

## Mitochondrial Dysfunction and Congenital Heart Disease

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### Abstract

The pulmonary vasculature in infants born with congenital heart defects that cause increased Pulmonary Blood Flow (PBF) is subjected to pathologic mechanical forces, including chronically increased shear stress, resulting in early functional abnormalities of the vascular endothelium. These functional abnormalities occur prior to the development of well-described morphologic changes. However, little is known about the factors that transduce the abnormal shear forces associated with increased PBF into abnormal vascular function and reactivity. Recently the disruption of mitochondrial function has been identified as a new mechanism that leads to the development of pulmonary endothelial dysfunction. This is a complex process that involves post-translational regulation of multiple proteins and the mitochondrial redistribution of uncoupled endothelial nitric oxide synthase (eNOS) resulting in the disruption of carnitine metabolism and subsequently mitochondrial bioenergetics. As both eNOS and GTP cyclohydrolase I, the rate limiting enzyme in tetrahydrobiopterin (BH4) biosynthesis, are chaperoned by hsp90 this results in a "feed-forward" signaling cascade in which eNOS becomes progressively more uncoupled resulting in pulmonary endothelial dysfunction. This review will discuss the current knowledge in the field, the limitations in our understanding this complex process, and the potential for targeting mitochondrial function in the treatment of children born with congenital heart defects that result in increased pulmonary blood flow.

**Keywords:** Mitochondrial dysfunction; Congenital heart disease

### Abbreviations

AC: Acylcarnitine; ADMA: Asymmetric Dimethyl arginine; ATP: Adenosine Triphosphate; ASL: Arginino succinate Lyase; ASS: Argininosuccinate Synthetase; BH4: Tetrahydrobiopterin; CACT: Carnitine Acylcarnitine Translocase; cGMP: Cyclic Guanosine Monophosphate; CrAT: Carnitine Acetyltransferase; COT: Carnitine Octanoyl transferase; CPT: Carnitine Palmitoyl Transferase; CHD: Congenital Heart Disease; CHIP: C-Terminal Hsc70:Interacting Protein; CoA: Coenzyme A; DDAH: Dimethylarginine Dimethylamino hydrolase; eNOS: Endothelial Nitric Oxide Synthase; ER: Endoplasmic Reticulum; FC: Free Carnitine; GCHI: GTP Cyclohydrolase I; Hsp90: Heat Shock Protein 90; MnSOD: Manganese Superoxide Dismutase; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; NO: Nitric Oxide; OCT: Organic Cation Transporters; PBF: Pulmonary Blood Flow; PKC: Protein Kinase C; PKG: Protein Kinase G; PPAR: Peroxisome Proliferator Activated Receptor; PRMT: Protein Arginine N: Methyltransferase; sGC: Soluble Guanylyl Cyclase; SMC: Smooth Muscle Cell; TZD: Thiazolidinedione; UCP: Uncoupling Protein

### Introduction

Congenital heart defects with increased PBF are associated with the development of increased pulmonary vascular reactivity and pulmonary hypertension [1-4]. In the post-natal period, the presence of large communications at the level of the ventricles (e.g. ventricular septal defect) or great vessels (e.g. truncus arteriosus) exposes the pulmonary circulation to abnormal elevations in blood flow and

pressure, which results in progressive structural and functional abnormalities of the pulmonary vasculature [3,5-13]. Structural changes involve the progressive diminution of the pulmonary vascular cross-sectional area, ultimate obliteration of large portions of the vascular bed, and death secondary to hypoxia and/or myocardial failure. Although advanced structural changes are irreversible, surgical correction can reverse early changes, which is the basis for the repair of many congenital heart defects in the neonatal period [4]. Functional changes, however, are not as readily addressed by early surgical intervention. In fact, even children with reversible disease suffer significant morbidity and mortality in the peri-operative period secondary to increased pulmonary vascular reactivity [2,3,14-18].

Increased vascular reactivity may produce severe hypoxemia, acidosis, low cardiac output, and death if not treated immediately. In addition, infants and children with heart defects that result in single ventricular physiology requiring staged surgical palliation for survival may suffer significant morbidity and mortality from abnormal pulmonary vascular reactivity. In fact, in these patients, who often have periods of increased PBF, even early, clinically undetectable, pulmonary vascular alterations may severely impact their clinical course by limiting surgical options or complicating peri-operative management. Although, the precise mechanisms responsible for the early pulmonary vascular abnormalities associated with increased PBF remain unclear, alterations in NO-signaling is known to be a significant contributor [19-23]. The following sections will describe our latest understanding of the mechanisms that underlie the disruption in NO signaling in CHD associated with increased PBF.

Much of the animal studies referenced in this review utilize a lamb model of Congenital Heart Disease (CHD) with increased pulmonary blood flow, created by the in utero placement of a large

aortopulmonary graft between the main pulmonary artery and the ascending aorta. These Shunt lambs undergo the postnatal changes that occur at birth in children with congenital heart defects that result in increased PBF, mimicking the clinical condition. These include failure to thrive, early endothelial dysfunction, abnormal pulmonary vascular reactivity and remodeling [24,25] as well as enlargement of the heart. Although Shunt lambs exhibit a burst in angiogenesis, likely induced in an attempt to decrease shear forces through the lung vasculature that results in an increase in blood vessel number in the lung at 4-weeks of age, under electron microscopy there are already signs of endothelial damage. This unique, powerful model facilitates the ability to investigate early mechanisms of disease.

## Arginine Homeostasis

NO is generated from L-arginine within the endothelium through the activity of the Ca<sup>2+</sup> dependent flavin enzyme NOS predominantly localized in caveolar structures within the plasma membrane [26]. Factors that change the ability of eNOS to obtain sufficient levels of L-arginine can have a significant negative impact on the ability of eNOS to generate NO (Figure 1).

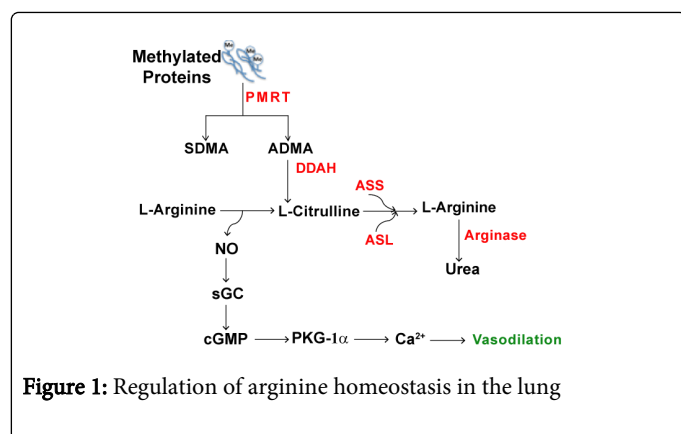


Figure 1: Regulation of arginine homeostasis in the lung

Methylated proteins within the cell are degraded by the action of the Protein Arginine N-Methyltransferase (PMRT) family of proteins resulting in the generation of symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA). ADMA is converted to L-citrulline by Dimethylarginine dimethylaminohydrolase (DDAH). L-citrulline is also generated by the action of nitric oxide synthase (NOS) in the enzymatic reaction that generates NO and consumes L-arginine. L-citrulline is converted back to L-arginine by the arginine recycling enzymes, argino succinate synthase (ASS) and argino succinate lyase (ASL). L-arginine can also be consumed by arginase to generate urea.

This leads to a situation in which the enzyme becomes uncoupled. Uncoupling occurs when electrons obtained from nicotinamide adenine dinucleotide phosphate (NADPH) are redirected to molecular oxygen to generate the superoxide anion. The presence of NO and superoxide in close proximity within the caveolae then leads to their rapid interaction and the subsequent generation of the reactive nitrogen species, peroxynitrite. Peroxynitrite in turn can interact with aromatic residues with protein, especially tyrosine residues, to induce protein nitration. This can induce changes in the function of the protein.

Further, nitration is a covalent modification that requires new protein synthesis to restore normal signaling. Over the last decade it has become apparent that the nitrative stress associated with increased

peroxynitrite generation plays a key role in the development of pulmonary endothelial dysfunction associated with CHD and increased PBF. Indeed studies have shown in both cultured Pulmonary Arterial Endothelial Cells (PAEC) and Shunt lambs that disruptions in arginine homeostasis play a major role in eNOS uncoupling and peroxynitrite generation.

L-arginine levels are decreased as early as 2-weeks after birth in Shunt lambs [27]. This is associated with decreases in the activity of the arginine recycling enzymes, Arginino succinate Synthetase (ASS) and Arginino succinate Lyase (ASL). ASS and ASL utilize the byproduct of the NOS reaction, L-citrulline to regenerate L-arginine [28-30]. In addition to a reduction in L-citrulline recycling, L-arginine degradation is also enhanced secondary to an increase in arginase activity [27]. Shunt lambs also exhibit increases in the amino acid, asymmetric dimethylarginine (ADMA) an endogenous NOS [31].

ADMA is generated by the turnover of methylated proteins and its levels are regulated both by its generation through the activity of the protein arginine N- methyltransferase (PRMT) family of proteins and through its degradation via the dimethylarginine dimethylaminohydrolase, DDAH-1 and DDAH2. Using our lamb model of CHD associated with increased PBF (Shunt) it appears that ADMA levels become elevated secondary to a reduction in DDAH activity rather than increases in ADMA generation [31].

Interestingly, the decrease in the activity of ASS, ASL and DDAH as well as the increases in arginase activity are not associated with changes in the respective proteins suggesting that as yet unidentified, post-translation events are involved in the dysregulation of arginine homeostasis associated with increased PBF.

## Mitochondrial Redistribution of eNOS

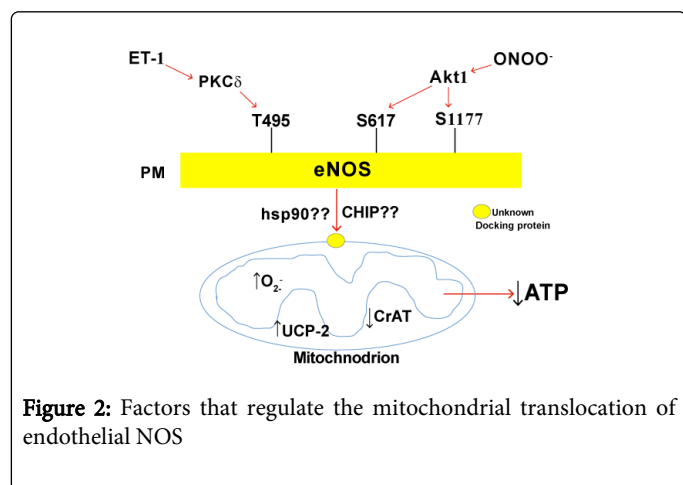
To exert its physiologic effect on Smooth Muscle Cell (SMC) relaxation, endothelial NOS is localized to the plasma membrane. This occurs through a regulated process that involves an initial myristoylation event at the glycine residue located at amino acid (aa) position 2 which allows eNOS to enter and traffic through the Golgi-endoplasmic reticulum (ER) where it becomes palmitoylated at multiple sites. When it exits the ER, eNOS becomes localized to the special invaginations within the plasma membrane called caveolae. There is evidence that Enos trafficking through the Golgi-ER complex is enhanced by its interaction with caveolin-1, which is the major structural protein of the caveolus [32,33].

Within the caveolus, its interaction with caveolin-1 keeps eNOS in a quiescent state and the enzyme becomes active when intracellular calcium levels raise leading to the generation of a calcium-calmodulin complex that dislodges caveolin-1 from eNOS allowing NO levels to rise.

However, data have shown that eNOS can also be located on the mitochondrion through a sequence located within aa627-631 (RRKRK) that has been designated the mitochondrial translocation loop [34].

A mutant eNOS in which this sequence was deleted prevents its mitochondrial accumulation when expressed in COS-7 cells [34]. More recently nitration and phosphorylation events have been shown to be involved in the redistribution of eNOS to the mitochondria. The available data indicate that the phosphorylation of eNOS at T495, via ET-1 mediated activation of protein kinase C delta (PKCδ), leads to

uncoupling of the enzyme and that the subsequent nitration events result in mitochondrial redistribution [35] (Figure 2).



Endothelin-1 stimulates PKC $\delta$  activity that in turn binds to, and phosphorylates, eNOS at T495. The nitration mediated activation of Akt1 also results in the sequential phosphorylation of eNOS at S1177 and S617. In both cases this results in eNOS mitochondrial translocation. The proteins that are involved in the redistribution are unknown but hsp90 and/or c-terminal of hsp70 interacting protein (CHIP) may be involved. The mitochondrial docking protein is also unknown.

In addition, ADMA-mediated uncoupling of eNOS has been shown to result in the nitration-mediated activation of Akt1 and this leads to the sequential phosphorylation of eNOS at S1177 and S617 [36] (Figure 2). Molecular modeling studies indicate that the phosphorylation of S617 exposes the mitochondrial translocation loop, leading to mitochondrial redistribution of the enzyme [36]. Interestingly, these two divergent kinase pathways do not appear to overlap, as ET-1 does not stimulate Akt1 signaling although eNOS becomes uncoupled. Why this is the case and what are the relative roles of each pathway during the development of pulmonary endothelial dysfunction remains unclear.

In addition, it remains to be resolved whether these phosphorylation events are sufficient to drive the mitochondrial redistribution of eNOS. However, it is more likely that there are other proteins that recognize the mitochondrial translocation loop and act as chaperones to guide the process (Figure 2). The identities of these putative factors are unclear, but we have postulated that this protein may be the molecular chaperone, C-terminal Hsc70-interacting protein (CHIP). This is based on the following evidence:

- (1) CHIP activity is stimulated both by nitritative stress in PAEC and in Shunt lambs [31];
- (2), CHIP can be recruited to heat shock protein 90 (hsp90) chaperoned proteins and eNOS is an hsp90 interacting protein [37];
- (3) Molecular chaperones have been shown to play key roles in the translocation of nuclear-encoded proteins into the mitochondrion [38,39];
- (4) CHIP facilitates the assembly of translocation complexes [40] and;
- (5), CHIP has been shown to associate with eNOS, and this leads to and alteration in its sub-cellular distribution [41].

Further, CHIP contains a region located between aa185-190 (EGDEDD) that is highly negatively charged [42] which could interact with the positively charged eNOS mitochondrial translocation loop. Alternatively, it is possible that hsp90 could itself be the protein that chaperones eNOS to the mitochondria. Indeed studies over the last decade have identified an important role for hsp90 in targeting proteins for mitochondrial import through the TOM40 complex [43,44]. However, a counter argument for hsp90 being important in the mitochondrial redistribution of eNOS comes from data that indicate that eNOS-hsp90 interactions decrease in Shunt lambs in a time-dependent manner, secondary to reduced levels of ATP. As the chaperone activity of hsp90 is dependent on ATP, decreases in cellular ATP would attenuate its activity and thus reduce eNOS mitochondrial redistribution [45-48]. However, it is also possible that both CHIP and hsp90 could both be involved with hsp90 playing a role in the early stages and then as its activity declines, CHIP takes over.

## Mitochondrial Dysfunction

The redistribution of uncoupled eNOS to the mitochondria leads to the development of mitochondrial dysfunction and reductions in the endothelial levels of ATP (Figure 2). Again this is a complex process that involves increased oxidative stress within the mitochondria, a decrease in mitochondrial membrane potential and a reduction in  $\beta$ -oxidation secondary to a disruption of the carnitine homeostasis. The increase in oxidative stress within the mitochondria appears to be due to a decrease in manganese superoxide dismutase (MnSOD) activity. This is a rapid process occurring within 2h of the induction of nitritative stress and correlates with a reduction in MnSOD protein levels [49].

The rapid pace of this process suggests that it is independent in changes in gene expression and that perhaps there is enhanced degradation of MnSOD within the mitochondria itself. The trigger for this process is unclear but could be related to nitration of the MnSOD protein. MnSOD protein is nitrated in rats with advanced forms of pulmonary hypertension [50]. The mitochondrial membrane potential appears to be attenuated secondary to increased levels of uncoupling protein-2 (UCP-2). The uncoupling proteins are a multi-gene family whose action short-circuits the proton motive force that is used by the mitochondria to allow ATP generation. UCP-1 is the prototypical UCP and is involved in heat generation in brown fat tissue [51]. However, it should be noted that the action of UCP-2 is controversial which published studies suggesting that besides changes in expression, UCP-2 requires a further unidentified post-translational event before it becomes active [52,53].

$\beta$ -oxidation is dependent on carnitine that acts as a carrier to allow activated fatty acids to cross the inner mitochondrial membrane. In addition, Carnitines are involved in the elimination of fatty acyl-CoA metabolites and are important in maintaining the ratio of free and acyl-CoA. Carnitine is present as a non-esterified free carnitine (FC) or an esterified acylcarnitine form (AC) and the A: FC ratio can be used to determine mitochondrial health. With a low AC: FC ratio indicating a cell with normal mitochondrial function and a high AC: FC ratio correlating with mitochondrial dysfunction. Carnitine is delivered to the mitochondria through the action of a family of organic cation transporters (OCT).

The important members of this family are OCTN1- a pH-dependent and sodium-independent multi-specific organic cation carrier [54], OCTN2- a sodium-dependent organic cation transporter

[55]; and OCTN3- a mammalian peroxisomal membrane carnitine transporter [56]. Inside the mitochondria, carnitine is acted upon by a group of enzymes that are collectively known as carnitine acyltransferases which act in concert to allow the reversible transfer of the acyl group from acyl-CoA to carnitine. These enzymes have been identified based on both the specificity towards the acyl and where their localization. The carnitine palmitoyl transferase (CPT) system comprises three proteins: carnitine palmitoyl transferase 1 (CPT1) which is located in the outer mitochondrial membrane, the carnitine-acylcarnitine translocase (CACT), which is an integral inner membrane protein, and carnitine palmitoyl transferase 2 (CPT2) which is located on the inner mitochondrial membrane [57].

These enzymes work in concert to allow the transport of long-chain fatty acids from the cytosolic compartment into the mitochondrial matrix allowing  $\beta$ -oxidation to occur.

There are two other carnitine acyltransferases that work in conjunction with the CPT system: carnitine octanoyl transferase (COT) [58,59] and carnitine acetyltransferase (CrAT) [60]. CrAT is able to utilize only short-chain acyl groups (C1-C4) as substrate while COT is able to utilize both medium and long-chain fatty acids (C5-C12) [61]. By acting together these enzymes form the “carnitine shuttle” which is responsible for regulating cellular carnitine homeostasis. Shunt lambs have an elevated AC: FC ratio indicating that there is a decrease in  $\beta$ -oxidation [62].

The disruption of  $\beta$ -oxidation is complex and involves both transcriptional and post-transcriptional events. Published studies indicate that Shunt lambs have decreased expression of 3 important carnitine-dependent enzymes (CPT1, CPT2 and CrAT) [62] that appears to be due to reduced peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) signaling [63,64].

In addition, to changes in expression there is also a post-translational inhibition of carnitine acetyl transferase (CrAT) that is mediated by nitration. The link between carnitine homeostasis and NO signaling has been further strengthened by data demonstrating that siRNA-mediated down-regulation of CrAT is sufficient to attenuate the shear-mediated increases in NO signaling in PAEC [65].

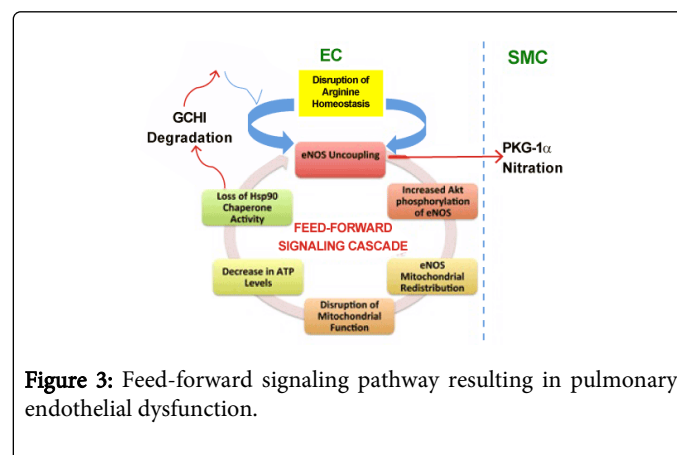
## Hsp90 and NO Signaling

The 90 kD heat shock protein (hsp90) is part of a family of proteins that act as molecular chaperones to modulate protein activity. Hsp90 is known to increase eNOS activity [66]. Hsp90 is ATP dependent and the ATPase site of the chaperone is responsible for the auto-phosphorylation required to enable hsp90 to interact with client proteins [67,68]. Several studies have shown that disruption of hsp90-eNOS interactions attenuates NO production [66,69-72].

Further, decreased hsp90 binding results in eNOS “uncoupling” i.e., consumption of NADPH is uncoupled from NO synthesis, resulting in the production of superoxide [73]. Sessa’s group has shown that hsp90 interacts with aa300-400 of eNOS, while Pritchard’s group, using an elegant overlapping decoy peptide strategy, identified aa301-325 as being important for hsp90 interaction with eNOS [74].

Previous work has shown that eNOS-hsp90 interactions are progressively disrupted during the development of the endothelial dysfunction associated with increased PBF [75]. In addition to eNOS, hsp90 also chaperones other proteins involved in NO signaling. GTP cyclohydrolase I (GCHI), the key BH4 biosynthetic enzyme, has been shown to be a client protein of hsp90 [31] and that the decrease in

hsp90 activity in Shunt lambs [76] results in the proteasomal degradation of GCHI [31] (Figure 3).



**Figure 3:** Feed-forward signaling pathway resulting in pulmonary endothelial dysfunction.

Initial disruption in arginine homeostasis results in eNOS uncoupling and its redistribution to the mitochondria. This in turn results in a disruption in mitochondrial bioenergetics resulting in mitochondrial dysfunction and reductions in ATP generation. The decrease in ATP leads to an attenuation of hsp90 activity, reducing its ability to chaperone GTP cyclohydrolase I (GCHI) and eNOS resulting in progressive eNOS uncoupling. When NO signaling is sufficiently compromised this results in pulmonary endothelial dysfunction.

Thus, the loss of ATP due to mitochondrial dysfunction attenuates hsp90 activity resulting in a “feed-forward” signaling cascade in which eNOS becomes ever more uncoupled leading to less NO- and more peroxynitrite-being produced driving pulmonary endothelial dysfunction [75] (Figure 3). In addition, the NO receptor, soluble Guanylyl Cyclase (sGC) is also found in a complex with hsp90 and Inhibition of hsp90 activity using geldanamycin stimulates its proteasomal degradation again via CHIP reducing the ability of the cell to produce cyclic guanosine monophosphate (cGMP) [77-80]. Although in Shunt lambs increased BNP-mediated activation of particulate GC may be able to compensate [81]. In addition, although it does not appear to be chaperoned by hsp90, the target of cGMP, protein kinase G-1 $\alpha$  (PKG-1 $\alpha$ ) is also compromised in Shunt lambs [82] through the nitration of Y247 that reduces the ability of the protein to bind cGMP [83] (Figure 3). Thus, in addition to an increase in NO generation the downstream effectors of NO are also compromised.

## The Mitochondrion as a Therapeutic Target

As discussed above, carnitine homeostasis is disrupted in Shunt lambs, at least in part, through a loss of PPAR $\gamma$  signaling [64]. PPAR $\gamma$  signaling is an attractive target due to the availability of the thiazolidinediones (TZD’s or glitazones) family of drugs initially introduced to treat type 2 diabetes [84,85]. TZDs act by mimicking the endogenous ligands of PPARs leading to the nuclear localization of the receptor and enhanced DNA binding in conjunction with the Retinoid X Receptor (RXR) [86]. This results in either transactivation or trans-repression of a number of genes. Treating Shunt lambs with the TZD, rosiglitazone (Avandia) has been shown to preserve carnitine homeostasis, mitochondrial function and ATP levels. This in turn preserves NO signaling by preventing the loss of hsp90 chaperone activity [64,87]. However, moving these studies forward into children with CHD should be approached with caution due to the checkered

past that the TZDs have exhibited in clinical trials. Early studies implicated rosiglitazone use with an increased risk of coronary heart disease and heart attacks [88,89] and led to safety concerns [90]. However, the RECORD trial, specifically designed to assess cardiac outcomes, concluded that rosiglitazone use in diabetic patients does not increase the risk of overall cardiovascular morbidity or mortality compared to standard glucose lowering agents [91]. However, concerns still exist [92]. It has also been suggested that different TZDs, e.g., pioglitazone (Actos) or troglitazone (Rezulin), may have a reduced likelihood for inducing adverse effects. But this remains unproven.

Although L-carnitine has been utilized extensively over a number of decades to improve mitochondrial function in children born with various mitochondrial myopathies its therapeutic potential in treating cardiovascular disease has only been explored more recently [93,94]. L-carnitine has been shown to be cardio protective in a number of animal and human studies [95-99]. We have recently carried out a prevention trial in which Shunt lambs were treated for 4-weeks with 100 mg/kg/day of L-carnitine or a vehicle. L-Carnitine-treated lambs had increased expression of the carnitine shuttle enzymes CPT1 and CPT2 protein levels, increased CrAT enzyme activity and an improved AC: FC ratio [100]. This preservation of mitochondrial function correlated with improved hsp90 function, increased NO generation and reduced NOS uncoupling [100]. Most importantly the loss of endothelial dependent vasodilation was prevented [100]. These data confirm the therapeutic possibilities of targeting the mitochondria to prevent the development of pulmonary endothelial dysfunction in children born with CHD that results in increased PBF.

## Conclusion

Over the last decade our work using a lamb model of CHD with increased PBF has linked the loss of NO signaling and subsequent pulmonary endothelial dysfunction to a loss of carnitine homeostasis. Further, our prevention studies indicate that maintaining the carnitine homeostasis, using the TZD, rosiglitazone to maintain expression of the carnitine homeostasis enzymes or by chronic ingestion of L-carnitine can prevent the mitochondrial dysfunction in the lung exposed to chronic high levels of PBF. This in turn preserves hsp90 function and NO signaling. Thus, there is potential clinical utility in these types of interventions, especially for L-carnitine that has been used for several decades to treat in born errors of metabolism with no obvious side effects. However, before proceeding to clinical trials it will be important to confirm that the changes in carnitine homeostasis observed in Shunt lambs are recapitulated in children born with CHD that result in increased PBF.

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