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Early Determinants of Pulmonary Vascular Remodeling in Animal Models of Complex Congenital Heart Disease

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INTRODUCTION

In this article, we review the current state of knowledge about early changes in the pulmonary vasculature resulting from persistent systemic-to-pulmonary arterial shunting in newborn lambs. Data generated in this model system may be important in children with various forms of congenital heart disease (CHD), but perhaps most so in those with single ventricle anomalies. Children born with a functional single ventricle (e.g. hypoplastic left heart syndrome, tricuspid atresia, pulmonary atresia) represent a subgroup of patients with congenital heart disease with the poorest outcome, with 5-year mortality rates up to 50%¹. Although functionally single ventricle heart disease comprises many structural variants, a shared physiologic feature is that both systemic and pulmonary circulations are supplied in

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None.

parallel by a functionally single pumping chamber. Immediately after birth, the pulmonary vasculature of these infants is exposed to abnormal conditions such as increased flow and/or pressure²⁻⁴. Over time, if these abnormal forces are not modified, they can lead to progressive functional and morphologic abnormalities in the pulmonary vasculature that are characterized by altered reactivity, increased resistance, and structural alterations (remodelling)³. Clinically, these abnormalities can have an important impact on surgical options and peri-operative outcome. The current approach to neonates with a functional single ventricle is to establish a controlled, low-pressure source pulmonary blood flow that facilitates systemic oxygen delivery sufficient to permit somatic growth and development without excessive ventricular volume loading, typically by means of a surgical systemic-to-pulmonary artery or ventricle-to-pulmonary artery conduit, or by restricting the main or branch pulmonary arteries². The pulmonary-to-systemic blood flow balance can be difficult to achieve, and the infant runs the gauntlet between pulmonary blood flow that is too low (resulting in chronic hypoxemia and the potential consequences thereof) and pulmonary blood flow that is too high (potentially leading to chronic heart failure, compromised systemic blood flow, and abnormal pulmonary vascular remodeling). Since subsequent stages of surgical palliation (bidirectional or partial cavopulmonary connection, PCPC, followed by Fontan-type surgery or total cavopulmonary connection, TCPC), result in passive pulmonary blood flow in the absence of a pumping ventricle, a normal low pulmonary vascular resistance is critical for maintaining low central venous pressure, overall health, and long-term survival⁵. The underlying mechanisms by which some patients with single ventricle physiology suffer from increased pulmonary vascular resistance resulting in failed surgical palliation while others do not still remains unclear. We speculate that these differences in outcome are related to *early*, clinically undetectable, pulmonary vascular abnormalities. For example, age at surgery correlates with increased transpulmonary gradient post-operatively in infants with a functional single ventricle undergoing and surviving PCPC (Figure 1). This may be due to the longer duration over which the pulmonary vasculature was exposed to increased flow. Figure 1 suggests that a relatively large surgical shunt with relatively high pulmonary blood flow (therefore allowing older age at PCPC) may lead to a subtly higher transpulmonary gradient (which suggests higher pulmonary vascular resistance) that may be detrimental in the long-term. This suggestion remains speculative as it is unknown if subtly increased resistance leads to demonstrably worse outcome. However, it is likely that there are subtle differences in signaling pathways and/or gene expression variations between patients. These signaling pathways, acted upon by the increases in pulmonary blood flow, can then lead to subtle differences in pulmonary vascular resistance to dramatically affect the surgical outcome. The underlying pathways in these newborns with single ventricle heart disease are only just beginning to be resolved. In this review we will focus on the work over the last decade that has begun to elucidate the molecular pathways that appear to be important in the initial pulmonary vascular remodelling in newborns with single ventricle heart disease.

METHODS

A lamb model of neonatal increased pulmonary blood flow

Although many animal models have been used to study increased blood flow and/or pressure on the pulmonary vasculature, most models use older or adult animals⁶. Therefore these models neglect the **initial** postnatal effects of increased blood flow and/or pressure on the developing pulmonary vasculature during the natural course of postnatal transition from fetal to adult pulmonary blood flow. A model to study the **initial** onset of pulmonary vascular remodelling associated with single ventricle physiology needs to have a large aorto-pulmonary shunt from birth so that the natural course of postnatal transition from fetal to adult pulmonary blood flow can be considered in the pathophysiology. To our knowledge,

the only existing animal model that can be used to study the **initial** onset of neonatal pulmonary vascular remodelling associated with single ventricle physiology is the neonatal lamb model with an aorto-pulmonary shunt placed *in-utero* (Figure 2)⁷. It should be noted that this model does have its limitations as a true mimic of single ventricle physiology. The Shunt model allows studies into the effects of increased pulmonary blood flow and pressure on pulmonary vascular signaling and gene expression. However, it may not take into account the influence of ventricular compliance or function that likely plays a role in the pulmonary vascular remodeling observed neonates with single ventricle physiology. However, it is the best model available for these type of investigations and over the last decade this model has been used extensively to study the **early** sequential evolution of pulmonary vascular remodeling mimicking the pathophysiological relevant elements of neonatal pulmonary blood flow in single ventricular heart disease. In the following sections we will briefly describe the current knowledge regarding the well-described nitric oxide (NO) and endothelin-1 (ET-1) signaling pathways as well as newly emerging pathways that may have potential for therapeutic intervention.

RESULTS & DISCUSSION

1. Established signaling pathways

A. The NO-cGMP Signaling Cascade—NO production by the vascular endothelium is integral to the maintenance of the low resistance state of the pulmonary vasculature, and dynamic alterations in NO production modulate vascular relaxation and constriction in response to various stimuli. Once formed, NO diffuses into vascular smooth muscle cells where it activates soluble guanylate cyclase, leading to increases in the second messenger, cyclic guanosine monophosphate (cGMP). The resulting activation of cGMP-dependent protein kinase in the pulmonary vascular smooth muscle layer leads to a decrease in intracellular calcium and relaxation of the vessel⁸. Extensive temporal investigations in the Shunt model over the first two months of life have shown that there is a complex pattern of regulation in the NO-cGMP signalling cascade that occurs both before, and after, overt alterations in endothelial function or vascular remodelling. As shown in Table 1 over the first two months of life there is a progressive loss of NO signalling. Loss of NO signalling is also found in humans with advanced forms of pulmonary vascular remodeling^{9, 10}. Decreases in NO metabolites have also been found in children with CHD and increased PBF^{11, 12} as well as after cardiopulmonary bypass^{13, 14}. Interestingly a genetic polymorphism (894G>T) has also been identified with CHD¹⁵. This polymorphism has been associated with decreased NO generation in humans^{16, 17}. Accordingly, inhalation of NO gas¹⁸ and therapies aimed at enhancing NO signalling have already become a common treatment strategy for newborns with increased pulmonary resistance^{19, 20}.

B. The Endothelin system—The hemodynamic effects of Endothelin-1 (ET-1), are mediated by at least two distinctive receptor populations, ET_A and ET_B, the densities of which are different depending on the vascular bed studied. ET_A receptors are located on vascular smooth muscle cells and mediate vasoconstriction, whereas ET_B receptors are located on endothelial cells and mediate vasodilation^{7, 21, 22}. In addition, a second subpopulation of ET_B receptors is located on smooth muscle cells and mediate vasoconstriction²³. The vasodilating effects of ET-1 are thought to be associated with the release of NO and potassium channel activation^{22, 24, 25}. The vasoconstricting effects of ET-1 are associated with phospholipase activation, the hydrolysis of phosphoinositol to inositol 1,4,5-triphosphate and diacylglycerol, and the subsequent release of Ca²⁺²⁶. In addition to its vasoactive properties, ET-1 is mitogenic for pulmonary arterial smooth muscle cells via ET_A receptor-mediated superoxide generation, and therefore may participate in vascular remodeling²⁷. Patients with advanced forms of pulmonary vascular

remodelling have increased lung and circulating ET-1 levels²⁸. Furthermore, pulmonary blood pressure and not flow is associated with net endothelin-1 production in patients with congenital heart disease and normal pulmonary vascular resistance^{29,30}. As with the NO-cGMP signaling pathway, Shunt lambs exhibit temporal alterations in the ET-1 cascade. As shown in Table 1, within one week of life there is enhanced ET-1 mediated signaling in Shunt lambs while at 4-weeks of age the enhanced circulating levels of ET-1 leads to increased vasoconstriction through increases in the expression of ET_A receptor and decreases in ET_B receptor. By two months of age the expression of ET_B receptor has returned to control levels but its localization is altered being predominantly on the SMC where it now mediates vasoconstriction. Therapies aimed at decreasing the vasoconstrictor and proliferative effects of ET-1 are being pursued for newborns with increased pulmonary resistance^{20, 31}. However, it should be noted that the fact that there are changes in the expression and localization of the ET receptors in the Shunt lambs during the temporal progression of both the endothelial dysfunction and the vascular remodelling means that the correct receptor antagonist strategy (ET_A vs. ET_B vs. dual) is still controversial. As Table 1 suggests the best therapeutic option may depend on where in the disease process the patient is.

2. Emerging signaling pathways

Recent studies in Shunt lambs have identified five signalling pathways that may represent therapeutic targets: PPAR γ , arginine metabolism, oxidative and nitrosative stress, thrombin, and carnitine. Furthermore, microarray analyses have identified new classes of genes that appear to be associated with *early* pulmonary vascular remodelling and may prove to be amenable to therapeutic interventions.

A. Peroxisome proliferator-activated receptors (PPAR)—Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor family. Recent evidence has established a role for altered PPAR signaling in the development of both systemic and pulmonary vascular disease³². Recent experimental studies suggest that the PPAR- γ isoform may have a role in advanced pulmonary hypertension^{33, 34}. PPAR- γ signaling is attenuated in Shunt lambs³⁵. A microarray analysis has been carried out in ovine pulmonary endothelial cells exposed to the PPAR- γ inhibitor, GW9662, to mimic the loss of PPAR- γ signaling. This analysis identified over 100 genes that were either up- or down-regulated (Table 2)³⁵. The upregulated genes are broadly classified into four categories: cell cycle-related genes, angiogenesis-related genes, ubiquitin-related genes, and zinc finger proteins. Furthermore, these *in vitro* results were confirmed in the Shunt lamb by Western blot analysis³⁵. Therefore, potential targets that may be good candidates for therapeutic intervention in patients with *early* neonatal pulmonary vascular remodelling associated with single ventricle physiology may soon be identified.

B. Arginine Metabolism—L-arginine bioavailability plays a key role in the generation of NO in the pulmonary vasculature. L-arginine is actively transported into endothelial cells through the cationic amino acid transporter-1 (CAT-1) where it is then used as a substrate by eNOS to form NO and L-citrulline or metabolized by arginase to form urea and ornithine. Thus, eNOS and arginase are in direct competition for L-arginine. In addition, there resides within the caveolus a recycling pathway for L-arginine, in which the enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) recycle L-citrulline back to L-arginine. L-arginine metabolism is disrupted in Shunt lambs through a number of mechanisms³⁶. L-arginine recycling is impaired due to decreased activity of ASS and ASL and L-arginine catabolism is enhanced due to increases in arginase activity³⁶. Although the expression of CAT-1 is increased which would enhance L-arginine levels in the cell, the

decrease in L-citrulline recycling combined with increased L-arginine catabolism results in a decrease in relative NO signalling³⁶. Thus, it is likely that the progressive loss of NO signaling in Shunt lambs⁸ is mediated at least in part by decreases in available L-arginine. This new description of the L-arginine-NO pathway fits well with previous studies in a rat model of hypoxia induced pulmonary hypertension³⁷. Taken together, these studies suggest that treatments strategies to enhance L-arginine bioavailability in neonates with early pulmonary vascular remodelling associated with single ventricle physiology may have clinical utility.

C. Oxidative and nitrosative stress—Excessive production of reactive oxygen species (ROS), outstripping endogenous antioxidant defence mechanisms, has been implicated in many disease processes in of the vasculature³⁸. Decreases in bioavailable NO can be related to increases in oxidative stress. In Shunt lambs, oxidative stress occurs secondary to both increased production of ROS³⁹ and decreases in their scavenging⁴⁰. The source of the increased superoxide is multi-factorial involving the upregulation of vascular xanthine oxidase and NADPH oxidase as well as an uncoupling of eNOS^{8, 41, 42}. The mechanisms underlying the uncoupling of eNOS, i.e., the consumption of NADPH becomes uncoupled from NO synthesis, are also complex and involve decreases in L-arginine³⁶ as well as alterations in biopterin metabolism³⁹. Superoxide and other ROS have also been shown to have signalling function in vascular cells promoting hypertrophy, proliferation, migration, matrix remodelling and even the formation of new vessels. The activation of NADPH oxidases with subsequent ROS production has been associated with increased proliferation of pulmonary artery smooth muscle cells isolated from both sheep²⁷ and humans⁴³.

Both NO and superoxide possess unpaired electrons, and react rapidly to form the reactive nitrogen species (RNS), peroxynitrite (ONOO⁻) decreasing NO bioavailability. In addition, ONOO⁻ can exert effects through the nitration of protein tyrosine residues. Tyrosine nitration, a covalent modification that adds a nitro group (-NO₂) to the ortho carbon of the phenolic ring, yields 3-nitrotyrosine (3-NT)⁴⁴. The nitration of tyrosine residues to form 3-NT is widely used as a marker of ONOO⁻ formation and recent studies have shown that there is a temporal increase in protein nitration in the lungs of Shunt lambs⁸. Further, eNOS itself is susceptible to nitration⁸ and mass spectroscopy studies have demonstrated that these nitrated residues are in regions of the protein that are important to its function⁴⁵. Thus, the potential of anti-oxidant therapies to reduce both ROS and RNS formation has been postulated for the treatment of early neonatal pulmonary vascular remodelling associated with single ventricle physiology. However, these studies highlight the limitations of current investigations: the lack of easily available means to identify modified proteins and residues. This is a roadblock in understanding the potential mechanistic contribution of these modifications and it is imperative that there is a greater focus on identifying the individual tyrosine residues targeted by nitration and the effect these nitration events have on the structure-function relationship of the protein. Only when these goals have been met will it be possible to develop directed therapies.

D. Thrombin—Recent studies have elucidated a novel positive feedback loop in which thrombin increase ROS levels via the consecutive activation of protease activated receptor 1 (PAR1), Rac1, and p21-activated kinase (PAK-1)⁴³. This also results in the activation of the transcription factor, NFκB which in turn leads to the activation of hypoxia inducible factor 1α (HIF-1α)^{46, 47}. Under these conditions, HIF-1 target genes such as plasminogen activator inhibitor-1 (PAI-1) or vascular endothelial growth factor are upregulated. The upregulation of PAI-1, which is the primary physiological inhibitor of tissue plasminogen activator and urokinase plasminogen activator, can then lead to reductions in fibrin clearance. In addition, increases in PAI-1 can also promote pulmonary artery SMC proliferation. Together, these anti-fibrinolytic and growth modulating activities have an immediate impact on pulmonary

vascular remodelling and PAK-1 levels are elevated in remodelled pulmonary vessels in patients with congenital heart disease and pulmonary vasculopathy^{43, 48}. There appears to be a secondary feed-forward loop activated by HIF-1 leading to further increases the expression of Rac1 and PAK-1 producing enhanced cytoskeletal remodelling and proliferation of pulmonary artery SMC which further contributing to the medial hypertrophy and vascular remodelling⁴³. As part of this feed-forward mechanism, ROS levels are stimulated due to the increased abundance of Rac1 and PAK-1 producing sustained activation of the HIF-1 pathway and increased pulmonary vascular remodelling. This novel pathway may play an important role in early neonatal pulmonary vascular remodelling associated with single ventricle physiology and therapeutic treatments strategies targeting thrombin blockade in these patients may have clinical utility.

E. Carnitine Metabolism and Mitochondrial Dysfunction—L-carnitine is a trimethylated amino acid is involved in the transport of long chain fatty acids across the inner mitochondrial membrane (Figure 3). Carnitine is present in the form of either free carnitine (non-esterified molecule; FC), or acylcarnitines (esterified form; AC). Acylcarnitines are products of the reaction in which acyl moieties are transferred to carnitine from acyl-CoA. This reaction is catalyzed by acyltransferases. Thus, the AC/FC ratio is a measure of acylated carnitines versus the free carnitines. A low AC/FC ratio indicates a healthy mitochondria and a high AC/FC ratio a mitochondria with a reduced capacity for ATP production. Data in Shunt lambs indicate that there is a disruption in carnitine metabolism involving decreased expression of carnitine palmitoyltransferase 1 and 2 as well as carnitine acetyltransferase (CrAT). In addition, RNS appear to be involved in the disruption of carnitine homeostasis as nitrated CrAT is elevated in Shunt lambs while the exposure of purified CrAT to authentic ONOO⁻ decreases its activity⁴⁹. Together these alterations result in mitochondrial dysfunction as demonstrated by decreased superoxide dismutase (SOD) 2-, increased uncoupling protein-2 (UCP-2)-expression, and an increased lactate/pyruvate ratio⁴⁹. Decreased expression of SOD2 has also been recently shown in SMC isolated from patients with PA hypertension (PAH)⁵⁰. It is now becoming apparent that mitochondria play a key role in the development of various cardiovascular diseases including heart disease⁵¹, diabetes⁵², and atherosclerosis⁵³. Studies have shown that mitochondrial targeted antioxidants have a beneficial effect in stroke prone rats⁵⁴, sepsis induced cardiac dysfunction⁵⁵, and cardiac ischemia-reperfusion injury⁵⁶ and there is increasing interest in the potential of mitochondrial targeted antioxidants in treating cardiovascular disease^{57, 58}. It remains to be seen if this is a potential therapeutic strategy for children with single ventricle physiology.

Mitochondria are the major site of cellular ATP production. ATP can stimulate NO release via the activation of eNOS⁵⁹. Studies have also demonstrated a key role for the 90kD heat shock protein (Hsp90) in the activation of eNOS⁶⁰ and the disruption of the Hsp90/eNOS complex adversely affects eNOS activation and induces eNOS uncoupling⁶¹. Hsp90 is ATP dependent and there is a temporal decrease in eNOS/Hsp90 interactions in Shunt lambs correlating with a decline in mitochondrial function⁴⁹. Data indicate that the mitochondrial dysfunction may result from an increase in the nitration of mitochondrial proteins due to a asymmetric dimethylarginine (ADMA) mediated redistribution of eNOS to the mitochondria⁶². Increased ADMA levels are implicated in the pathogenesis of pulmonary hypertension^{63, 64} and in a single study of children and young adults with congenital heart disease and increased pulmonary blood flow⁶⁵. As studies support the view that the ratio between L-arginine and ADMA is a key component in the regulation of endothelial NOS activity and it is possible that L-arginine could be a therapy for children with single ventricle physiology.

F. The identification of new genes involved in early neonatal pulmonary vascular remodeling—Previous studies have identified a burst of pulmonary angiogenesis in Shunt lambs that peaks at 4-weeks of age and is then lost. However, it is unclear how this angiogenic and apparent anti-angiogenic signaling is regulated. Micro-array analysis has been used to identify gene changes in Shunt lambs at an early time point prior to the angiogenic burst (3 days of age) and at 4-weeks of age to determine if there are differences in gene expression between the two ages and between the age matched control lambs¹³⁰. The data indicate that there are a large number of gene categories that are regulated between the Shunt lambs and the age-matched controls at both 3 days and 4-weeks of age (Table 3). Further, when only genes directly related to angiogenesis and the extracellular matrix are examined the data indicate that these genes are predominantly up-regulated at 3 days of age and down-regulated at 4-weeks of age (Table 3). However, relevance Network analyses in the 3-day old Shunt lambs reveals just how complex and inter-connected these signalling pathways are (Figure 4). Thus, although these studies will likely lead to novel signalling pathways that can be investigated to produce exciting new results it is unlikely to lead to new therapeutic strategies in the near future.

CONCLUSIONS

In summary, utilizing an animal model allowing us to study the *initial* onset of neonatal pulmonary vascular remodelling associated with single ventricle physiology, new pathways for further mechanistic studies have been identified. New potential targets for therapeutic intervention have been identified and are now being tested: L-arginine, anti-oxidants, thrombin, and L-carnitine. In the longer term we can hope that the use of newer techniques such as metabolomics, proteomics, and gene profiling may lead to new therapeutic targets to address the *initial* onset of neonatal pulmonary vascular remodelling associated with single ventricle physiology.

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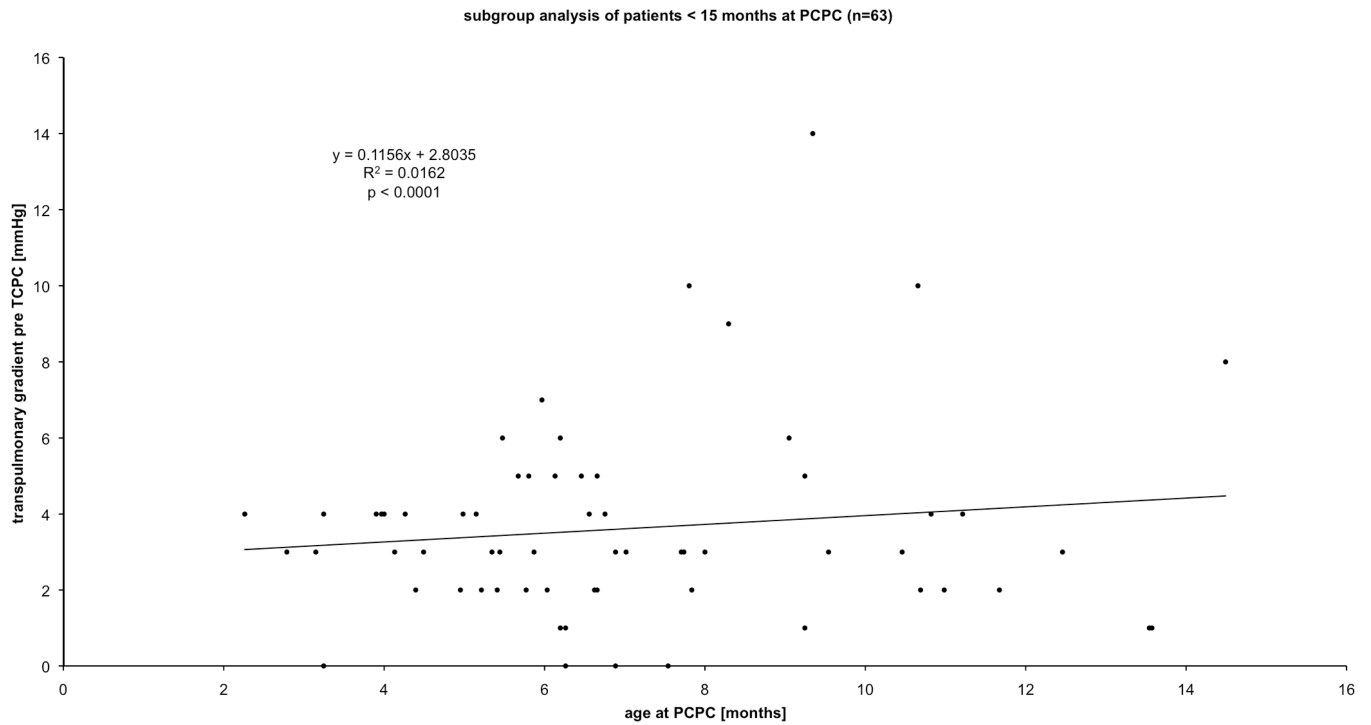


Figure 1. Retrospective analysis of n= 69 consecutive patients younger than 15 months (subgroup of reference 9) with single ventricle physiology who underwent and survived partial cavo-pulmonary connection (PCPC). The X-axis depicts age at PCPC in months. The Y-axis depicts transpulmonary gradient defined as left atrial pressure minus pulmonary artery pressure during heart catheterization before total cavo-pulmonary connection (TCPC) surgery.

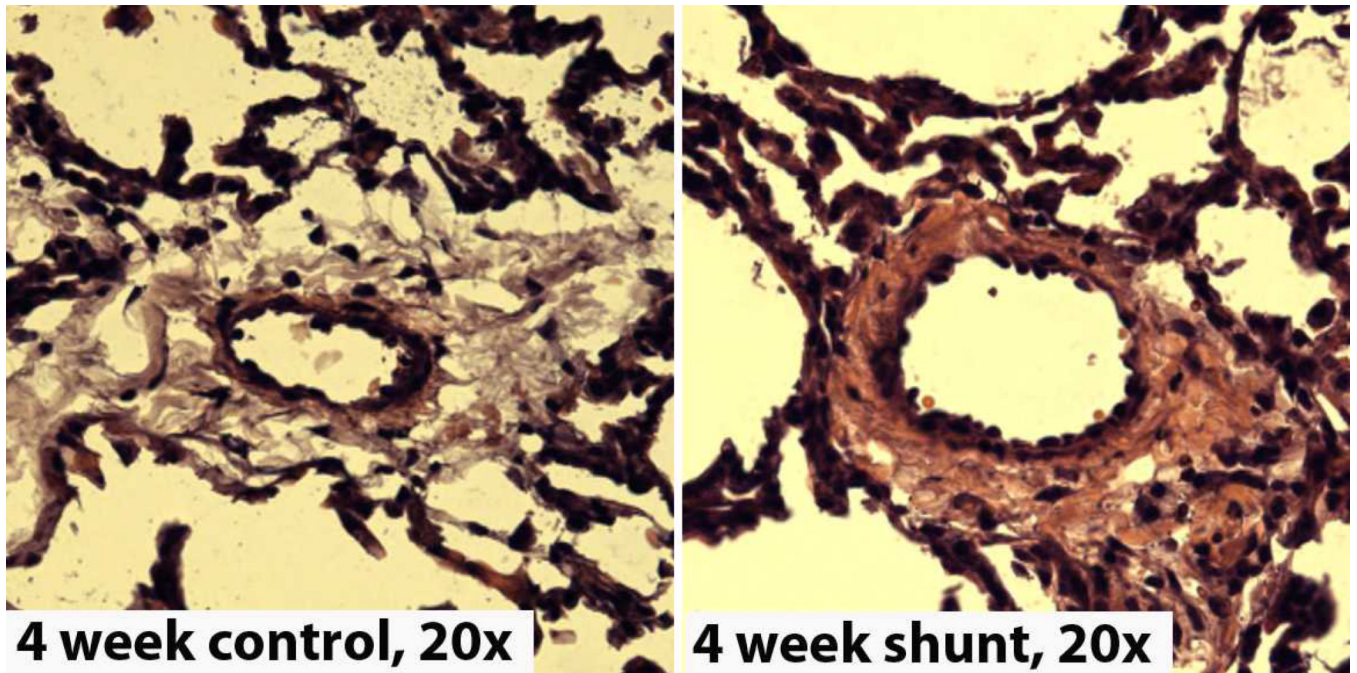


Figure 2. Representative H & E stains of peripheral lung fixed in 4% paraformaldehyde and sectioned at 7 microns, from 4-week control (left) and Shunt (right) lambs. Note the increase in medial thickness in the Shunt lambs. Magnification=20 \times .

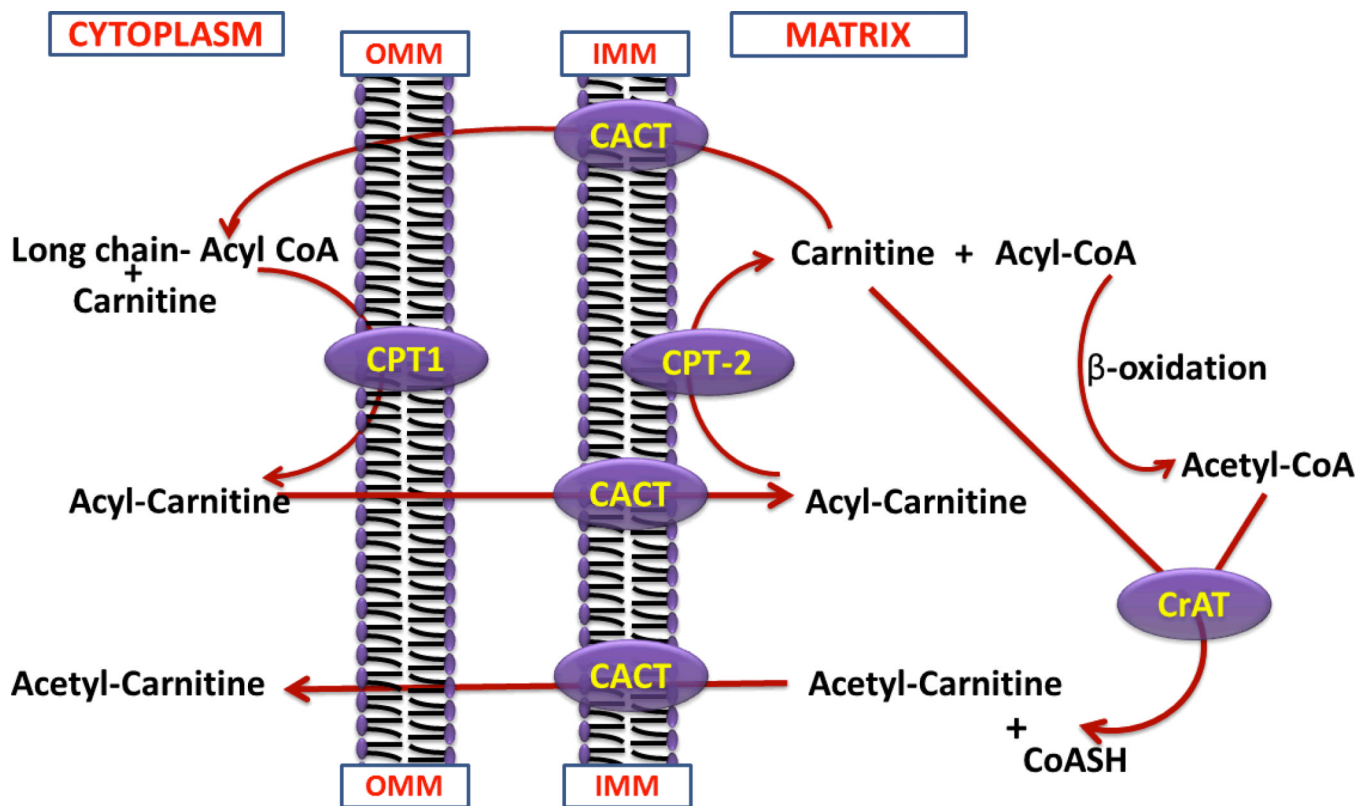


Figure 3. The Carnitine Shuttle and role of carnitine in the mitochondrial oxidation of fatty acids. Abbreviations: CPT-I, carnitine palmitoyltransferase-1; CPT-II, carnitine palmitoyltransferase-2; CACT, carnitine acylcarnitine translocase; CrAT, carnitine acetyl transferase; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

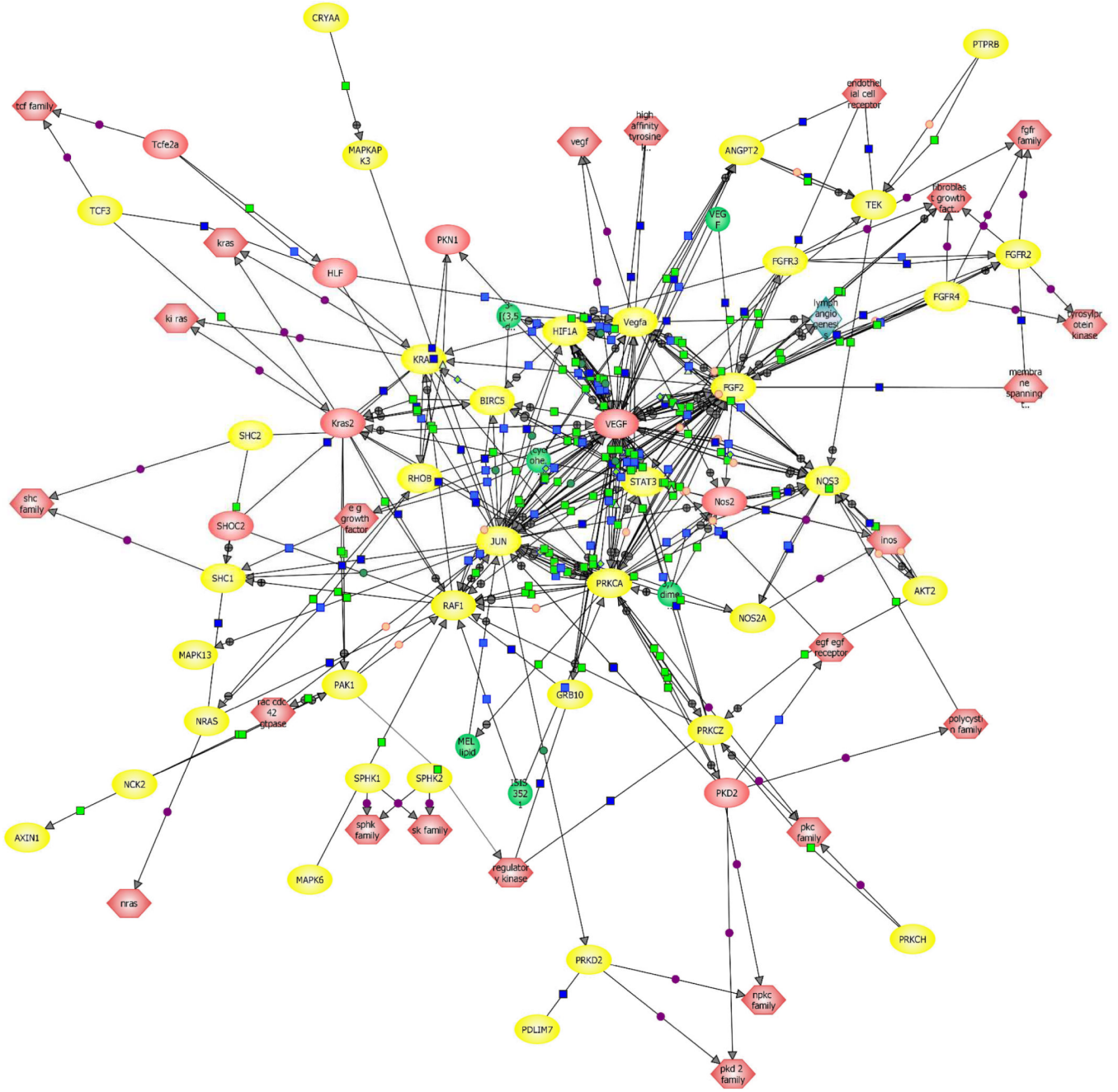


Figure 4. Relevance network of genes belonging to angiogenesis related signaling pathways identified using Pathway Architect software. Yellow indicates the genes that are altered between Shunt and Control lambs at 3-days of age.

Table 1

Developmental changes in NO and ET-1 signaling in Shunt lambs

NO Signaling	1 week	2 week	4 week	8 week
eNOS protein levels	↔	↔	↑	↔
Relative NOS activity	↔	↓	↓↓	↓↓↓
Plasma NO _x levels	nd	↑	↔	↔
Lung Tissue NO _x levels	nd	↑	↔	↔
Nitrated eNOS	nd	↑	↑↑	↑↑↑
Hsp90 protein levels	nd	↔	↔	↔
eNOS bound Hsp90	nd	↓	↓↓	nd
Plasma cGMP levels	↔	↑	↑	↑
Lung tissue cGMP levels	nd	↑	↑	↑
sGC-α protein levels	nd	↑	↑	↔
sGC-β protein levels	nd	↔	↑	↔
PDE5 protein levels	nd	↔	↑	↔
PDE5 activity	nd	nd	↑	nd
Plasma BNP levels	nd	↔	↑	↑
ET signaling	1 week	4 week	8 week	
Plasma ET-1	nd	↑	nd	
Immunoreactive ET-1	↑	nd	nd	
preproET-1 mRNA	↔	↔	nd	
preproET-1 protein levels	↔	↔	nd	
ECE-1 mRNA	nd	↑	nd	
ECE-1 protein levels	↑	↑	nd	
ET _A receptor protein levels	↔	↑	↑	
ET _B receptor protein levels	↓	↓	↔	

* nd=not determined

Table 2Endothelial cell genes regulated by PPAR γ

Functional classifications	Number of genes up-regulated	Number of genes down-regulated
Cell cycle related genes	7	0
Angiogenesis related genes	11	1
Ubiquitin related genes	9	0
Zinc finger genes	17	1
unclassified	56	18
total	100	20

Table 3

Developmental Changes in Gene Expression in Shunt lambs Gene functional classification in 3-day Shunts

Functional classifications	Number of genes up-regulated	Number of genes down-regulated
cell cycle related genes	7	0
cell proliferation related genes	5	0
angiogenesis related genes	6	2
extracellular matrix and cell adhesion related genes	6	2
transcription factors	4	0
unclassified	90	15
total	118	19

Gene functional classification in 4-wk Shunts		
Functional classifications	Number of genes up-regulated	Number of genes down-regulated
cell proliferation related genes	8	0
extracellular matrix related genes	3	28
angiogenesis related genes	0	6
specific cell signaling pathways	6	0
transcription factors	6	0
unclassified	132	62
total	155	96