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Systemic-to-pulmonary shunt malfunction due to neointimal hyperplasia in children with complex cyanotic congenital heart disease - Exploration of clinical, genetic and histopathological risk factors

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Owed to my family

Presented on

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Abbreviation list

Abbreviation	Expanded Form
ACE	Angiotensin-Converting-Enzyme
ASD	Atrial Septal Defect
α-SMA	Alpha Smooth Muscle Actine
AVF	Arteriovenous Fistula
CAD	Coronary Artery Disease
CS	Central Shunt
DES	Drug Eluting Stent
DILV	Double Inlet Left Ventricle
DKSA	Damus-Kaye-Stansel Anastomosis
DORV	Double Outlet Right Ventricle
EGFR	Epidermal Growth Factor Receptor
EGF	Epidermal Growth Factor
HLHS	Hypoplastic Left Heart Syndrome
IQR	Interquartile Range
LR	Left-Right
MAPCA	Major Aortopulmonary Collateral Artery
mBTA	Modified Blalock-Taussig Anastomosis
MMP-9	Matrix Metalloproteinase-9
PA + VSD	Pulmonary Atresia with Ventricular Septal Defect
PDA	Ductus Arteriosus
PCPC	Partial Cavopulmonary Connection
PLINK	PUTTY Link
PTFE	Polytetrafluoroethylene
RL	Right-Left
RVOT	Right Ventricular Outflow Tract
RVPA	Right Ventricle to Pulmonary Artery
SMC	Vascular Smooth Muscle Cell
SNP	Single Nucleotide Polymorphism
SP	Systemic-to-Pulmonary
TA	Tricuspid Atresia
TGA	Transposition of the Great Arteries
TIMP-1	Tissue Inhibitor of Metalloproteinases-1
TOF	Tetralogy of Fallot
VSD	Ventricular Septal Defect

Abstract

OBJECTIVES:
The goal of this dissertation was to quantify neointimal hyperplasia in systemic-to-pulmonary shunts in infants with complex cyanotic congenital heart disease, which are at risk for shunt obstruction prior stage II palliation, corrective or other follow-up procedures. Specifically, we aimed to determine the relationship between neointimal proliferation and early interventions, and we sought to identify clinical risk factors associated with neointimal formation. Moreover, our goal was to quantify epidermal-growth-factor-receptor (EGFR) and matrix-metalloproteinase-9 (MMP-9) in systemic-to-pulmonary shunts by immunohistochemistry and to identify risk alleles encoding related proteins.

METHODS:
Removed shunts were stained with Hematoxylin/Eosin and Richardson. Immunohistochemistry was performed with anti - EGFR, anti - MMP-9, anti - alpha-smooth-muscle-actin, and anti - CD68 antibodies. Non-parametric analysis and univariable regressions were conducted to identify clinical risk factors. Whole-genome single nucleotide polymorphism genotyping was performed on DNA extracted from patient blood samples, and allele frequencies were compared to identify risk alleles between the group of patients with severe shunt stenosis (≥40%) and the remaining group.

RESULTS:
Fifty-seven shunts were analyzed, including 39 modified Blalock-Taussig-Anastomoses, 8 right-ventricle to pulmonary-artery anastomoses, and 10 central shunts. Neointimal hyperplasia and shunt stenosis were found to be significantly correlated with each other and were higher in the group that required early interventions. Univariate linear regression revealed an association between smaller shunt size, lower acetylsalicylic acid (ASA) dosage, and enhanced neointimal proliferation.
EGFR and MMP-9 both correlated positively with the area of neointimal formation. Certain alleles in the epidermal growth factor (EGF) and tissue inhibitor of metalloproteinases-1 (TIMP-1) genes were found to be associated with increased neointimal hyperplasia.

CONCLUSIONS:
Neointimal hyperplasia in systemic-to-pulmonary shunts has been found to be associated with early interventions. Additionally, smaller shunt size, lower aspirin dosage and the proteins EGFR and MMP-9 have been identified as contributing factors to increased neointimal proliferation. Systemic-to-pulmonary shunts from patients carrying certain risk alleles in the genes encoding for EGF and TIMP-1 were found to exhibit increased neointima formation.

Publication List

1st Publication

“Neointimal hyperplasia in systemic-to-pulmonary shunts of children with complex cyanotic congenital heart disease”
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Introduction

Congenital heart diseases are the result of abnormal development of the heart during embryogenesis. They can occur as isolated anomalies or as part of a syndrome, and are thought to arise from genetic and/or environmental factors (Sun et al. 2015). The incidence of congenital heart defects is approximately 1% of all live births, and they represent the most common birth defect, with an increasing trend (van der Linde et al. 2011, Liu et al. 2019) . Each year, around 6,000 children are born with congenital heart disease in Germany (Bauer 2006). Today, more than 90% of patients with this condition reach adulthood (Speer 2019). Approximately 300,000 children, adolescents, and adults in Germany live with this underlying condition (Bauer 2006).

Congenital heart defects can affect any aspect of the cardiovascular system, including the heart valves, chambers and blood vessels. The clinical presentation and prognosis of these defects vary widely, ranging from mild or asymptomatic forms to severe and life-threatening conditions. Besides the more common non-cyanotic heart malformations, the cyanotic heart defects are less in prevalence but display generally more severe cardiac anomalies, which are primarily associated with a right-left shunt. This applies, for example, to the hypoplastic left heart syndrome, in which the systemic circulation can only be supplied via a persistent ductus arteriosus arising from the pulmonary circulation. This severe congenital heart defect displays the most frequent cause of cardiogenic mortality in neonates. (Haas 2019)

Complex cyanotic heart defects often require similar therapeutic procedures - without surgical intervention, these defects show uniformly high mortality. Surgical therapy is divided into corrective and palliative measures, depending on whether the heart defect can be corrected or a circulation adjustment is necessary. The best-known palliative procedure is probably the Norwood palliation, which has been successfully practiced since 1981 (Norwood et al. 1981). The traditional three-staged palliation consists of the first palliation at birth, a partial or bidirectional cavopulmonary compound (PCPC/BCPC) in stage II and finally a total cavopulmonary anastomosis (TCPC) at stage III (Ohye et al. 2016). (Haas 2021)

Systemic-to-pulmonary (SP) shunts are implanted in stage I palliation to ensure systemic and/or pulmonary perfusion until stage II palliation, corrective surgery, or other follow-up procedures. Norwood palliation continues to be one of the riskiest and most expensive surgical interventions performed, with a high reported in-hospital

mortality of 20% and overall-interstage mortality of 12 % after stage I palliation (Ghanayem et al. 2012, Ohye et al. 2016, Hamzah et al. 2020)

Several patient characteristics such as prematurity, low birth weight and unplanned reoperation have been determined as risk factors for interstage morbidity. However, the occurrence of neointimal hyperplasia in SP shunts and its impact on interstage morbidity has not yet been described (Siffel et al. 2015, Alsoufi et al. 2018, Erikssen et al. 2018, Mechak et al. 2018).

Neointimal hyperplasia is a pathological vascular remodeling due to migration and proliferation of vascular smooth muscle cells (SMCs) after vascular injury, which leads to intimal narrowing of biological and/or artificial grafts after their implantation (Bonatti et al. 2004). SP shunt stenosis due to neointimal formation in Norwood patients can lead to pulmonary malperfusion, causing severe cyanosis and oxygen desaturations (Wells et al. 2005, Haas 2021). Neointimal hyperplasia is the major cause of vascular access dysfunction in hemodialysis patients, which leads to higher morbidity and mortality of this critical patient cohort (Bonatti et al. 2004). Neointimal formation also causes “in-stent restenosis” in coronary arteries after cardiovascular interventions as balloon dilatation and stent implantation (Kleinedler et al. 2012, Lee and Ul Haq 2015). Artificial shunts used for arteriovenous fistulas (AVFs) in hemodialysis patients and SP shunts in children with complex cyanotic congenital heart defects are made of the exact same material – Polytetrafluoroethylene (PTFE). While AV-Fistula failure and in-stent-restenosis have received significant attention in the medical literature, the topic of shunt malfunction caused by neointimal hyperplasia in children with complex cyanotic heart disease has only been marginally reported. We assume similar mechanisms behind the formation of neointima in AVFs, in-stent-restenosis and SP shunts. Even though the pathophysiology is not fully understood, medical drug therapies as mTor-inhibitors in drug eluting stents (DES) target the post-interventional inhibition of inflammatory and proliferative pathways to prevent neointimal growth in coronary arteries (Costa and Simon 2005).

Currently, there is no drug therapy addressing neointimal hyperplasia in SP shunts in children with complex cyanotic congenital heart disease. We assume that the expression of neointimal hyperplasia has an impact on the outcome of interstage morbidity of children with SP shunts between stage I and II palliation.

The objective of our study is to improve our understanding of the pathophysiology behind the formation of neointimal hyperplasia in SP shunts in order to counteract the issue of high interstage mortality with new therapeutic modalities in the future.
Therefore, we quantified neointimal hyperplasia histologically and determined its influence on interstage morbidity reflected by unplanned early interventions. Further, we attempted to identify clinical risk factors associated with greater neointimal formation. As part of our second study, we aimed to determine the role of EGFR and MMP-9 in neointimal formation in SP shunts by immunohistochemistry. Moreover, we aimed to identify risk alleles of single nucleotide polymorphisms (SNPs) in related genes that might predispose for neointimal growing.

Complex cyanotic congenital heart defects

Congenital heart defects are categorized into cyanotic and non-cyanotic heart defects. A cyanotic heart defect is a complex congenital cardiac abnormality in which a right-to-left shunt causes venous blood to flow into the systemic circulation instead of flowing to the lungs. This results in the mixing of venous deoxygenated and arterial oxygenated blood, leading to central and peripheral cyanosis. Tetralogy of Fallot (TOF) and transposition of the great arteries (TGA) are among the most common cyanotic heart defects. Also, the group of univentricular heart malformations is considered to belong to the group of cyanotic heart defects. (Haas 2019, Speer 2019, Haas 2021)
The univentricular heart describes a heart defect in which the patient is born with only one ventricle that must supply both systemic and pulmonary circulation. The single ventricle can be the right or left ventricle, whereby in some cases, it cannot be clearly assigned as a right or left ventricle by morphologic criteria. The majority of patients have an accessory outlet chamber as a remnant of the other ventricle, which is only rudimentarily formed. The outlet chamber is connected to the main ventricle by a ventricular septal defect (VSD). In most cases, one great artery originates from the main ventricle and the other from the rudimentary outlet chamber. In addition, functionally univentricular hearts are described. These are cardiac defects in which two ventricles are basically present, but the function of the heart is equivalent to an univentricular heart and requires equal therapeutic procedures. Univentricular heart defects are among the cardiac anomalies with the worst prognosis. The clinical picture

of hypoplastic left heart syndrome (HLHS) is the most common heart defect represented in this study and will be described and discussed in detail. (Haas 2019, Haas 2021)

In the following, we will focus on the therapeutic modalities of cyanotic heart defects. The surgical treatment will be discussed more intensively for the individual heart defects, since it can differ significantly for each heart defect.

Drug therapy

Drug therapy can improve pulmonary perfusion by keeping the ductus arteriosus (PDA) artificially patent to ensure the vital left-right (LR) shunt. This is done with immediate postpartum prostaglandin infusion. Immediate prostaglandin injection also makes it possible to reopen the PDA secondarily after it has primarily occluded. (Haas 2021)

Catheter Interventional Therapy

If the ductus arteriosus is at risk to occlude despite prostaglandin administration, interventional or surgical measures must be taken immediately, because, without a LR shunt, the newborn is not viable due to not perfused lungs. In this case, stenting of the PDA is indicated. Catheter interventional creation of a shunt at the atrial level is also possible and describes the “Raskin's Balloon Atrioseptostomy”. Here, a catheter is inserted into the right atrium via a large vein and the ventricular septum is perforated thereon. This is appropriate in case of hypoplastic pulmonary vascularization and thus insufficient pulmonary perfusion to stimulate pulmonary vascular growth prior to definitive corrective surgery. (Haas 2021)

Surgical therapy

The primary therapy for the listed heart defects with complex cyanotic congenital heart defects is surgical therapy. The heart transplantation is one possibility for causal therapy. However, due to lack of availability of donor organs, in most cases, surgical conversion of the circulation or a correction is pursued. The different surgical modalities are described in the following for each heart defect individually. (Haas 2021)

Hypoplastic Left Heart Syndrome

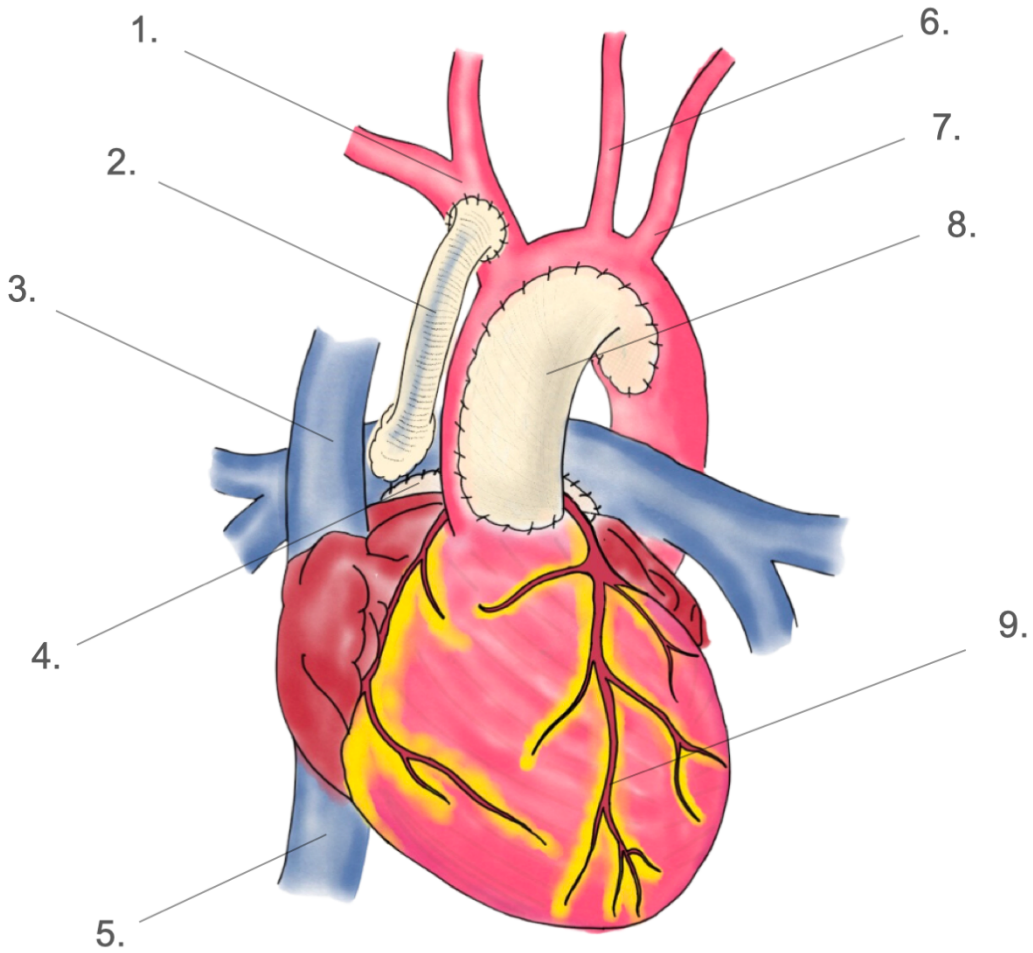
The hypoplastic left heart syndrome (HLHS) involves hypoplasia of the left ventricle in association with critical stenosis or atresia of the aortic and/or mitral valves. It is associated with hypoplasia of the ascending aorta and the aortic arch. The left ventricle is unable to supply sufficient antegrade blood to the aorta and to pump an adequate cardiac output for the systemic circulation. The right ventricle is responsible for perfusing the pulmonary and systemic circulation and supplying the coronary arteries. The left atrium can only empty via a sufficiently large atrial gap. Systemic perfusion depends on a right-to-left shunt throughout an open ductus arteriosus. The ascending aorta and coronaries are retrogradely perfused. (Haas 2019, Haas 2021)
A nonrestrictive atrial shunt and an open ductus arteriosus are essential for survival.
HLHS is evident in 1-2% of all cardiac defects and remains one of the defects with the worst prognosis. The Norwood procedure is the established surgical treatment that involves a three-staged surgical series based on Fontan's concept of converting the blood circulation. In the subsequent explanation, we will elaborate on the details of this procedure. (Haas 2019, Haas 2021)

Norwood I palliation: Standard surgery is performed on 5-7 days of life. To ensure systemic circulation, a "neo-aorta" is first formed and usually, the aortic arch is plastically dilated. The aorta, which is hypoplastic in this condition, is anastomosed with the pulmonary artery. Thereupon, systemic perfusion is ensured. Since the pulmonary artery and the aorta are now anastomosed, the heart no longer carries blood to the pulmonary vessels. This pulmonary perfusion is now secured by an artificially inserted PTFE shunt, which is implanted during the same surgery. In case of the modified-Blalock-Taussig Anastomosis (mBTA), the PTFE shunt is built between the right subclavian artery/brachiocephalic trunk and the right pulmonary artery branch. In addition, the Right-Ventricle-to-Pulmonary-Artery Anastomosis (RVPA) method shunt exists. Here, as the name suggests, a PTFE shunt is connected between the right ventricle and the right branch of the pulmonary artery. The advantage of this method is the increased diastolic pressure and improved coronary perfusion. The ventriculotomy, which can cause cardiac arrhythmias in the long term, and the increased volume load of the pulmonary circulation are disadvantages of this method. (Ghanayem et al. 2012, Haas 2021)

The PDA is closed during surgery and the atrial septum is resected for better blood mixing (atrioseptectomy).

The Hybrid Procedure is regarded as an alternative to Norwood I palliation (a combination of surgical and catheter interventional therapy). In this procedure, a stent is first implanted in the PDA instead of the SP shunt. In addition, the atrial gap can be optionally stented. To avoid pulmonary overflooding, pulmonary artery banding is performed in the same session. The Hybrid Procedure is also used to bridge the time until heart transplantation. (Haas 2021)

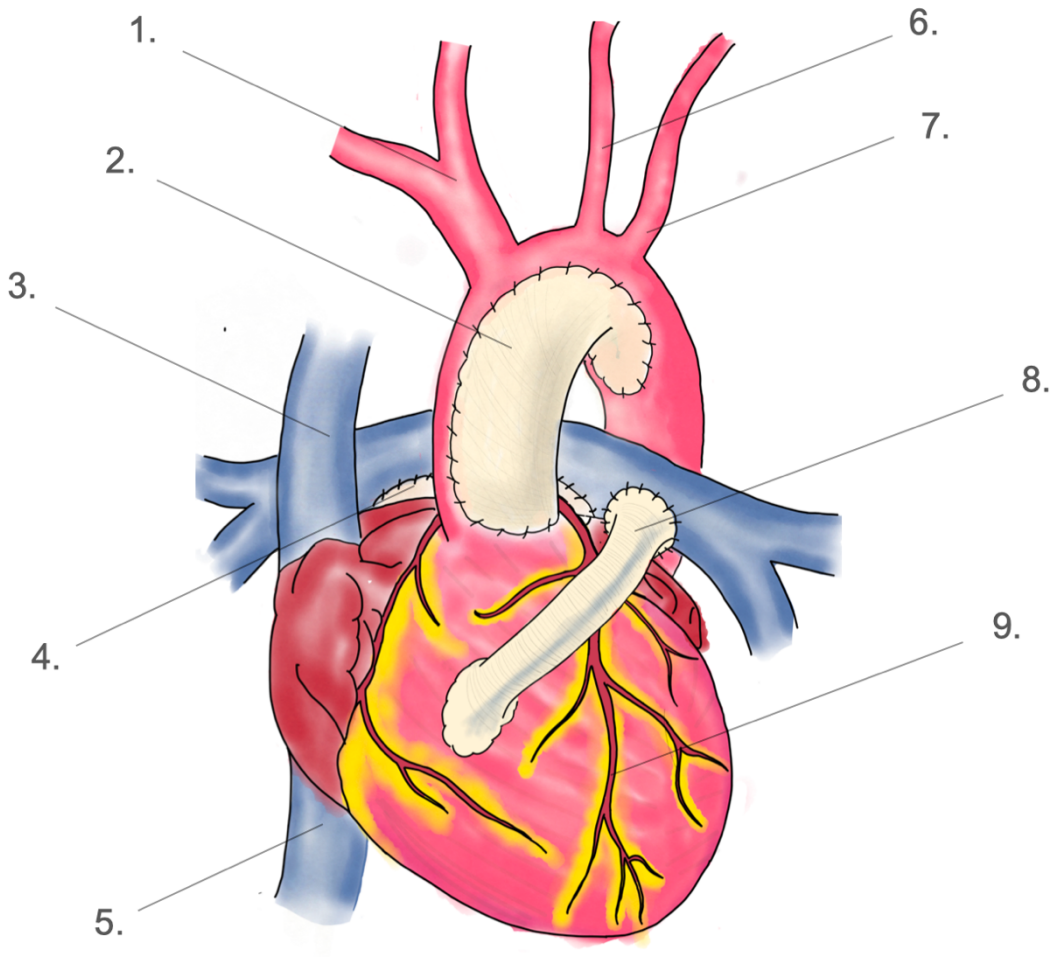
Figure 1. Modified-Blalock-Taussig Anastomosis – “mBTA”



Picture (Kottmann et al. 2022)

Figure 1: Schematic picture of a heart after Norwood stage I palliation with a modified-Blalock-Taussig Anastomosis. *1) Truncus brachiocephalicus 2) modified-Blalock-Taussig Anastomosis 3) Vena Cava Superior 4) Upper pulmonary artery trunk closed with patch 5) Vena Cava inferior 6) Arteria carotis communis 7) Arteria subclavia 8) Aortic patch with neo aorta 9) Ramus interventricularis anterior*

Figure 2. Right-Ventricle-to-Pulmonary-Artery Anastomosis – “RVPA”



Picture (Kottmann et al. 2022)

Figure 2: Schematic picture of a heart after stage I palliation of Norwood with a Right-Ventricle-to-Pulmonary Artery Shunt (RVPA). *1) Truncus brachiocephalicus 2) Aortic patch with neo aorta 3) Vena Cava Superior 4) Upper pulmonary artery trunk closed with patch 5) Vena Cava inferior 6) Arteria carotis communis 7) Arteria subclavia 8) Right-Ventricle-to-Pulmonary Anastomosis 9) Ramus interventricularis anterior*

Norwood II: Also called bidirectional cavopulmonary anastomosis (BCPC) or "Glenn operation”. The surgery is timed at the age of 4-6 months. During this surgery, the

superior vena cava is connected to the equilateral pulmonary artery. Since blood from the upper half of the body now flows passively to the lungs, the SP shunt can be removed. (Haas 2021)

Norwood III: This procedure is also called Total Cavopulmonary Anastomosis and is usually performed at the age of 2-3 years. Here, the inferior vena cava is now also connected to the pulmonary artery. All venous blood from the superior and inferior vena cava flows now passively into the lungs. This condition represents the Fontan circulation. (Haas 2021)

Pulmonary atresia with ventricular septal defect

Pulmonary atresia is a complex cyanotic congenital heart defect describing an obstruction of the pulmonary valve, in which a membranous structure is usually found in place of the pulmonary valve. It is often accompanied with a maldevelopment of the RVOT (Right ventricular outflow tract) and MAPCAs (Major aortopulmonary collateral arteries). Further, pulmonary atresia is usually associated with a large VSD. If there is no connection between systemic and pulmonary circulation, the situation is life-threatening. (Haas 2019, Haas 2021)

The purpose of surgery is to restore continuity between the right ventricle and the pulmonary arteries in one correction and to close the VSD. The surgical objective is defined based on the presence of pulmonary vascular hypoplasia. Sometimes a correction cannot be performed until adequate growth of the pulmonary vessels has been achieved. In this case, with ductus-dependent pulmonary perfusion, an SP shunt in the form of a central shunt (CS) is usually implanted and the VSD is closed. Another option is to expand the RVOT using a patch without closing the VSD. When an adequate size of the pulmonary vessels has been achieved by the two previous palliative measures, the "Rastelli Surgery" is performed. This involves an implantation of a conduit between the right ventricle and the pulmonary artery, VSD closure, and eventual SP shunt takedown. (Haas 2019, Haas 2021)

In the less common multifocal pulmonary perfusion, the multiple aortopulmonary collaterals are anastomosed to a single vessel corresponding to the pulmonary artery in an operation called "unifocalization". In the same session, a flap-bearing conduit is

placed between the RV and PA. In this variant, the VSD is not initially occluded, as it still functions as an overflow valve due to increased pulmonary resistance.

Pulmonary atresia with intact ventricular septum

In pulmonary atresia with intact ventricular septum, the RVOT is completely occluded. Instead of the valve opening, a membranous structure usually occludes the RVOT or it is muscularly atretic, resulting in an equivalent pathology. Because the ventricular septum is intact, the right ventricle has no normal outlet. It can empty only retrogradely via tricuspid regurgitation or, less commonly, via a connection between the right ventricle and the coronary arteries (myocardial sinusoids). At the atrial level, a right-to-left shunt is necessary for survival. Pulmonary perfusion is dependent on an open ductus arteriosus. Rarely, pulmonary perfusion occurs via MAPCAs. (Haas 2019, Haas 2021)

If the right ventricle is large enough, this heart defect can be treated with a commissurotomy of the pulmonary valve and, if necessary, with an infundibulectomy. If the infundibulum cannot be reconstructed, a valve-bearing homograft can be used. In addition, the creation of a central SP shunt is often indicated, since the usually untrained right ventricle cannot carry the pulmonary perfusion on its own. This PTFE shunt connects the aorta and the pulmonary artery. In this case, the shunt and the atrial septum of some patients can be closed again and a biventricular heart anatomy can be established. (Haas 2021)

If the right ventricle is not large enough, a SP shunt is placed to ensure pulmonary perfusion. In most cases, the RVOT and the pulmonary valve are also dilated by commisurectomy. If the pulmonary arteries are large enough, conversion to Fontan is then performed out in a two-stage principle by BCPC and TCPC. In favorable cases, a so-called "1.5 Fontan situation" can be attempted. Here, the pulmonary artery originating from the right ventricle is not occluded and only an upper cavopulmonary anastomosis is created. In this case, the pulmonary circulation is supplied via both the cavopulmonary anastomosis and the right ventricle. This results in volume decompression of the right ventricle. In some cases, by opening the RVOT and implanting a CS, an increased size of the right ventricle can be achieved, so that a subsequent biventricular correction can be attained after closure of the shunt.

Likewise, a Fontan principle is indicated in "right ventricular dependent coronary perfusion". In this case, the coronary arteries are supplied only by myocardial sinusoids or fistulas. Here, opening the RVOT and/or pulmonary valve would cause a pressure reversal in the right ventricle so that the coronaries are no longer supplied and myocardial ischemia occurs. (Haas 2021)

Tetralogy of Fallot

Tetralogy of Fallot (TOF) is one of the most common congenital heart defects, in which the combination of the following pathologies is present: VSD, obstruction of the RVOT, aorta "riding" over the VSD and right ventricular hypertrophy. Absence of fusion and incorrect placement of the conus septum and ventricular septum during embryonic development results in a VSD and an obstruction of the RVOT. In addition, a shift of the aortic root towards the right ventricular outflow tract is present, causing the aorta to "ride" on the edge of the ventricular septum. Right ventricular hypertrophy develops due to the pressure load on the right ventricle that occurs as a result of right ventricular outflow tract obstruction and the VSD. (Haas 2019, Haas 2021)

Therapeutically, surgical correction is the first priority; palliative measures are rarely necessary. During the correction, the VSD is closed with a patch so that the overriding aorta is assigned to the left ventricle. The RVOT is unloaded with infundibulostomy or valvulostomy/commissurotomy if infundibular stenosis or pulmonary stenosis is present. A patch dilatation of the pulmonary annulus is considered in some cases. A palliative approach with a SP shunt is indicated when the pulmonary vessels are too hypoplastic to provide pulmonary perfusion. This procedure bridges the time to definitive correction until the pulmonary vessels have reached the minimum size to ensure pulmonary perfusion. (Haas 2019, Haas 2021)

Transposition of the great arteries

After tetralogy of Fallot, Transposition of the great arteries (TGA) is the second most frequent cyanotic heart defect. In the most common form of this complex congenital heart defect, called the “dextro-TGA”, the aorta originates from the right ventricle, whereas the pulmonary artery originates from the left ventricle. The ascending aorta and pulmonary artery run parallel in their proximal segments and the usual crossing of

those vessels is missing. The aorta runs to the right/anterior to the pulmonary artery. Large and small circulations are connected in parallel in a d-TGA, that's why survival is only possible through lateral connections between the two circulations (e.g. ASD, VSD, PDA). If none of these LR shunts are present, prostaglandin administration, catheter-interventional stenting of the PDA, or Rashkind balloon atrial septostomy is indicated. (Haas 2021)

Possible procedures that will only be mentioned due to the scope of the presented dissertation are: "Rastelli operation", "Reparation a l'etage ventriculaire", "Atrial reversal according to Mustard or Senning", "Damus-Kaye-Stansel operation" and the most commonly practiced "Switch operation" with VSD closure. Again, if pulmonary perfusion is too low in hypoplastic pulmonary vessels, SP shunt implantation may be considered as an interim solution to definitive correction. (Haas 2021)

Tricuspid atresia

Tricuspid atresia (TA) ranks third in the list of cyanotic heart defects after tetralogy of Fallot and d-TGA. In TA, there is anatomically no direct connection between the right atrium and the right ventricle. There is only a fibromuscular bulge, or more rarely, a fibrous membrane instead of the tricuspid valve. Blood supply to the right ventricle can only be obtained through a VSD. A right-to-left shunt at the atrial level is necessary for survival. The classification is based on the position of the great vessels and the extent of pulmonary perfusion. (Haas 2021)

The therapeutic goal of TA is usually a palliative approach with circulatory conversion according to the Fontan principle. The extent of lung perfusion determines the palliative approach. In case of decreased lung perfusion, a SP shunt is implanted first, which is commonly the mBTA. In case of increased pulmonary perfusion, the Damus-Kaye-Stansel Anastomosis (DKSA) is usually considered. During the DKSA the ascending aorta and pulmonary artery are anastomosed. The pulmonary artery is separated from the trunk. Again, a mBTA is usually implanted for pulmonary perfusion. If there is a decrease in pulmonary resistance after these surgeries, further procedures enabling the Fontan circulation are indicated (BCPC, TCPC). (Haas 2021)

Double Outlet Right Ventricle

A double outlet right ventricle (DORV) describes an anatomic condition in which the pulmonary artery and the aorta originate entirely or mainly from the right ventricle by the constant presence of a VSD. The DORV is not a uniform clinical presentation and the hemodynamic situation can vary depending on the location of the VSD in relation to the great vessels and the presence or absence of pulmonary stenosis. The occurrence of a RVOT obstruction is variable. The hemodynamics can resemble those of other heart defects such as d-TGAs, TOFs, or a large VSDs. Usually, a correction surgery is performed in case of a DORV. This is largely dependent on the position of the VSD in relation to the great arteries, the presence of pulmonary stenosis, associated cardiac anomalies and the position of the great arteries in relation to each other. The resulting different variants of DORV result in many therapeutic surgical options for correcting this heart defect. Possible surgeries include: "Switch operation with VSD closure", "Rastelli operation", "Reparation a l'etage ventriculaire", “Atrial reversal according to Mustard or Senning", “Damus-Kaye-Stansel Anastomosis”. (Haas 2021)

Double Inlet Left Ventricle

A double inlet left ventricle (DILV) is a type of congenital heart defect where both the left and right atria empty into the left ventricle, which serves as the single functioning pumping chamber for the systemic perfusion. This condition is classified as a single-ventricle lesion, meaning that only one ventricle is effectively pumping blood throughout the body. The right ventricle is hypoplastic or absent and functions only as an outlet chamber which drains directly into the aorta or pulmonary artery. Both atriae communicate with the ventricle through a single atrioventricular valve. The two chambers are connected to each other with a VSD called “Foramen bulboventriculare”. There is a large LR shunt with rapidly developing pulmonary hypertension. The DILV with the aorta originating from the hypoplastic right ventricle is the most common form of single-ventricle-lesions (75%). The therapeutic procedure is similar to the procedure for HLHS. (Haas 2021)

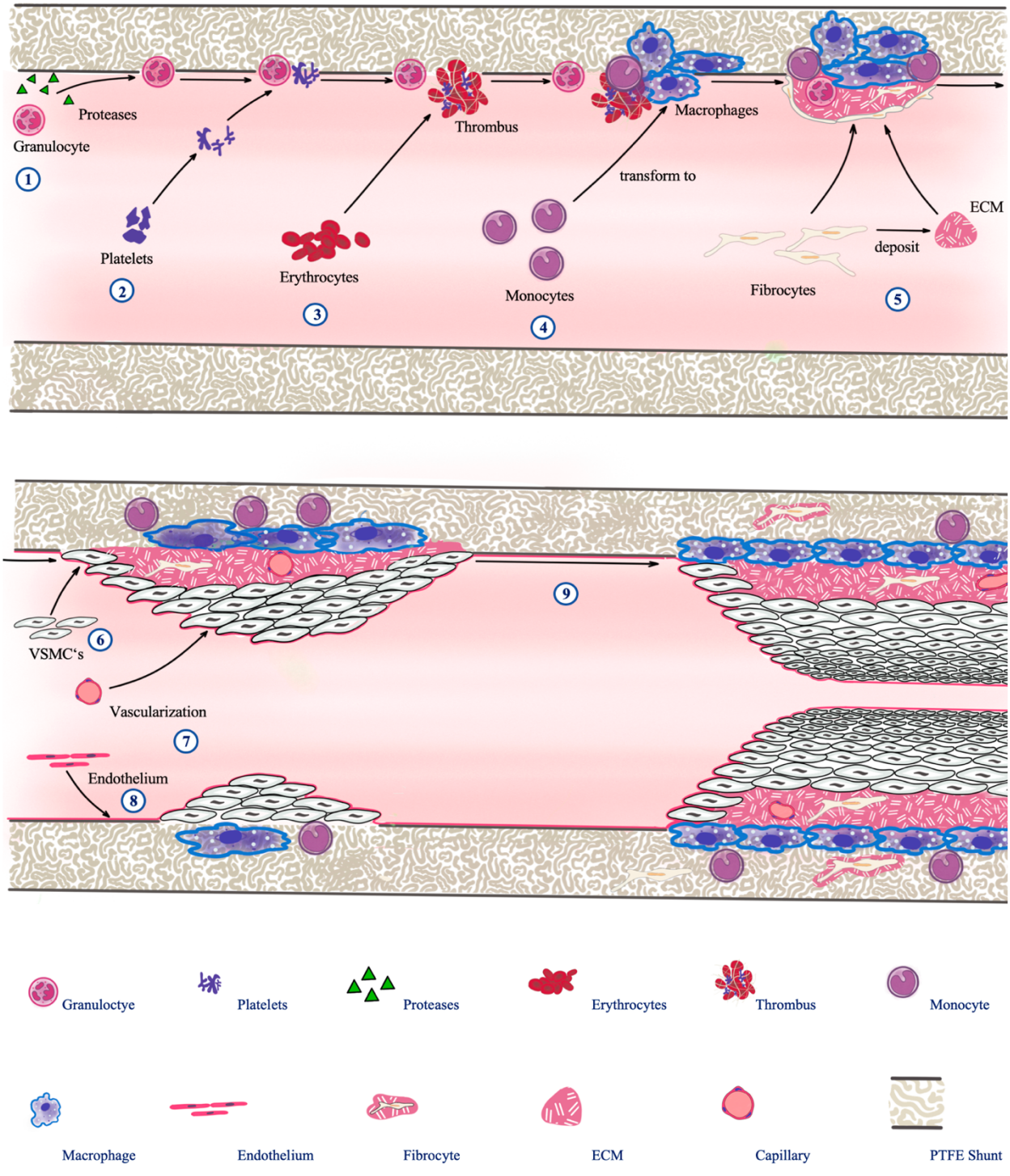
Neointimal Hyperplasia

Neointimal hyperplasia is a type of tissue formation in blood vessels or artificial grafts, such as PTFE shunts, which occurs in response to vascular irritations or injuries, such as surgical or catheter interventions, increasing the risk of vascular obstruction (Stracke et al. 2002, Li et al. 2007). The development of neointimal hyperplasia is thought to be a complex multifactorial process involving multiple cell types and signaling pathways (Stracke et al. 2002, Li et al. 2007). It is characterized by the migration and proliferation of tissue cells and extracellular matrix proteins within the tunica intima (Stracke et al. 2002, Li et al. 2007). Smooth muscle cells and myofibroblasts, which are responsible for the contractile function of blood vessels, play a key role in the pathophysiology of the formation of neointimal hyperplasia. On biomaterial such as PTFE grafts, the patient's body reacts against the foreign shunt with protein adsorption to the biomaterial surface after vascular anastomosis (Mora et al. 2009). Consequently, a thrombogenic surface within the shunt occurs, resulting in platelet aggregation, activation, and thrombus formation (Angelini et al. 1990, Verheye et al. 2000, Bonatti et al. 2004, Zain et al. 2020). This is followed by leukocyte and macrophage recruitment which then infiltrate the biomaterial causing thrombus degradation, which results in fibrosis and inflammatory processes (Chamberlain et al. 2013) (Angelini et al. 1990). Impaired biomaterial phagocytosis triggers increased secretion of cytokines, reactive species, and enzymes by macrophages, exacerbating the inflammatory process (Baker et al. 2014). Multinucleated foreign body giant cells, formed through the aggregation of macrophages (Yang et al. 2014), are known to play a key role in both the inflammatory and proliferative processes (Yu et al. 2015). They facilitate the recruitment of myofibroblasts and smooth muscle cells, which subsequently migrate into the shunt to complete the process of wound healing (Stracke et al. 2002, Li et al. 2007). The degradation of the extracellular matrix by metalloproteinases promotes the enhanced movement and growth of smooth muscle cells (SMCs) and monocytes, ultimately leading to the accumulation of these cells within the lumen of the shunt (Rotmans et al. 2004). When specific conditions are present, the migrated smooth muscle cells (SMCs) shift from a dormant, non-proliferative type to an active, multiplying type. This shift leads to an escalation of excessive cell growth within the shunt lumen (Campbell and Campbell 1990, Thyberg et al. 1995). Other cell types, such as endothelial cells, can also contribute to the development of neointimal growth. Endothelial cells, which line the inner surface of

blood vessels, are responsible for maintaining the integrity of the arterial wall and regulating the vascular tone (Lüllmann-Rauch 2015). Endothelial dysfunction in response to vascular injury can lead to the activation of endothelial cells resulting in the production and secretion of proinflammatory mediators that stimulate smooth muscle cell proliferation (Mora et al. 2009, Lüllmann-Rauch 2015).

Consequently, there is an accumulation and proliferation of the aforementioned cells within the shunt, exacerbating extracellular matrix deposition (Bakker et al. 1988). This gradual stenosis progression within the shunt can ultimately lead to fatal clinical shunt malfunction (Clowes and Reidy 1991, Casscells 1992). An overview of the possible pathophysiology of neointimal hyperplasia can be seen in Figure 3.

Figure 3. Formation of neointimal hyperplasia



Picture: (Kottmann et al. 2023)

Figure 3: Formation of neointimal hyperplasia 1) *Granulocyte reacts against the foreign biomaterial with protein adsorption due to the secretion of proteases* ***2)*** *Proteases and vascular shear stress create a thrombogenic surface initiating the adhesion, activation and aggregation of platelets on the biomaterial surface.* ***3)***

Erythrocytes and fibrin fibers accumulate and a wall-standing thrombus originates ***4)*** *Monocytes invade the shunt tissue transforming into macrophages trying to degrade the thrombus as well as the PTFE tissue. Failed phagocytosis of the PTFE material forces the macrophages to further secrete cytokines and reactive species which exacerbates the inflammation and attracts other cell types* ***5)*** *Cytokines attract myofibroblasts and vascular smooth muscle cells which continue the process of wound healing throughout the deposition of extracellular matrix creating neointimal hyperplasia* ***6)*** *Throughout the migration and proliferation of SMCs, the body creates vascular-like conditions in the PTFE shunt* ***7)*** *Vascularization is initiated promoting the expansion and proliferation of mentioned cells* ***8)*** *Parallelly, reendothelialization takes place to reduce blood-flow turbulences and to avoid further platelet aggregation* ***9)*** *The consequence is an accumulation of SMCs in the shunt lumen depositing extracellular matrix which leads to severe shunt stenosis over time.*

Neointimal hyperplasia in AV-Fistulas

Arteriovenous fistulas (AVFs) comprise a surgically created anastomosis between an artery and a vein allowing the direct flow of blood between these two vessels. AVFs are commonly used in hemodialysis patients to provide access to the circulation for dialysis procedures. PTFE (polytetrafluoroethylene) shunts are small grafts that are inserted into the AVF to increase its diameter and improve blood flow. PTFE shunts are commonly used in patients with small or narrow vessels or in those with insufficient blood flow (Roy-Chaudhury et al. 2001).

Neointimal hyperplasia is a common complication of AVFs and PTFE shunts in hemodialysis patients (Roy-Chaudhury et al. 2001). Hemodialysis is a life-sustaining treatment for patients with end-stage kidney disease, and AV malfunction can disrupt this treatment and cause a number of serious complications increasing morbidity and mortality (Roy-Chaudhury et al. 2001). These include decreased blood flow, thrombosis and infection (Kotsis et al. 2016).

Neointimal hyperplasia typically occurs within the first few months after AVF and shunt creation (Li et al. 2007). It can occur anywhere within the AVF or the shunt but is most commonly found in the anastomotic region and the distal portion of the shunt (Roy-Chaudhury et al. 2001).

Several growth factors, including transforming growth factor-beta (TGF-beta) (Stracke et al. 2002, Lee and Wadehra 2012), platelet derived growth-factor (PDGF) (Roy-

Chaudhury et al. 2001), and vascular endothelial growth factor (VEGF) (Misra et al. 2008), have been identified as potential players in the pathogenesis of neointimal hyperplasia in AVFs. These growth factors stimulate the proliferation and migration of smooth muscle cells and collagen production within the blood vessel wall, leading to the formation of a neointima.

There are several therapies that have been performed in hemodialysis patients to prevent or reduce neointimal hyperplasia in AVFs. These treatments include drug therapy and interventional therapies. Statins (Chang et al. 2016), ACE inhibitors (Chen et al. 2016), and aspirin combined with dipyridamole (Dixon et al. 2009) have been shown to reduce the risk of neointimal hyperplasia in AVFs (Chang et al. 2016). Stenting and balloon angioplasty have also been shown to be effective in reducing stenosis due to neointimal hyperplasia in AVFs (Haskal et al. 2010).

In summary, neointimal hyperplasia is a common complication of PTFE shunts in AVFs in hemodialysis patients and can lead to significant morbidity and mortality of this cohort (Roy-Chaudhury et al. 2001). It is thought to be mediated by several growth factors that stimulate the proliferation and migration of smooth muscle cells and collagen within the blood vessel wall.

Neointimal hyperplasia after cardiovascular interventions

Coronary artery disease (CAD) is a leading cause of morbidity and mortality worldwide, and balloon dilatation and stent implantation are commonly used interventions to treat coronary stenosis caused by CAD or myocardial infarction. However, a significant problem after such interventions is the development of ”in-stent restenosis”, which is the reoccurrence of stenosis within the stent itself. This is often caused by neointimal hyperplasia, which is a process of abnormal growth and thickening of the innermost layer of cells lining the blood vessel (called the "tunica intima") (Kibos et al. 2007).

The pathophysiology of in-stent restenosis is complex and involves a number of factors, including inflammation, oxidative stress and SMC proliferation. The resulting formation of a neointimal layer within the stent itself can cause severe stenosis by narrowing the lumen of the blood vessel, leading to reduced blood flow and impaired perfusion to the myocardium. (Kibos et al. 2007)

In-stent restenosis is a significant complication after coronary artery intervention and often requires reintervention to correct the stenosis and restore normal blood flow.

Reintervention can be associated with significant morbidity and mortality, and there is a need to develop strategies to reduce the incidence of in-stent restenosis and the associated need for reintervention. (Thuesen et al. 2006, Vemmou et al. 2021)
One approach that has been shown to be effective in reducing the incidence of in-stent restenosis is the use of drug-eluting stents (DES). Those are stents coated with anti-inflammatory agents as Sirolimus that are released over time to inhibit SMC proliferation and, consequently, reduce the formation of neointimal tissue. Several randomized controlled trials have demonstrated that DES are associated with a significantly lower rate of in-stent restenosis compared to bare-metal stents, and they have become the standard of care for the treatment of coronary stenosis. (Suttorp et al. 2006, Thuesen et al. 2006, Falkowski et al. 2009).

Scientific problem

The interim period between stage I and stage II palliation is a time span with high interstage morbidity and mortality in infants with complex cyanotic congenital heart disease. Interstage mortality is reported with 12% in a multicenter study (Ghanayem et al. 2012). Risk factors associated with increased mortality are low birth weight, prematurity or unplanned re-operations (Alsoufi et al. 2018). While AVF malfunction and “in-stent-restenosis” due to neointimal hyperplasia are well studied, SP shunt malfunction in infants with complex cyanotic congenital heart defects due to neointimal hyperplasia is only marginally researched.

Research question

In the first phase of the study, the aim was to quantitatively evaluate neointimal hyperplasia through histopathological analysis. Additionally, the objective was to investigate the potential association between increased neointimal formation and interstage morbidity, as reflected by early interventions such as balloon dilatation, stent implantation or shunt revision. Moreover, we sought to identify clinical risk factors that are associated with increased neointimal proliferation.
In the second phase of the study, immunohistochemical analysis was conducted to identify specific proteins associated with neointimal hyperplasia. Previous research has implicated epidermal growth factor receptor (EGFR) and matrix metalloproteinase-

9 (MMP-9) as factors contributing to neointimal formation in other diseases (Newby 2005, Sanchez-Guerrero et al. 2013). Therefore, the objective of this study was to quantitatively assess EGFR and MMP-9 levels in SP shunts using immunohistochemistry. Additionally, our aim was to explore the presence of specific risk alleles in single nucleotide polymorphisms (SNPs) within relevant genes that may predispose individuals to neointimal hyperplasia development.

Methods

Study design and patients

A retrospective single-center study was conducted, collecting clinical data from patients with preserved explanted SP shunts available at the biomaterial bank of the German Heart Center Munich. Demographic and clinical information, including birth details, shunt implantation and shunt removal was extracted. The dosage of acetylsalicylic acid (ASA) was calculated based on weight and recorded at the time of shunt implantation and removal.

Wet lab procedures

The shunts were directly collected after stage I palliation and fixed in formalin. The formalin-fixed shunt was divided into two parts. One piece was embedded in synthetic resin (methyl methacrylate, Technovit 9100, Kulze Wehrheim, Germany). The other part of the shunt was put in paraffin after dehydration. The cured resin-embedded samples were cut with a diamond saw (300 CP, Exakt GmbH, Norderstedt, Germany), and the resulting sections were cut with a rotary grinder (400 CS; Exakt GmbH, Norderstedt, Germany) to a thickness of 10–30 µm. The paraffin-embedded samples were sectioned as 3 µm-thick slices with using a standard microtome. The ground sections were deplastinated by incubation in a series of xylenes, 2-methoxyethyl acetate, acetone, and water to apply a histopathological protocol. The microtome slides were deparaffinized using acetone, a series of ethanol and water. Routine histochemical stains Richardson, Hematoxilin/Eosin, and Elastica van Gieson were prepared according to standard protocols which are described in (Quentin et al. 2009, Mulisch 2010).

Immunohistochemical stainings were optimized for each antibody (see Table 1.) Briefly, after blocking endogenous peroxidases and an antigen-dependent antigen retrieval, slides were incubated with the first antibody overnight (4°C), washed and then incubated with a horseradish peroxidase (HRP) coupled secondary antibody. Finally, Diaminobenzidin (DAB) was added to the slides to visualize the areas with bound antibodies in brown. Counterstaining was performed using hematoxylin staining nuclei in violet.

All stained sections were masked, digitized with the Dotslide system (Olympus Europa Holding GmbH, Hamburg, Germany), and visualized with Olyvia software (Olympus Center Valley, PA, USA).

Table 1. Antibodies

Antigen	Antibody	Company	Pre-treatment	Secondary antibody
α-SMA	Monoclonal Mouse Anti Actin (Smooth Muscle)	DAKO M0851	Citrat Puffer pH 6.0DAKO S2031 90°C, 20min	Rabbit Anti Mouse 1:100 Immunglobulin DAKO P0260
CD68	Monoclonal Mouse Anti-Human CD68	DAKO M0876	Target Retrieval Buffer high pH DAKO S3307 90°C, 20min	EnVision dual link DAKO K4063 1 drop
EGFR	Monoclonal Mouse Anti Human EGFR	DAKO M3563	Proteinase K, DAKO S3004, 0,05 M TRIS/HCl pH 7,6	Zytochem plus HRP/Polymer System (Mouse/Rabbit), POLHRP-100
MMP-9	Monoclonal Mouse Anti-MMP9 (5G3)	abcam ab119906	Target Retrieval Puffer high pH DAKO S3307	Zytochem plus HRP/Polymer System (Mouse/Rabbit), POLHRP-100

Table 1: Antibodies used in this study *Including exact antibody information, dilutions, pre-treatments and detection system used*

Histopathologic evaluation

ImageJ was used for quantification of histological parameters (US National Institutes of Health, Bethesda, MD, USA). Area of thrombi, cellular infiltration into PTFE-material and neointimal hyperplasia were measured with the manual measuring function and the greatest value was recorded. Neointima was defined as the tissue protruding into

the lumen of the shunt. Relative shunt stenosis was assessed by dividing the cross-sectional area of neointimal hyperplasia by the cross-sectional area of the potential shunt lumen. Likewise, relative PTFE infiltration was calculated by dividing the cross-sectional area of PTFE infiltration by the cross-sectional area of the complete PTFE biomaterial.

$$Shunt\ stenosis[\%] = Neointima[mm^2]/Potential\ shunt\ lumen[mm^2]$$

$$PTFE\ infiltration[\%] = PTFE\ infiltration\ [mm^2]/Full\ PTFE\ biomaterial\ [mm^2]$$

By using the color threshold function of ImageJ, the stained area of EGFR, MMP-9, α-SMA and CD68 was determined.

Identification of single nucleotide polymorphisms

Written consent was obtained from the parents of the affected patients for the collection of blood samples. Following the manufacturer's guidelines, high-quality DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen; Hilden, Germany). The concentration of DNA was determined using a NanoDropTM spectrophotometer, and gel electrophoresis was conducted to verify the integrity of the genomic DNA. DNA analysis for single nucleotide polymorphisms (SNPs) was performed using the Infinium OmniExpress Kit (Illumina, Inc., San Diego, CA, USA). The Infinium high-density DNA analysis solution, which combines the Infinium assay with BeadChip microarrays, enabled a comprehensive genome-wide investigation of genetic DNA variations. Genotyping was performed using the standard genome-wide SNP method. PLINK (PUTTY Link, (Purcell et al. 2007)) was used for the bioinformatic analysis of the Infinium BeadChip data.

Based on the extent of lumen narrowing resulting from neointimal hyperplasia, two distinct clusters were established: Group 1, comprising cases with 0-39.9% lumen stenosis (n=26), and Group 2, including cases with ≥ 40% lumen stenosis (n=5). Initial analysis revealed that a shunt stenosis exceeding 40% was linked to more cardiac

interventions, such as balloon dilatation, stenting and shunt revision (Kottmann et al. 2022).

The PLINK software determined which SNPs differed significantly between the two groups (p-value of <0.01, Chi-square test).

In order to select the most significant SNPs, semi-quantitative criteria were applied, including statistical significance in the PLINK cluster analysis, gene associations with the EGFR and MMP-9 pathways and previous publication of the individual SNPs. The study utilized the web-based tool “SNPnexus” (www.snp-nexus.org) to gather bioinformatic data from diverse genome databases and associate them with the provided SNP query. This allowed the analysis of potential overlaps with structural DNA elements, prediction of functional consequences on proteins and identification of connections to previous studies on genetic diseases. (Chelala et al. 2008, Dayem Ullah et al. 2012) (Dayem Ullah et al. 2018). The reference genome “GRCh/hg19” was used for the analysis of the human genome. Genotypes associated with neointimal formation and relative shunt stenosis were identified by stratifying allele frequencies within the study population.

Statistics

IBM Statistics SPSS Version 28 was used for the statistical analysis. The data are presented as median and interquartile range (IQR). The Mann-Whitney-U test was used to analyze nominal variables with two categories in non-parametric analysis. The Kruskall-Wallis test was applied if the number of categories exceeded two. The strength of the association between two variables was measured using Spearman's Rho non-parametric test.

The present study employed univariable linear regressions to identify if there was an association between distinct clinical factors and increased neointimal hyperplasia or relative shunt stenosis. Fisher's exact and Chi's Square tests were used to compare nominal variables.

The p-value is stated raw unless it falls below p = 0.001, in which case it is corrected to p<0.001.

Figure 4. Methods of 1st Publication

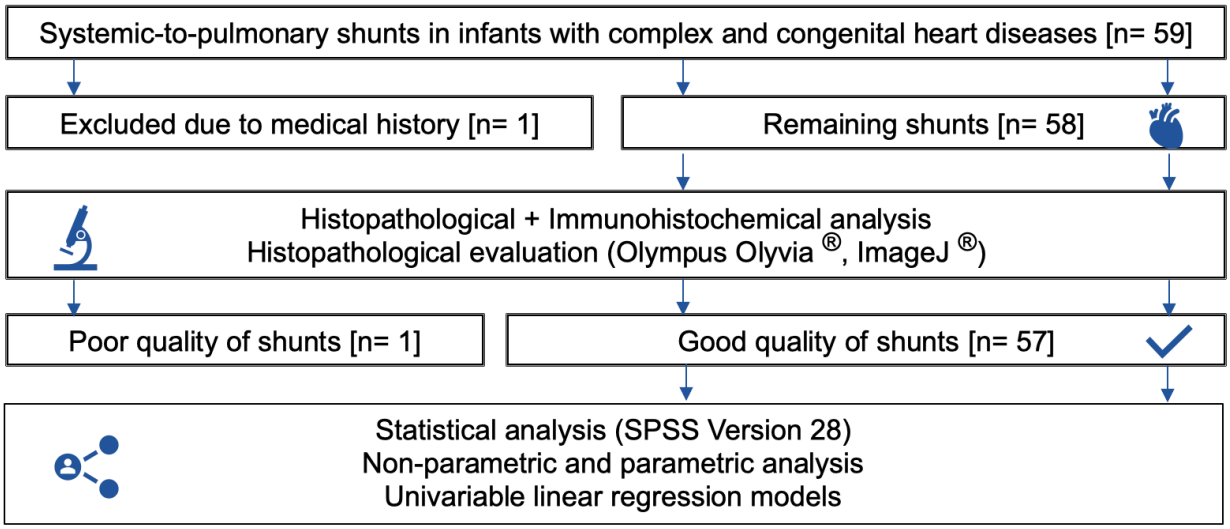
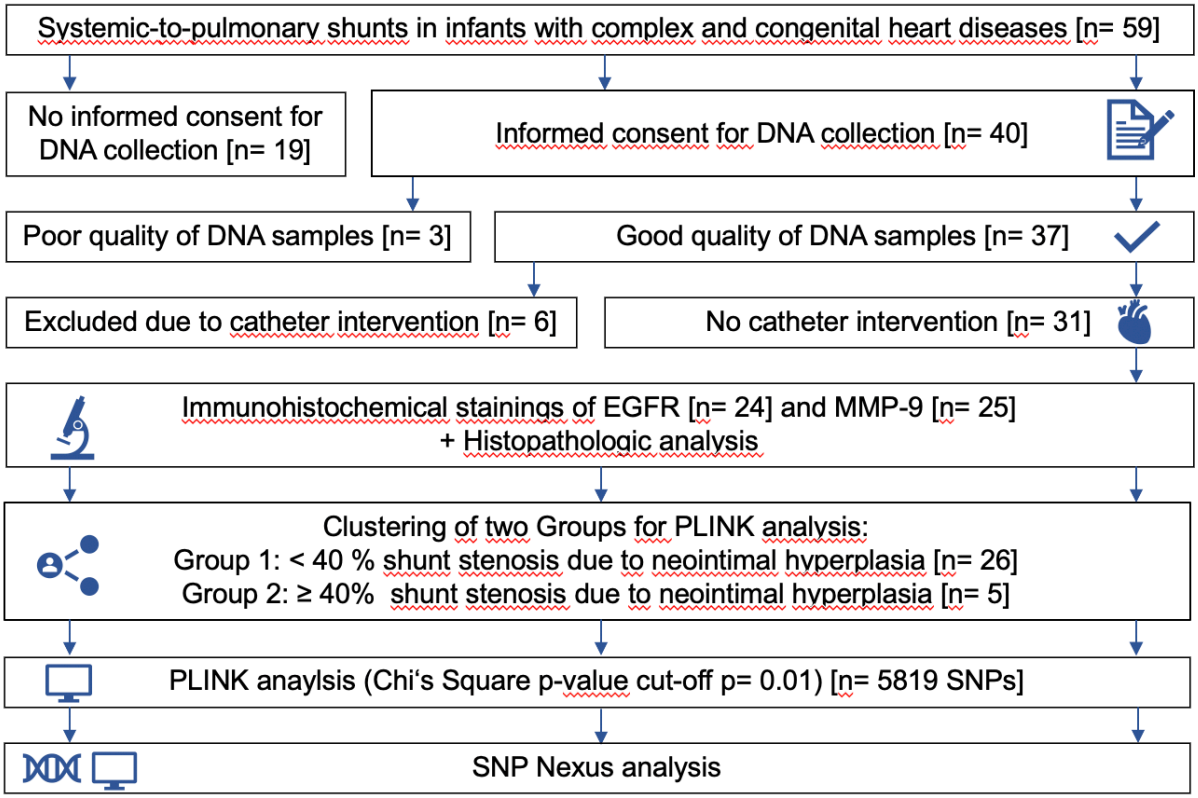


Figure 4: Schematic flowchart of methods: "Neointimal hyperplasia in systemic-to-pulmonary shunts of children with complex cyanotic congenital heart disease" (Kottmann et al. 2022)

Figure 5. Methods of 2nd Publication



Picture: (Kottmann et al. 2023)

Figure 5: Schematic flowchart of methods: "EGFR and MMP-9 are associated with neointimal hyperplasia in systemic-to-pulmonary shunts in children with complex cyanotic heart disease"*(Kottmann et al. 2023)*

Summary of Publications

Summary of 1st Publication

"Neointimal hyperplasia in systemic-to-pulmonary shunts of children with complex cyanotic congenital heart disease" (Kottmann et al. 2022)

Systemic-to-pulmonary (SP) shunts are commonly used in neonates with single-ventricle physiology and other complex cyanotic congenital heart diseases in order to ensure proper systemic and/or pulmonary perfusion until further treatment can be administered. These shunts are artificial grafts that allow the transfer of blood between the systemic and pulmonary circulations, and they can be created using various surgical techniques such as modified Blalock-Taussig anastomosis, right ventricle to pulmonary artery anastomosis or central shunts.

However, the long-term success of these shunts may be compromised by the occurrence of neointimal hyperplasia, a process in which the inner layer of the artificial graft becomes thickened and narrowed over time. This gradual stenosis of the graft can lead to shunt malfunction in infants with complex cyanotic congenital heart disease which can lead to fatal consequences. The objective of this study was to investigate the histopathological alterations in polytetrafluoroethylene SP shunts and assess the potential correlation between enhanced neointimal formation and early interventions such as balloon dilatation, stent implantation and shunt revision. Furthermore, the study aimed to identify clinical risk factors that might contribute to increased neointimal proliferation.

To achieve these objectives, we examined a total of 57 removed shunts that had undergone stage I palliation. The removed shunts underwent histopathological processing, with subsequent staining of slides using Hematoxylin/Eosin and Richardson techniques. Immunohistochemistry was performed, utilizing antibodies against alpha-smooth-muscle-actin and CD68. Non-parametric analysis and univariate regressions were employed to investigate potential clinical factors associated with neointimal hyperplasia and shunt stenosis.

The results of the study showed that the median area of neointimal proliferation within the shunts was 0.75 mm^2, with an interquartile range of 0.3 - 1.57 mm^2. The relative shunt stenosis was found to be in the median at 16.7%, with an interquartile range of

6.7 - 30.8%. Neointimal hyperplasia and shunt stenosis were found to be significantly greater in the group that required early interventions.
Univariable linear regression analysis identified smaller shunt size and lower acetylsalicylic acid (aspirin) dosage as clinical risk factors that were associated with greater neointimal proliferation and shunt stenosis.
In this study, we found that neointimal hyperplasia in systemic-to-pulmonary shunts was significantly associated with the need for early interventions in infants with complex cyanotic congenital heart disease. These early interventions included balloon dilatation, stent implantation, and shunt revision. Additionally, smaller shunt size and lower aspirin dosage were found to be associated with increased neointimal proliferation and shunt stenosis. These findings may be useful in the management and long-term care of neonates with single-ventricle physiology and other complex cyanotic congenital heart diseases, as they can help to identify potential risk factors for shunt failure and guide the implementation of appropriate preventive measures.

Author contributions:

In this study, the doctoral candidate Philip Kottmann contributed to the following procedures: Study design, retrospective data collection, histopathological and immunohistochemical evaluation, data analysis and manuscript writing.

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19th May 2023

Dear Philip Kottmann,

RE. Philip Kottmann et al. 'Neointimal hyperplasia in systemic-to-pulmonary shunts of children with complex cyanotic congenital heart disease.' *European Journal of Cardio-Thoracic Surgery* (62) 6 (2022): ezac431, https://doi.org/10.1093/ejcts/ezac431

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Summary of 2nd Publication

"EGFR and MMP-9 are associated with neointimal hyperplasia in systemic-to-pulmonary shunts in children with complex cyanotic heart disease" (Kottmann et al. 2023)

The malfunction of systemic-to-pulmonary (SP) shunts is a significant source of morbidity in infants with complex cyanotic heart disease undergoing palliative and/or corrective procedures. It has been suggested that neointimal hyperplasia may contribute to the pathogenesis of this issue, increasing the risk of shunt obstruction (Kottmann et al. 2022). Previous research has identified epidermal growth factor receptor (EGFR) and matrix metalloproteinase - 9 (MMP-9) as potential contributing factors to neointimal formation in other diseases (Newby 2005, Sanchez-Guerrero et al. 2013).

This study aimed to determine the expression of EGFR and MMP-9 in systemic-to-pulmonary (SP) shunts obtained from children undergoing stage II palliation using immunohistochemistry, as well as identify risk alleles in genes encoding related proteins. To do this, explanted SP shunts were fixed in formalin after shunt removal and later stained with Hematoxylin/Eosin and immunohistochemistry using anti-EGFR and anti-MMP-9. Images were captured using a microscope camera and corresponding software systems (CCD-color camera Color View II and Software Analysis 3.2, Olympus Europa Holding GmbH, Hamburg, Germany), and the percentage of shunt stenosis was calculated by dividing the area of neointimal hyperplasia by the potential shunt lumen with ImageJ (NIH, Bethesda, MD, USA). We then analyzed the expression of EGFR and MMP-9 by using the color threshold function of Image J to measure the stained area in relation to the area of neointimal tissue.

Written consent was obtained from the parents to collect peripheral blood samples from the patients, which were subsequently stored in the cardiovascular biobank at the German Heart Center Munich (KaBi-DHM). DNA was extracted from the blood samples, and whole-genome genotyping of single nucleotide polymorphisms (SNPs) was performed using the semi-custom HumanOmniExpress array (Illumina, San Diego, CA, USA).

The genetic data were analyzed using the PLINK program (Purcell et al. 2007) and the web-based tool SNPNexus (www.snp-nexus.org). We categorized and compared the allele frequencies of single nucleotide polymorphisms between the subgroup of patients with shunts exhibiting severe stenosis (≥40% shunt stenosis) and the remaining subgroup. In addition, we collected demographic and clinical data from the medical charts at the timepoints of birth, shunt implantation and shunt takedown.
Our results showed that EGFR and MMP-9 were primarily detected in the luminal area of the shunt in immunohistochemistry, forming a ring-like structure at the border between PTFE material and neointimal formation. The median cross-sectional area of EGFR and MMP-9 was measured as 0.19 mm^2 (IQR, 0.1-0.3 mm^2) and 0.04 mm^2 (IQR, 0.03-0.09 mm^2), respectively. These measurements exhibited a positive correlation with the area of neointimal hyperplasia ($r=0.729$, $p<0.001$ and $r=0.0479$, $p=0.018$). Furthermore, we discovered that specific risk alleles on SNP's located in the epidermal growth factor (EGF) and tissue inhibitor of metalloproteinases-1 (TIMP-1) genes were associated with increased stenosis and neointimal hyperplasia within SP shunts.
To summarize, our findings suggest that EGFR and MMP-9 play a role in neointimal proliferation in SP shunts among children with complex cyanotic heart disease. SP shunts derived from patients carrying particular risk alleles in the genes encoding for EGF and TIMP-1 displayed increased neointimal formation and shunt stenosis.

Author contributions:

In this study, the doctoral candidate Philip Kottmann contributed to the following procedures: Study design, retrospective data collection, histopathological and immunohistochemical evaluation, performing of PLINK and SNPNexus analysis, data analysis and manuscript writing.

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EGFR and MMP-9 are associated with neointimal hyperplasia in systemic-to-pulmonary shunts in children with complex cyanotic heart disease

Author: Philip Kottmann et al
Publication: Mammalian Genome
Publisher: Springer Nature
Date: Mar 3, 2023

Discussion

Interim morbidity and mortality of children with complex cyanotic congenital heart defects between stage I and stage II palliation remain high (Ghanayem et al. 2012) and is directly influenced by the patency of the implanted shunt system to ensure systemic and/or pulmonary perfusion. The aim of this dissertation was to examine how neointimal hyperplasia is associated with increased interim morbidity between stage I and stage II palliation and to find clinical risk factors predisposing for its formation. Furthermore, we aimed to elucidate the role of the proteins EGFR and MMP-9, which showed to influence the formation of neointimal proliferation in other studies (Newby 2005, Sanchez-Guerrero et al. 2013). In a following SNP identification model, we examined whether certain polymorphisms in related genes of EGFR and TIMP-1 are prevalent and associated with increased neointimal formation.

After quantification and histopathological examination of the SP shunts neointimal hyperplasia occurred in our collected shunt specimen with a frequency of 74% and a median shunt stenosis of 17% in all shunts. Based on our histopathological analysis, the primary contributor to relative shunt stenosis was neointimal hyperplasia, while thrombi only marginally contributed to luminal narrowing. The results of detailed histopathological analysis, including immunohistochemical staining for α-SMA, indicated that the neointimal hyperplasia is primarily composed of myofibroblasts and smooth muscle cells. Macrophages, endothelial cells and vascularization in form of smaller vessels were also prevalent in our histopathologic sections, when neointima comprised a larger area. (Kottmann et al. 2022)

Our findings are consistent with other studies in which myofibroblasts and vascular smooth muscle cells migrate and proliferate from the anastomotic margin into the shunt as a result of vascular connection (Li et al. 2007, Kottmann et al. 2022).

We showed that an independent predictor causing greater neointimal formation was smaller shunt size (Kottmann et al. 2022), which was also found by others (Wells et al. 2005). According to other researchers, smaller shunts are associated with a higher incidence of shunt thrombosis (Fenton et al. 2003) a higher incidence of shunt-related mortality (Vitanova et al. 2019) and an increased incidence of interventions (O'Connor et al. 2011). According to Hagen-Poiseuille's law, one explanation for this could be that flow velocity, and thus shear stress in the shunt is significantly favored by a smaller shunt, promoting the formation of neointimal hyperplasia throughout endothelial dysfunction and platelet activation (Kottmann et al. 2022).

Another independent predictor for neointimal hyperplasia and shunt stenosis was a lower ASA dosage per kilogram body weight at the time of shunt takedown (Kottmann et al. 2022). In a multicenter study, the postoperative rates of death and shunt thrombosis were examined and ASA-receiving patients had a significantly lower risk of shunt thrombosis and death compared with those not receiving ASA (Li et al. 2007).
This data and our findings indicate that a gradual adjustment of ASA dosage in relation to body weight during the interim period could provide a benefit in terms of reduced neointimal narrowing, and therefore, a better outcome for children receiving SP shunts (Kottmann et al. 2022).
Another finding of ours is that neointimal hyperplasia and shunt stenosis are significantly more pronounced in the patient cohort that received early shunt intervention (Kottmann et al. 2022). A study showed that in children who underwent Norwood palliation, long-term survival was significantly lower in the group that required early shunt intervention (O'Connor et al. 2011). Therefore, it can be assumed that increased neointimal proliferation could not only affect interstage morbidity reflected by early shunt interventions but also impact interstage mortality in this critically ill population (Kottmann et al. 2022). However, since infants with fatal disease progression were not included in this work, we cannot state an association between severe shunt malfunction, neointimal formation and interstage mortality in children with complex congenital heart disease.

EGFR/EGF

In our second study, utilizing EGFR staining, we observed a significant correlation between EGFR levels and the extent of neointimal hyperplasia. Additionally, we identified specific SNPs in the EGF gene that were associated with an increased risk of severe shunt stenosis in affected patients (Kottmann et al. 2023).
EGFR is produced by multiple types of cells such as macrophages, vascular smooth muscle cells and endothelial cells (Bagheri-Yarmand et al. 2000, Tamura et al. 2001, Lamb et al. 2004). EGF, its ligand, plays a key role in the development of cell differentiation and proliferation (Sanchez-Guerrero et al. 2013). An imbalance of this tightly regulated system has been associated with hyperproliferative diseases (Huang and Harari 1999, Olayioye et al. 2000, Trieu et al. 2000, Chan et al. 2003). Rat models demonstrated significantly less restenosis due to neointimal hyperplasia by inhibiting or blocking EGFR after carotid artery injury (Trieu et al. 2000, Chan et al. 2003). Others showed that EGFR blockade in rat aortic SMCs inhibited

cell proliferation and migration in vitro (Nicholl et al. 2005). In a recent work, Foth et al. reported an upregulation of EGFR in the thickened walls of bioprosthetic valved conduits, suggesting its involvement in conduit stenosis due to chronic inflammatory processes (Foth et al. 2021). On the basis of our results and mentioned studies, the integrity of the EGFR pathway in the pathophysiology of neointimal formation in SP shunts can be hypothesized. Targeted blockade of EGFR, as previously demonstrated in preclinical models with suppressing neointimal proliferation (Trieu et al. 2000, Chan et al. 2003, Nicholl et al. 2005) may represent a valuable therapeutic option for the treatment of cyanotic heart defects in infants receiving SP shunts, particularly in infants with reduced shunt diameter given their greater risk for obstruction (Wells et al. 2005, Kottmann et al. 2022).

MMP-9/TIMP-1

Immunohistochemical analysis of our SP shunts indicated that the stained area of MMP-9 correlated significantly with the area of neointimal hyperplasia. Moreover, a risk allele on TIMP-1 (rs6609533), a strong inhibitor of MMP-9, was found to be associated with greater neointimal formation and shunt stenosis in explanted SP shunts.

MMP-9 regulates tissue remodeling (Watanabe et al. 2018). Monocyte invasion, neovascularization, and neointimal proliferation depend on the protease activity of MMP-9, implicating it as a potential therapeutic target for inhibiting intimal proliferation (Yabluchanskiy et al. 2013). For example, Song et al. showed a potent reduction of intimal hyperplasia, by stents eluting a potent inhibitor of MMP-9, in a porcine model (Song et al. 2020). Matrix-metalloproteinases are potently inhibited by TIMP-1. TIMP-1 has been associated with a variety of functions including cell growth, apoptosis, angiogenesis and recruitment of leukocytes and macrophages (Ouyang et al. 2003, Cabral-Pacheco et al. 2020) (Alpízar-Alpízar et al. 2016). Rat models showed significantly reduced neointimal formation in arteries of rats after gene transfer of TIMP-1 (Dollery et al. 1999, Furman et al. 2002, Ramirez Correa et al. 2004).

COPD emphysema is characterized by a thickened bronchiolar wall due to peribronchiolar fibrosis and an increased smooth muscle thickness, similar to the pathophysiology of neointimal hyperplasia (Siafakas et al. 2007). Similar to our study, Kumar et al. identified the SNP rs6609533 of the TIMP-1 gene as a risk variant for the development of COPD by downregulation and lower concentrations of TIMP-1.

According to Kumar, this led to a higher expression of MMPs, which thereby raised the migration of SMCs and facilitated neointimal growth. (Kumar et al. 2011)
According to our study results and Kumar et al. findings, the SNP rs6609533 could have led to reduced suppression of MMP-9 and therefore facilitated the growth of neointimal hyperplasia in SP shunts.
To conclude our second scientific paper, it appears that EGFR and MMP-9, possibly regulated by EGF and TIMP-1, might play a role in the development of neointimal hyperplasia in SP shunts in children with cyanotic heart disease. Preclinical studies targeting these genes to suppress their signaling pathways showed a reduction in hyperplasia in various shunt models. In the future, novel approaches could significantly reduce neointimal hyperplasia in SP shunts via the use of agents specifically suppressing pathways around EGF/EGFR, TIMP-1/MMP-9 and, consequently, improve the outcome of children with complex and congenital heart defects.

Overall, the findings of this work suggest that neointimal hyperplasia is a significant complication in SP shunts potentially leading to interstage morbidity by causing early interventions in infants with complex cyanotic congenital heart disease. Neointima is influenced by a range of factors including shunt size, aspirin dosage, key-player proteins and genetic risk alleles. These results contribute to our understanding of the complex pathogenesis of shunt malfunction. Further, our findings can help to identify potential risk factors for shunt failure and guide the implementation of appropriate preventive measures to provide a basis for identifying potential therapeutics and to prevent the formation of neointimal hyperplasia in SP shunts of children with complex cyanotic congenital heart disease. Further research is needed to better understand the underlying mechanisms leading to neointimal hyperplasia in SP shunts and to develop strategies for the prevention and/or mitigation of this complication.

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Appendix

1st Publication

2nd Publication

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ORIGINAL ARTICLE

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Neointimal hyperplasia in systemic-to-pulmonary shunts of children with complex cyanotic congenital heart disease

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Key question

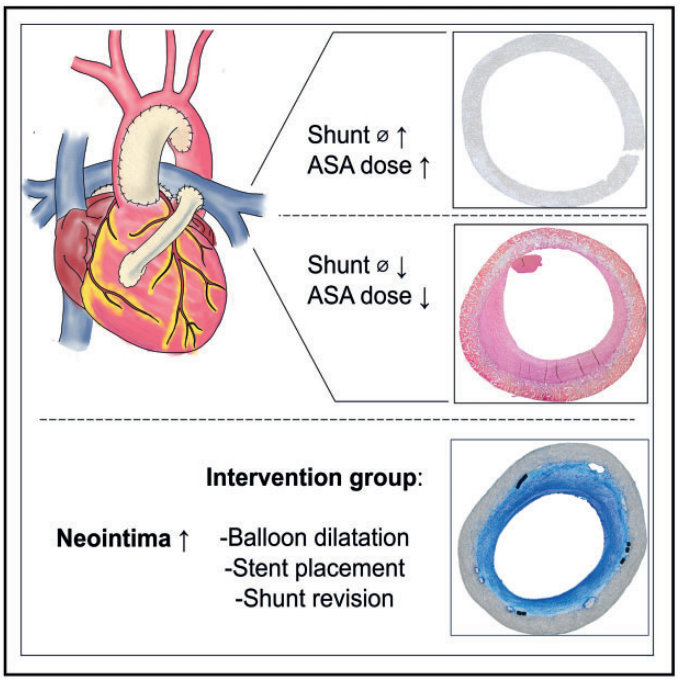
How does neointimal hyperplasia influence systemic-to-pulmonary shunts in children with cyanotic heart disease?

Key finding(s)

More neointima in the early intervention group. Smaller shunt size & lower ASA dose associated with increased neointima

Take-home message

Neointimal proliferation contributes to interstage morbidity and is influenced by shunt size and ASA dosing



Presented at the AEPC 2022—55th Annual Meeting of the Association for European Paediatric and Congenital Cardiology, Geneva, Switzerland, 25–28 May 2022.

Abstract

OBJECTIVES: Neointimal hyperplasia might affect systemic-to-pulmonary shunt failure in infants with complex cyanotic congenital heart disease. The aim of this study was to elucidate histopathologic changes in polytetrafluoroethylene shunts and to determine whether increased neointimal formation is associated with early interventions comprising balloon dilatation, stent implantation and shunt revision. Furthermore, we intended to identify clinical factors associated with increased neointimal proliferation.

METHODS: Removed shunts were processed for histopathological analysis. Slides were stained with hematoxylin/eosin and Richardson. Immunohistochemistry was performed with anti-alpha-smooth muscle actin and anti-CD68. Non-parametric analysis and univariable regressions were performed to identify clinical factors associated with neointimal hyperplasia and shunt stenosis.

RESULTS: Fifty-seven shunts (39 modified Blalock–Taussig anastomosis, 8 right ventricle-to-pulmonary artery anastomosis, 10 central shunts) were analysed. Area of neointimal proliferation within the shunt was in median 0.75 mm^2 (interquartile range, 0.3–1.57 mm^2) and relative shunt stenosis in median 16.7% (interquartile range, 6.7–30.8%). Neointimal hyperplasia and shunt stenosis correlated with each other and were significantly greater in the group that required early interventions and shunt revision. Univariable linear regression identified smaller shunt size and lower acetylsalicylic acid dosage as factors to be associated with greater neointimal proliferation and shunt stenosis.

CONCLUSIONS: In infants with complex cyanotic congenital heart disease, neointimal hyperplasia in systemic-to-pulmonary shunts is associated with early interventions comprising balloon dilatation, stent implantation and shunt revision. Smaller shunt size and lower aspirin dosage are associated with increased neointimal proliferation.

Keywords: Neointimal hyperplasia • Systemic-to-pulmonary shunt • Shunt malfunction • Norwood procedure • Hypoplastic left heart syndrome • Cyanotic heart defects

ABBREVIATIONS

ASA	Acetylsalicylic acid
CS	Central aortopulmonary shunt
HLHS	Hypoplastic left heart syndrome
IQR	Interquartile range
mBTA	Modified Blalock–Taussig anastomosis
PTFE	Polytetrafluoroethylene
RV–PA	Right ventricle-to-pulmonary artery anastomosis
SP	Systemic to pulmonary
α-SMA	Alpha-smooth muscle actin

INTRODUCTION

Systemic-to-pulmonary (SP) shunts are implanted in neonates with single-ventricle physiology at stage I of Norwood palliation and in children with other complex cyanotic congenital heart disease to ensure systemic and/or pulmonary perfusion until stage II palliation, corrective surgery or other follow-up procedures. Despite major advances in clinical care, imaging, interventional and surgical technologies, infants with this palliative circulation remain with high interstage morbidity and mortality.

While several patient characteristics like prematurity, low birth weight or unplanned re-operations are considered to be proven risk factors contributing to increased interim morbidity, the occurrence and impact of neointimal hyperplasia in SP shunts has not been described as a risk factor yet [1].

Neointimal hyperplasia describes a pathological vascular remodelling due to the migration and proliferation of smooth muscle cells and myofibroblasts, leading to intimal thickening and gradually shunt malfunction over time [2].

Neointimal hyperplasia is the major cause of 'in-stent restenosis' in coronary arteries after catheter interventions and vascular access dysfunction of arterio-venous grafts in haemodialysis patients [2, 3]. While already well researched and treated in these medical disciplines, shunt failure due to neointimal hyperplasia in children with complex and congenital heart disease is only marginally reported [4]. We assume similar mechanisms behind neointimal formation in SP shunts implanted in those infants.

The aim of this study was to quantify neointimal hyperplasia histologically and to determine its influence on interstage morbidity reflected by unplanned early interventions such as balloon angioplasty, stenting or shunt revision. Furthermore, we attempted to identify clinical factors associated with greater neointimal formation.

MATERIALS AND METHODS

Ethics statement

The use of biomaterial for the purpose of this study was approved by the institutional review board (ethics committee, Technical University Munich, Germany, approval 27 November 2015, number 223/15) and all legal representatives of participants gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Study design and patients

In a single-centre study, clinical data were collected retrospectively from all patients of which preserved explanted SP shunts were available at the German Heart Center biomaterial bank. Demographic and clinical information was extracted at the timepoints of birth, shunt implantation and shunt takedown. Acetylsalicylic acid (ASA) dose was calculated per weight and noted at shunt implantation and shunt takedown.

Wet laboratory procedures

After the removal of the shunt, tissue was prepared as previously described [5]. Richardson, hematoxylin/eosin and Elastica van Gieson stainings were performed by standard protocols [5, 6].

Immunohistochemical stainings were applied as described in [5] and optimized for each antibody (Supplementary Material, Table S1).

Stained sections were digitized using the Dotslide system (Olympus) and visualized with the Olyvia software (Olympus Center Valley, PA, USA).

Evaluation of the tissue

Quantification was done using ImageJ (ImageJ, US National Institutes of Health, Bethesda, MD, USA). Neointima was defined as the cellular proliferation located inside the lumen and was morphologically distinguished from thrombi. Cellular and fluid components inside the polytetrafluoroethylene (PTFE) material describe the variable PTFE infiltration. All variables were measured manually and the greatest value was noted.

Relative shunt stenosis was calculated by dividing the cross-sectional area of neointimal hyperplasia by the cross-sectional area of the potential shunt lumen. Relative PTFE infiltration was calculated by dividing the cross-sectional area of PTFE infiltration by the cross-sectional area of the full PTFE biomaterial.

$$\text{Shuntstenosis}\ (\%) = \text{neointima}\ (\text{mm}^2)/\text{potentialshuntlumen}\ (\text{mm}^2)$$

$$\text{PTFEinfiltration}\ (\%) = \text{PTFEinfiltration}\ (\text{mm}^2)/\text{fullPTFEbiomaterial}\ (\text{mm}^2)$$

Alpha-smooth muscle actin (α-SMA) and CD68-positive areas were detected using the colour threshold in ImageJ.

Statistics

Statistical analysis was performed using IBM Statistics SPSS Version 28. Data are reported as median and interquartile range (IQR). For non-parametric analysis, nominal variables with 2 categories were compared with Mann–Whitney *U* tests. If the number of categories exceeded 2, the Kruskal–Wallis test was applied. Spearman's Rho non-parametric test was used to measure the strength of association between 2 variables.

Univariable linear regression was performed to identify associations of distinct clinical factors with greater neointimal hyperplasia or relative shunt stenosis.*P*-values are stated raw unless a number below $P = 0.001$, in which case they are corrected to $P < 0.001$.

RESULTS

Demographics

Fifty-nine SP shunts explanted from infants and children undergoing stage II palliation or corrective surgery between February 2011 and August 2016 were studied. Two shunts per patient were explanted in 2 patients: one of them had 2 central shunts implanted simultaneously and the other patient underwent a shunt revision and both shunts were considered in the analysis. Excluded were 2 shunts: 1 shunt showed insufficient histopathologic quality and the other shunt was from a patient whose medical history made clinical interpretation with the other patients impracticable. The remaining 57 shunts from 55 infants (36 males, 11 premature born) were analysed. The median age at implantation of shunts was 10 days (IQR, 7–21 days). Most common structural heart defects were hypoplastic left heart syndrome (HLHS) in 24 (44%) and pulmonary atresia in 13 patients (24%). Shunts included PTFE grafts connecting the subclavian artery or truncus brachiocephalicus [modified Blalock–Taussig anastomosis (mBTA)], the right ventricle [Right ventricle-to-pulmonary artery anastomosis (RV–PA)] or the aorta (central) to the pulmonary artery in 39, 8, and 10 cases, respectively. Shunt size was 3.0 mm in 9 (16%), 3.5 mm in 34 (60%) and >3.5 mm in 14 shunts (24%). Patients received either ASA dosed to efficacy based on thrombocyte-functioning test at the time of implantation, or coumadin with target INR of 2–3 or both agents. Six infants did not receive any platelet inhibition or anticoagulation. Takedown of shunt was performed after a median duration of 112 days (IQR, 84–172 days, Table 1).

Histopathologic evaluation of systemic-to-pulmonary polytetrafluoroethylene shunts

Thrombus formation. Wall-standing thrombi were present in 24 shunts (42%) (Fig. 1a, b, c and f) with a median area of 0.1 mm^2 (IQR, 0.04–0.24 mm^2, Table 1). Thrombus formation in our specimen occurred independently from neointima, as there was no statistical correlation to the occurrence of shunt stenosis and neointimal proliferation (Supplementary Material, Table S2). Non-parametric analysis between clinical variables and area of thrombi (mm^2) yielded no significant results (Supplementary Material, Tables S3 and S4). Free thrombi located in the lumen could not be assessed throughout wash-out and histological preparation.

Assessment of neointima. Neointima was found in 42 of 57 assessed shunts (74%). The cross-sectional area of the neointima measured in median 0.75 mm^2 (IQR, 0.3–1.57 mm^2), resulting in a median shunt stenosis of 16.7% (IQR, 6.7–30.8%) (Table 1 and Fig. 1b–h). The extend of neointima and relative shunt stenosis of all patients correlated ($P < 0.001$, $r = 0.968$, Spearman's Rho, Supplementary Material, Table S2). α-SMA quantified to a median area of 0.26 mm^2 (IQR, 0.09–0.63 mm^2) within neointima (Fig. 1g and Table 1). There was a strong correlation between neointima and α-SMA (Fig. 1g; $P < 0.001$, $r = 0.760$, Spearman's Rho, Supplementary Material, Table S2).

Observed endothelialization and vascularization were particularly prevalent in shunts with greater neointimal formation (Fig. 1b and e).

Assessment of PTFE shunt material. Infiltrations of cells and fluid into the PTFE-material were present in nearly all shunts (95%), resulting in a median area of 1.86 mm^2 (IQR, 1.22–2.79 mm^2) (Table 1 and Fig. 1d). Tissue infiltrated in median 53% (IQR, 35–68%) of the cross-sectional PTFE biomaterial (Table 1). The amount of tissue infiltrating the PTFE material did not correlate with neointimal hyperplasia or with resulting shunt stenosis (Supplementary Material, Table S2). There seemed to be a greater degree of neointimal hyperplasia and shunt stenosis in shunts without PTFE infiltration ($n = 3$), but this did not reach statistical significance (Supplementary Material, Table S5).

Macrophages, as discovered by positive stain for CD68, covered a median area of 0.05 mm^2 (IQR, 0.006–0.117 mm^2) within the PTFE material but were also found inside the neointima. They often fused into multinucleated foreign body giant cells 'FBGC',

Table 1: Patient characteristics and histopathological findings

Clinical parameters	Characters	*n* (%)/median [IQR]
Gender	Male	36 (63.2)
	Female	21 (36.8)
Gestational age (weeks)		38 [37–39]
Premature born	No	43 (79.6)
	Yes	11 (20.4)
Birth weight (g)		3040 [2650–3340]
Age at shunt implantation (days)		10 [7–21]
Weight at shunt implantation (g)		3232.5 [2800–3560]
Diagnosis	HLHS	24 (44.4)
	PA + VSD	11 (20.4)
	PA	2 (3.7)
	TOF	4 (7.4)
	DORV	2 (3.7)
	TA	6 (11.1)
	TGA	1 (1.9)
	DILV	1 (1.9)
	Others	3 (5.6)
Shunt type	mBTA	39 (68.5)
	RVPA	8 (14.0)
	CS	10 (17.5)
Shunt diameter	3 mm	9 (15.8)
	3.5 mm	34 (59.6)
	4 mm	5 (8.8)
	5 mm	8 (14.0)
	6 mm	1 (1.8)
Duration of shunt implanted (days)		111.5 [84–172]
Anticoagulation	No anticoagulation	6 (11.1)
	ASA	33 (61.1)
	Coumadin	13 (24.1)
	Both	2 (3.7)
Early intervention	No intervention	35 (71.4)
	Balloon angioplasty	4 (8.2)
	Stent	6 (12.2)
	Shunt revision	4 (8.2)
Neointimal hyperplasia	Yes	42 (73.7)
	No	15 (26.3)
Area of neointimal hyperplasia (mm^2)		0.75 [0.3–1.57][a]
Relative shunt stenosis (%)		16.71 [6.66–30.83][a]
Adherent thrombus	Yes	24 (42.1)
	No	33 (57.9)
Area of adherent thrombi (mm^2)		0.1 [0.04–0.24][a]
PTFE infiltration	Yes	54 (94.7)
	No	3 (5.3)
PTFE infiltration (mm^2)		1.86 [1.22–2.79][a]
Relative PTFE infiltration (%)		53 [35–68][a]
α-SMA (mm^2)		0.26 [0.09–0.63][a]
$CD68^+$ (mm^2)		0.05 [0.01–0.12][a]

n (%): number of shunts (percentage); others: other single-ventricle physiologies.

ASA: acetylsalicylic acid; CD68: cluster of differentiation 68; CS: central aortopulmonary shunt; DILV: double inlet left ventricle; DORV: double outlet right ventricle; HLHS: hypoplastic left heart syndrome; IQR: interquartile range; mBTA: modified Blalock–Taussig anastomosis; PA: pulmonary atresia; PA + VSD: pulmonary atresia with septal ventricular defect; PTFE: polytetrafluoroethylene; RVPA: right ventricle-to-pulmonary artery anastomosis; α-SMA: alpha-smooth muscle actin; TA: tricuspid atresia; TGA: transposition of great arteries; TOF: tetralogy of Fallot.

[a]Median of histopathological calculated only from shunts displaying measurable expression of parameters.

mostly located on the margin of the foreign PTFE biomaterial (Fig. 1d).

Association between clinical variables and neointimal hyperplasia

A reverse correlation between birth weight and weight at shunt implantation and the amount of neointimal hyperplasia were identified (Fig. 2a and b). There was a significantly greater area of neointima in shunts from premature born compared to full-term born infants (Fig. 3a).

Comparing neointimal formation between patients with distinct structural heart diseases, the area of neointima was less in infants with HLHS compared to children with other complex heart defects (Fig. 3b). Neointima was more pronounced in smaller shunts (Fig. 3c), or in mBTA and central aortopulmonary shunt (CS) as compared to RV–PA shunts (Fig. 3d). Data are shown separately for mBTA, CS and right ventricle-to-pulmonary artery anastomosis conduit (Supplementary Material, Table S6). Lower dose of ASA per body weight at shunt explantation was associated with higher neointimal formation (Fig. 2d; also see Supplementary Material, Tables S3, S4, S6 and S7 for detailed clinical data).

Univariable linear regressions were performed to identify clinical predictors to be associated with greater neointimal hyperplasia and the percentage of relative shunt stenosis. Metric and nominal variables identified in non-parametric analysis were included in the regression model and comprised shunt size (mm), ASA dose at explantation (mg/kg) and diagnosis (HLHS versus other single-ventricle lesions). The variables' weight at implantation (g), birth weight (g), prematurity (≥37 weeks; <37 weeks) and shunt type (mBTA versus right ventricle-to-pulmonary artery anastomosis versus CS) were excluded from the regression analysis, since these variables determine the choice of shunt size. Smaller shunt diameter and lower ASA per weight dose at explantation were significant predictors of higher expression of neointimal hyperplasia and relative shunt stenosis in the univariable linear regression (Supplementary Material, Tables S8–S13).

Clinical relevance of neointimal hyperplasia in systemic-to-pulmonary shunts

Fourteen patients (29%) required early intervention during interstage period. All patients had clinically presented with decreased oxygen saturations and shunt narrowing was evident on catheter angiography (Fig. 4). Early interventions included interventional stent implantation in 6, balloon angioplasty in 4 and surgical shunt revision in 4 patients. A significantly greater area of neointima and shunt stenosis was detected in shunts deriving from patients requiring early intervention compared to the remaining patients ($P = 0.012$ and $P = 0.027$, respectively, Fig. 5). Considering the 6 shunts requiring stent implantation, histological sections of the stent material were obtained in 3 specimens (Fig. 6a and d), where neointima manifested itself in 2 cases above the stent struts (Fig. 6b) and in the other shunt below the stent material (Fig. 6d). In the remaining 3 shunts, surgical preservation of the stents was not possible.

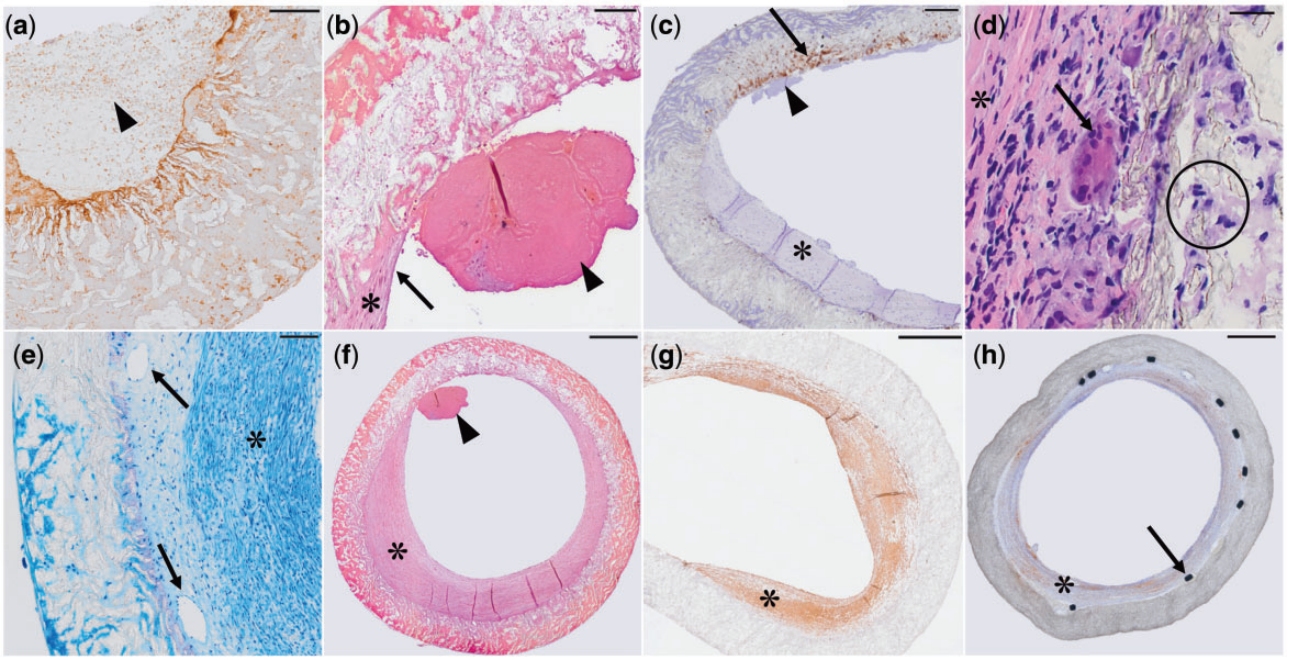


Figure 1: Histopathology of systemic-to-pulmonary shunt. (**a**) Young thrombus formation (triangle); staining: Von Willebrand factor; scale bar, 100 μm; (**b**) organized thrombus (triangle), (*) shows neointimal hyperplasia and arrow points to endothelium; staining, van Gieson; scale bar, 100 μm; (**c**) macrophage phagocytosis of thrombus; arrow points to macrophages, triangle points to thrombus; (*) shows neointimal hyperplasia; staining: $CD68^{+}$; scale bar, 200 μm; (**d**) Macrophages assembling into multinucleated foreign body giant cells 'FBGC' (arrow) and fibroblasts infiltrating the polytetrafluoroethylene material releasing collagen (circle); (*) shows neointimal hyperplasia; staining, Hematoxylin/ Eosin; scale bar, 20 μm; (**e**) neointimal hyperplasia (*) and 2 incised vessels (arrows); staining, Richardson; scale bar, 200 μm; (**f**) neointimal hyperplasia (*) and thrombus (triangle); staining; van Gieson; scale bar, 500 μm; (**g**) cells of neointimal hyperplasia (*) stained with alpha-smooth muscle actin-antibody; scale bar, 500 μm; and (**h**) shunt severely affected by neointimal hyperplasia (*) requiring catheter intervention via stent placement (arrow); staining, alpha-smooth muscle actin-antibody; scale bar, 500 μm

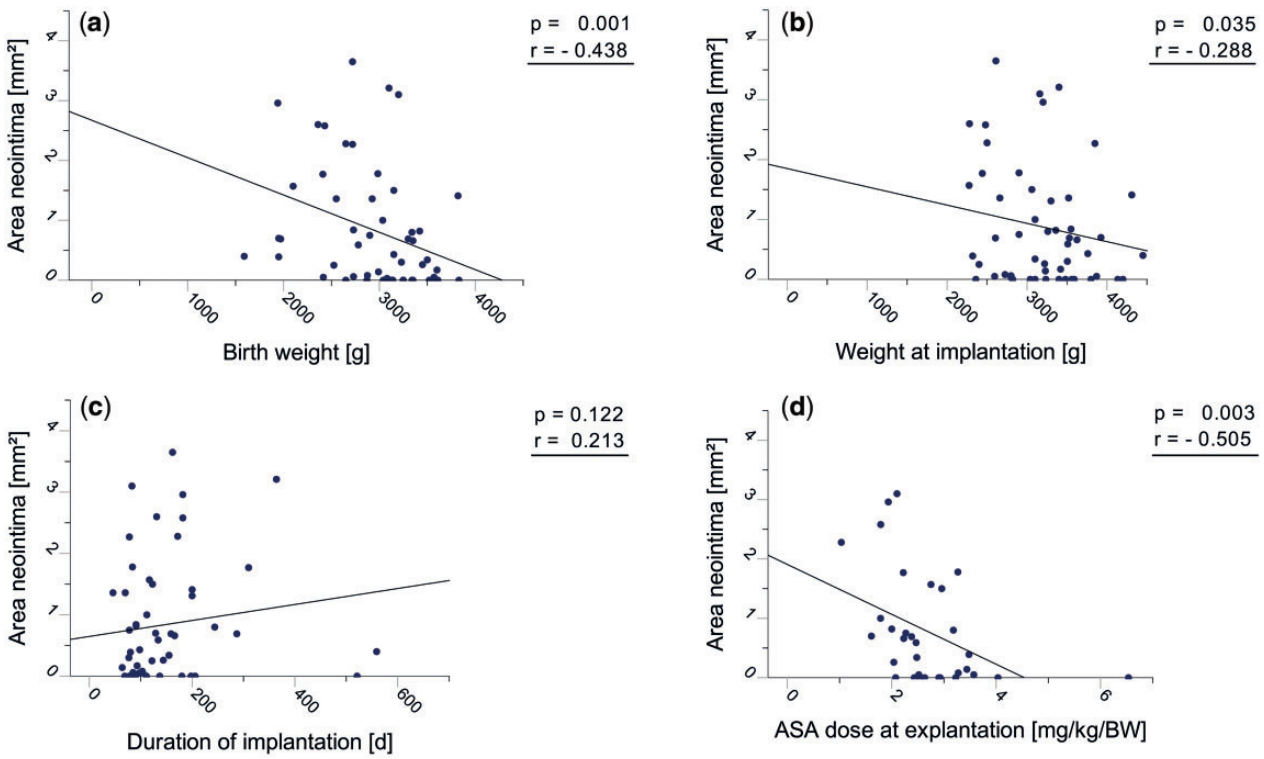


Figure 2: Clinical variables influencing neointimal hyperplasia. Spearman correlations demonstrating association between clinical variables and neointimal hyperplasia; *r*, correlation coefficient.

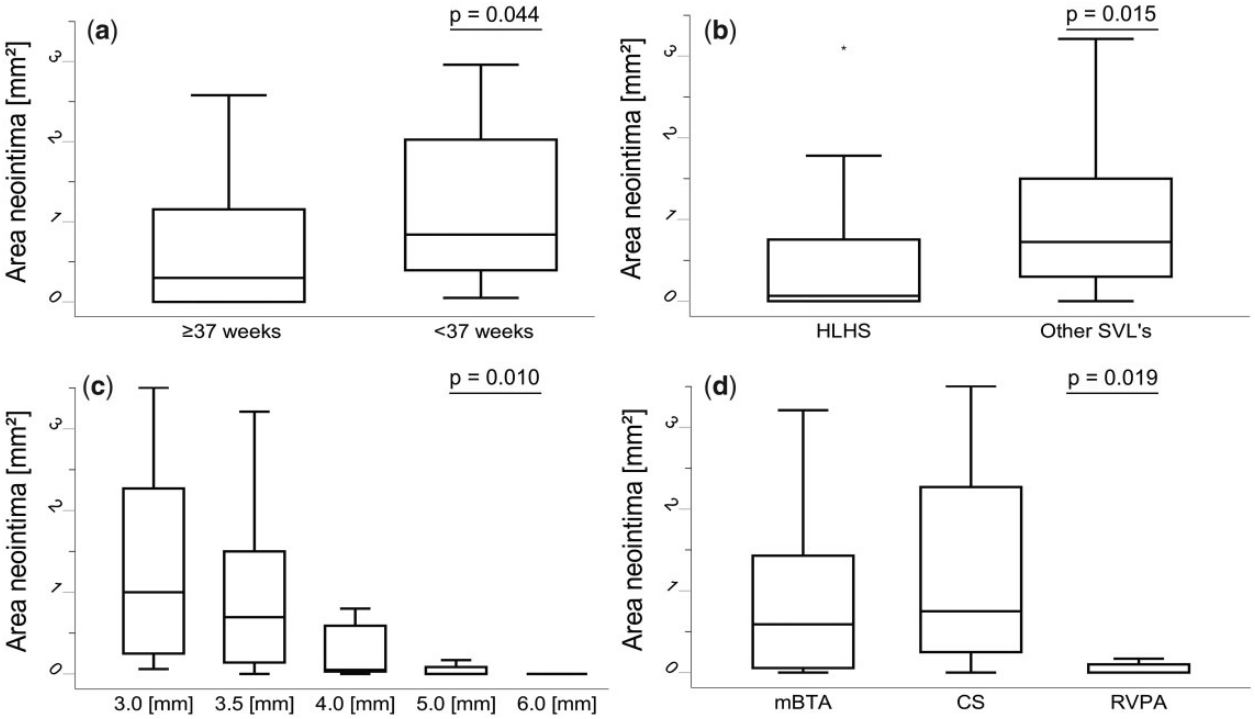


Figure 3: Association of clinical factors and neointimal proliferation. Neointimal hyperplasia was more pronounced in patients born prematurely (**a**) with structural heart disease other than hypoplastic left heart syndrome (**b**), with smaller shunt sizes (**c**) and differed between shunt types (**d**); SVL: single-ventricle lesions; *P*-values: Mann–Whitney *U* test and Kruskal–Wallis test, respectively

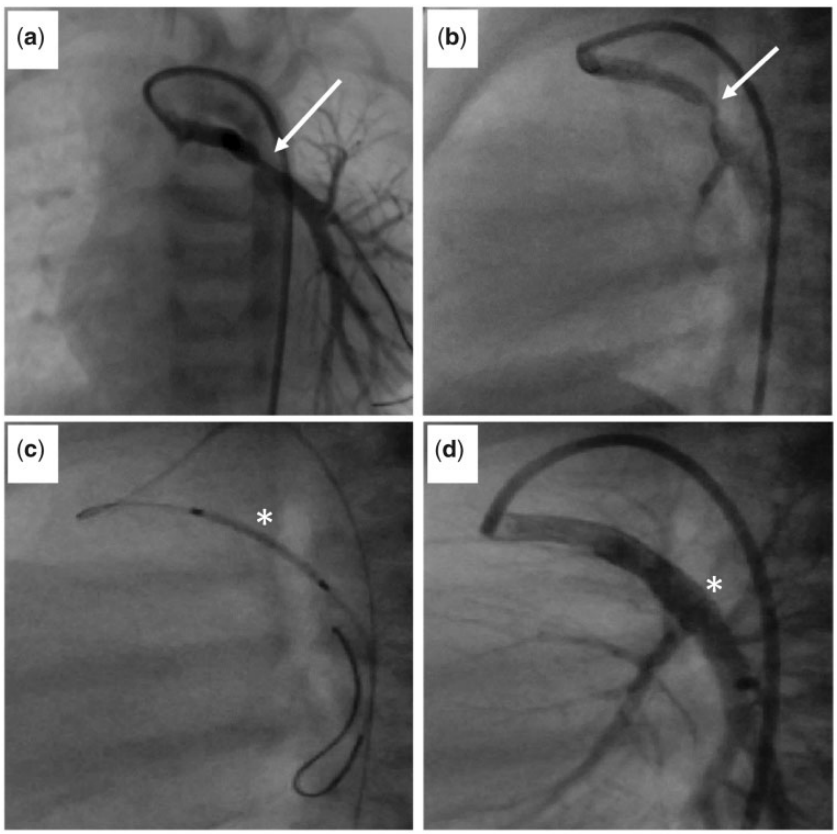


Figure 4: Catheter angiography of systemic-to-pulmonary shunt malfunction before stent placement. Patient with central shunt placement (3 mm). Angiography shows distal narrowing of the shunt (**a** and **b**, arrows). Unrestricted flow over the shunt after successfully implantation of a 3.5 mm × 13 mm Coroflex-Stent (**c** and **d**, stars).

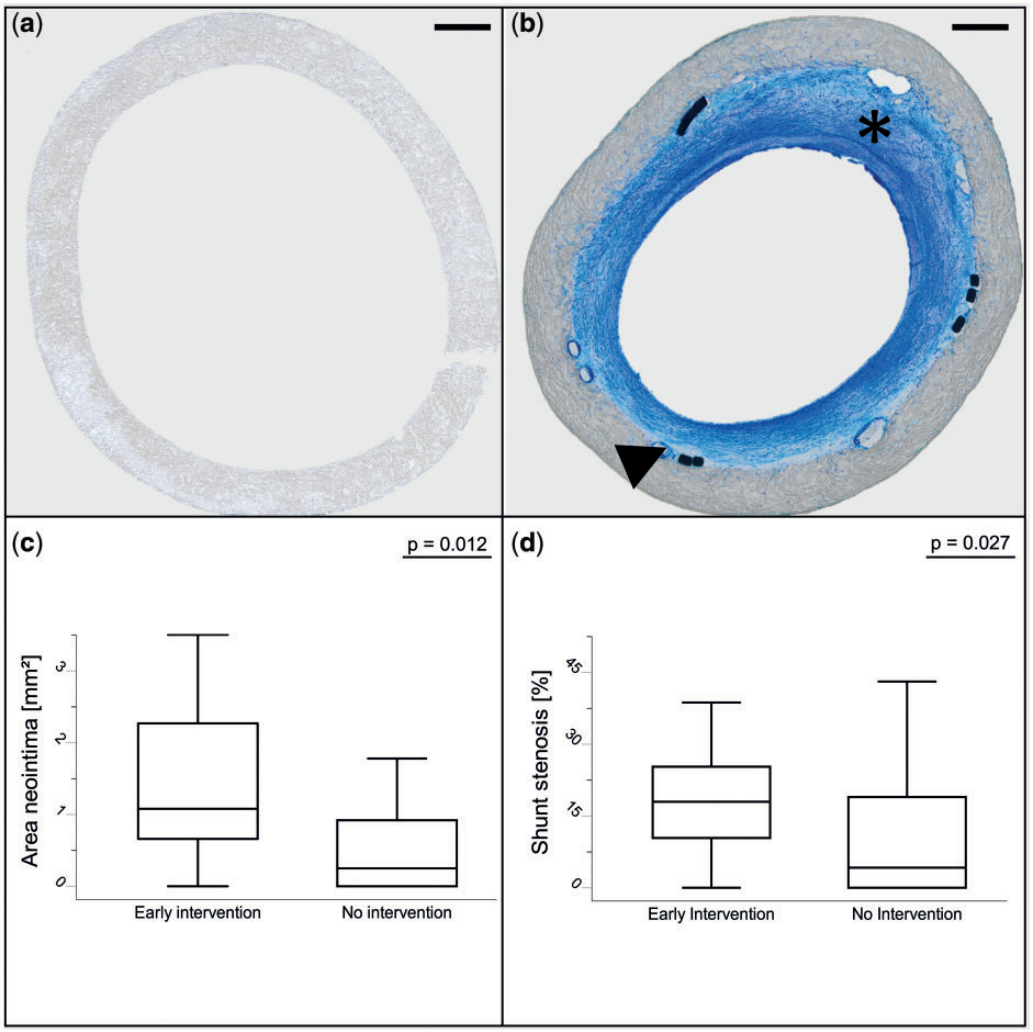


Figure 5: Neointimal hyperplasia and shunt stenosis in the early intervention group. (**a**) Shunt without neointimal formation and polytetrafluoroethylene infiltration (**b**) shunt with stent implantation; (*) neointimal hyperplasia; (triangle) stent; staining, Richardson; scale bar, 500 μm; and (**c** and **d**) boxplots showing distributions of neointima and relative shunt stenosis between groups: early intervention versus no intervention; early intervention included balloon angioplasty; stent implantation; and shunt revision (Mann–Whitney *U* test).

DISCUSSION

Data demonstrated that neointimal hyperplasia and wall-standing thrombi are frequently found in our SP shunts explanted from infants and children with complex cyanotic congenital heart disease after palliative surgery.

Histopathologic examination revealed neointima as the major contributor of relative shunt stenosis, whereas wall-standing thrombi only marginally accounted to the luminal narrowing. Shunt malfunction due to thrombi, however, was described previously [7]. In addition, associations between perioperative thrombocyte transfusion and the growth of neointimal hyperplasia [4] or later shunt malfunction [8] have been described. The differences in those findings compared to the present study might be that first, evaluation of thrombi is biased by histological wash out, fixation and preparation and that second, the cohort studied did not include infants with fatal shunt malfunction.

In the present study, histopathological analysis including immunohistochemical stain for α-SMA revealed that neointimal hyperplasia consists mainly of myofibroblasts and vascular smooth muscle cells. However, when neointima comprises a large area, various other cells such as macrophages, endothelial cells and smaller vessels were found in our histopathologic sections.

We hypothesize that the migration of different types of cells plays a crucial role in the pathomechanism of neointimal formation, which can be supported by several lines of evidence in our study. Involved cells are illustrated in Fig. 1. Consistent with other studies, we assume that myofibroblasts and vascular smooth muscle cells migrate and proliferate from the anastomotic margins into the shunt in response to vascular connection [9], as shown exemplary in Fig. 1g. The patient's body could react against the foreign shunt with protein adsorption to the biomaterial surface after implantation [10]. This creates a thrombogenic surface in the shunt, leading to platelet aggregation and activation [11], which was also demonstrated in the examined histopathologic specimens (Fig. 1a, b, c and f). A thrombogenic surface is known to attract inflammatory cells such as neutrophils and macrophages, which then infiltrate into the biomaterial [12]. In our study, we detected inflammatory cells within neointima as well

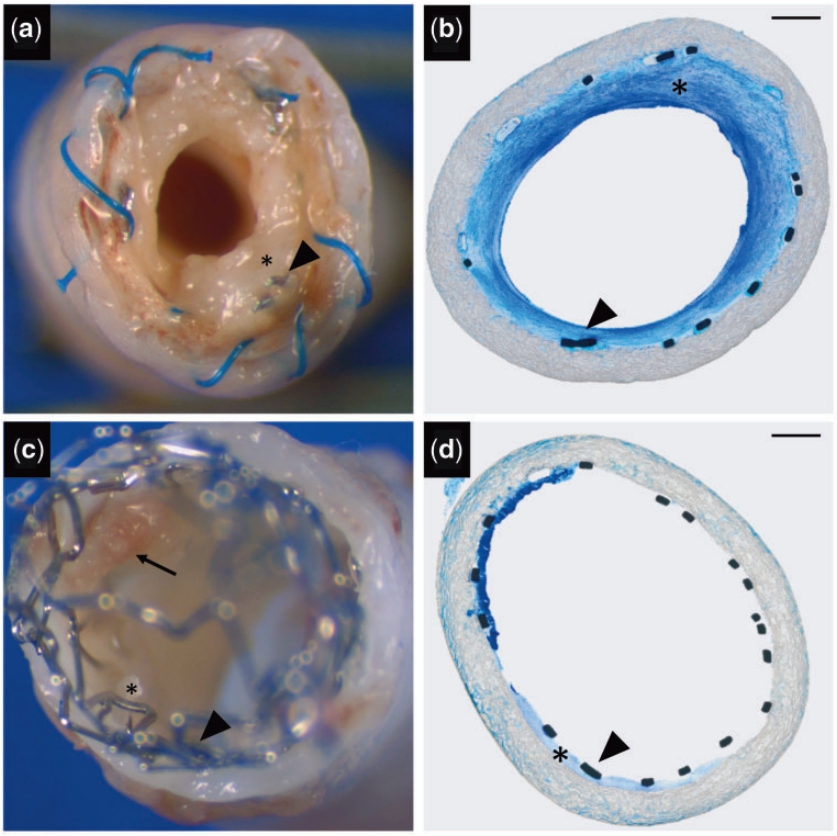


Figure 6: Neointimal hyperplasia in shunts with stent implantation. (**a** and **c**) Macroscopical images; (**b** and **c**) histological sections of shunts with stent implantation (staining, Richardson; scale bar, 500 μm); (*) neointima; triangle, stent; arrow, thrombus. (**b**) Neointimal hyperplasia over and (**d**) under the stent strut.

as infiltrated within the biomaterial by a CD68 immunohistochemical staining (Fig. 1c). A possible mechanism for this is the body's attempt to degrade the biomaterial and newly formed thrombi [12] (Fig. 1c). Failed phagocytosis of the biomaterial forces macrophages to further secrete cytokines, reactive species and enzymes, which exacerbates the inflammation [13]. Macrophages assemble into multinucleated foreign body giant cells [14], as depicted in Fig. 1d, which are reported to be the mediators of the inflammatory and proliferative process [15]. They recruit myofibroblasts and smooth muscle cells, which migrate into the shunt to complete the process of wound healing as demonstrated in Fig. 1g. The final consequence is an accumulation and proliferation of above-mentioned cells in the shunt, aggravating the deposition of extracellular matrix [16] causing gradual stenosis within the shunt with subsequent possible clinical shunt malfunction.

In addition, we aimed to identify clinical factors associated with neointimal hyperplasia. We demonstrated that smaller shunt size was associated with increased neointimal formation on non-parametric analysis and univariable regression. This goes in line with correlations of smaller shunts with the higher incidence of shunt thrombosis [17] and higher shunt-related mortality [8] and increased incidence of interventions [18] described by others. Wells *et al.* quantified mBTA stenosis histopathologically and correlated it with demographic and clinical risk factors. In their study, smaller shunt size (<4 mm) significantly contributed to a stenosis of >50%, due to neointima [4]. One explanation may be that according to Hagen Poiseuille's law of fluid mechanics, flow velocity increases with smaller shunt diameters, which may lead to turbulent flow. Those shear forces on the shunt material may directly or indirectly contribute to the development of neointimal formation.

Another finding of our study was that lower ASA dosage per kilogram body weight by the time of shunt takedown was associated with greater neointimal formation and shunt stenosis. This goes in line with findings of a multicentre prospective study of 1004 infants undergoing bidirectional Glenn/Hemi-Fontan surgery, in which patients who received ASA had a significantly lower risk of shunt thrombosis and death compared with those not receiving ASA [7].

Because ASA is blocking the thrombocyte aggregation, it is possible that higher ASA dosages prevented thrombus formation and, therefore, the initial event of the development of neointimal hyperplasia. In addition, there is recent evidence in preclinical models that ASA, independently from thrombus formation, reduces neointimal growth [19].

Another aim of this study was to investigate the association between the formation of neointima and the need for early shunt intervention. Our data provide evidence that higher expression of neointima and shunt stenosis is significantly more prevalent in the patient group who required early shunt interventions. It has been demonstrated that long-term survival in children who underwent Norwood palliation was significantly worse in the group that required shunt intervention [18]. Thus, increased neointimal proliferation could not only contribute to increased morbidity but might indirectly also impact mortality in this critically ill patient population. Since shunts explanted from children that died of shunt malfunction were not included, our results cannot provide a direct explanation of the

significance of neointima on fatal shunt malfunctions. Our data also indicated that neointima occurred either beneath or over the stent struts on histological sections. Based on those findings, our study cannot provide a definitive conclusion on whether the neointima is the trigger for early intervention or vice versa. Nevertheless, there is evidence that neointimal proliferation accounts for shunt malfunction and the associated early intervention, also supported by the fact that patients without shunt malfunction and thus without intervention displayed significantly fewer neointima. Furthermore, shunts that presented neointima over the stent struts became clinically conspicuous due to exacerbations of oxygen desaturation and fatal luminal shunt narrowing in catheter angiography prior to stent placement, which indicates the presence of neointima before the intervention. We therefore believe that neointima is the trigger for shunt dysfunction and the associated clinical exacerbation of the patients.

Currently, no targeted drug therapy addressing neointimal hyperplasia in SP shunts exists. Additional investigations are necessary to study the effect of coating of PTFE shunts with antiproliferative or antiplatelet agents as sirolimus, paclitaxel or heparin in this setting [20–24].

Limitations

Major limitation of this study is the sample size given the overall rarity of the disease and interventions. Also, our study is descriptive and hypothesis regarding the possible patho-mechanisms of neointimal formation need to be interpreted with caution. Although we can provide evidence for myofibroblasts, smooth muscle cells, thrombi and macrophages by applying standard and immunohistochemical staining methods to our histological specimens, we are not able to provide solid evidence for the speculated early platelet activation and thrombi formation given wash-out phenomena. Although we were able to gather information about the influence of stent placement on neointimal proliferation, our methods did not allow us to describe cause and sequelae given that histopathologic changes were determined at only 1 point in time. We are aware of variation in shunt size and distinct haemodynamics in the various shunt types (specifically central/mBTA versus RV–PA) limiting the interpretation of data. Although we demonstrated more neointima within smaller shunts, a recommendation for the implantation of larger shunts is impracticable. Surgeons must consider various other patient characteristics as avoiding pulmonary overflooding when choosing the correct shunt size.

Even though we demonstrated an association between neointimal proliferation and low ASA dosage, the study is not powered to allow a general statement regarding an optimal ASA dosage. However, it seems of benefit to adjust ASA dosage for increasing weight during the interstage period, once the initial optimal dose has been determined by performing an ASPI test.

Further imaging techniques such as X-ray phase contrast tomography, micro-computer-tomography and Synchrotron could be applied to avoid the shrinkage of the tissue and to analyse unsectioned lumen. However, this was not performed given the retrospective setting of this study and the scarce availability of these advanced imaging techniques.

CONCLUSION

In conclusion, our data provide evidence that neointima in SP shunts is associated with early interventions and therefore contributes to morbidity in infants and children with complex cyanotic congenital heart disease after palliation surgery. Smaller shunt size and lower per weight ASA dosage at shunt takedown are associated with greater neointimal proliferation.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *EJCTS* online.

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Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.

Author contributions

Philip Kottmann: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing—original draft; Writing—review & editing. **Julie Cleuziou:** Investigation; Methodology; Resources; Writing—review & editing. **Julia Lemmer:** Data curation; Resources; Writing—review & editing. **Katja Eildermann:** Methodology; Resources; Validation; Writing—original draft; Writing—review & editing. **Keti Vitanova:** Resources; Writing—review & editing. **Maria von-Stumm:** Resources; Writing—review & editing. **Luisa Lehmann:** Formal analysis; Investigation; Writing—original draft; Writing—review& editing. **Juergen Horer:** Resources; Writing—review & editing. **Peter Ewert:** Resources; Supervision; Writing—original draft; Writing—review & editing. **Matthias Sigler:** Methodology; Resources; Visualization; Writing—original draft; Writing—review & editing. **Cordula M. Wolf:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing—original draft; Writing—review & editing.

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EGFR and MMP-9 are associated with neointimal hyperplasia in systemic-to-pulmonary shunts in children with complex cyanotic heart disease

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Abstract

Systemic-to-pulmonary shunt malfunction contributes to morbidity in children with complex congenital heart disease after palliative procedure. Neointimal hyperplasia might play a role in the pathogenesis increasing risk for shunt obstruction. The aim was to evaluate the role of epidermal growth factor receptor (EGFR) and matrix-metalloproteinase 9 (MMP-9) in the formation of neointimal within shunts. Immunohistochemistry was performed with anti-EGFR and anti-MMP-9 on shunts removed at follow-up palliative or corrective procedure. Whole-genome single-nucleotide polymorphisms genotyping was performed on DNA extracted from patients´ blood samples and allele frequencies were compared between the group of patients with shunts displaying severe stenosis (≥ 40% of lumen) and the remaining group. Immunohistochemistry detected EGFR and MMP-9 in 24 of 31 shunts, located mainly in the luminal area. Cross-sectional area of EGFR and MMP-9 measured in median 0.19 mm^2 (IQR 0.1–0.3 mm^2) and 0.04 mm^2 (IQR 0.03–0.09 mm^2), respectively, and correlated positively with the area of neointimal measured on histology ($r = 0.729$, $p < 0.001$ and $r = 0.0479$, $p = 0.018$, respectively). There was a trend of inverse correlation between the dose of acetylsalicylic acid and the degree of EGFR, but not MMP-9, expression within neointima. Certain alleles in epidermal growth factor (EGF) and tissue inhibitor of metalloproteinases 1 (TIMP-1) were associated with increased stenosis and neointimal hyperplasia within shunts. EGFR and MMP-9 contribute to neointimal proliferation in SP shunts of children with complex cyanotic heart disease. SP shunts from patients carrying certain risk alleles in the genes encoding for EGF and TIMP-1 displayed increased neointima.

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Introduction

Systemic-to-pulmonary (SP) shunts are artificial polytetrafluoroethylene (PTFE) grafts implanted in children with complex congenital cyanotic heart defects in order to secure the pulmonary perfusion between Norwood stage I and II palliation, corrective surgery or another follow-up procedure. Interstage mortality of these infants remains high and is directly influenced by the patency of these grafts, which are at risk of obstruction due to neointimal formation or thrombosis (Agarwal et al. 2017; Fenton et al. 2003; Monagle 2005; Vitanova et al. 2019). Neointimal hyperplasia is associated with interstage morbidity in children with complex and congenital heart disease (Kottmann et al. 2022) and is caused by the foreign body response, which involves the infiltration of immune cells, the formation of granulation tissue, and the generation of a fibrous capsule around foreign material. The resulting pathological vascular remodeling and tissue deposition within the shunt lead to gradual shunt dysfunction (Lee and Ul Haq 2015).

Epidermal growth factor receptor (EGFR) and matrix-metalloproteinase 9 (MMP-9) are proteins linked to hyperproliferative diseases such as bronchial carcinoma (Gong et al. 2016; Pao et al. 2005) and are associated with the formation of neointimal hyperplasia in numerous studies (Newby 2005; Sanchez-Guerrero et al. 2013). In separate rat and porcine study models, targeted suppression of EGFR (Chan et al. 2003; Trieu et al. 2000) and MMP-9 (Song et al. 2020) has been demonstrated to significantly reduce the formation of neointimal hyperplasia after vascular injury.

Since the pathophysiology of neointimal hyperplasia in SP shunts of infants with complex congenital heart disease remains largely unknown, we aimed to identify the role of EGFR and MMP-9 in the formation of neointima in SP shunts. Previous research has suggested that acetylsalicylic acid (ASS) might play a role in neointimal growth (Kottmann et al. 2022). Therefore, we also examined the possible association between ASA and expression of the two proteins.

EGFR and MMP-9 were quantified by immunohistochemistry (IHC) in SP shunts explanted during follow-up surgery and the positively stained area was correlated with the area of neointimal hyperplasia. In addition, we aimed to discover alleles of single-nucleotide polymorphisms (SNPs) in related genes that may predispose to the formation of neointimal hyperplasia.

Currently, there is no drug therapy addressing neointimal hyperplasia in SP shunts of children with cyanotic heart disease. The findings of this study shall contribute to understand the parts of the complex pathogenesis of shunt malfunction and shall provide a basis to identify potential therapeutics to prevent the formation of neointimal hyperplasia in SP shunts.

Methods

Patients and patient material

SP shunts were fixed and stored in formalin immediately after explantation and until further processing. Peripheral blood was collected from respective patients and stored in the cardiovascular biobank at the German Heart Center Munich (KaBi-DHM).

Demographic and clinical data were collected from medical charts at the timepoints date of birth, shunt implantation, and shunt takedown.

Histological processing of the shunt material

Explanted, formalin-fixed shunts were cut into two pieces. One piece was embedded in synthetic resin (methyl methacrylate, Technovit 9100, Kulze Wehrheim, Germany) as described (Quentin et al. 2009); the other in paraffin after dehydration. Standard protocols were applied for the preparation and histochemical stainings Richardson, Hematoxylin/Eosin, and Elastica van Gieson (Mulisch 2010; Quentin et al. 2009). IHC stainings were performed as previously described (Quentin et al. 2009) and optimized for each antibody (Online supplementary, Table 1).

Dotslide system (Olympus) was used to digitize all stained sections and the Olyvia software (Olympus Center Valley, PA, USA) for visualization.

Evaluation of the tissue

ImageJ (ImageJ, US National Institutes of Health, Bethesda, MD, USA) was used for histopathological quantification. For the determination of neointimal hyperplasia, the tissue protruding into the lumen of the shunt and the area of thrombi was measured manually and the greatest value was used for analysis. The ratio of the cross-sectional area of neointimal hyperplasia divided by the cross-sectional area of the potential shunt lumen amounts to the relative shunt stenosis.

$$\text{Shunt stenosis}[\%] = \text{Neo intima}\left[\text{mm}^2\right] / \text{Potential shunt lumen}\left[\text{mm}^2\right]$$

EGFR and MMP-9 were analyzed by measuring the stained area using the color threshold function of ImageJ. Stained area was then related to the area of neointimal tissue.

Identification of single-nucleotide polymorphisms coding for neointimal hyperplasia

Blood samples of the affected patients were obtained after written consent by the parents. High-quality DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen;

Hilden, Germany) according to the manufacturer's recommendation. Concentration was determined by NanoDrop™ spectrophotometer and the integrity of genomic DNA was confirmed by gel electrophoresis. DNA was analyzed for SNPs using the Infinium Omniexpress Kit (Illumina, Inc., San Diego, CA, USA). The Infinium high-density DNA analysis solution combines the Infinium assay with BeadChip microarrays to perform a large genome-wide query of genetic DNA variations. Standard genome-wide SNP genotyping was performed.

Bioinformatic analysis of the Infinium BeadChip data was carried out using PLINK (PUTTY Link). Two categories of clusters were formed based on the shunt stenosis caused by neointimal hyperplasia (Group 1 = 0–39.9% lumen stenosis ($n = 26$); Group 2 = ≥ 40% lumen stenosis ($n = 5$)). A cut-off of 40% was chosen based on a preliminary analysis suggesting clinical relevance in that a shunt stenosis of greater than 40% was associated with an increased risk for cardiac interventions, such as balloon dilatation or shunt stenting (Kottmann et al. 2022). PLINK calculated the SNPs that significantly differed in the two groups (p-value < 0.01, Chi's Square).

Significant SNPs were selected semi-quantitatively based on the following criteria: statistical significance in PLINK cluster analysis, gene associations with EGFR and MMP-9 pathways, and previous presence of the individual SNPs in scientific publications.

SNP data were analyzed with the web-based SNPnexus tool (www.snp-nexus.org), which provides bioinformatic data from different genome databases and assigns them to the entered SNP query, enabling to allocate overlaps with structural DNA elements to predict functional gene protein consequences and to retrieve links with previous genetic disease studies (Chelala et al. 2008; Dayem Ullah et al. 2012, 2013; Dayem Ullah et al. 2018). GRCh/hg19 was used for the analysis of the human genome. Allele frequencies of the study population were stratified and genotypes linked with neointimal formation and shunt stenosis were determined as alleles in association with neointimal hyperplasia and relative shunt stenosis.

Statistics

SPSS Version 27 was used for all statistical analysis. Data are provided as median and interquartile range (IQR). Mann–Whitney U tests were applied for nominal variables with two categories for non-parametric analysis. Kruskall–Wallis test was used for more than two categories. To determine the strength of the association between two variables, Spearman's Rho non-parametric test was performed. For the comparison of nominal variables, Chi's Square and Fisher's exact tests were used, respectively. A multivariate linear regression was conducted to examine the association between EGFR [mm^2], MMP-9 [mm^2], and ASA dosage per bodyweight [mg/kg/BW] and their impact on neointimal hyperplasia. p-values are stated raw unless a number below $p = 0.001$, in which case they are corrected to $p < 0.001$.

An overview of the study design can be found in Fig. 1.

Results

Patient characteristics

SP shunts and blood for DNA extraction were collected between February 2011 and August 2016. 40 patients with Caucasian background that underwent palliative correction using a PTFE shunt gave written consent to the study and were initially included into the study. Three samples had to be excluded due to poor quality of extracted the DNA. Another six patients were excluded because of previous stent placement or balloon dilatation prior to shunt explantation, which could have influenced neointimal proliferation. SP shunt neointimal hyperplasia, SNP analysis from patient blood DNA, and clinical data interpretation were analyzed from 31 patients (18 (58%) male), as outlined in Fig. 1. The most common underlying structural heart disease included hypoplastic left heart syndrome in 15 patients (48%) and pulmonary atresia with and without ventricular septal defect in 7 patients (23%) (Table 1). At the time of implantation, children were in median 9 (IQR 7–14) days old. SP shunt was explanted after a median of 117 (IQR 85–198) days due to a Glenn procedure, corrective surgery, or shunt revision. The diameter of the implanted shunts was 3 mm in 3 (9.7%), 3.5 mm in 17 (54.8%), 4 mm in 2 (6.6%), and 5 mm in 9 (29%) patients (Table 1).

Platelet inhibition was performed in the form of ASA dosed to efficacy based on thrombocyte-functioning test prior to stage I palliation and measured in median 2.5 mg / kg/BW [2–3 mg/kg/BW] at the time of shunt removal.

Morphometric measurements of shunt lumen and neointimal hyperplasia

Neointimal hyperplasia occurred in 20 out of 31 shunts (65%). The median of shunt stenosis was 17% (IQR 2–34%) and greater than 40% in five patients. The area of neointima was in median 0.84 mm^2 (IQR 0.14–2.54 mm^2) (Table 2, Asterix in Fig. 2). A comparison of macroscopy and microscopy of neointimal hyperplasia is shown in Fig. 2.

Immunohistochemical detection and measurement of EGFR and MMP-9

Due to insufficient quality, seven samples of the IHC for EGFR and six samples for MMP-9 were not included in the analysis.

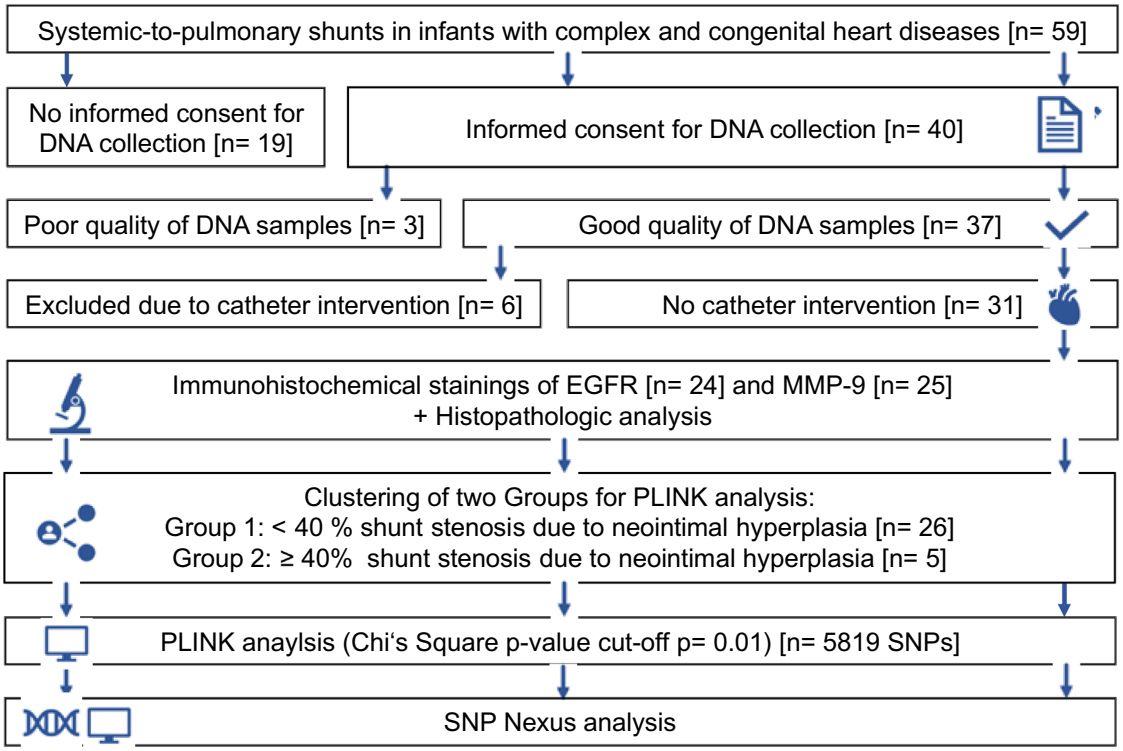

Fig. 1 Visual abstract. Flow chart depicting the study design; *EGFR* epidermal growth factor receptor; *MMP-9* matrix-metalloproteinase-9; *SNP* single-nucleotide polymorphism; *PLINK* PUTTY Link

The stained area after EGFR detection was in median 0.19 mm^2 (IQR 0.1–0.3 mm^2, $n=24$), per cross-section, that of MMP-9 in median 0.04 mm^2 (IQR 0.03–0.09 mm^2, $n=23$) (Table 2). The area of EGFR and MMP-9 significantly correlated with the area of neointimal hyperplasia (Fig. 3). EGFR and MMP-9 were mainly detected in the luminal region of the PTFE material and showed a ring-like structure (see Fig. 4). Cell morphology was that of macrophages and foreign body giant cells, which partly migrated into the shunt material and assembled between neointima and PTFE biomaterial (Fig. 4). Figure 5 visualizes the correlation from Fig. 3 by showing shunts with severe neointimal proliferation and a greater amount of EGFR and MMP-9 next to those with mild expression and no tissue formation.

There was a trend of negative correlation between the ASA dosage per kilogram of body weight and the cross-sectional area of EGFR ($p=0.073$, $r=-0.381$, Spearman-Rho). There was no correlation between ASS dosage and MMP-9 expression ($p=0.719$, $r=-0.077$, Spearman-Rho).

Multivariate regression

In a multivariate regression, we examined the influence of the variables EGFR [mm^2], MMP-9 [mm^2], and acetylsalicylic acid (ASA) dosage [mg/kg/BW] on the variable neointimal hyperplasia [mm^2]. The variables MMP-9 [mm^2] and EGFR [mm^2] remained as significant predictors for greater neointimal formation on regression analysis ($p=0.001$; adjusted $R^2=0.690$, online supplementary tables 2–6).

Single-nucleotide polymorphisms

PLINK analysis from patient DNA resulted in 5819 SNPs that differed significantly between shunts with severe stenosis (> 40%) and shunts with mild to no stenosis (Chi's Square, p-value cut-off = 0.01). Out of these, 2270 SNPs located in protein coding genes and of these, 86 were nonsynonymous, meaning that they alter the amino acid sequence of the encoded protein**.** Remaining SNPs were either located in noncoding intron variants or in the following transcript structures coding variants: 5′-UTR, 5′ upstream, 3′-UTR, 3′ upstream, and 3′ downstream transcript variant isoforms.

Evaluation of all SNPs for their relationship to genes and pathways around EGFR returned the SNPs, rs2237051, rs2298989, and rs2298999 which were all located within the EGF gene that on chromosome 4 between the bases 109970517 and 109980042. Related to MMP-9 was the SNP

Table 1 Demographic parameters

Clinical parameters	Characters	No. (%)/median [range]
Gender	Male	18 (58.1%)
	Female	13 (41.9%)
Diagnosis	HLHS	15 (48.4%)
	PA + VSD	6 (19.4%)
	PA	1 (3.2%)
	TOF	3 (9.7%)
	DORV	1 (3.2%)
	TA	2 (6.5%)
	TGA	1 (3.2%)
	Other	2 (6.5%)
Shunt type	mBTTS	21 (67.7%)
	RVPA	7 (22.6%)
	CS	3 (9.7%)
Shunt diameter [mm]	3	3 (9.7%)
	3.5	17 (54.8%)
	4	2 (6.5%)
	5	9 (29%)
Birth weight [grams]		3060 [2650–3340]
Age at implantation [days]		9 [7–14]
Days of implantation [days]		117 [85–198]
ASA dosage [mg/kg/BW]		2.50 [2.09–2.98]

No. (%), number of patients (percentage)

HLHS hypoplastic left heart syndrome; *TOF* tetralogy of Fallot; *PA + VSD* pulmonal atresia with ventricular septal defect; *PA* pulmonal atresia; *TGA* transposition of great arteries; *DORV* double outlet right ventricle; *TA* tricuspidal atresia; *mBTTS* modified Blalock-Taussig-Thomas Shunt; *RVPA* right-ventricle-to-pulmonary-artery shunt; *CS* central shunt; *ASA dosage [mg/kg/BW]* ASA dosage per kilogram bodyweight at the time of shunt removal

rs6609533 within the TIMP-1 gene on the X-chromosome at base position 47585887 (Table 4).

Allele stratification revealed allele "C" in rs2298989 [EGF] and rs2298999 [EGF] to be associated with higher expression in neointimal hyperplasia and shunt stenosis. For the polymorphisms rs2237051 [EGF] and 6609533 [TIMP-1], allele "G" was associated with greater neointima and relative shunt stenosis (Table 3 and Fig. 6).

Functional annotations of selected SNPs by SNPnexus

Due to alternative splicing and thus various transcript isoforms, each SNP can take different forms of transcript modifications. All functional annotations of transcripts are summarized in Table 4. Transcript isoforms of rs2237051 [EGF] and rs6609533 [TIMP-1] are classified as nonsynonymous, leading to a missense variation, which in the first case manifests as an amino acid exchange from methionine to isoleucine and in the second case from threonine to alanine or from threonine to serine. In different transcript variants, rs2237051 [EGF] and rs6609533 [TIMP-1] are considered to be in the 5′ upstream 3′ upstream region, respectively. rs2298999 [EGF] and rs2298989 are located on intronic and non-coding-intronic DNA elements (see online supplementary tables 7–9 for detailed information about each transcript isoform and whole PLINK analysis). SNPnexus analysis can be reproduced by entering the PLINK analysis (online supplementary table 9) in the query function of the web-based tool www.snp-nexus.org.

Table 2 Histopathological and immunohistochemical parameters

Histopathological parameters	Categories	No. (%)/median [range]
Neointimal hyperplasia	Yes	20 (64.5%)
	No	11 (35.5%)
Area of neointimal hyperplasia [mm^2]		0.84 [0.14–2.58]
Shunt stenosis due to neointimal hyperplasia [%]		16.93 [2.16–34]
Thrombi	Yes	12 (61.3%)
	No	19 (38.7%)
Area of thrombi [mm^2]		0.13 [0.08–0.48]
Shunt stenosis due to thrombi [%]		2.07 [0.41–6.46]
EGFR [mm^2]		0.19 [0.1–0.3]
MMP-9 [mm^2]		0.04 [0.03–0.09]

No. (%), number of patients (percentage)

EGFR epidermal growth factor receptor; *MMP-9* matrix-metalloproteinase-9; *mm* millimeter; mm^2 square millimeter

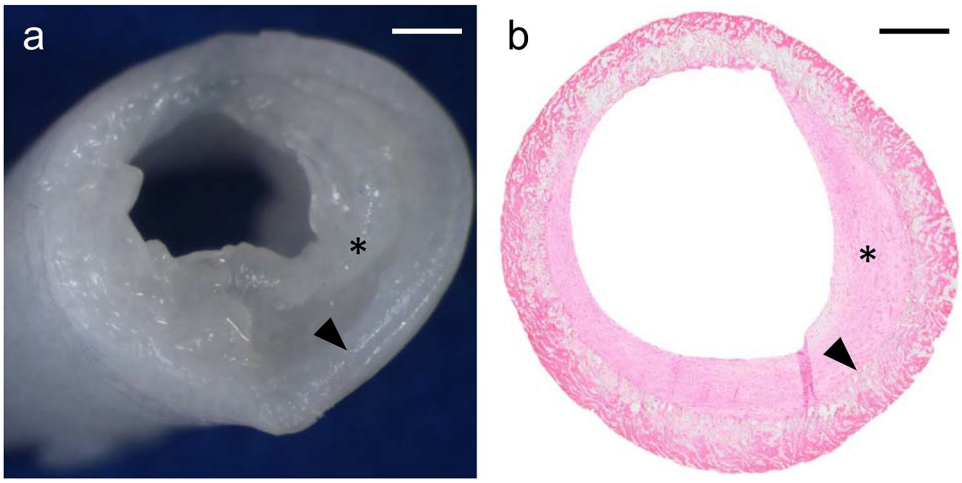

Fig. 2 Macroscopical and microscopical shunt image. **a** macroscopical image of a systemic-to-pulmonary PTFE shunt; scale bar 500 µm. **b** Hematoxylin/Eosin (HE) image showing the cross-section of the identical shunt; scale bar 500 µm. In both panels, the star [*] depicts the neointimal formation and the [triangle] shows the border between PTFE material and neointimal hyperplasia

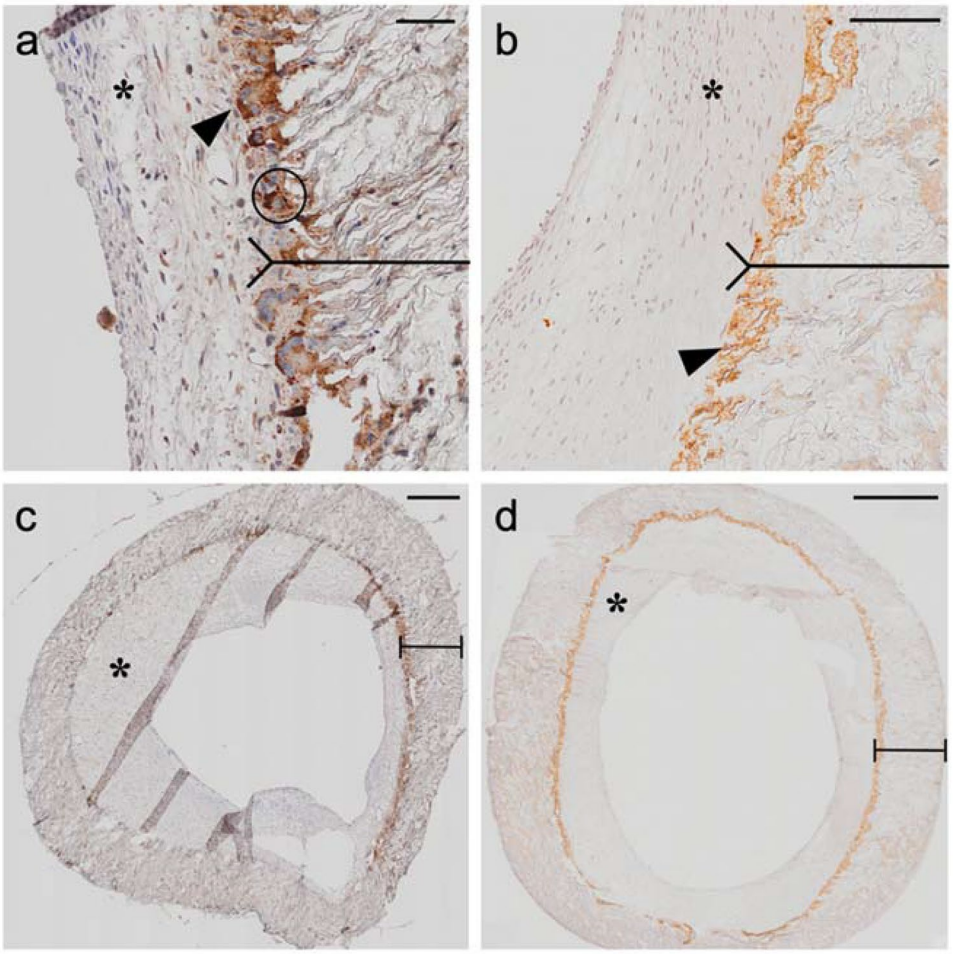

Fig. 3 Immunohistochemical staining of EGFR and MMP-9. Cross-sectional images of systemic-to-pulmonary shunts implanted in children with complex and congenital heart disease. In section **a** and **b**, the reverse arrow points to the border between PTFE material and neointimal formation, whereas the star [*] displays neointimal hyperplasia. Scale bar **a** = 20 µm, **b** = 50 µm. In section **c** and **d**, the double-ending line represents the PTFE material. The triangles point toward the targeted proteins of the immunohistochemical staining which resemble MMP-9 in panel **a** and **c** and EGFR in panel **b** and **d**. Scale bars in **c**–**d** = 500 µm. Both stainings demonstrate that the proteins are located mainly in the luminal area of the PTFE material, which is illustrated by the line in panel **a** and **b**. Morphology identifies stained cells as macrophages (Circle, panel **a**). *EGFR* epidermal growth factor receptor; *MMP-9* matrix-metalloproteinase-9

Discussion

Neointimal hyperplasia in systemic-to-pulmonary (SP) shunts has recently been associated with interstage morbidity in children with complex and congenital heart defects (Kottmann et al. 2022). Understanding the complex pathophysiology of neointimal formation could identify potential drug targets to reduce cardiovascular interventions in SP shunts in this critical ill population. To our knowledge, this is the first study to examine the

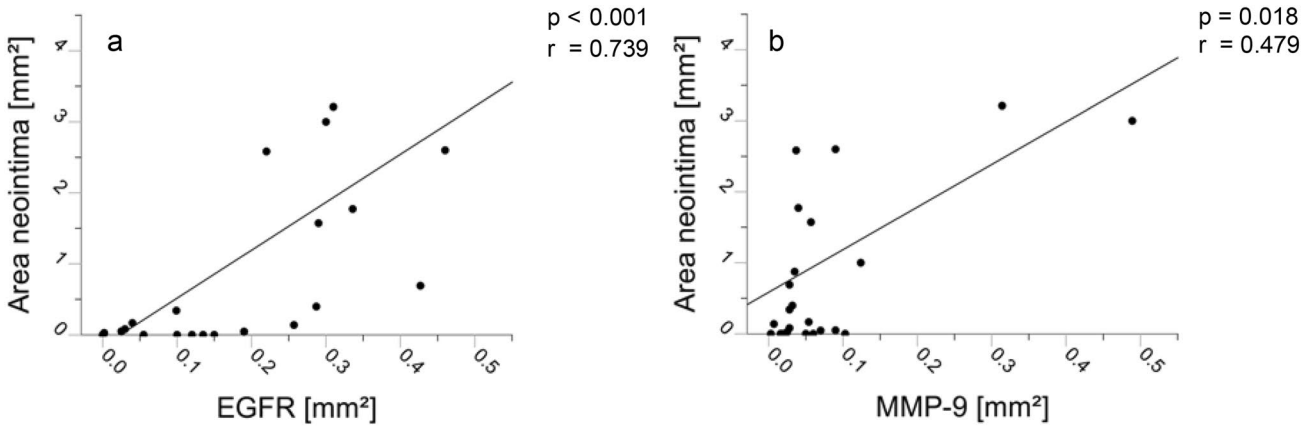

Fig. 4 EGFR [mm^2] and MMP-9 [mm^2] are associated with neointima hyperplasia [mm^2]. Dot-Plots. Spearman correlations showing the correlations between the variables MMP-9 [mm^2] and EGFR [mm^2] and neointimal hyperplasia [mm^2], [mm^2] = square millimeters; *r* correlation-coefficient; *EGFR* epidermal growth factor receptor; *MMP-9* matrix-metalloproteinase-9

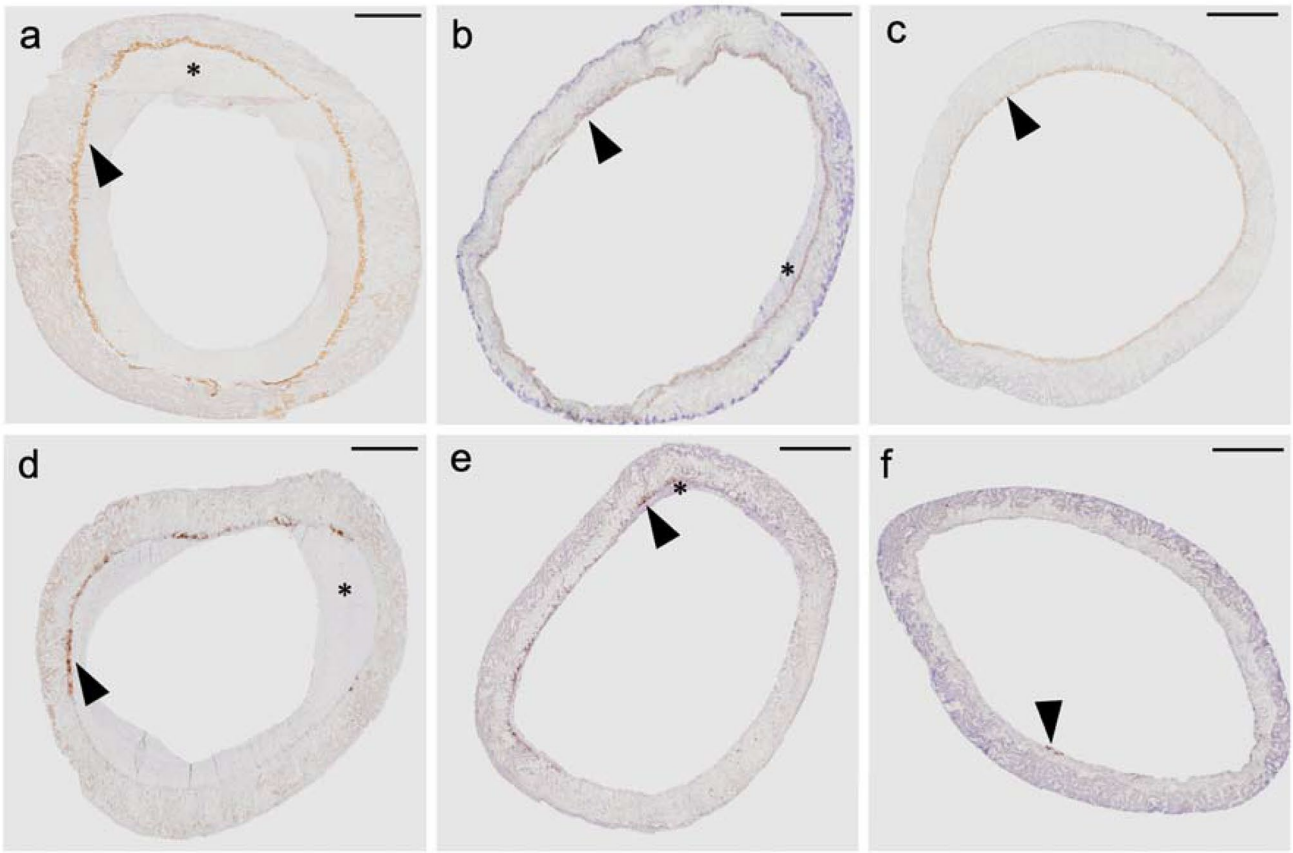

Fig. 5 EGFR and MMP-9. Comparison of systemic-to-pulmonary shunts with respect to neointimal proliferation and immunohistochemical (IHC) stainings of EGFR (panel **a**, **b**, **c**) and MMP-9 (**d**, **e**, **f**). Panelsl (**a**, **d**) show severe neointimal proliferation and greater distribution of the IHC staining EGFR and MMP-9 compared to panels (**b**, **e**) showing mild and panel (**c**, **f**) presenting zero neointimal proliferation and fewer IHC stainings. The star [*] displays neointimal hyperplasia and the triangles point toward EGFR or MMP-9-positive areas. Scale bar in all panels = 500 µm. *EGFR* epidermal growth factor receptor; *MMP-9* matrix-metalloproteinase-9

Table 3 Single-nucleotide polymorphisms and allele-specific distribution of neointimal hyperplasia and shunt stenosis in systemic-to-pulmonary PTFE shunts

Gene	ID	REF	ALT	Minor	MAF	Allele	Frequency No. (%)	Stenosis [%] Median [IQR]	p_1	Neointima [mm^2] Median [IQR]	p_2
EGF	rs2237051	A	A	G	0.382188	AA	8 (25.8%)	0.34 [0–3.95]	0.049	0.02 [0–0.19]	0.028
						AG	14 (45.2%)	4.73 [0–29.13]	0.936	0.28 [0–1.57]	0.872
						GG	9 (29%)	17.04 [2.16–48.67]	0.071	1 [0.06–3]	0.053
EGF	rs2298999	T	C	T	0.332268	CC	11 (35.5%)	17.04 [0.17–48.67]	0.087	1 [0.03–3]	0.066
						CT	12 (38.7%)	4.73 [0.51–23.09]	0.935	0.28 [0.04–1.26]	0.870
						TT	8 (25.8%)	0.34 [0–3.95]	0.49	0.02 [0–0.19]	0.028
EGF	rs2298989	T	C	T	0.357628	CC	8 (25.8%)	21.18 [9.49–49.82]	0.032	1.98 [0.38–3.11]	0.022
						CT	14 (45.2%)	2.1 [0–17.06]	0.601	0.15 [0–0.95]	0.658
						TT	9 (29%)	0.69 [0–6.66]	0.293	0.05 [0–0.34]	0.201
TIMP-1	rs6609533	A	G	G	0.473377	AA	17 (54.8%)	0.17 [0–6.66]	0.001	0.03 [0–0.34]	0.002
						AG	7 (22.6%)	34 [6.63–48.67]	0.01	1.57 [0.4–3.21]	0.019
						GG	7 (22.6%)	25.32 [1.25–42.28]	0.197	1.77 [0.05–2.96]	0.197

Each single-nucleotide polymorphism (SNP) and the corresponding overlapping gene with genomic coordinates. Distribution of neointimal hyperplasia [mm^2] measured in shunts from patients with the respective genotype (e.g., AA; AG; GG) is shown. Additionally, the table shows the reference (REF)-, altered (ALT)- , and minority alleles (MA) with the belonging minor allele frequencies (MAF) of each polymorphism. ID, rs number; REF, reference allele; ALT, observed allele; Minor, minor allele; MAF, minor allele frequency (1000 Genomes); No. (%) patients in percent; p_1, *p*-value of Mann–Whitney U tests comparing the different genotypes against distribution of shunt stenosis; p_2, *p*-value of Mann–Whitney *U* tests comparing the different genotypes against the area distribution of neointimal hyperplasia; EGF, epidermal growth factor; TIMP-1, tissue inhibitor of metalloproteinases 1; A, Adenine; G, Guanine; C, Cytosine; T, Thymine; p-value Mann–Whitney-U test comparing one allele frequency with two other frequencies (e.g., AA vs. AG + GG, etc.)

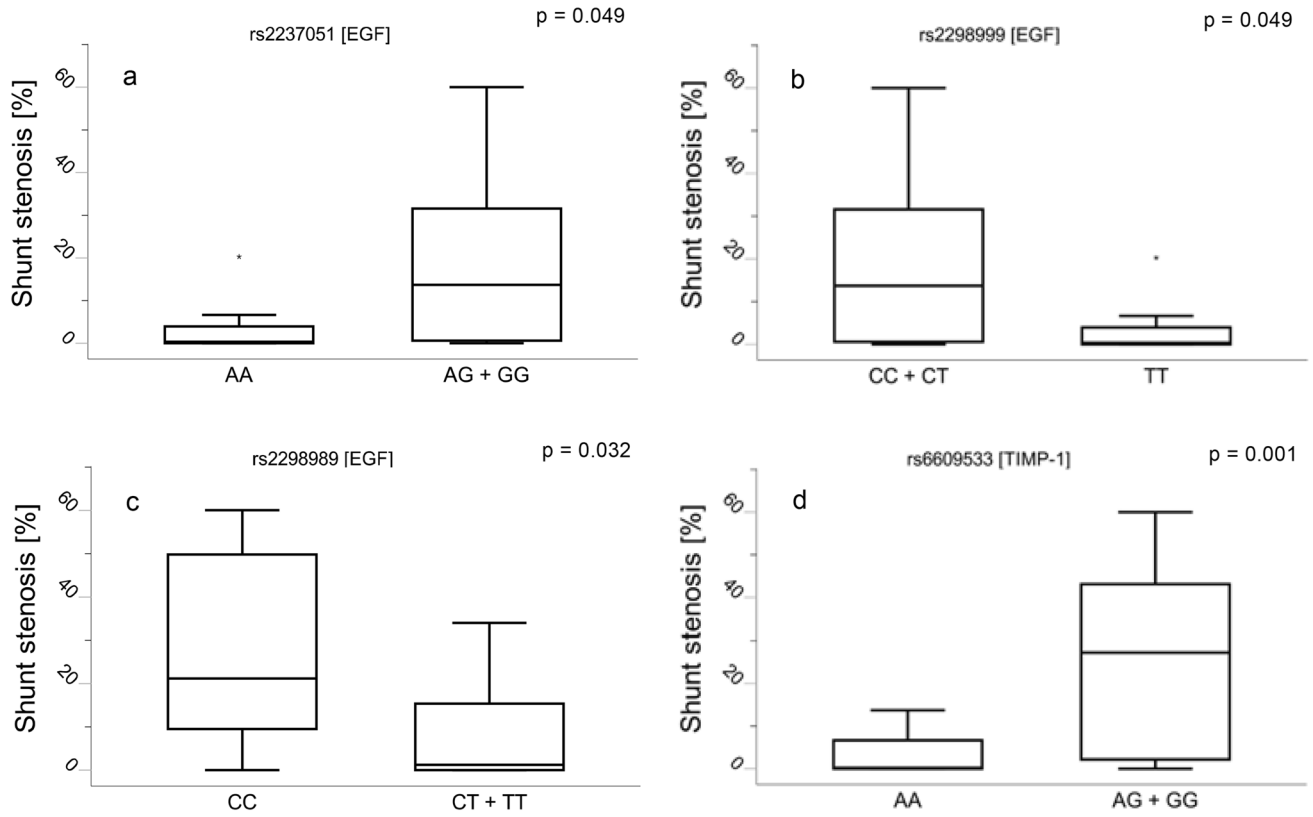

Fig. 6 Alleles associated with relative shunt stenosis due to neointimal hyperplasia. Distribution of shunt stenosis and neointimal hyperplasia (*y*-axis [%]) is shown in patients grouped for distinct genotypes. *EGF* epidermal growth factor; *TIMP-1* tissue inhibitor of metalloproteinase-1; *A* adenine; *G* guanine; *C* cytosine; *T* thymine; * extruders

Table 4 Detailed information about respective single-nucleotide polymorphisms (SNPs)

Gene	rs number	Chr: base position	Annotations of transcript isoforms	AA change
EGF	rs2237051	4:109980042	Coding nonsyn, 5upstream	M < I
EGF	rs2298999	4:109990751	Non coding intronic, intronic	
EGF	rs2298989	4:109970517	Non coding intronic, intronic	
TIMP-1	rs6609533	X:47585887	Coding nonsyn, 3upstream, intronic, 3utr	T > A|T > S

Transcript modifications resulting in various predicted protein consequences are shown in the "Annotation" column [ENSEMBL]. Due to alternative splicing and thus various transcript isoforms, each SNP can take different forms of transcript modifications. Chr, chromosome number; 3utr, 3′ untranslated region of transcript; 3upstream, within 2 kb upstream of the 3′ end of a transcript; 5upstream, within 2 kb upstream of the 5′ end of a transcript AA change [ENSEMBL], reference amino acid(s),">", observed amino acid(s); M, methionine; I, isoleucine; T, threonine; S, serine; EGF, epidermal growth factor; TIMP-1, tissue inhibitor of metalloproteinases 1

expression of EGFR and MMP-9 and to assess the presence of risk alleles for neointimal formation in SP shunts from children with complex cyanotic congenital heart disease. Neointimal hyperplasia is subject to a multifactorial genesis: initial endothelial dysfunction and activation of the connected vessel are accompanied by platelet activation, aggregation, and thrombus formation (Angelini et al. 1990; Bonatti et al. 2004; Zain et al. 2020). This is followed by leukocyte and macrophage recruitment (Angelini et al. 1990) causing thrombus degradation, which in turn results in fibrosis. Metalloproteinase-induced extracellular matrix degradation leads to facilitated migration and proliferation of SMCs and monocytes, causing enrichment of these cells in the shunt lumen (Rotmans et al. 2004). Under certain conditions, these migrated SMCs undergo a phenotype switch from the contractile-silent into the secretory-proliferative phenotype aggravating the hyperproliferation inside the shunt lumen (Campbell and Campbell 1990; Thyberg et al. 1995). The final consequence is an accumulation of SMCs and myofibroblasts in the shunt lumen depositing extracellular matrix and collagen, which leads to gradual progression of shunt stenosis to a total shunt occlusion (Casscells 1992; Clowes and Reidy 1991).

The aim of the study was to quantify EGFR and MMP-9 in explanted SP shunts of children with complex cyanotic heart disease and to identify the risk alleles in related genes which might impact signaling pathways promoting the formation of neointimal hyperplasia. A schematic representation of the possible pathophysiology and pathways of the formation of neointimal hyperplasia in SP shunts are shown in Fig. 7.

EGF/EGFR

EGF plays a major role as growth factor in the regulation of cell proliferation and differentiation (Sanchez-Guerrero et al. 2013). Its receptor EGFR is expressed by numerous cells such as vascular smooth muscle cells, macrophages, and endothelial cells (Bagheri-Yarmand et al. 2000; Lamb et al. 2004; Tamura et al. 2001). EGF binds with high affinity to its membrane-bound receptor that is located on the cell surface and triggers intrinsic tyrosine kinase activity (Johnson et al. 2004). The tyrosine kinase initiates a signaling cascade influencing cell metabolism and thus stimulates the expression of genes enhancing DNA synthesis, cell proliferation, and angiogenesis (Bagheri-Yarmand et al. 2000; Johnson et al. 2004; Okada et al. 1997).

Dysregulation of this tightly balanced system by overexpression, amplification, or mutation has been associated with hyperproliferative diseases such as cancer and the presence of neointimal hyperplasia (Chan et al. 2003; Huang and Harari 1999; Olayioye et al. 2000; Trieu et al. 2000).

Trieu et al. and Chan et al. demonstrated in separate rodent models significantly less restenosis from neointimal hyperplasia by inhibition of EGFR with an EGFR inhibitor (EGFR genistein) and by blocking the receptor with a monoclonal IgG antibody after carotid artery injury (Chan et al. 2003; Trieu et al. 2000).

Nicholl et al. showed that blocking EGFR in rat aortic SMCs suppresses not only cell proliferation but also migration of vascular smooth muscle cells in vitro (Nicholl et al. 2005).

In a recent study, Foth et al. found an upregulation of EGFR by 2.8-fold in the thickened walls of bioprosthetic valved conduits and demonstrated that the interaction of macrophages and EGFR in particular might play a role in conduit stenosis (Foth et al. 2021). In line with other studies, they report that EGFR-positive macrophages possibly reflect an activated state responsible for inflammatory processes and thus the formation of neotissue (Hardbower et al. 2017; Hoyer et al. 2019). A blockade of EGFR reduces the chronic inflammatory process, potentially resulting in decreased stenosis in conduits (Foth et al. 2021; Tang et al. 2019).

In our study, the cross-sectional amount of EGFR significantly correlated with the area of neointimal hyperplasia and was an independent predictor for its formation in multivariate analysis. Additionally, we identified SNPs

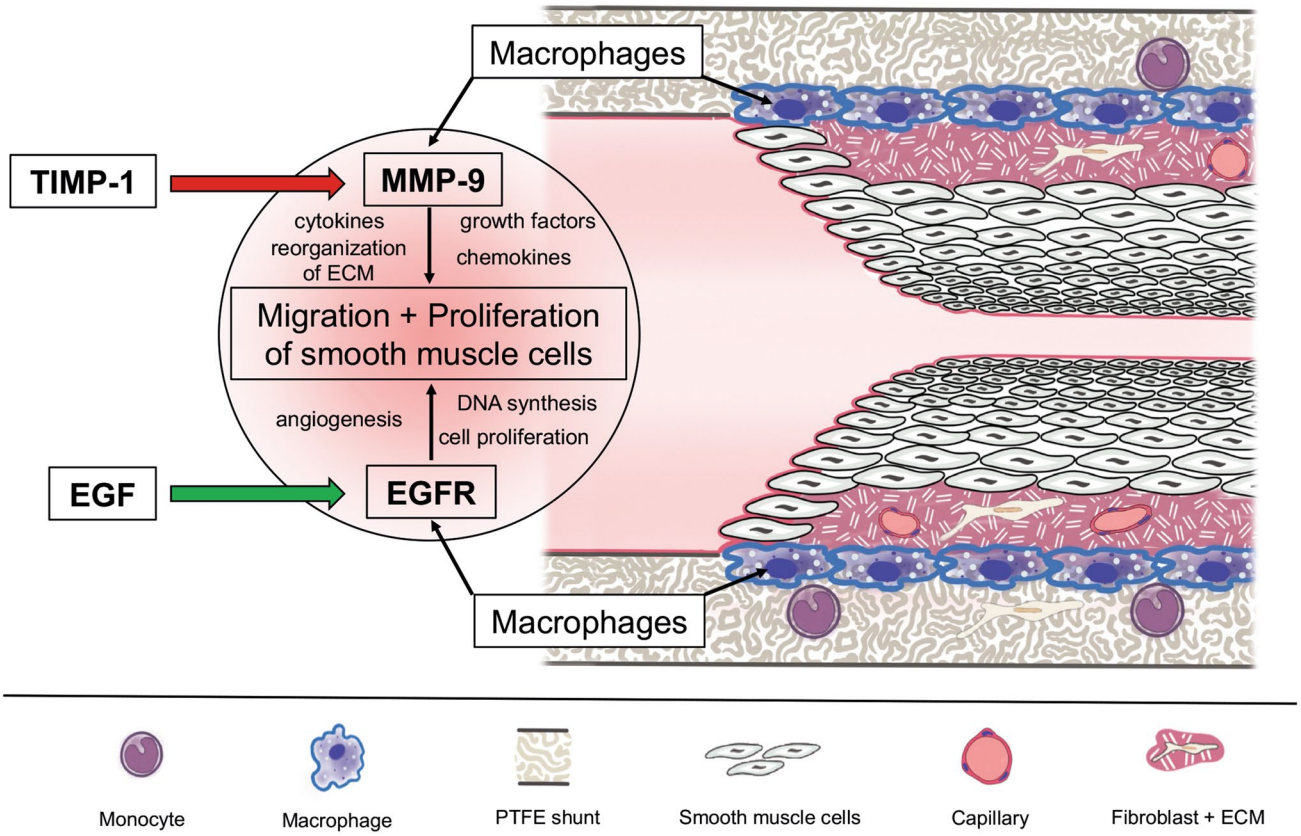

Fig. 7 Possible pathways influencing neointimal hyperplasia in systemic-to-pulmonary shunts EGFR and its ligand EGF play a crucial role in the regulation of cell proliferation and differentiation. Upregulation of the receptor was associated with the growth of neointimal hyperplasia. By binding to EGFR on the cell surface of, e.g., macrophages, tyrosine kinase cascade activity stimulates the expression of genes involved in DNA synthesis, cell proliferation, and angiogenesis. Therefore, upregulation of this pathway by, e.g., single-nucleotide polymorphisms could promote the enhanced formation of neointimal hyperplasia. MMP-9 secreted from macrophages promotes the migration and proliferation of smooth muscle cells and monocytes by the degradation and reorganization of extracellular matrix and the regulation of cytokines, chemokines, and growth factors. TIMP-1 serves as a strong inhibitor of the metalloproteinases, so that a loss of function of TIMP-1, as indicated in our SNP analysis, could lead to a predominance of MMP-9 and the formation of neointimal hyperplasia. *EGF* epidermal growth factor; *TIMP-1* tissue inhibitor of metalloproteinase-1; *EGFR* epidermal growth factor receptor; *MMP-9* matrix-metalloproteinase-9; *ECM* extra cellular matrix

with risk genotypes of *EGF* in patients with severe shunts stenosis. rs2237051 leads to a missense variant and amino acid change whereas rs22989999 and rs229898989 lead to intronic alterations, which could have consequences on protein function or expression. Our data provide evidence for the integrity of the EGFR signaling pathway in the pathophysiology of neointimal formation in SP shunts from children with complex congenital heart disease. Suppression of neointimal hyperplasia by targeted blockade of the EGFR signaling pathway as shown in preclinical models with distinct pathophysiologies (Chan et al. 2003; Nicholl et al. 2005; Trieu et al. 2000) might serve as a possible therapeutic option for children with cyanotic heart disease requiring placement of SP shunts, specifically in the smallest size shunts given their high risk for obstruction (Wells et al. 2005).

MMP-9/TIMP-1

MMP-9 degrades proteins of the extracellular matrix directly and stimulates cytokines and chemokines to regulate tissue remodeling (Yabluchanskiy et al. 2013). Invasion of monocytes, neovascularization, and neointimal growth all require the protease activity of MMP-9, identifying this enzyme as a possible therapeutic target to suppress intimal proliferation (Watanabe et al. 2018). In a porcine model, the use of stents eluting the MMP-9 inhibitor "GM6001" showed potent inhibition of intimal hyperplasia, an increase in luminal area, and no obvious thrombosis in explanted arteries (Song et al. 2020). On the basis of our immunohistochemical staining, we hypothesize that MMP-9 is also involved in the pathophysiology of neointimal proliferation in SP shunts.

TIMP-1 is a strong inhibitor of the matrix-metalloproteinases. In the form of a cytokine, TIMP-1 has been linked to numerous effects such as cell growth, cell differentiation, apoptosis, and angiogenesis. TIMP-1 was also found to be involved in the pathways "Interleukin Signaling" and "Signaling by IL-10," which both play a crucial role in the recruitment of leukocytes and macrophages (Ouyang et al. 2003).

Three separate studies demonstrated a significant reduction of neointimal hyperplasia in a rat model of injured arteries after adeno-associated gene transfer of AAV-TIMP-1 compared with untreated arteries in rats without gene transduction (Dollery et al. 1999; Furman et al. 2002; Ramirez Correa et al. 2004).

Similar to the pathophysiology of neointimal hyperplasia, COPD emphysema is a result of thickened bronchiolar wall due to airway remodeling throughout peribronchiolar fibrosis and an increase of airway smooth muscle mass (Siafakas et al. 2007). Kumar et al. identified the SNP rs6609533 of the *TIMP-1* gene as a risk variant for the development of COPD. In this study, COPD patients carrying the SNP rs6609533 showed significantly lower concentrations of TIMP-1 compared to controls (Kumar et al. 2011). In our study, the SNP rs6609533 with allele G was found to be associated with a greater extent of hyperplasia and shunt stenosis. In different transcript isoforms, this polymorphism was annotated to result in changes at the mRNA level in the exon region, as well as in the 3′ UTR and intronic region which may have regulatory impact on gene expression. In our study, immunohistochemistry showed that MMP-9 correlated significantly with the area of neointimal hyperplasia and was an independent predictor for its formation in multivariate analysis. Like EGFR, MMP-9 formed a ring-like structure in the luminal area of the PTFE layer of the shunt. In line with the results of our study, Kumar et al. demonstrated the downregulatory function of rs6609533 in *TIMP-1* in shunt tissue, resulting in enhanced expression of MMPs and thereby facilitating the migration of SMCs and the formation of neointimal hyperplasia.

Children with complex cyanotic heart disease are often treated with platelet inhibition like ASA dosed to efficacy based on thrombocyte-functioning test prior to stage I palliation. In a previous study, we identified clinical factors associated with increased neointimal formation and found a significant inverse correlation between ASA dosage and neointimal hyperplasia, meaning that a lower per kilogram body weight ASA dosage was related to greater neointimal formation (Kottmann et al. 2022). In this study, we see a trend of greater expression of EGFR in shunts removed from patients receiving lower per weight ASS dosage, with no correlation observed on MMP-9 expression. Despite the small sample size limiting statistical significances, this finding might suggest that ASS exhibits its protective effect via reduced EGFR and not MMP-9 expression.

In our study, the immunohistochemical staining for EGFR and MMP-9 showed a significant association to neointimal hyperplasia in multivariate and correlation analysis. SNP evaluation on DNA extracted from peripheral blood of affected patients identified four risk alleles on the genes *EGF* and *TIMP-1* associated with greater neointimal hyperplasia and shunt stenosis. Alterations in the amino acid sequence of the proteins may have a functional impact on protein function and / or protein expression.

In summary, our findings indicate a potential role of EGFR and MMP-9 possibly regulated by EGF and TIMP-1 in the formation of neointimal hyperplasia of SP shunts from children with cyanotic heart disease. Evidence from preclinical studies that targeted these genes to suppress their pathways indicated a reduction of hyperplasia in distinct shunt models. Novel approaches, such as coating PTFE shunts with agents specifically suppressing pathways around EGF/EGFR and TIMP-1/MMP-9, might significantly reduce neointimal hyperplasia in SP shunts and therefore improve the outcome of children with complex and congenital heart defects.

Limitations

The main limitation of the present study is the restricted sample size given the rarity of the disease studied. Also, the association between protein expression and amount of neointimal proliferation does not conclude causality. Furthermore, the exact influence of the polymorphisms on protein function was not assessed within in the scope of this study.

Methodology of this analysis in a retrospective setting did not allow to specifically examine the effect of hypoxemia on immunohistopathological findings. As reduced oxygen saturation is a strong stimulus for vasculature (Forsythe et al. 1996)), it would have been interesting to investigate the correlation of transcutaneous oxygen saturation and the degree of stenosis, EGFR, and MMP-9 expression. However, documentation of oxygen saturation in medical charts was biased by the fact that many patients received supplemental oxygen and for the majority of cases, it was not possible to evaluate the native oxygen saturation, in the absence of oxygen supplementation. Due to limited shunt material available, only selected stainings could be performed. Other possible signaling pathways were therefore not explored within the scope of this analysis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00335-023-09982-3.

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Author contributions CMW, PK, SRM, and JC designed the study. PK and JL collected the clinical data. RL, JC, JH, and KV collected the specimens (shunts removed at surgery) and reviewed the data. CMW and JC collected the blood samples. HL and MD provided biomaterial from the KaBi-DHM and clinical data. MS and KE performed histopathologic and immunohistopathologic analysis. PL, HL, MD, SRM, CMW, PL, and PK conducted the PLINK and SNP analysis. CMW, PK, LL, SRM, KE, and PE analyzed the data and wrote the manuscript. All authors reviewed the manuscript and agreed to the submitted version of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Competing interests The authors declare no competing interests related to this study.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. The institutional review board (ethics committee, Technical University Munich, Germany, approval 11/27/2015, number 223/15) approved the use of the biomaterial (KaBi-DHM project number 5943/13) for the purpose of this study.

Consent to participate Written informed consent was obtained from all legal representatives of the participants.

Accession IDs Homo sapiens; 9606.

Accession ID of the core genes studied EGFR; EGF; MMP-9; TIMP-1.

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