples in AF, as seen in graph C of figure 4.11. However, in CF, graph C of figure 4.12, half of the samples rise with culture and the other half decreases.

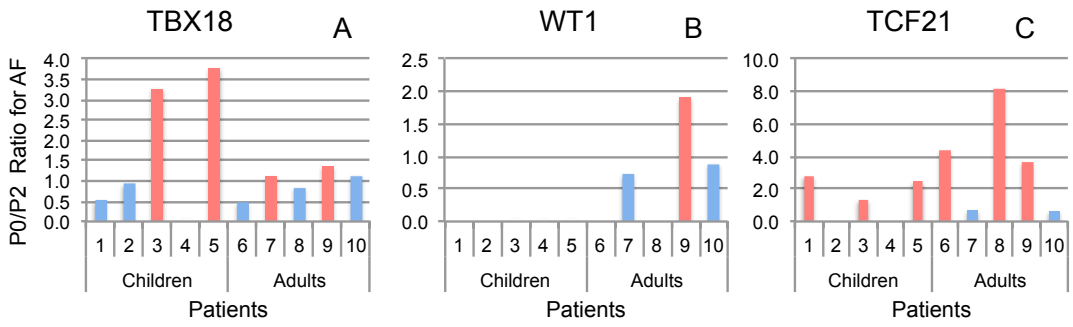

Figure 4.11 - P0/P2 ratio analysis of relative gene expression of the following epicardial genes *TBX18* (A), *WT1* (B), and *TCF21* (C), for AF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; AF, adipose derived fibroblasts.

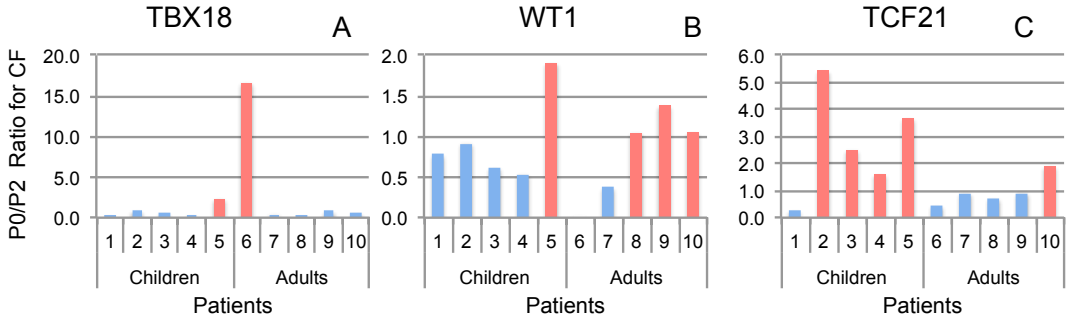

Figure 4.12 - P0/P2 ratio analysis of relative gene expression of the following cardiac genes *TBX18* (A), *WT1* (B), and *TCF21* (C), for CF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; CF, cardiac fibroblasts.

Moving on to fibroblasts markers, displayed in table 4.3, expression of *CD90* shows for AF, in graph A of figure 4.13, a higher expression in P0. The opposite occurs for CF, as shown in graph A of figure 4.14. For *DDR2*, it is mostly higher expressed in P0 in AF (graph B of figure 4.13), however in CF, (graph B of figure 4.14), the expression rises in only half of the samples and decreases in the other half, during culture. *VIM* has a higher expression in P2 in CF, as seen in graph C of figure 4.14. In AF, most samples show, however, a higher expression in P0, shown in graph C of figure 4.13.

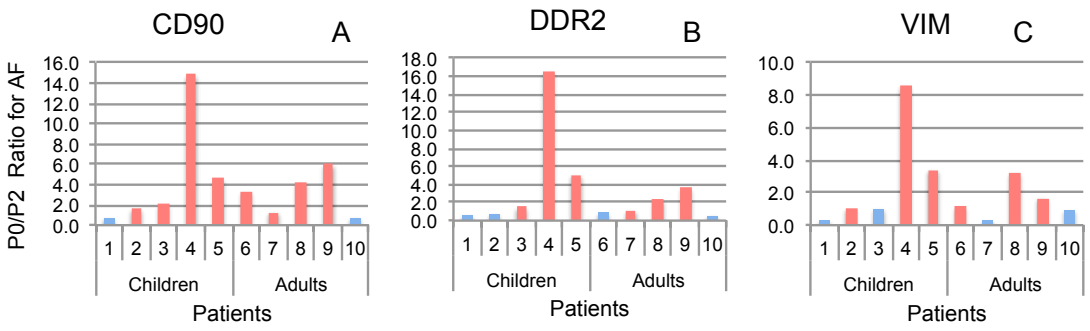

Figure 4.13 - P0/P2 ratio analysis of relative gene expression of the following fibroblasts markers *CD90* (A), *DDR2* (B), and *VIM* (C), for AF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; AF, adipose derived fibroblasts.

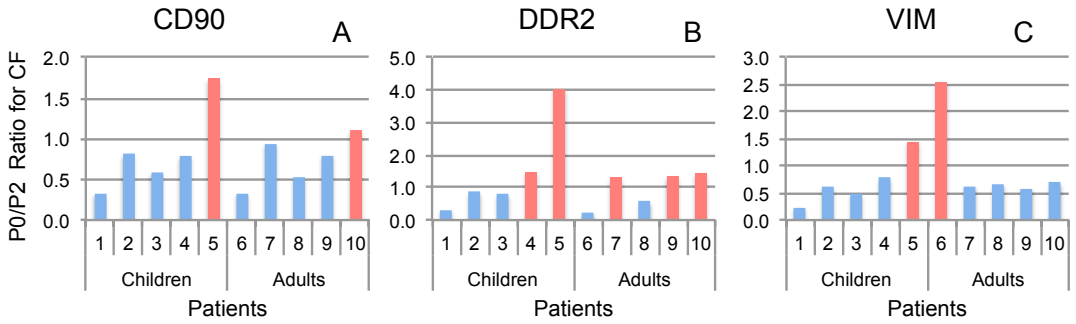

Figure 4.14 - P0/P2 ratio analysis of relative gene expression of the following fibroblasts markers *CD90* (A), *DDR2* (B), and *VIM* (C), for CF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; CF, cardiac fibroblasts.

At last, the results of the other markers are displayed in table 4.4. *CD105*, endothelial marker, is mostly expressed in P2 for CF, as seen in graph A of figure 4.16. For AF, represented in graph A of figure 4.15, the expression rises in half of the samples and drops in the other half with culture. Regarding the mesenchymal marker *PDGRFA*, half of the samples show an increase of expression CF, as seen in graph B of figure 4.16. For AF, represented in graph B of figure 4.15, is mostly expressed in P0. As for the other mesenchymal marker *POSTN*, for AF, it is also mostly expressed in P2, as shown in graph C of figure 4.15. However, in CF, represented in graph C of figure 4.16, in more than half of the samples, expression rises with culture.

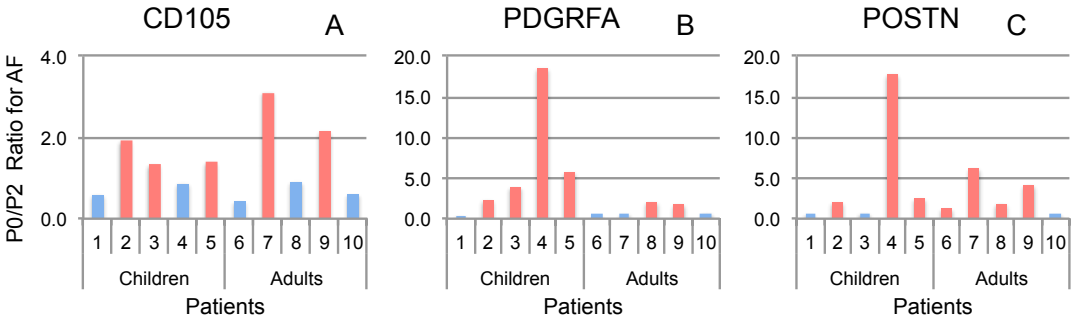

Figure 4.15 - P0/P2 ratio analysis of relative gene expression of the following endothelial and mesenchymal markers *CD105* (A), *PDGRFA* (B), and *POSTN* (C), for AF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; AF, adipose derived fibroblasts.

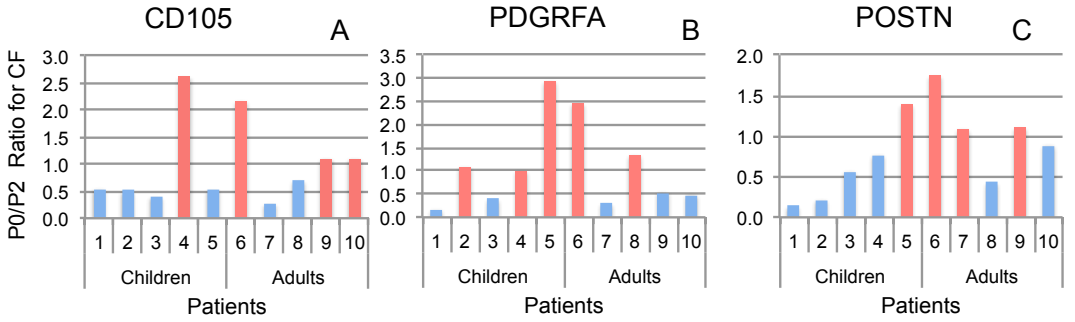

Figure 4.16 - P0/P2 ratio analysis of relative gene expression of the following endothelial and mesenchymal markers *CD105* (A), *PDGRFA* (B), and *POSTN* (C), for CF. In red, expression is higher in CF than AF. In blue, expression is higher in AF. The patients are numbered from 1 to 10. Patients 1 to 5 represent children, and 6 to 10, adults. Abbreviations: P0, passage zero; P2, passage two; CF, cardiac fibroblasts.

4.3.3. Comparison between Children and Adults

Regarding the children all genes tested are expressed in CF. Only the following ones are not expressed in AF: *GATA4, GATA5* and *WT1* as presented in tables 4.1 and 4.2. In regards to the adult patients, all genes are expressed in CF and the only genes that are not expressed in AF are *GATA5*, as demonstrated on tables 4.1.

Concerning cardiac genes *GATA4* is not expressed in AF samples from children. It is expressed in AF samples from adults, but the values are rather low, as shown in table 4.1 and graph A from figure 4.5. *GATA5* is not expressed in AF samples from either age group. *GATA6* appears to be more expressed in CF in children and in AF in adults, as seen in graph B of figure 4.5.

Regarding epicardial genes, *TBX18*, represented in graph A of figure 4.6, is mainly highly expressed in AF than CF, in adults. As for *WT1* it is absent in all AF samples from children, as seen in graph B of figure 4.6, and two from adult patients. In CF, expression tends to rise in mainly children samples, during culture (graph B figure 4.12) Expression of *TCF21*, is lower in AF children samples, resulting in high CF/AF ratio values, shown in graph C of figure 4.6 and in table 4.2. Regarding expression of *TCF21* in CF, shown in graph B of figure 4.12, adult samples rise with culture and children samples decrease.

Regarding fibroblasts markers, for *DDR2*, as far as expression is concerned, it is mostly higher in P0 in AF (graph B of figure 4.13), however in CF, (graph B of figure 4.14), the expression of mainly children samples increases with culture, as the of most adult samples decreases.

Concerning mesenchymal marker, *POSTN*, in CF, represented in graph C of figure 4.16, in more than half of the samples expression rises with culture (in most child samples it is more highly expressed in P2, and the adult samples in P0).

Genes not mentioned above were analysed but did not present conclusive results regarding this comparison of different age groups.

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5. DISCUSSION

Relating to the objectives of this work, having presented the ratio results, the analysis of different tissue derived fibroblasts and passages will be discussed.

However, to have a general overview of gene expression in cardiac and adipose fibroblasts and in heart tissue of the tested genes in all patients (mean of all children values, and the same for adults, disregarding different pathologies, explained in methods 3.5.5.), a heatmap presented in Figure 5.1. The colour red represents a higher expression, and blue lowest expression values, including zero.

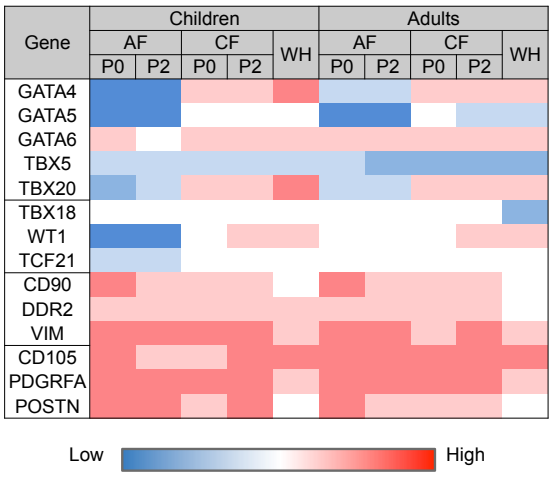

Figure 5.1 - Heatmap of gene expression of all tested genes in all patients, including both cell cultures (in both passages), and tissue control. Expression is displayed as low by the colour blue, and high by the colour red. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero; P2, passage two; WH, whole heart control.

5.1. Analysis of Comparison between AF and CF

Generally, out of the 10 patients in 14 genes, more expression is found in AF rather than in CF. Analysing the four ratio tables presented in the results, one third of samples (considering only the samples in which genes are expressed in AF) show a higher of expression in CF. It is interesting to conclude that the genes are expressed differently in different tissues. The percentages were calculated from ratio results from tables 4.1, 4.2, 4.3 and 4.4.

In figure 5.1, it is visible that cardiac and epicardial genes present an overall low expression. This is expected because cardiac genes are mainly active during embryonic development, according to literature. They show, however, a much lower expression in AF than in CF. This

was expected, as these genes are expressed in the heart, they were not expected to be present in AF. However, *GATA6* and *TBX18* have a slight higher expression in AF. *WT1* and *GATA4* stand out as being present only in half of samples. *GATA5* is absent in all AF samples.

Regarding table 4.1, of cardiac genes, more less half of the genes present a higher expression in CF. Note that *GATA5* is not expressed in AF in both age groups and *GATA4* is absent in AF in children. Not having values to compare with, because they are null, as they are dark blue in the heatmap of figure 5.1, this percentage is only considering the samples in which genes are expressed in both AF and CF, thus bringing *TBX20* a bigger contribution to gene expression. TBX5, on the other hand, exhibits a higher expression in AF.

Expression of all cardiac genes was expected in CF, but not in AF. However, *GATA6*, *TBX5*, *TBX20* and *GATA4* are expressed in AF. *TBX20* presents extremely low expression values, that could be considered null. However, having a specific peak at Ct 37 there is low expression and the activity may still be sufficient. Another point to consider is that these genes are mainly active during embryonic cardiac development, and their function postnatal is not yet known. This could explain the low expression values of genes of this group. In a study, samples from adult patients that underwent coronary artery bypass surgery were collected (64). A qualitative analysis was performed and results are in good agreement with the results for *GATA5* and *GATA6* in the present analysis. In contrast, a general expression of *GATA4* in all samples was found, which may be due to their approach, which only shows the endpoint of the PCR analysis but does not include a quantitative analysis (64).

As for the epicardial genes, less than half present a higher expression in CF, as shown in table 4.2. Note that WT1 is not expressed in AF from children.

Epicardial genes were tested to analyse the different CF epicardial lineage, as both have important functions in cardiogenesis. Expression values of epicardial genes are higher, compared to cardiac genes. This could be a clue for a further understanding of fibroblasts function, since they express, in different levels, the cardiac genes, meaning that they may play other roles in cardiac tissue than being just fibroblasts.

Comparing cardiac and epicardial markers to the rest of the genes - fibroblasts, endothelial and mesenchymal markers – a difference is visible in figure 5.1. They display a higher expression, as mostly squares are red and light red. Most even are higher expressed in AF than in CF, in contrast to cardiac and epicardial markers, except *DDR2*. This was expected because these are usually used to mark fibroblasts and the others are described as cardiac markers, not fibroblast markers.

Fibroblasts markers show that only 3 samples are more expressed in CF than in AF, as presented in table 4.3. As for endothelial and mesenchymal markers, shown in table 4.4, 5 samples are higher expressed in CF.

These two groups of genes were tested due to their ability to mark fibroblasts, regardless of their histogenic origin, since they mostly encode proteins present in the cell membrane, cytoplasm and ECM (table 5.1). In comparison, cardiac and epicardial genes, which are only present in the nucleus, were tested to compare their expression in CF. However, regarding fibroblasts, endothelial and masenchymal markers, it is interesting to observe that an overall higher expression is noticeable in AF. Besides similar morphology, this shows that fibroblasts exhibit topographical differentiation. And general fibroblasts markers, present in most fibroblast cells, gene expression patterns vary, depending on location, genes involved in lipid metabolism, cell signalling pathways that control proliferation, cell migration, and cell fate determination (22).

5.2. Analysis of Comparison between Passages zero and two

Generally, out of the 10 patients in 14 genes, more expression is found in P2 rather than in P0. Analysing the four ratio tables presented in the results, in AF, particularly 40% of samples (considering only the samples in which genes are expressed in AF) show a rise of expression when culturing the cells. In contrast, in CF (considering only the samples in which genes are expressed in CF), 60% show a rise of expression from P0 to P2. It is interesting to conclude that the different types of cell populations respond differently to culture *in vitro*. The percentages were calculated from ratio results from tables 4.1, 4.2, 4.3 and 4.4.

In figure 5.1, regarding the comparison between P0 and P2, there is not much variation between passages for cardiac and epicardial genes in children in both cell populations. With the exception of *TCF21*, that rises during culture in AF and *TBX20* and *TCF21*, that drop from P0 to P2 in CF. In adults' samples in AF do not show colour variation. In CF, there are more evident variations between the two passages, except for *GATA5* and *TBX5*.

Taking a closer look at the cardiac gene group, displayed in table 4.1, there is not a significant difference between AF and CF, having 50% and 60% respectively, where expression rises with culture. In AF, the main contributor for this percentage is *TBX20*, although most values are very low and 3 samples do not show expression at all. *TBX5*, on the other hand, brings an opposite contribution as almost all samples a higher expression of this gene in P0. It's worth noting that *GATA5* is not expressed in any AF sample and *GATA4* expression is absent in all AF cultures derived from children. In CF, all genes contribute quite evenly to this result, unless we take both age groups into consideration. Then, adults from *GATA4* and *GATA6* seem to strengthen expression with culture.

Regarding the epicardial group, presented in table 4.2, CF shows a 60% of rise of expression during culture, a similar value to that of cardiac genes group. In this case *TBX18* is the main contributor, along with children expressing *WT1* and adults *TCF21*, shown in graphs B from figure 5.4 and graph C from figure 5.5. In AF, again the expression of some samples of *WT1* was not detected, however all three genes contribute evenly, presenting almost half. Like in CF, the value is not far from the cardiac genes group.

Regarding fibroblasts and other markers, they display in figure 5.1 more different expressions between the two passages. In AF in children, expression of *VIM*, *CD105* and *POSTN* rise during culture. *PDGRFA* drops and *CD90* and *DDR2* do not vary. However, in CF, all markers remain stable, with the exception of expression of *CD90* that drops. In the case of adults both *CD90* and *DDR2* do not significantly vary in both cell populations. *CD105* and *PDGRFA*, do not show a change between passages in AF, as well as in CF. Vim drops during culture in AF, but rises in CF. The opposite happens to *POSTN*.

As for the fibroblasts markers group, there is a bigger discrepancy between tissues, as in CF, expression of 70% of samples rise with culture and in AF only a third rise, as demonstrated in table 4.3.

Concerning the last gene group, presented in table 4.4, 40% of AF samples have more expression in P2 and 50% in CF. Endothelial and mesenchymal genes *CD105* and *POSTN* from children biopsies have a greater weight in the CF percentage, as seen on graphs A and C, respectively, of figure 5.8. As for AF, *POSTN* shows a major expression in P0, rather than P2.

Having used mRNA, gene expression of RNA corresponds to what is expressed in the cell at the time of extraction, thus RNA extraction was accomplished to determine if there is a change in gene expression in fibroblast from P0 to P2. In conclusion, there is a change in gene expression from P0 to P2. But no general expression pattern was detected considering either cell populations (AF and CF) or age groups (children and adults). However, considering gene by gene it is possible to find specific patterns regarding age groups, namely in gene expression of *TCF21*, *DDR2* and *POSTN*, presented in table 5.1, all in CF samples. Maybe this could be a starting point for further studies, including a larger number of patients.

Table 5-1 - Age dependent gene expression patterns found.

Gene	Tissue	Age group	Expression during culture	Figure
TCF21	CF	Adults	↑	graph B of figure 4.12
		Children	↓	graph B of figure 4.12
DDR2	CF	Adults	↓	graph B of figure 4.14
		Children	↑	graph B of figure 4.14
POSTN	CF	Adults	↑	graph C of figure 4.16
		Children	↓	graph C of figure 4.16

5.3. Comparison between Children and Adults

In culture, a difference between child and adult samples is already established, as children's fibroblasts grow more rapidly than adult ones, due to cellular metabolism. Having this in mind, gene expression in children and adults is compared and analysed.

Concerning cardiac genes, *GATA4* is not expressed in AF samples from children. It is expressed in AF samples from adults, but the values are rather low, as shown in table 4.1 and graph A from figure 4.5. In a study, a general expression of *GATA4* in all samples was found, however only adult samples were used (64). Expression of this cardiac gene was only expected in CF, however in adults, it is expressed in AF. This could mean that this gene may have a function in AF in adults. The zinc-finger transcription factor coding gene *GATA6*, is higher expressed regarding other cardiac genes. It also appears to be higher expressed in CF in children and in AF in adults, as seen in graph B of figure 4.5.

Regarding epicardial genes, *WT1* it is absent in all AF samples from children, as seen in graph B of figure 4.6, and two from adult patients. Expression of *TCF21*, is lower in AF children samples, resulting in high CF/AF ratio values, shown in graph C of figure 4.6 and in table 4.2.

CHD are complex multifactorial diseases, in which the environment and genes interact. Purely genetic diseases are however caused by gene mutations and are generally simpler than multifactorial ones. However, in congenital disease, gene contribution weighs more than environment. Note that this may be due to a specific gene, or other genes that interact or influence the first one. Thus, it is expected to see more interesting results in children, than in adults. Even tough, in most cases, the genetic reasons are also still completely unknown, due to a variability of cardiac gene mutations and interactions with other genes, some genes are already known to be associated to certain CHDs, like *GATA4* for ASDII, for instance (48) (49) (50) (51). However still in many cases, the mutated genes are unknown and causal association has not been fully proven yet.

Regarding adult samples, the clinical diagnosis is important, since coronary and valvular heart disease are also multifactorial diseases, but heavily influenced by the environment, associated with aging and risk factors like high blood pressure (65), diabetes (66), high blood cholesterol (67) obesity or lack of exercise (68), poor diet, smoking (69) and excessive alcohol consumption (70). There is also an influence of the genetic background, that predispose people to suffer from coronary heart disease, though the exact variations remain poorly understood.

Considering the used genes, and taking into account the literature review, in the introduction, since some genes are associated with a couple of diagnoses, in particular of the children, it was decided to detail a heatmap for each of the cases. Considering these interrelationships, and the fact that CHDs are complex and more genes may be involved, a diagram, shown in figure 5.2,

depicts a brief overview of subsets of interaction of transcription factors and proteins of this study. The genes were divided into groups with distinct colouring.

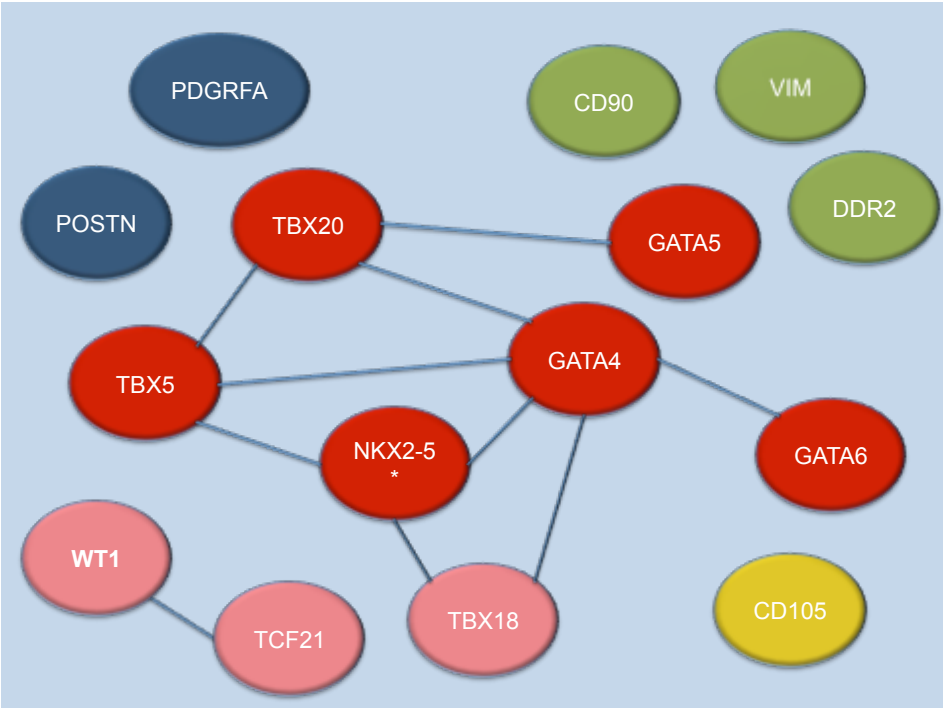

Figure 5.2. – Genes coloured in red are cardiac factors that drive cardiac differentiation during embryonic development. *GATA4* interacts with *NKX2-5*; *TBX5*; *TBX20* and *GATA6*. The pink genes represent epicardial factors. *WT1* interacts with *TCF21* and *TBX18* interacts with *NKX2-5* and *GATA4*. The blue genes represent mesenchymal markers and the yellow one an endothelial marker. Green stands for known fibroblasts markers. It is worth noting that the asterisk for *NKX2-5* means that this gene was not used in this study. However, since it has interactions with other tested genes, it was decided to include it in the diagram.

These interactions are included in the table in attachment D, complemented by more information from consulted literature. The table 5.1 presents a literature review of what each gene encodes, its role and cellular location. It also shows what types of cells or where in the body the gene is expressed. Also to which disease the gene is associated and with what other genes it interacts. Finally, for the genes expressed in the heart, it is shown at what embryonic stage it is expressed during cardiac development.

However, this work does not go into the molecular gene-to-gene detail, just simple finding that there is expression. Since this expression may be different in CF and AF and may present different values for each patient, it was considered interesting to detail the initial heatmap, shown in figure 5.1, and present information for all patients. For the sake of better visualisation, the heatmap was divided into groups of genes: cardiac, epicardial, and fibroblast, endothelial,

and mesenchymal markers, corresponding to figures 5.3, 5.4, 5.5 and 5.6, respectively. Adults and children were considered, with the respective diagnoses. Differences found were marked with green circles.

In attachment E, are presented some suggestions for future studies, discussed in the next paragraphs.

Cardiac genes

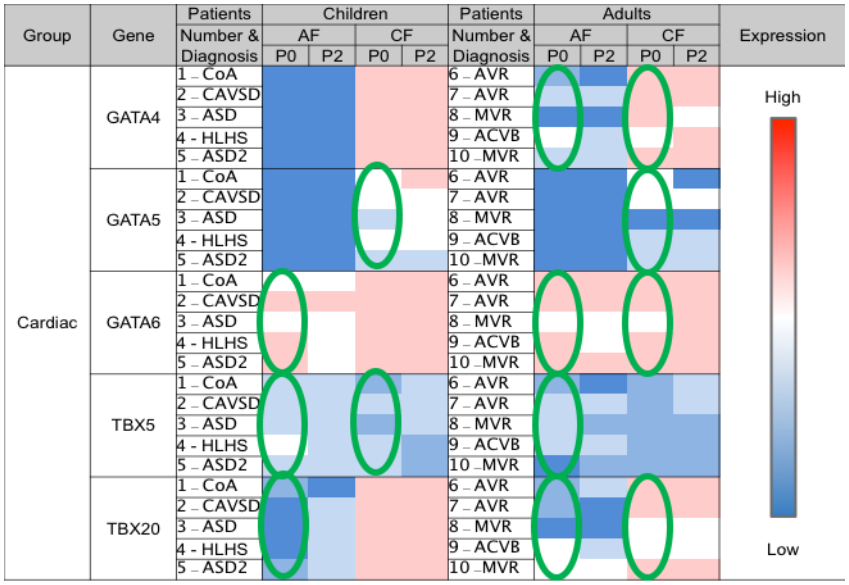

Figure 5.3 – Heatmap of gene expression of cardiac genes in all patients, considering their diagnoses. Expression is displayed as low by the colour blue, and high by the colour red. The green circles mark differences of gene expression found in one gene for different pathologies. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero; P2, passage two.

Concerning *GATA4*, all younger patients show in both cell populations, in figure 5.3, a spectrum of colour that matches the heatmap represented in figure 5.1. As for adults, clear different expressions are present, especially in AF, for each diagnosis. As for *GATA5*, dark blue colour remains for AF in both groups, confirming the absence of this gene. Expression of *GATA5* for children, remains stable overall, matching the white colour, except for patient 5, diagnosed with ASDII, presenting a lower expression with light blue colour in figure 5.3. Adult results are generally in agreement with the general heatmap. Concerning *GATA6*, it is interesting that HLHS and ASDII stand out with higher expression values. Patient 8, MVR, also is higher expressed than other patients. Regarding *TBX5*, both age groups are agreement with figure 5.1, varying mostly between passages. For *TBX20*, children's diagnoses present agreeable results. In adults, both AVR patients show the same colours relating to general heatmap. Both MVR and ACVB show differences.

Generally, ASD and ASDII show a similar expression of cardiac genes. However, in CF, ASDII shows a rise of *GATA4* and *TBX20*, shown in values of attachment C. A study demonstrated that *GATA4* and *NKX2.5* specifically cooperate in activating atrial natriuretic factor and other cardiac promoters, and physically interact both in vitro and in vivo (45). Also, *GATA4* is known to be directly associated to ASDII. As presented in figure 5.2, *GATA4* does interact with other cardiac genes like *TBX20* (47) (17). It could be possible to justify the different expression of *TBX20* in ASDII due to interaction of *GATA4* and *TBX20*. In the future, further studies of patients with ASDII could be conducted analysing these genes in parallel.

A different study reported mutations within human *TBX20* that were associated with a family history of CHD and a complex spectrum of developmental anomalies, including defects in septation, chamber growth, and valvulogenesis (53). Results from another study, present a significantly enhanced transcriptional activity of *TBX20*, which was further increased in the presence of co-transcription factors *GATA4/5* and *NKX2-5* (47). Another study raised the possibility that *GATA4*, *NKX2.5*, and *TBX5* function in a complex to regulate a subset of genes required for cardiac septal formation (48). Future studies may analyse gene expression of patients with diagnoses, mainly affecting cardiac chamber formation and septation, like HLHS and CAVSD, paying closer attention to *TBX20*, *GATA4/5* and *NKX2-5*. Regarding HLHS, the patient shows a moderately invariable expression of cardiac genes, having a higher expression of *TBX5* in comparison to other patients it is directly associated with *NKX2-5*. Although it was not tested in the present study, this difference could be explained by the interaction of *NKX2-5* with *TBX5* (46), shown in figure 5.1.

Concerning adult patients, *TBX20*, shown in figure 5.3, it has more expression in both AVR patients and in patient 10, diagnosed with MVR. A study on *TBX20,* by in-vivo cardiac functional analysis of *Tbx20* heterozygous mutant mice, shows that haploinsufficiency of this gene induced atrial dilation in all adult mice (15). This study points out the question whether it is just developmental in origin or if also reflects specific adult functions for *TBX20*. This reminds researchers and doctors to think of acquired cardiac disease in adults, instead of only behaviour and environment influence also of a possible a gene influence in the cardiac function. Also, not to only look to cardiac genes for embryonic development but to study their pathway in the adult life.

Epicardial genes

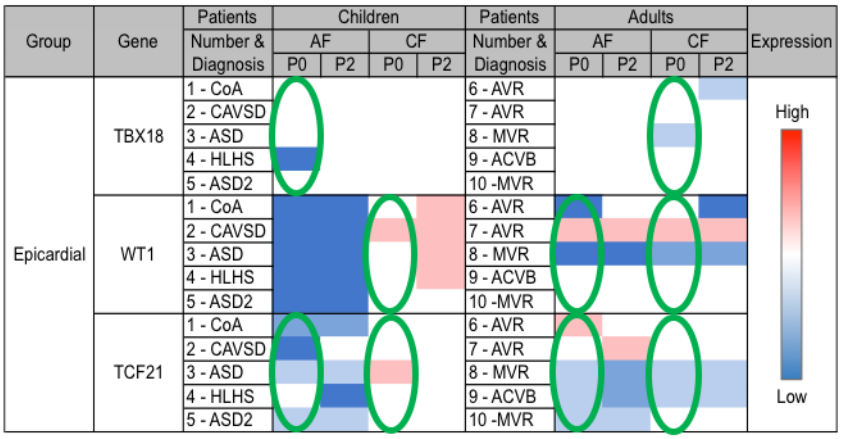

Figure 5.4 - Heatmap of gene expression of epicardial genes in all patients, considering their diagnoses. Expression is displayed as low by the colour blue, and high by the colour red. The green circles mark differences of gene expression found in one gene for different pathologies. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero; P2, passage two.

Starting with *TBX18* shows, in figure 5.4, an overall expression in agreement with heatmap of figure 5.1, for both age groups. Concerning AF, the value for HLHS was not detected, as it appears in dark blue. Concerning *WT1*, the children results agree with the general heatmap. ASDII stands out showing less expression than other patients. As for adults, more variable results are observable in figure 5.4. *WT1* was not detected in some passages of patient 6 (AVR) and AF samples of patient 8 (MVR). Patient 7 (AVR) shows the highest expression in both cell populations, as it stands out from other patients by the light red colour. On the contrary, patient 8, shows the lowest expression regarding this gene. Finally, analysing *TCF21*, detailed patient results are in agreement with the general heatmap of figure 5.1. In children, ASD stands out with the highest expression in CF and HLHS in AF. In adult patients 8 to 10 present an overall lower expression as seen in figure 5.4. Curiously, both AVR patients show a higher value of expression in AF than other diagnoses.

TBX18 gene codes for a member of an evolutionary conserved family of transcription factors that plays a crucial role in embryonic development (28). TBX18 can directly bind to the homeodomain transcription factor coding gene *NKX2–5* and the zinc finger protein coding gene *GATA4*, during heart development (71), as presented in figure 5.1. Knowing this and *GATA4* being associated with ASDII, as previously mentioned, future studies on this pathology may be conducting analysing these genes in parallel. A larger number of samples of children with ASDII would be required.

An interesting detail is that *WT1* was not expressed in AF in children, but it was expressed in AF in adults. In a different study, *WT1* expression was found in all of the visceral fat depots in mice, including epicardial in the thoracic cavity, but it was not detected in the subcutaneous adipose tissue, which is outside the body cavity (72). So, it is known that *WT1* is expressed in adipose tissue. The possible explanation for this curious absence of *WT1* in adipose tissue derived fibroblasts, is that the samples harvested from children are subcutaneous. The adipose tissue samples from adults must have been collected from deeper sites of the body, like visceral depots.

According to a recent study, *TCF21* should be used as a visceral white (epididymal) adipose tissue marker as it has an absolute distinctive ability primary cell culture in mouse (73). Expression of *TCF21* is however very low in AF either in both age groups. An explanation for that is that gene is more related to the anatomical positioning of a certain tissue, rather than to its function. Another cause would be the connection to *WT1*. Studies have demonstrated that *TCF21* expression in the proepicardial organ is dependent on *WT1*, meaning that in the absence of *WT1*, *TCF21* is also absent (74) (75). However, this interaction hasn't been proved for adipose tissue.

Regarding adults, *TCF21* transcription factor plays an important role in cell fate and differentiation in the developing embryonic coronary vasculature, as presented in table 5.2. This gene promotes cardiac fibroblast differentiation also in the postnatal and adult heart. (30) However increased expression of *TCF21* after myocardial injury has not been reported (27). In the present analysis, *TCF21* is expressed in all patients. Since the patients that went through mitral valve repair and most importantly a coronary bypass, stand for diagnosis that may indicate some art of failure at the coronary level, both these diagnoses may indicate a flaw at the activation and tissue repairing level once *TCF21* is involved in the process of coronary vascularisation. However, there is no further information available about these patients having suffered myocardial injury. Nonetheless, further studies on this type of behaviour in different diagnoses are necessary not only for understanding of the *TCF21* regulatory interaction but also towards repair and healing as this source of cells could enhance coronary revascularization after cardiac injury.

Fibroblasts Markers

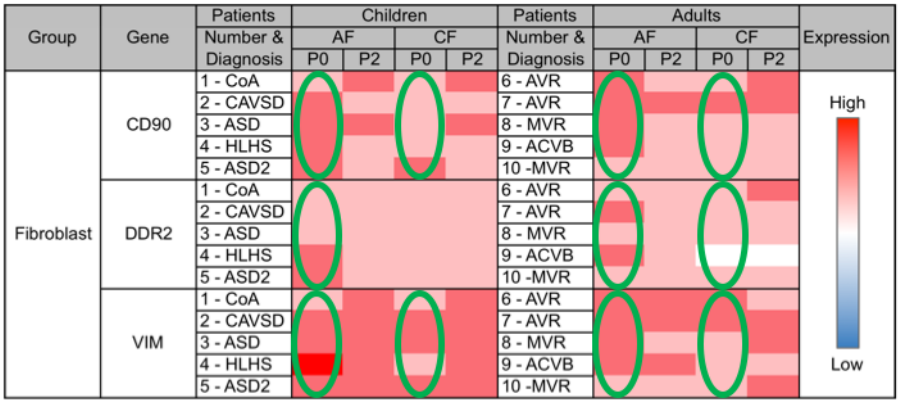

Figure 5.5 - Heatmap of gene expression of fibroblasts markers in all patients, considering their diagnoses. Expression is displayed as low by the colour blue, and high by the colour red. The green circles mark differences of gene expression found in one gene for different pathologies. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero; P2, passage two.

Taking a closer look at fibroblasts markers, *CD90* expression values for both age group patients agree with the heatmap of figure 5.1, as it is more highly expressed in AF. *DDR2* values are considerably stable, as presented in figure 5.5. In child patients 4 and 5, diagnosed with HLHS and ASDII respectively, stand out with higher expression values in AF. The same happens for adult patients 7 and 9, diagnosed with AVR and ACVB. This last one emerges from the age group with a lower expression in CF. Lastly, analysing expression of intermediate filament marker *VIM*, in children the CoA patients shows a lower expression and the HLHS patient a much higher expression regarding other patients, in AF, as presented in figure 5.5. Both show a lower expression in CF. Concerning adults patient 10 (MVR) stands out with an overall lower expression of *VIM*.

In conclusion, all three markers present higher expression values for AF than other cardiac and epicardial genes. Inside the group, *DDR2* has the lowest expression, *CD90* follows and VIM shows more expression.

The HLHS patient shows a higher gene expression pattern in AF only, relatively to other diagnoses. This could be an indicator that these cardiac diseases may interfere with these genes, not only at cardiac level, but also in fibroblasts from other tissues.

Endothelial and Mesenchymal markers

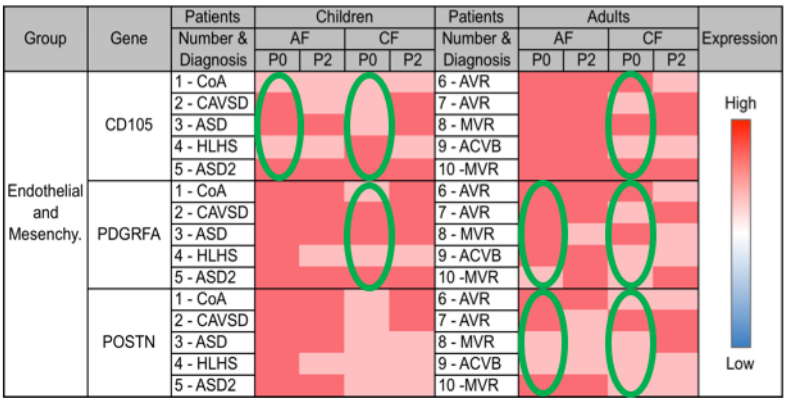

Figure 5.6 - Heatmap of gene expression of endothelial and mesenchymal markers in all patients, considering their diagnoses. Expression is displayed as low by the colour blue, and high by the colour red. The green circles mark differences of gene expression found in one gene for different pathologies. Abbreviations: CF, cardiac fibroblasts; AF, adipose derived fibroblasts; P0, passage zero; P2, passage two.

Starting with endoglin, it is higher expressed in HLHS and ASDII patients in CF, as presented on figure 5.6. Regarding adults, expression is agreeable to heatmap from figure 5.1, however ACVB patient exhibits a lower expression in CF than other patients. Concerning mesenchymal marker *PDGRFA*, overall expression matches the one in the general heatmap of figure 5.1. In children HLHS, present a lower expression in comparison to other patients. In adults, the same occurs for ACVB patient. In adults, there is a variation in CF regarding passages, as light red and red colour intercalate in figure 5.6. Finally, regarding periostin, overall expression in children matches the one in the general heatmap of figure 5.1. As for adults, patients 8 and 9 (MVR and ACVB) show lower expression in AF. In CF, patient 7 (AVR) stands out expressing higher values of *POSTN* in comparison to other patients.

The Endoglin gene, is expressed during early gestation (indicating human heart development and angiogenesis) but is also present during and even after birth (33). In our results, it is expressed after birth even up to 73 years. ASDII has four times more expression of *CD105* than ASD. This shows how genetic discrepancies at a genetic expression level are important and can distinguish two diagnostics with the same physical malformation, thus underlining again the relevance of genetic studies and how significant the malformations range is, even in the same diagnosis. However more samples would be necessary to withdraw conclusions, since this is derived that from two single values. Another point of view regarding endoglin, born infants have their cardiac vasculature already organized, but since they are children and they are still growing, the gene may still be active to facilitate vascularization. On the other hand, regarding

adults, this is a gene that might be activated when necessary to revascularization – in sequence of any heart condition - repair of cardiac tissue, thus it is expected that the expression in adults is relatively higher than children.

CD105 encodes a homodimeric transmembrane glycoprotein of the vascular endothelium involved in angiogenesis and regulation of endothelial cell proliferation (31) and (32). The lower values in CoA could point out the role exerted by this protein, since this diagnosis affects the cardiac vasculature, namely the aorta, and this protein is responsible for the formation of vessels during embryonic cardiac development, as presented in table 5.2.

In a different study, *PDGRFA* expression and secretion were up-regulated in obese mice and found to stimulate collagen synthesis (35). It contributed to fibrosis, although, while inflammation is frequently a major driver of fibrosis, *PDGRFA* activation was not associated with inflammation (35). Although last study is directed to patients with obesity, it can be concluded that *PDGRFA* stimulates collagen synthesis, the main component of fibroblasts. It is overall expressed in fibroblasts, especially in AF.

As for adults, in another study, fibroblasts did not seem particularly enriched with *PDGRFA* expression compared with whole atrial appendage (human atrium) or ventricular septum (human ventricle), but it was expressed in both tissues (cardiac and foreskin) (64).

Cardiac expression of *POSTN* stands out, as it appears to be constant in all diagnosis in children, figures 5.1, 5.6 and expression values in attachment C. This could be a good marker for future studies, as it seems that periostin not only is present in the heart but also is not affected by different pathologies in children. Since lack of *POSTN* would induce cardiac valve disease (in mice) (36) we could expect a lack of expression of this gene in CF in HLHS and CAVSD. However, this is not the case, since it is expressed in all patients. However, these two diagnoses show the highest values of expression in AF. Other than cardiac development, *POSTN* was identified to be potential novel mediator in adipogenesis, however its role has not been reported yet. Exploring its role in adipocyte biology might provide more insights in functions of adipocytes and if this gene (not altered in the heart) may be an indicator related to CHD HLHS.

Expression of *POSTN* in CF in adults behaves differently than in children. Since lack of *POSTN* would induce cardiac valve disease (in mice) (36) we could expect a lower expression of this gene in CF in AVR and MVR patients, however this doesn't happen. Again, these patients are expected to have these diseases due to age and not due to genetic mutation. This ECM component known fibroblast marker it is expressed in both heart and foreskin cells in another project (64). This gene encodes a secreted extracellular matrix protein that functions in tissue development and regeneration, including wound healing (fibroblasts) and ventricular

remodelling after myocardial infarction (76), however none of the tested patients had that diagnosis, thus not showing high levels of expression of activated fibroblasts, but resting ones.

6. CONCLUSION

6.1. AF vs. CF

Origin influence

Fibroblasts are heterogeneous and exhibit topographical differentiation, meaning fibroblasts from different anatomic sites have distinct characteristics and phenotypes, which can subsequently be maintained in vitro when fibroblasts are isolated from their surrounding environment and the influence of other cells. Additionally, fibroblasts from different anatomic sites have distinct transcriptional patterns. Of particular interest, ECM gene expression patterns can vary based on location of fibroblast harvest, as well as genes involved in lipid metabolism, cell signalling pathways that control proliferation, cell migration, and cell fate determination (22).

Influence of origin in CF

Cardiac fibroblasts represent one major cell population in the heart, and are responsible for many important cardiac functions. They also arise from different cardiac lineages, suggesting that they are primed to transdifferentiation, and may be directly involved in cardiogenesis. The objective of this study was to achieve a detailed analysis of the transcriptional activity of human cardiac fibroblasts, derived from right and left atrium, assuming a possible contribution of the fibroblast population in cardiac development. Of special interest in CF is that they express certain cardiac genes (*GATA4*, *GATA5*, *TBX20*) but also other markers, like epicardial genes *WT1* or *TCF21*, which are not or only at a very low level expressed in AF. This clearly underlines that fibroblasts of different histogenetic origin represent distinct populations, which are identified by a discrete gene expression profile.

Current problem in fibroblast studies

Regarding fibroblasts, there is a lack of specific markers that complicates the study of this lineage (6 p. 1) Research focussing on cardiac fibroblast is required to improve our understanding of cardiac function in normal and pathophysiological states (21). With this, techniques to model these cells for potential therapeutic applications, specialized therapies to treat congenital and acquired heart disease may be further developed (3). In conclusion, fibroblasts are perhaps the most underestimated cell population in the heart, and other tissues (21).

Control

Both tissues are affected by the same pathology, meaning that a heart condition cannot only affect cardiac derived fibroblasts but also adipose tissue derived fibroblasts. Thus, adipose samples do not serve as a control. A conclusion is reached towards development of the relationship between fibroblasts from these two different tissues, also at a cellular maintenance level, even though they are distinct differentiated cell types as they display distinct and characteristic transcriptional patterns (77).

Age group

So initially the main goal of this project was to compare fibroblast from adipose tissue and cardiac tissue from patients of the hospital, both children and adults. However, after using these genes, in children's cells, it was detected that, as expected, some were expressed only in CF, as it is the case for *GATA4* and *WT1* for children and *GATA5* for both age groups, and some in both cell populations, all the rest of tested genes.

Pathology

The pathology these patients have, especially in congenital heart disease, accuse the gene responsible for it, like *GATA4* for ASDII. And studies have mainly discussed expression of these cardiac genes in heart tissue, especially during embryonic development, and no other tissues. As cardiac genes were expected to be expressed only in the heart, and expression in adipose tissue derived fibroblasts was also found, an interesting proposal for future studies would be to analyse patterns of these genes in other fibroblast locations in parallel with heart fibroblasts from patients with the same diagnosis. As these genes may reveal patterns in other locations than the heart and by behaving differently they may serve as different indicators for the same pathology. Thus, an adipose tissue sample could be harvested for diagnostic instead of cardiac tissue. This could have a major impact on clinical; diagnostic or research utility and even later support therapeutic practice. As a more available tissue can be harvested and invasive intervention can be prevented. However, in the future, a much higher number of samples of specific diagnosis is required for this study.

However, in adults it is different. Further analysis of embryonic as well as adult cardiac genes with respect to their derivation, molecular regulation, and relationships to one another promises to enhance our understanding of heart function and repair. In the future, potential studies of gene influence and patterns in the heart may lead to new development of treatments, either of congenital heart disease and adult cardiac diseases. Since some cardiac genes are present in adult cardiomyopathies (15), this, questions whether these genes are expressed due to developmental in origin or if also present in specific adult functions.

6.2. Culture

Culture influence

The results have also clearly shown that gene expression may change dramatically during *in vitro* culture. With the alteration between P0 and P2, a conclusion is reached that there is in fact a genetic alteration, in which genes are higher or lower expressed. In AF, there is a tendency for fibroblasts markers to be higher expressed in P0 than in P2. In cardiac tissue the expression appears to remain rather stable during culture, however a higher expression in P2 is shown in the results.

Possible reasons

From P0 to P1, culture is cleaning the toxicity (also at DNA level) of the cells from the coming tissue, like potential contamination of other types of cells that will not be able to proliferate in culture as fibroblasts do, or even considering that, such young children that underwent surgery, must have been heavily medicated, thus cell culture could allow a stabilised medium to fibroblast growth. In P2, culture is stabilized thus allowing the possibility of having more biological material for further studies. Only if too many passages were to be done in culture there would be the risk to introduce DNA level mutations. It makes sense to make culture because I get stable genetic material and a more cell lineage homegeneity. Since the sample comes from a patient, and these cells are being grown cultured, the DNA stays the same and that allows a genetic study of that patient, with a specific diagnostic.

Analysis performed in this project – future studies

In this study, CF vs. AF was analysed in P0, since it is the passage closest to the *in vivo* situation. Therefore, in the future, it might be useful to obtain the cell population directly by FACS (Fluorescence-activated cell sorting) sorting *ex vivo* from a biopsy to determine their *in vivo* gene expression profile.

Future specific media optimization

Since the difference of the gene expression between both passages in each cell type is different, a suggestion for future studies would be to bet on an optimization of specific culture protocols for both adipose and cardiac tissue fibroblasts. For instance, in culture medium choice of extra ingredients for specific stimulus for each type of cell could be considered, as well different growing conditions, if necessary, in order to develop more specific cell lineages.

Technique used in this project – future studies

Concerning culture technique used in this project, methodology makes sense if we want to understand the influence of the gene. The gene is the one that changes culture. The utility of using methodology to understand gene expression in certain tissues adds value to clinical part. Cells do not change throughout culture; however, the gene may be more or less expressed depending on the type of gene (transcription factor, on/off, healing activated, etc.) and the patient (pathology). Medium and technique was repeated, gene expression is the one that changes. Testing different genes, because they are expressed in different quantities, times and tissues. This approach may bring clues to pathologies, analysing different genes for each diagnosis, since in most CHD cases the genetic reasons are also still not understood or unknown. Undoubtedly a study with a large number of samples for each diagnosis will have to be conducted for more consistent results. It makes sense to do cell culture, because, possibly, there is more availability of gene material and biological material availability, i.e. after analysis we can conclude that instead of cardiac samples, for some genes there are more accessible tissue for the same patient, like adipose tissue.

6.3. Children vs. Adults

Age group influence

Most noticeable difference within pathologies is the expression of *GATA4* and *WT1* for children and *GATA5* for both age groups, only in CF.

For a more detailed study and a bigger number of patients with same diagnostics is needed independently of the age. Apart from the main objective, there was the opportunity, having studied all 14 genes in further detail, to link certain pathologies. This interesting differential expression could bring another instrument to take the study of genetic etiology further.

CHD

Regarding the children, the type of information given by the results, serve as an argument to bet on genetic studies, in case there is a congenital syndrome with a directly associated gene. In that way, the different gene expression levels observed could point for candidate genes, or specific molecular genetic testing (like sequencing or comparative genomic hybridization) and contribute to understand the genetic disease etiology. Since the diagnostic and treatment are most of the time clinical if the genetic history is known, therapeutic treatment can be altered and improved for that specific disease.

Nowadays it is known that embryonic heart development is dependent on the regulated and complex activity of encoding members of the zinc-finger, homoeodomain, T-box and basic helix–loop–helix domain families (17). Future studies, concerning genetic pathways in the developing heart can lead to the understanding of the genetic basis of CHD (78). Along with other methodology and other techniques, networks and pathways that define the complex cardiac biology and behaviour must be studied and connected in order to better understand the cardiogenesis and enhance therapies directed toward cardiac repair and regeneration. (1)

An early diagnosis for children with cardiac malformations is another point to take into consideration. Cardiac anomalies can be detected in pregnant women by echocardiogram or ecodoppler as a clinical diagnosis. An amniocentesis, a procedure in which amniotic fluid is sampled for further genetic tests, may help know what type of cardiac malformation the foetus presents, other than visual clinical diagnosis. This may help regarding interruption of pregnancy if the diagnosis is severe, also with the child's life quality. Other than that, it may also inform parents of a genetic predisposition regarding future pregnancies.

Cardiovascular disease

These gene studies, may also apply to adults. It might bring clues to understand differential diagnostics in the adult, because genetic changes in children are major and minor in adults, but they might cause problems later on. Understanding the biology of the heart during cardiogenesis would allow studies of the adult heart to whether cardiac genes remain in the postnatal heart or may be reactivated during repair after cardiac injury (18). Especially regarding direct-reprogramming (79). Taking advantage of the wound healing aspect of fibroblasts, towards the adult population allowing the treatment for heart disease.

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ATTACHMENTS

Attachment A - List of consumables, software and used primers

Consumables

Material	Manufacturer
Disposable gloves (Powder-free 100)	Semper
Disposable gloves (Purple Nitrile powder-free	Kimberly-Clark
Erlenmeyer flasks (200ml, 500ml)	Neubert-Glas
Filter (30µm, 70µm)	BD Falcon
Cryo tubes (1ml, sterile)	Thermo Scientific
Petri dishes (150x15mm)	Omnilab
Tweezers	
Cell culture multiwell plates (6 well, 12 well)	Greiner Bio One
Surgical Disposable Scalpels	B.Braun
Pipette tips (0,1-10µl S, 0,1-10µl M, 2-20µl, 10-100µl, 100-1000µl)	Eppendorf
Safe-Lock Tubes (1,5ml, 2ml)	Eppendorf
Serological pipettes (sterile, 5ml, 10ml, 25ml)	Kisker
Syringe (2ml, 5ml, 10ml)	B.Braun
Sterile filter (0,22µm)	Carl Roth GmbH & Co. KG
Centrifuge tubes PP (sterile, 15ml, 50ml)	Corning Incorporated
Cell culture flasks (25cm^2, 75cm^2)	SPL Life Sciences
Falcon Tubes (15ml, 50ml)	BD Falcon
pLenti6.2-GW/EmGFP Expression Control Vector	Invitrogen
LightCycler® 480 Multiwell Plates 96	Roche
Ceramic Beads Kit 1.4 mm, 2 mL tubes	Precellys®

2mL tubes with 1.4mm ceramic (zirconium oxide) beads

Software

Designation	Manufacturer
Axio Vision (Version 4.8.2)	ZEISS
NanoDrop 2000/2000c	Thermo Scientific
Quantity One Chemidoc XRS (Version 4.6.9)	Bio-Rad
ABI Prism 7000 SDS 1.0	Applied Biosystems
LightCycler Software (Version 3.5)	Roche
Microsoft Excel 2010	Microsoft
SPSS 22.0	IBM

Primers Used

hu β-Actin_F382	5’-CCA ACC GCG AGA AGA TGA-3’
hu β-Actin_F478	5’-CCA GAG GCG TAC AGG GAT AG-3’
hu CD90_F1330	5’-AGG ACG AGG GCA CCT ACA C-3’
hu CD90_R1436	5’-GCC CTC ACA CTT GAC CAG TT-3’
hu CD105_F593	5’-AAT GCC ATC CTT GAA GTC CA-3’
hu CD105_R687	5’-GTG CCA TTT TGC TTG GAT G-3’
hu DDR2_F1528	5’-TAT GGC ACC CAC AAC CTA TG-3’
hu DDR2_R1628	5’-TGG CCA GGA GGA TAA AGA TG-3’
hu GATA4_F1513	5’-GGA AGC CCA AGA ACC TGA AT-3’
hu GATA4_R1607	5’-GCT GGA GTT GCT GGA AGC-3’
hu GATA5_F738	5’-CTC GTT CGG CCT CAG AAG-3’
hu GATA5_R813	5’-TGT TGG TCG TGT GGC AGT-3’
hu GATA6_F1524	5’-GCG GGC TCT ACA GCA AGA T-3’
hu GATA6_R1601	5’-TGG CAC AGG ACA ATC CAA G-3’
hu PDGRFA_F3163	5’-CCA CCT GAG TGA GAT TGT GG -3’
hu PDGRFA_R3245	5’-TCT TCA GGA AGT CCA GGT GAA -3’
hu POSTN_F989	5’-ATG GGA GAC AAA GTG GCT TC-3’
hu POSTN_R1076	5’-CTG CTC CTC CCA TAA TAG ACT CA-3’
hu TBX5_F888	5’-TGA TCA TAA CCA AGG CTG GA-3’
hu TBX5_R953	5’-GAT TAA GGC CCG TCA CCT TC-3’
hu TBX18_F1136	5’-CCT GGA ATT CCC AAG CAA G-3’
hu TBX18_R1236	5’-GAG GAG CCA GAC AAA AGG TG-3’
hu TBX20_F993	5’-AGT CAG ACA ACC CCA AAT CG-3’
hu TBX20_R1086	5’-ACC CAG GAA AAC TGG AAG AAG-3’
hu TCF21_F6	5’-CTC CTC TAC GGC CAC GAC T-3’
hu TCF21_R97	5’-TCG CAG AGT TGT GAG AAG GA-3’
hu VIM_F954	5’-AAA GTG TGG CTG CCA AGA AC-3’
hu VIM_R1027	5’-AGC CTC AGA GAG GTC AGC AA-3’
hu WT1_F1045	5’-GAG GAC GCC CTA CAG CAG-3’
hu WT1_R1133	5’-AGG TGG CTC CTA AGT TCA TCT G-3’

Attachment B - Standard curve for CD90

Standard curve for ABI Prism 7000

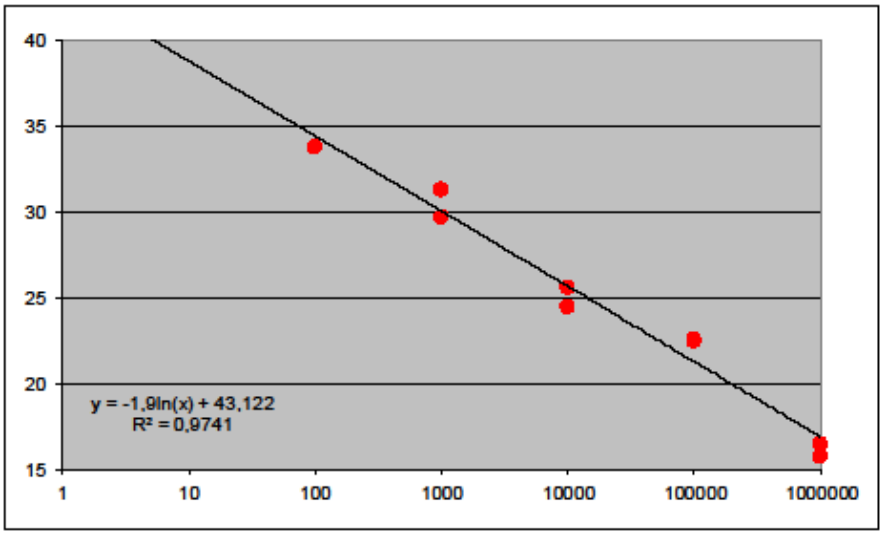
hu CD90 standard curve

Noise band set to 0.2

Date: 11.05.2016 Run: CC_007_2016-11-05_CD90:STCURVES

Primer forward: hu CD90_F1330 Primer reverse: hu CD90_R1436

sample	dilution	arbit. Units	ct value
1	undiluted	1000000	15,76
2	undiluted	1000000	16,49
3	1:10	100000	22,57
4	1:10	100000	22,44
5	1:100	10000	24,48
6	1:100	10000	25,58
7	1:1.000	1000	31,21
8	1:1.000	1000	29,61
9	1:10.000	100	
10	1:10.000	100	33,72
11	1:100.000	10	
12	1:100.000	10	
13	1:1.000.000	1	
14	1:1.000.000	1	



Efficiency = $10^{(-1/slope)}$ 2,05 Slope: -3,209

$AU = e^{(ct\ value - zp)/-slope)}$

		theoret. value
1	1795964	1000000
2	1223030	1000000
3	49853	100000
4	53384	100000
5	18244	10000
6	10225	10000
7	528	1000
8	1226	1000
9		100
10	141	100
11		10
12		10
13		1
14		1

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Attachment C - RT-qPCR Gene Expression Results

				AF		CF	
Gene	Age Group	Diagnosis	Age	P0	P2	P0	P2
GATA4	Children	CoA	6d	0.00E+00	0.00E+00	1.40E-02	4.92E-02
		CAVSD	7d	0.00E+00	0.00E+00	2.44E-02	2.21E-02
		ASD	33m	0.00E+00	0.00E+00	1.09E-02	3.89E-02
		HLHS	54m	0.00E+00	0.00E+00	1.29E-02	2.20E-02
		ASD2	8y	0.00E+00	0.00E+00	2.80E-02	1.29E-02
	Adults	AVR	60y	7.58E-05	6.90E-06	1.10E-02	8.96E-02
		AVR	60y	1.71E-04	3.52E-04	1.99E-02	6.48E-02
		MVR	66y	0.00E+00	0.00E+00	1.19E-02	3.68E-03
		ACVB	69y	1.91E-03	4.42E-04	7.65E-03	1.68E-02
		MVR	73y	4.97E-04	7.88E-04	2.92E-02	3.08E-02
GATA5	Children	CoA	6d	0.00E+00	0.00E+00	1.52E-03	1.26E-02
		CAVSD	7d	0.00E+00	0.00E+00	3.52E-03	3.11E-03
		ASD	33m	0.00E+00	0.00E+00	4.66E-04	1.00E-03
		HLHS	54m	0.00E+00	0.00E+00	1.92E-03	2.45E-03
		ASD2	8y	0.00E+00	0.00E+00	3.73E-04	2.67E-04
	Adults	AVR	60y	0.00E+00	0.00E+00	1.43E-03	0.00E+00
		AVR	60y	0.00E+00	0.00E+00	2.92E-03	2.29E-03
		MVR	66y	0.00E+00	0.00E+00	0.00E+00	0.00E+00
		ACVB	69y	0.00E+00	0.00E+00	2.64E-04	1.70E-04
		MVR	73y	0.00E+00	0.00E+00	7.48E-04	5.41E-04
GATA6	Children	CoA	6d	3.62E-03	7.89E-03	1.52E-02	5.93E-02
		CAVSD	7d	1.20E-02	2.20E-02	3.30E-02	2.11E-02
		ASD	33m	7.07E-03	8.57E-03	2.52E-02	3.40E-02
		HLHS	54m	4.37E-02	2.46E-03	2.10E-02	2.49E-02
		ASD2	8y	2.12E-02	5.34E-03	2.37E-02	1.70E-02
	Adults	AVR	60y	4.59E-02	1.93E-02	3.12E-02	6.34E-02
		AVR	60y	3.20E-02	5.34E-02	3.01E-02	4.92E-02
		MVR	66y	3.22E-03	2.01E-03	9.61E-03	1.16E-02
		ACVB	69y	1.64E-02	9.68E-03	1.17E-02	1.48E-02
		MVR	73y	1.41E-02	3.24E-02	1.89E-02	2.20E-02
TBX5	Children	CoA	6d	1.83E-04	3.09E-04	7.50E-05	1.72E-04
		CAVSD	7d	2.05E-04	2.22E-04	1.11E-04	1.62E-04
		ASD	33m	4.27E-04	3.85E-04	9.49E-05	2.41E-04
		HLHS	54m	1.54E-03	1.11E-04	2.11E-04	9.87E-05
		ASD2	8y	5.01E-04	1.01E-04	1.03E-04	4.10E-05
	Adults	AVR	60y	8.57E-05	8.20E-06	5.17E-05	1.11E-04
		AVR	60y	4.85E-04	2.88E-04	6.91E-05	1.62E-04
		MVR	66y	1.09E-04	3.42E-05	3.67E-05	3.68E-05
		ACVB	69y	1.42E-04	1.09E-04	2.56E-05	2.14E-05
		MVR	73y	5.30E-06	3.70E-05	7.72E-05	7.22E-05
TBX20	Children	CoA	6d	3.68E-05	0.00E+00	1.97E-02	5.16E-02
		CAVSD	7d	1.10E-06	3.59E-04	3.17E-02	1.98E-02
		ASD	33m	0.00E+00	2.39E-04	2.39E-02	5.42E-02
		HLHS	54m	0.00E+00	2.44E-04	1.84E-02	1.85E-02
		ASD2	8y	9.34E-05	3.33E-04	5.13E-02	1.40E-02
	Adults	AVR	60y	3.82E-05	2.05E-04	1.20E-02	5.76E-02
		AVR	60y	3.24E-05	0.00E+00	1.85E-02	3.35E-02
		MVR	66y	0.00E+00	0.00E+00	4.44E-03	2.93E-03
		ACVB	69y	1.56E-03	7.28E-04	3.43E-03	4.65E-03
		MVR	73y	1.04E-03	1.20E-03	2.89E-02	2.52E-02

				AF		CF	
Gene	Age Group	Diagnosis	Age	P0	P2	P0	P2
TBX18	Children	CoA	6d	1.43E-03	2.82E-03	2.05E-03	7.11E-03
		CAVSD	7d	3.07E-03	3.29E-03	3.72E-03	4.31E-03
		ASD	33m	7.01E-03	2.14E-03	3.46E-03	5.72E-03
		HLHS	54m	0.00E+00	1.93E-03	1.49E-03	4.01E-03
		ASD2	8y	7.12E-03	1.88E-03	6.17E-03	2.68E-03
	Adults	AVR	60y	1.32E-03	2.75E-03	2.03E-03	1.23E-04
		AVR	60y	4.13E-03	3.65E-03	3.67E-03	7.93E-03
		MVR	66y	1.81E-03	2.13E-03	5.65E-04	1.90E-03
		ACVB	69y	6.97E-03	5.13E-03	1.93E-03	2.52E-03
		MVR	73y	3.11E-03	2.71E-03	2.38E-03	4.06E-03
WT1	Children	CoA	6d	0.00E+00	0.00E+00	9.56E-03	1.23E-02
		CAVSD	7d	0.00E+00	0.00E+00	1.16E-02	1.26E-02
		ASD	33m	0.00E+00	0.00E+00	6.56E-03	1.07E-02
		HLHS	54m	0.00E+00	0.00E+00	6.16E-03	1.14E-02
		ASD2	8y	0.00E+00	0.00E+00	8.20E-03	4.31E-03
	Adults	AVR	60y	0.00E+00	2.19E-03	6.09E-03	0.00E+00
		AVR	60y	2.17E-02	3.00E-02	2.12E-02	5.60E-02
		MVR	66y	0.00E+00	0.00E+00	9.02E-05	8.70E-05
		ACVB	69y	9.87E-03	5.17E-03	5.11E-03	3.71E-03
		MVR	73y	3.47E-03	3.90E-03	9.35E-03	8.92E-03
TCF21	Children	CoA	6d	6.39E-05	2.27E-05	1.73E-03	6.35E-03
		CAVSD	7d	0.00E+00	1.92E-03	5.85E-03	1.08E-03
		ASD	33m	1.63E-04	1.23E-04	1.51E-02	6.03E-03
		HLHS	54m	3.59E-03	0.00E+00	2.93E-03	1.84E-03
		ASD2	8y	4.81E-04	1.94E-04	8.26E-03	2.25E-03
	Adults	AVR	60y	1.60E-02	3.66E-03	2.46E-03	5.61E-03
		AVR	60y	7.10E-03	1.03E-02	5.28E-03	5.88E-03
		MVR	66y	2.74E-04	3.35E-05	2.57E-04	3.66E-04
		ACVB	69y	3.48E-04	9.52E-05	6.71E-04	7.40E-04
		MVR	73y	5.13E-04	8.20E-04	2.60E-03	1.37E-03

				AF		CF	
Gene	Age Group	Diagnosis	Age	P0	P2	P0	P2
CD90	Children	CoA	6d	7.81E-02	1.19E-01	4.18E-02	1.25E-01
		CAVSD	7d	1.27E-01	7.82E-02	6.37E-02	7.87E-02
		ASD	33m	2.32E-01	1.14E-01	7.04E-02	1.20E-01
		HLHS	54m	5.61E-01	3.79E-02	4.85E-02	6.14E-02
		ASD2	8y	4.24E-01	9.28E-02	1.48E-01	8.50E-02
	Adults	AVR	60y	2.12E-01	6.41E-02	6.81E-02	2.04E-01
		AVR	60y	2.61E-01	1.99E-01	1.01E-01	1.08E-01
		MVR	66y	1.17E-01	2.76E-02	5.06E-02	9.55E-02
		ACVB	69y	1.13E-01	1.88E-02	1.22E-02	1.53E-02
		MVR	73y	6.01E-02	9.00E-02	4.14E-02	3.75E-02
DDR2	Children	CoA	6d	3.60E-02	5.50E-02	2.16E-02	6.66E-02
		CAVSD	7d	3.44E-02	4.73E-02	2.71E-02	3.20E-02
		ASD	33m	5.62E-02	3.64E-02	3.25E-02	4.14E-02
		HLHS	54m	1.68E-01	1.02E-02	2.07E-02	1.41E-02
		ASD2	8y	1.03E-01	2.00E-02	4.72E-02	1.17E-02
	Adults	AVR	60y	4.65E-02	5.16E-02	2.70E-02	1.10E-01
		AVR	60y	1.08E-01	9.10E-02	3.49E-02	2.66E-02
		MVR	66y	3.08E-02	1.31E-02	2.12E-02	3.79E-02
		ACVB	69y	1.24E-01	3.35E-02	8.95E-03	6.61E-03
		MVR	73y	2.53E-02	4.61E-02	3.07E-02	2.14E-02
VIM	Children	CoA	6d	8.11E-02	2.76E-01	7.57E-02	3.19E-01
		CAVSD	7d	1.78E-01	1.71E-01	1.23E-01	1.98E-01
		ASD	33m	1.82E-01	1.85E-01	1.17E-01	2.39E-01
		HLHS	54m	1.05E+00	1.22E-01	7.96E-02	1.02E-01
		ASD2	8y	3.64E-01	1.06E-01	1.56E-01	1.08E-01
	Adults	AVR	60y	1.29E-01	1.12E-01	1.16E-01	4.55E-02
		AVR	60y	1.09E-01	3.49E-01	1.37E-01	2.18E-01
		MVR	66y	1.91E-01	5.97E-02	7.89E-02	1.17E-01
		ACVB	69y	2.60E-01	1.55E-01	5.83E-02	9.83E-02
		MVR	73y	7.51E-02	8.14E-02	8.68E-02	1.26E-01

				AF		CF	
Gene	Age Group	Diagnosis	Age	P0	P2	P0	P2
CD105	Children	CoA	6d	1.99E-02	3.37E-02	2.58E-02	4.66E-02
		CAVSD	7d	1.73E-01	9.00E-02	6.06E-02	1.20E-01
		ASD	33m	1.48E-01	1.10E-01	6.02E-02	1.46E-01
		HLHS	54m	8.38E-02	9.76E-02	1.14E-01	4.36E-02
		ASD2	8y	1.65E-01	1.18E-01	1.85E-01	3.45E-01
	Adults	AVR	60y	1.52E-01	3.51E-01	1.26E-01	5.79E-02
		AVR	60y	6.11E-01	1.96E-01	5.79E-02	2.09E-01
		MVR	66y	1.93E-01	2.12E-01	1.27E-01	1.80E-01
		ACVB	69y	5.04E-01	2.35E-01	4.66E-02	4.25E-02
		MVR	73y	1.78E-01	2.91E-01	2.00E-01	1.81E-01
PDGRFA	Children	CoA	6d	1.12E-01	2.65E-01	7.02E-02	4.15E-01
		CAVSD	7d	8.16E-01	3.49E-01	2.85E-01	2.61E-01
		ASD	33m	9.50E-01	2.41E-01	1.90E-01	4.87E-01
		HLHS	54m	7.13E-01	3.84E-02	7.58E-02	7.57E-02
		ASD2	8y	5.86E-01	1.01E-01	3.48E-01	1.19E-01
	Adults	AVR	60y	2.03E-01	4.15E-01	1.84E-01	7.45E-02
		AVR	60y	3.01E-01	5.01E-01	8.89E-02	2.97E-01
		MVR	66y	1.21E-01	5.87E-02	1.24E-01	9.19E-02
		ACVB	69y	3.95E-01	2.25E-01	2.36E-02	4.37E-02
		MVR	73y	9.00E-02	1.21E-01	8.63E-02	1.76E-01
POSTN	Children	CoA	6d	3.27E-01	6.00E-01	4.92E-02	3.44E-01
		CAVSD	7d	4.92E-01	2.46E-01	5.25E-02	2.39E-01
		ASD	33m	1.19E-01	1.65E-01	3.64E-02	6.34E-02
		HLHS	54m	9.71E-01	5.47E-02	4.27E-02	5.57E-02
		ASD2	8y	2.92E-01	1.16E-01	8.16E-02	5.88E-02
	Adults	AVR	60y	1.27E-01	1.02E-01	5.32E-02	3.04E-02
		AVR	60y	1.97E-01	3.18E-02	1.24E-01	1.14E-01
		MVR	66y	9.44E-02	5.22E-02	6.59E-02	1.53E-01
		ACVB	69y	9.47E-02	2.32E-02	4.00E-02	3.61E-02
		MVR	73y	1.19E-01	2.16E-01	2.10E-02	2.34E-02

Attachment D – Review of 14 studied genes

Markers	Gene	Encodes	Plays a role in	Cellular locations	Marks	Associated with disease	Interacts with	Embryonic days	Reference
Cardiac	*GATA4*	Zinc-finger transcription factor	Cardiac development	Nucleus	CMs; gut; primitive endoderm; gonads	ASD2, TOF, VSD1, AVSD4	NKX2-5, TBX5, TBX20, GATA6	E7.5-E10.5	8, 11, 12, 17, 45, 46, 47, 48, 49, 50, 51, 57, 58, 65
	GATA5	Zinc-finger transcription factor	Cardiovascular development and in smooth muscle cell diversity	Nucleus	Endocardium	Congenital heart malformation	TBX20	-	9, 10, 12
	GATA6	Zinc-finger transcription factor	Gut, lung, and heart development	Nucleus	Heart	ASD9; AVSD5; TOF	GATA4	E13.5	8, 12, 13, 14, 54
	TBX5	T-box Transcription factor	Heart development and specification of limb pattern formation	Nucleus	Heart	Holt-Oram syndrome; congenital heart malformation	NKX2-5, GATA4, TBX20	E8-E10.5	7, 15, 17, 45, 46, 47, 48
	TBX20	T-box Transcription factor	Cardiac progenitor differentiation pathway and heart development	Nucleus	Heart,	ASD4; general congenital heart malformation, co-arctation of the aorta	GATA4, GATA5, TBX5	-	15, 16, 17, 45, 46, 47, 48, 53
Epicardial	*TBX18*	Transcription factor	Crucial role in embryonic cardiac development	Nucleus	Epicardium, in adult in SMCs of coronary vessels	Congenital anomalies of kidney and urinary tract	GATA4, NKX2.5	-	27, 28, 65, 71
	WT1	Krueppel-like Zinc-finger protein	Cellular development and cell survival	Nucleus	Kidney, heart, epicardial cells	Wilms tumor	TCF21	-	25, 66, 67, 68, 69, 75
	TCF21	Transcription factor of basic helix-loop-helix family	Cell fate and differentiation in the developing coronary vasculature	Nucleus	Epicardial cells, adipose tissue , kidney, lung	-	WT1	E9.5	29, 30, 67, 68, 69, 70, 73, 74
Fibroblast	*CD90*	Glycoprotein	cell adhesion and cell communication	Cell membrane	Mixed fibroblasts		-	-	6, 23
	DDR2	Tyrosine kinase receptor	communication of cells with their microenvironment, regulation of cell growth, differentiation, and metabolism	Cell membrane	SMCs; activated epithelial cells and specific bone marrow-derived cells	Ppondylometaepiphyseal dysplasia, short limb-hand type	-	-	22, 38, 39
	VIM	Intermediate filament protein	Structural function	Cytoplasm	Fibroblasts, SMCs, myoepithelial cells, pericytes and even neurones	-	-	-	6, 38
Endothelial and Mesenchymal	*CD105*	Homodimeric membrane glycoprotein	Angiongenesis	Cell membrane	Endocardium, epicardial and myocardial vessels	hereditary hemorrhagic telangiectasia	-	E14.5	31, 32, 33, 34
	PDGRFA	Tyrosine kinase receptor	organ development, wound healing, and tumor progression	Cell membrane	Fibroblasts,adipose tissue fibroblasts, cardiac fibroblasts, SMCs, CMs	Gastrointestinal stromal tumor, somatic	-	-	23, 35
	POSTN	Protein	Cell adhesion; tissue regeneration and healing	ECM	Fibroblasts, Aorta, In fibrous cardial skeleton and endocardial cushions	cardiac valve disease	-		6, 22, 38, 64, 72

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Attachment E – Some future studies suggestions

	Study	Gene	Diagnosis	Age Group	Description
1.	Periostin	*POSTN*	all	Children	Since same expression level in all pathologies → candidate for fibroblast marker, independent of diagnosis
2.	Tissue availability	Cardiac genes	CHD	Children	Study expression of genes in CF and AF from skin – discover pattern between CF and AF for determined gene in different pathologies
3.	Wilms' Tumor	WT1	-	Children and Adults	More detailed study, about why *WT1* is not expressed in AF in children, but is expressed in AF in adults
4.	GATA4	*GATA4*	-	Children and Adults	More detailed study, about why *GATA4* is not expressed in AF in children, but is expressed in AF in adults
5.	GATA5	*GATA5*	-	Children and Adults	More detailed study, about why *GATA5* is expressed in CF but not in AF in children and adults
6.	Coronary vasculature	*TCF21*	Coronary	Adults	Analysis of revascularisation after myocardial injury
7.	Fibroblast marker	*TCF21*	-	-	Gene expression analysis in AF (visceral, instead of dermal) and CF
8.	Endoglin	*CD105*	CoA, HLHS	Children	Study gene associated to angiogenesis and its behaviour in CHD which have malformations in the aorta, for instance
9.	Cardiac genes	*GATA4, NKX2.5, TBX5, TBX20* and associated genes)	-	-	How these genes correlate with each other in various CHD (separated) – different locations in the heart
10.	Septal formation	*GATA4, NKX2.5, TBX5* (and associated genes)	HLHS, ASD (and variations), CAVSD, etc	Children	Study gene expression in CHD affecting the septum and compare to results of embryonary development (of other studies)

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