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# Toll-like receptor 4 mutation suppresses hyperhomocysteinemia-mediated hypertension.

Anastasia Familtseva

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TOLL- LIKE RECEPTOR 4 MUTATION SUPPRESSES  
HYPERHOMOCYSTEINEMIA- MEDIATED HYPERTENSION

By

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M.D., Omsk State Medical Academy, 2010

A Dissertation

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A Dissertation Approved on

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## DEDICATION

This dissertation is dedicated to my mother, Mrs. Svetlana Kuznetsova, for believing in me and continuously inspiring me.

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## ABSTRACT

### TOLL- LIKE RECEPTOR 4 MUTATION SUPPRESSES HYPERHOMOCYSTEINEMIA- MEDIATED HYPERTENSION

Anastasia Familtseva

June 1, 2016

**Background:** Hyperhomocysteinemia (HHcy) has been observed to promote hypertension, but the mechanisms are unclear. Toll-like receptor 4 (TLR-4) is a cellular membrane protein that is ubiquitously expressed in all cell types of the vasculature. TLR-4 activation has been shown to promote inflammation that has been associated with pathogenesis of hypertension. In this study, we hypothesize that HHcy induces hypertension by TLR-4 activation that promotes inflammatory cytokine up-regulation (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) and initiation of mitochondrial dysfunction leading to cell death and chronic vascular inflammation.

**Methods:** To test this hypothesis, we used C57BL/6J mice (WT); Cystathionine- $\beta$ -synthase deficient mice (CBS+/-) with genetic mild HHcy; C3H/HeJ (C3H)



mice, with TLR-4 mutation and mice with combined genetic CBS enzyme deficiency and TLR-4 mutation (CBS+/-/C3H). Arterial blood pressure was measured using non-invasive tail-cuff method. Ultrasonography of the superior mesenteric artery was performed to assess resistance to blood flow and to measure wall/lumen ratio. Collagen deposition in the SMA was analyzed using Masson's trichrome staining. The levels of oxidative stress markers and endothelial nitric oxide synthase (eNOS) were measured by western blotting and the expression of endothelial cell markers analyzed by immunohistochemistry (IHC). The proteins of mitochondrial dynamics were assessed by western blot, qPCR and IHC. The levels of inflammatory markers were analyzed by qPCR and IHC. Mitochondrial apoptosis protein expression was measured by western blot, qPCR and IHC. DNA fragmentation in the SMA was assessed by TUNEL assay.

**Results:** Ultrasonography of the SMA detected an increase of wall-to-lumen ratio and resistance to blood flow in CBS+/- mice that was associated with arterial blood pressure elevation in the same mouse model detected by tail-cuff measurement. The SMA from CBS+/- mice expressed elevated markers of inflammation, oxidative stress, mitochondrial fission and mitochondrial apoptosis. The increased collagen accumulation was observed in the SMA of CBS+/- mouse model. However, all these changes were attenuated in CBS+/-/C3H mouse model.

**Conclusions:** We conclude that HHcy promotes TLR-4-driven chronic vascular inflammation and mitochondria-mediated cell death inducing peripheral vascular

remodeling and hypertension. TLR-4 mutation attenuates vascular inflammation and cell death that prevents vascular remodeling and suppresses hypertension.

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## CHAPTER I

### INTRODUCTION

Homocysteine (Hcy) is a non-protein-coding  $\alpha$ -amino acid that is synthesized from dietary protein-derived methionine. The excess of Hcy in plasma is cleared via two essential pathways remethylation and transsulfuration pathways [1]. In remethylation, cobalamin-dependent methionine synthase (MS) and cobalamin-independent betaine-homocysteine methyltransferase (BHMT) enzymes are used to convert Hcy back to methionine [2]. In transsulfuration that occurs only in the small intestine, liver, pancreas and kidney a cofactor- vitamin B<sub>6</sub> is required to convert Hcy to cystathionine by cystathionine  $\beta$ - synthase (CBS) and cystathionine to cysteine by cystathionine gamma-lyase (CSE) [3]. Cysteine is further utilized as a precursor for synthesis of antioxidant glutathione or vasodilator hydrogen sulfide. Mutations in MTHFR, CBS and CSE enzymes and nutritional deficiencies in vitamin cofactors (B<sub>12</sub>, B<sub>9</sub>, and B<sub>6</sub>) are the common causes of HHcy. The “normal” range for plasma tHcy is about 5-15  $\mu$ M/L and hyperhomocysteinemia (HHcy) is defined as an elevation of plasma tHcy level above 15 $\mu$ M/L that is classified on moderate (16-30  $\mu$ M/L), intermediate (31-100  $\mu$ M/L) and severe (more that 100  $\mu$ M/L) HHcy [4]. Epidemiological studies have described HHcy as an independent risk factor of atherothrombotic vascular disease complications of which include stroke, myocardial infarction, peripheral

vascular disease, miscarriage, pulmonary embolism and coronary heart disease [5, 6]. A significant amount of other studies have shown a positive correlation between Hcy plasma levels and hypertension [7, 8]. It has been implicated that the possible mechanisms that are involved in HHcy-mediated hypertension could be reactive oxygen species-induced vascular endothelium damage that causes impairment in vasodilation, vascular remodeling with elastin degradation and collagen deposition and facilitation of vascular inflammation [6, 9]. Several in vitro studies have shown inflammatory markers up-regulation in HHcy, including activation of NF- $\kappa$ B that is inflammatory cytokine transcription factor [10]. Zhang et al. have reported that circulating pro-inflammatory cytokine levels (IL-6, TNF- $\alpha$  and MCP-1) are positively correlated with Hcy serum levels [11]. Scherer et al., have also observed mild HHcy-mediated augmentation of inflammatory cytokine production (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MCP-1) in serum and different organs [12]. Although HHcy is known to promote downstream pro-inflammatory cytokine elevation, the precise mechanism of triggered inflammatory response is not clearly defined until now. Pathogen recognition receptor, and in particular Toll-like receptor 4 (TLR-4) is a foreign antigen sensor that plays role in innate immune system activation and has recently gained a significant attention in the field of hypertension. TLR-4 is ubiquitously present within the vasculature (endothelial cells, VSMC) and its activation promotes inflammatory cytokine up-regulation facilitating vascular inflammation that is the hallmark of hypertension [13, 14].

Mitochondrial dysfunction and mitochondria-dependent apoptosis have



been shown to promote endothelial cell loss leading to endothelial dysfunction [15, 16] that contributes to pathogenesis of hypertension [17, 18]. Impaired mitochondrial dynamics such as excessive mitochondrial fission and decreased mitochondrial fusion is associated with cardiovascular diseases and diabetes [19-22]. Studies have implicated that HHcy facilitates excessive mitochondrial fission [23] and mitochondria-mediated cell death [24] however, the mechanisms are not well described. In the current study, we aimed to explore whether HHcy activates TLR-4 with downstream pro- inflammatory cytokine elevation that facilitates vascular inflammation. These events subsequently induce mitochondrial dysfunction characterized by excessive mitochondrial fission and mitochondrial apoptosis contributing to vascular remodeling followed by hypertension. In addition, we elucidated the role of TLR-4 mutation in attenuation of HHcy-mediated vascular inflammation and mitochondria- dependent cell death that suppresses hypertension.

## CHAPTER II

### BACKGROUND

#### **Superior Mesenteric Artery**

##### Anatomy and Physiology of the Superior Mesenteric Artery

Superior mesenteric artery is one of the branches of abdominal aorta (inferior phrenic, celiac, superior mesenteric, middle suprarenal, renal, gonadal, lumbar, inferior mesenteric, median sacral, common iliac arteries) that arises usually at the level of L1, 1-1.5 cm below celiac artery and is just superior to the origin of the renal arteries [25]. The SMA supplies blood to the pancreas, lower part of the duodenum, jejunum, ileum, ascending colon and transverse colon, giving respective branches: inferior pancreaticoduodenal artery, jejunal arteries (usually 4-6 arteries), ileal arteries (usually 8-12 arteries), ileocolic artery, right colic artery and middle colic artery [25, 26]. Splanchnic blood flow at rest is about 25% of cardiac output, but can fluctuate between 10% -35% of cardiac output depending upon magnitude, time and composition of food ingestion. The mean blood flow in human celiac axis and SMA varies from 300- 1200 mL/min [27]. At rest, mesenteric flow has intermediate to high resistance with low diastolic flow.

With food ingestion, mesenteric vascular bed is maximally dilated with low resistance to blood flow. Mesenteric blood flow is controlled by extrinsic and intrinsic systems [25]. Extrinsic system regulates splanchnic blood flow through neural and hormonal axis. Mesenteric circulation is predominantly controlled by sympathetic nervous system through activation of  $\alpha_1$ -adrenoreceptors that are highly expressed in the intestinal vasculature. Hence, a significant reduction in intestinal blood flow is mediated by increased sympathetic nerve activity during strenuous exercise or pathologically low arterial blood pressure. Mesenteric vascular bed also contains  $\beta$ -adrenoreceptors that are less prevalent than  $\alpha$ -receptors. Stimulation of  $\beta$ -adrenoreceptors with isoproterenol produces vasodilation of the mesenteric vasculature. Apart from neural regulation, mesenteric blood flow is controlled by hormonal systems. The loss of extracellular volume stimulates renin-angiotensin axis, inducing mesenteric vascular bed vasoconstriction through the direct effect of angiotensin II as well as indirectly through adrenergic receptors [28]. In addition, the decrease in blood volume and hyperosmolarity stimulate vasopressin release that produces profound mesenteric vasoconstriction and venorelaxation. The intrinsic system includes myogenic and metabolic control mechanisms of the circulation [29]. Adenosine is one of the metabolites that plays role in mesenteric vasodilation by increasing intestinal perfusion. Local release of nitric oxide induces vascular smooth muscle cell relaxation, leading to vasodilation that helps to preserve sufficient intestinal perfusion compensating for systemic hypotension. The intrinsic system is poorly developed in the intestinal circulation and hence,

extrinsic mechanism is a predominant regulator.

SMA wall comprised of three layers: tunica intima, tunica media and tunica adventitia [30]. The most inner layer of the vascular wall is tunica intima that includes a single alignment of endothelial cells. The next layer of the arterial wall is tunica media that is comprised of vascular smooth muscle cells with some elastin and collagen and separated from tunica intima by internal elastic membrane. Tunica media of the SMA has well defined circular layer of smooth muscle cells that classifies this artery as muscular artery, allowing contraction or relaxation to redistribute splanchnic blood flow if needed. The most outward layer of the arterial wall is tunica adventitia that consists of collagen and elastin and is separated from tunica media by poorly defined external elastic membrane.

## **Peripheral Vascular Remodeling and Hypertension**

Peripheral Vascular Remodeling. Classification.

Arterial remodeling is the form of vessel wall adaptation to mechanical and hemodynamic stimuli, characterized by structural and functional changes of the vascular wall mediated by different mechanisms (hyperplasia of the arterial intima and media, redistribution of extracellular matrix components such as collagen and elastin, fibrosis, arterial calcification and endothelial dysfunction). Structural reorganization of the vascular wall architecture facilitates elevation of resistance to blood flow, contributing to amplification of total peripheral resistance

that is the hallmark of hypertension [31]. Arterial remodeling is classified based on changes in luminal diameter: outward remodeling (increased vessel diameter) and inward remodeling (decreased vessel diameter), and based on changes in wall thickness: hypertrophic (thickening of the vessel wall), eutrophic (constant wall thickness) and hypotrophic (thinning of the vascular wall) [32]. Outward hypertrophic remodeling is characterized by increased vessel diameter and thickening of intimal and medial layers of the vascular wall promoting stiffness of the large central elastic arteries that occurs in hypertension [31]. However, muscular peripheral arteries develop one of two types of arterial remodeling: inward eutrophic or inward hypertrophic remodeling. In the first type of remodeling, that is attributed to essential hypertension in humans and is also found in spontaneously hypertensive rat model, the lumen is reduced and the wall thickness is constant and similar to that of normotensive individuals [31]. The second type or inward hypertrophic remodeling is developed in secondary hypertension (renovascular hypertension, pheochromocytoma, diabetic, salt-dependent and mineralocorticoid hypertension) and is characterized by reduced lumen and enlarged media cross-sectional area [31].

#### Pathogenesis of Arterial Remodeling

Arterial remodeling occurs due to the complex of mechanisms (smooth muscle cell proliferation and differentiation, elastin degradation and collagen deposition, arterial calcification and endothelial dysfunction) that mediate reorganization of all layers of the vascular wall [32]. Tunica media that is comprised of smooth muscle cells (SMC) plays a major role in regulation of vascular tone and diameter

through smooth muscle contraction and relaxation. SMC content in the tunica media raises up to 85% with decrease in vessel diameter. In contrast to large central elastic arteries, SMC of the small arteries circumferentially arranged with a pitch angle smaller than  $2^{\circ}$ . Such an arrangement of SMC allows optimal resistance against vessel distention [33]. Under normal condition, SMC have contractile phenotype and express respective SMC proteins: smooth muscle 22-alpha (SM22 $\alpha$ ), alpha-smooth muscle actin ( $\alpha$ SMA) and smoothelin [34, 35]. However, studies have shown that in vascular injury or stress contractile SMC undergo phenotype switching, where SMC differentiate into synthetic phenotype that further gives two classes of SMC (migratory-proliferative or osteogenic phenotypes) [32]. Inflammation, oxidative stress, mechanical stretch, angiotensin II, transforming growth factor- $\beta$  (TGF- $\beta$ ), matrix metalloproteinases (MMPs) are the major stimuli for vascular smooth muscle cells (VSMC) phenotype switching. Synthetic VSMC secrete MMPs allowing the migration of SMC into intima by detaching cells from the basement membrane and extracellular matrix (ECM) [36]. The migrated SMC are involved in proliferation and hyperplasia of intima promoting thickening of the arterial wall. High extracellular levels of calcium and phosphate and the absence of inhibitors of calcification allow VSMC differentiation into osteogenic phenotype, where VSMC have the features attributed to osteoblasts or chondrocytes [37]. Osteogenic phenotype decreases the expression of SMC markers and induces intense calcification of elastic fibers in vascular wall.

The next event that contributes to arterial vascular remodeling is

redistribution of ECM proteins in the vascular wall. ECM occupies more than half of the vascular wall mass and contains elastin, collagen, fibronectin, fibrillins, proteoglycans and leucine- rich glycoproteins [38]. ECM maintains vascular function under normal and pathophysiological conditions. Interaction of vascular wall cells with ECM regulates cell migration, adhesion, proliferation and phenotype. Integrins are the ECM receptors that are engaged by cells to sense the ECM content change that is involved in tissue remodeling.

Elastin is mainly synthesized by SMC, however endothelial cells and adventitial fibroblasts are able to produce tropoelastin. Large resistance arteries contain internal and external elastic membranes and some elastic fibers located between smooth muscle cell fibers. In contrast to large elastic arteries, elastin of small arteries and arterioles is only limited to internal elastic membrane. Large central elastic arteries contain significant amount of elastin (111mg/g in the rat carotid artery) assisting in mitigation of pressure pulsations. The amount of elastin reduces with a decrease in vessel diameter (15mg/g in small mesenteric arteries) [33].

Collagen is a protein that is in a high content provides arterial stiffness and limits arterial compliance. Collagen distribution reduces with a decrease of vessel caliber (e.g. from 124 mg/g in carotid artery to 67mg/g in mesenteric arteries). Collagen vascular wall content reduces towards periphery from 20% to 9% of the wall volume over the mesenteric vasculature [33]. Collagen I and III are expressed in media and highly present in adventitia. The basement membrane is comprised of collagen IV.

The elastic fibers of the large elastic arteries provide an adequate arterial compliance during systole. With aging, elastic laminae fragmentation process occurs, redistributing mechanical load to collagen fibers that are stiffer by nature [39]. Such impairments in vascular wall amplify systolic and pulse pressures that provoke hypertension. Arterial remodeling in hypertension is characterized by increase of media/lumen ratio with or without wall thickening (hypertrophic, eutrophic) due to redistribution of SMCs or ECM proteins [40, 41]. Arterial wall thickening occurs due to elastin degradation and collagen deposition. It reduces arterial compliance and amplifies arterial fibrosis limiting distention of the vascular wall. Therefore, pressure elevation is required to surpass arterial wall stiffness.

Endothelial dysfunction is often, but not always, observed in arteries of hypertensive subjects and plays a significant role in pathogenesis of arterial remodeling [31]. Changes in blood flow and shear stress stimulate nitric oxide (NO) release from endothelial cells (EC) that produce relaxation of VSMC and vasodilation. EC layer damage facilitates impairments in endothelium- dependent vasodilation due to the loss of NO production. In oxidative stress, the presence of reactive oxygen species (ROS) decrease NO bioavailability due to the formation of peroxynitrite that further exacerbates oxidative environment and EC injury [42]. In inflammation, EC produce cytokines [43] and growth factors: monocyte chemotactic protein (MCP-1), TGF- $\beta$ , C-reactive protein, plasminogen activator inhibitor (PAI-1). They facilitate EC, VSMC, vascular pericytes proliferation. Altered morphology with disruption of endothelial layer integrity, impaired



vasodilation and inadequate vasoconstriction significantly affect vascular tone contributing to pathogenesis of hypertension [44].

## **Homocysteine**

### Homocysteine Metabolism

Homocysteine (Hcy) is a non-protein-coding  $\alpha$ -amino acid that is synthesized from dietary protein-derived methionine. Hcy is circulated in plasma in four forms: about 1% is presented as the free thiol, 70-80% of circulated Hcy is bound to plasma proteins (albumin), the rest of 20-30% of Hcy is bound to itself to form Hcy dimers or combined with other thiols including cysteine forming Hcy-cysteine mixed disulfide [4]. The term “total plasma (or serum) Hcy” (tHcy) is determined as all four circulating forms of Hcy combined together [4].

Within the cells methionine converts to S-adenosylmethionine that is a methyl group donor and is essential for various methylation reactions involving DNA, amino acids and proteins [45]. S-adenosylhomocysteine is formed by the methyl group donation of SAM to various substrates [45]. The excess of Hcy in plasma is cleared via two essential pathways: remethylation and transsulfuration pathways (Figure 1.). In remethylation pathway Hcy is converted back to methionine with implication of two different enzymes: cobalamin-dependent methionine synthase (MS) that is expressed in all tissues at very low levels and betaine-homocysteine methyltransferase (BHMT) that is specific for kidney and

liver and is produced at the very high levels [46]. The first enzyme utilizes cobalamin ( $B_{12}$ ) as a cofactor and uses 5-methyltetrahydrofolate (5-MTHF) as the methyl donor. 5-MTHF or active form of folate ( $B_9$ ) is synthesized from 5, 10-methylenetetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR). Betaine-homocysteine methyltransferase (BHMT) that is only expressed in the liver and kidney and is cobalamin-independent enzyme uses betaine as methyl donor to remethylate Hcy back to methionine [47]. In transsulfuration that occurs only in the small intestine, liver, pancreas and kidney a cofactor- vitamin  $B_6$  is required to convert Hcy to cystathionine by cystathionine  $\beta$ - synthase (CBS) [48]. Cystathionine is hydrolyzed by vitamin  $B_6$ - dependent cystathionine gamma-lyase (CSE) to cysteine that is used as a precursor for synthesis of antioxidant- glutathione or vasodilator-hydrogen sulfide [48]. Nutritional deficiencies in vitamin cofactors ( $B_{12}$ ,  $B_9$ , and  $B_6$ ) and mutations in MTHFR, CBS and CSE enzymes are the common causes of HHcy.

## Homocysteine Metabolism

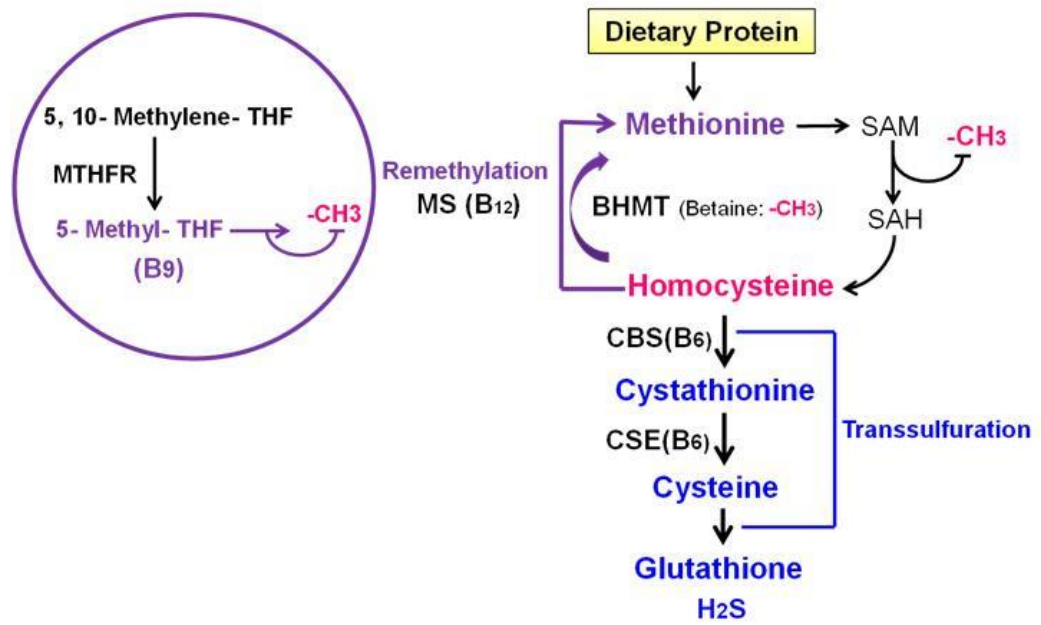


Figure 1. Schematic representation of homocysteine metabolism

## Hyperhomocysteinemia

### Epidemiology and Etiology of Hyperhomocysteinemia

The “normal” range for plasma tHcy is about 5-15 uM/L and hyperhomocysteinemia (HHcy) is defined as an elevation of plasma tHcy level above 15uM/L. HHcy is classified into moderate (16-30 uM/L), intermediate (31-100 uM/L) and severe (more than 100 uM/L) HHcy [4].

Studies have shown that the prevalence of HHcy varies in different countries and is estimated to be 6% in the Costa Rican population, 5% in the United States, 77% in India, 27.5% in China and similar to that estimate in Brazil and Lebanon [49, 50]. Patients with symptomatic atherosclerotic vascular disease have shown 13-47% of HHcy prevalence. Several studies have shown the elevation of tHcy plasma level with age due to age-associated decline in the activity of enzymes that are involved in Hcy clearance and reduction in renal function [51, 52]. The prevalence of HHcy is significantly higher in men than in women that could be explained by the difference in muscle mass, lifestyle, vitamin intake and sex hormones. A clinical study showed that four-month treatment with ethinyl estradiol of male-to-female transsexulas significantly reduced the Hcy plasma levels, however female-to-male transsexuals who received androgen therapy expressed high plasma levels of Hcy [53]. In addition, another clinical study confirmed the beneficial effect of estrogen replacement therapy in reduction of Hcy plasma levels in postmenopausal women [54]. A significant amount of studies have observed a strong positive correlation

between smoking, alcohol and coffee consumption and serum tHcy concentrations [49, 55, 56]. Vermaak et al. have shown that cigarette smokers have significantly lower levels of pyridoxal phosphate that contributes to Hcy metabolism compared to non-smokers [57].

Genetic defects in enzymes that are involved in Hcy metabolism are significant etiological factors that contribute to HHcy. The most common genetic disorder that causes severe HHcy and homocysteinuria is homozygous deficiency of CBS enzyme that is inherited as an autosomal recessive trait and characterized by 40-fold elevation of fasting tHcy [4]. It occurs in 1 in 100,000 live birth and defined by specific phenotype that includes lens disposition, bone impairments, intellectual disability and premature atherosclerosis [58]. There are 60 described CBS enzyme mutations with I278T and G307S are being the most common types [59]. The less common cases of severe HHcy are genetic mutation in MTHFR, MS enzymes and genetic disorders in cobalamin metabolism [60]. The most prevalent type of genetic enzyme disorder that has been associated with moderate increase of plasma tHcy levels is a single point mutation at nucleotide 677 (C-to-T substitution) in MTHFR gene that causes about 50% decline in enzyme activity [61]. There is about 10-13% of prevalence for this specific type of MTHFR mutation (TT genotype) in white population [62]. Nutritional deficiencies in vitamin cofactors such as folate (B<sub>9</sub>), cobalamin (B<sub>12</sub>) and pyridoxal phosphate (B<sub>6</sub>) contribute to the development of HHcy [63]. It has been reported that about two-thirds of HHcy is due to low blood concentrations of mentioned vitamin co-factors [64]. Other case that could affect tHcy plasma

levels is kidney dysfunction. Clinical studies have shown that patients with chronic renal diseases have elevated tHcy levels due to the impairments in Hcy clearance by renal enzymes [65].

Epidemiological studies have described HHcy as an independent risk factor of atherothrombotic vascular disease complications of which include stroke, myocardial infarction, peripheral vascular disease, miscarriage, pulmonary embolism and coronary heart disease [5, 6]. Several mechanisms have been implicated in pathogenesis of HHcy-induced vascular disease: reactive oxygen species activation, damage of vascular endothelium followed by endothelial dysfunction and promotion of atherosclerosis, impairment in the process of thrombolysis and hypercoagulation [66-68]. Sengwayo et al. have reported that Hcy elevation was significantly associated with increase in both systolic and diastolic blood pressure [6]. It has been implicated that the possible mechanisms that are involved in HHcy- mediated hypertension could be ROS- induced vascular endothelium damage that causes impairment in vasodilation, vascular remodeling with elastin degradation and collagen deposition and VSMC hyperproliferation [6, 9]. A significant amount of other studies have also shown a positive correlation between Hcy plasma levels and hypertension [7, 8, 69-73]. HHcy has been implicated to be involved in the initiation of vascular inflammation as one of the key mechanisms that contributes to the development of arterial vascular disease predisposing to hypertension [11, 74-76].

## Hyperhomocysteinemia and Inflammation

### The Innate Immune System and Toll- Like Receptors (TLRs)

Inflammatory response is the reaction of the immune system to the presence of antigens that involves two major systems innate and adaptive, that are closely interact with each other. The innate immunity is the immediate response of the immune system and the first line of defense against foreign antigens. The epithelial cell barrier, pathogen recognition receptors (PRR) including Toll-like receptors (TLRs), compliment system, monocytes and macrophages are the major constituents of the innate immune system response [77]. It has been proposed that TLRs and the innate immune system play a significant role in the development of cardiovascular diseases. Sustained or excessive activation of TLRs of the immune and vascular cells promote chronic vascular inflammation that contributes to cardiovascular diseases. TLRs are expressed on immune (macrophages, dendritic cells, monocytes) as well as non-immune cells (endothelial cells, smooth muscle cells, fibroblasts, epithelial cells). TLR is a type I glycoprotein receptor containing: 1) 20-27 extracellular leucine-rich repeat domains that are required for ligand recognition, 2) transmembrane domain, 3) intracellular Toll/interleukin-1 receptor (TIR) domain that activates downstream signaling pathways [78, 79]. TLR signal transduction starts from TIR-mediated recruitment of several adapter proteins: myeloid differentiation primary response gene 88 (MyD88), MyD88-like adapter protein or also known as Toll-Interleukin 1 receptor domain containing adaptor protein (TIRAP), TIR domain-containing

adaptor protein inducing interferon- $\beta$  (TRIF), TRIF related adaptor molecule (TRAM) and sterile  $\alpha$ - and armadillo-motif-containing protein (SARM) [80]. TIRAP is necessary for activation of MyD88-mediated pathway and connects TIR domain with MyD88 adapter protein. TRAM is required in TLR-4-mediated activation of TRIF pathway. TIR-mediated recruitment of different adaptor proteins promote: 1) the formation of “Myddosome” that involves MyD88-induced mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light chain-enhancer of activated B-cell (NF- $\kappa$ B) activation, 2) the assembly of “Trifosome” for the activation of interferon regulatory factors (IRFs) and other kinases that are involved in downstream signaling [81, 82]. In addition, TLR induces inflammasomes that trigger caspase-1-mediated proteolytic cleavage of pro-IL-1 $\beta$  and pro-IL-18 to activate mentioned cytokines (IL-1 $\beta$ , IL-18) [83]. Depending on the type of TLRs, they sense the presence of bacterial, viral and fungal motifs or pathogen-associated molecular pattern molecules (PAMPs). However, recent studies have described that in addition to PAMPs, TLRs get activated by circulating endogenous molecules or damage-associated molecular pattern molecules (DAMPs) (Table 1; Goulopoulou et al. 2015) that are released from damaged or dying cells [13, 14]. There are 13 TLRs that have been described in mammals (TLR1-TLR10 in humans and TLR1-TLR9; TLR11-TLR13 in mice): cell surface TLRs that sense the presence of microbial motifs (TLR- 1, 2, 4- 6 and 11) and TLRs that localized to intracellular membranes and recognize viral or bacterial nucleic acids (TLR- 3, 7- 9 and 13) [84-86].

The role of TLR- 4 that is ubiquitously present within the vasculature



(endothelial cells, VSMC, fibroblasts) has been recently highlighted in sterile inflammation [13, 14]. In contrast to others, TLR-4 is the most associated and well-defined TLR that has been involved in etiology of hypertension. Bonfim et al. have described the TLR-4 protein up-regulation in the mesenteric artery of spontaneously hypertensive rat model compared to control that was accompanied by increase in mean arterial blood pressure and circulating serum levels of IL-6. However, treatment with anti-TLR-4 antibody reduced TLR-4, IL-6 expression and blood pressure [87]. Hernanz et al. have shown Angiotensin II-mediated TLR-4 up-regulation that contributed to vascular inflammation, endothelial dysfunction and vascular remodeling that are the hallmarks of hypertension [88]. Dange et al. confirmed higher levels of TLR-4 in paraventricular nucleus of spontaneously hypertensive rat model that was correlated with inflammatory markers elevation and hypertension, but TLR-4 inhibition attenuated blood pressure, cardiac hypertrophy and reduced TNF- $\alpha$ , IL-1 $\beta$ , NF $\kappa$ B and iNOS levels [89]. Schneider et al. have reported that patients with TLR-4 single nucleotide polymorphism rs4986790 (TLR4 896A/G) had significantly lower systolic blood pressure as well as pulse pressure compared to TLR4 896A/A allele carriers [90]. Sollinger et al. have found that treatment with inhibitor of endothelial nitric oxide synthase L-NAME induced DAMPs up-regulation that was accompanied by ROS increase in TLR-4- dependent manner, however there was reduction in arterial contractility in TLR-4<sup>-/-</sup> model [91]. Pineda et al. in his study observed increase in TLR-4 in placentas of patients with preeclampsia and even higher expression of TLR-4 in placentas of patients with

chorioamnionitis compared to normal pregnancies. In addition, the authors have found an elevation of TLR- 2-4 and TLR- 9 in placental villous stroma in patients with preeclampsia [92]. There was an association confirmed between Asp299Gly polymorphism in TLR-4 gene and reduction in C-reactive protein, prevalence of coronary artery disease and diabetes [93]. A significant amount of studies have also highlighted the role of TLR-4 in pathogenesis of atherosclerosis such as den Dekker et al. have shown that TLR-4 signaling pathway plays role in mast cell-mediated VSMC apoptosis that contributes to atherosclerotic plaque destabilization [94]. Several other studies have observed that TLR-4 activation promotes macrophage differentiation into foam cells and apoptosis [95-97], induces SMC proliferation [98] and pro- inflammatory activation of endothelial cells [99-101].

DAMPs for TLR-4	Cell types	Disease	References
<b>C-reactive protein</b> <b>ECM proteins:</b> Biglycan Decorin Fibrinogen Hyaluronan <b>HMGB1</b> <b>HSP60</b> <b>HSP70</b> <b>Oxidized LDL</b>	VSMC Macrophages Macrophages, renal arteries Monocytes Macrophages, EC Neutrophils, Macrophages Monocytes, VSMC Monocytes, Macrophages EC, Macrophages	Atherosclerosis	Liu et al. 2010 Thompson et al. 2014 Merline et al. 2011 Smiley et al. 2001 Taylor et al. 2004 Hirata et al. 2013 De Graaf et al. 2006 Vabulas et al. 2002 Su et al. 2011
<b>ADMA</b> <b>Angiotensin II</b> <b>C- reactive protein</b> <b>Fibrinogen</b> <b>HMGB1</b> <b>HSP60</b> <b>Hyaluronan</b>	Adipocytes Arteries, PVN VSMC Cardiomyocytes EC, Cardiomyocytes VSMC EC	Hypertension	Yang et al. 2009 Dange et al. 2014 Liu et al. 2010 Liu et al. 2010 Mersmann et al. 2013 de Graaf et al. 2006 Taylor et al. 2004
<b>HMGB1</b> <b>Peroxiredoxin</b>	Neurons, EC, glial cells Macrophages	Stroke and Cerebrovascular Injury	Giu et al. 2008, 2010 Kuang et al. 2014

Table 1. Damage- associated molecular patterns (DAMPs) or toll- like receptor 4 (TLR- 4) specific ligands

Macrophages are significant constituents of the innate immune system that promote inflammation, vascular remodeling, engaging matrix metalloproteinases (MMPs) and impair vascular reactivity through increased ROS production. Macrophages play a major role in IL-1 $\beta$ , IL-6 and TNF- $\alpha$  cytokine release.

## Pro-inflammatory Cytokines that Contribute to Vascular Inflammation and Hypertension

### Interleukin-1 $\beta$ (IL-1 $\beta$ )

IL-1 $\beta$  is primarily released by immune cells (monocytes and macrophages) and by other cell types including vascular EC and renal tubular epithelial cells [102, 103]. IL-1 $\beta$  acts as pro-inflammatory cytokine through stimulation of specific cell surface receptor IL-1 type 1 receptor (IL-1RI) that is located on immune cells (macrophages, monocytes and lymphocytes) as well as on vascular cells (EC and SMC). Ligand- receptor (IL-1 $\beta$ - IL-1RI) interaction recruits accessory protein (IL-1RAcP) that provides stronger ligand-receptor connection. Several adapter proteins such as myeloid differentiation factor 88, TNF receptor-associated factor 6 and IL-1R-associated kinase are engaged by accessory proteins followed by JNK, p38 MAPK, activator protein-1 (AP-1) and transcription factor NF- $\kappa$ B activation that induce pro-inflammatory gene up-regulation [104]. IL-1 $\beta$  is an immediate response cytokine that promotes release of other downstream cytokines such as: IL-6 and IL-17 that stimulate proliferation of T helper 1 and T helper 17 cells contributing to pathogenesis of hypertension [105, 106]. Studies

have described the direct effect of IL-1 $\beta$  on vascular wall suggesting that IL-1 $\beta$  infusion facilitates impaired endothelial-dependent relaxation response to acetylcholine compared to vessels incubated with vehicle [107]. It was shown that IL-1 $\beta$  promotes reactive oxygen species production, as IL-1 $\beta$  treated vessels expressed higher levels of iNOS and superoxide than controls [108]. Dalekos et al. have shown that patients with essential hypertension have higher circulating levels of IL-1 $\beta$  compared to normotensive patients [109]. Monocytes isolated from peripheral blood of hypertensive patients produce higher levels of IL-1 $\beta$  in response to Angiotensin II or LPS stimulation compared to monocytes isolated from normotensive individuals [110]. Dorrance et al. have reported that IL-1 $\beta$  treatment induced greater vasoconstriction in aortas isolated from hypertensive rats compared to normotensive rats [111].

#### Interleukin 6 (IL-6)

IL-6 is a 21kDa glycoprotein that is released by variety of cells: dendritic cell, macrophages, T helper 1 cells, monocytes and vascular cells. The IL-6 interaction with IL-6 receptor induces several effects on target organs including neutrophil chemotaxis, polarization of CD4<sup>+</sup> T cells and bone resorption. IL-6 up-regulation has been associated with several conditions including atherosclerosis, hypertension, autoimmune diseases and malignancies. Studies have shown a positive correlation of IL-6 plasma levels with blood pressure elevation and IL-6 levels reduction after angiotensin II receptor blockade [112]. The Angiotensin II treatment up-regulates plasma level of IL-6 in humans and this is prevented by treatment with spironolactone [113]. Lee et al. have confirmed that IL-6<sup>-/-</sup> mice are

resistant to developing hypertension and albuminuria after angiotensin II treatment and excessive salt intake compared to control group [114].

Tumor necrosis factor- $\alpha$  (TNF-  $\alpha$ )

TNF-  $\alpha$  is released by diverse cells of the immune system including macrophages, T cells and by vascular cells such as endothelial cells, fibroblasts. TNF-  $\alpha$  activates TNF receptor 1 and TNF receptor 2 inducing activation of death/survival pathways, NADPH oxidase and nuclear factor kappa B (NF $\kappa$ B) signaling pathway [115]. TNF-  $\alpha$ -mediated NADPH oxidase and NF $\kappa$ B activation promote rise in chemokine and adhesion molecule expression, sodium retention by the kidney and vascular remodeling [116]. Studies have shown that TNF-  $\alpha$  inhibits endothelial nitric oxide synthase (eNOS) promoter affecting mRNA and protein expression of eNOS reducing the production of NO that impairs vasodilation and promotes vasoconstriction [117, 118]. In kidneys NO inhibits sodium reabsorption and the absence of NO promotes sodium retention. Studies suggest that Angiotensin II infusion stimulates T cells to release TNF-  $\alpha$  and inhibition of TNF-  $\alpha$  protects from Angiotensin II-mediated hypertension [119]. TNF-  $\alpha^{-/-}$  mice are resistant to Angiotensin II-induced blood pressure elevation and express reduction of left ventricular hypertrophy [120].

The Adaptive Immune System

Unlike the innate immune system, the adaptive immunity is highly specific to unique foreign antigen and is designed to create immunological memory that allows the enhanced immune defense against recognized pathogen. Antigen-

presenting cells (APCs: macrophages, dendritic cell, B-cells) represent foreign antigens in major histocompatibility complexes (MHC), such that MHC-I attracts CD8<sup>+</sup> T- cells and MHC-II activates CD4<sup>+</sup> T- cells [77]. T- cells express T- cell receptor on their surface that interacts with MHC of the APC, leading signal-1 T- cell activation. Signal-2 or T- cell co-stimulation is essential for the full T- cell activation and is induced by T- cell CD28 and APCs B7 ligands (CD80, CD86) interaction. Studies suggest that T- cell co-stimulation plays a major role in pathogenesis of hypertension such that hypertension has been associated with up- regulation of CD86 in spleen and lymph node dendritic cells and B7 ligand-deficient mice are resistant to blood pressure elevation [77, 121]. The full T- cell activation induces a complex of signaling events including effector T- cell proliferation, cytokine production and T- cell migration from secondary lymphoid organs (spleen, lymphoid nodes) to sites of tissue damage that is guided by cytokines and chemokines released from affected tissues. The effector T- cells that are arrived to peripheral tissues further orchestrate the inflammatory response releasing variety of mediators [122]. The effector T- cell response is limited in duration and the majority of the effector T- cells undergo programmed cell death, the rest are destined to become central memory cells that migrate back to secondary lymphoid organs or stay at the peripheral sites to become effector memory cells. CD4<sup>+</sup> effector T- cells have four specific phenotypes: T-helper 1 cells that are produced in defense to intracellular bacteria and virus and release interferon- $\gamma$ , T helper 2 cells produced against allergens and helminthes, T helper 17 cells that protect against extracellular bacteria and fungi and the

fourth phenotype includes regulatory T- cells that release anti-inflammatory cytokine IL-10 and suppress T helper 1 and 2- mediated immune responses [123]. CD8<sup>+</sup> effector T- cells release cytotoxic molecules (perforin, granzyme B), cytokines and are classified in the same manner as CD4<sup>+</sup> cells (i.e. T<sub>C</sub>1, T<sub>C</sub>2, T<sub>C</sub>17 and cytotoxic regulatory T- cells).

### The Role of Hyperhomocysteinemia in Inflammatory Response

A significant number of studies have described inflammation as one of the toxic effects of Hcy. Zhang et al. reported that plasma Hcy was positively correlated with plasma pro-inflammatory cytokine and chemokine (IL-6, TNF-  $\alpha$  and MCP-1) levels and promoted inflammatory monocyte differentiation [11]. Zanin et al. have shown that HHcy is involved in the synthesis and secretion of IL-1 $\beta$  in murine macrophages via NF-kB that is inflammatory cytokine transcription factor [124]. Han et al. have observed endothelial cell inflammatory injury through activation of NF-kB and cytokine IL-6 up regulation in HHcy [125]. Several in vitro studies have shown inflammatory markers up-regulation in HHcy, including activation of NF-kB [10, 126]. Scherer et al. have also observed mild HHcy-mediated augmentation of inflammatory cytokine production (IL- 1 $\beta$ , IL- 6, TNF-  $\alpha$ , MCP-1) in serum and different organs [12]. Wang et al. have reported Hcy treatment-induced monocyte chemoattractant protein- 1 (MCP-1) elevation in macrophages via NF-kB activation [127]. Lee et al. described that HHcy facilitates (MMP- 9) production in murine macrophages the activity of which is involved in vascular remodeling [128]. Gao et al. observed that combined treatment with Hcy and LPS led to macrophage polarization from anti-inflammatory (M2) to pro-inflammatory

subtype (M1) that promotes the progression of atherosclerosis [129].

Apart from being involved in innate immune response, HHcy has been also shown to play a role in the adaptive immune system. Feng et al. have observed that HHcy increases the production of ROS from T lymphocytes and reduces proliferation and function of regulatory T- cells that have anti-inflammatory properties [130]. Zhang Q et al. have described that Hcy elevation promotes T-cell and lipopolysaccharide-induced B-lymphocytes proliferation that contribute to chronic inflammatory progression of atherosclerosis [131, 132].

## **Hyperhomocysteinemia and Mitochondrial Dysfunction**

### **Mitochondrial Dynamics and Cardiovascular Diseases**

Mitochondria are the cell organelles that function to provide energy generation, regulation of cell survival (apoptosis), synthesis of reactive oxygen species and control of intracellular  $\text{Ca}^{2+}$  concentration [133]. Mitochondria preserve and constantly maintain their function in response to various changes in the cellular environment through mitochondrial dynamics. Mitochondrial dynamics is defined as mitochondrial adaptation to intracellular changes where mitochondria constantly undergo through fusion and fission processes. Mitochondrial fusion allows union of two or more mitochondria to form one and as a result mitochondria acquire tubular or elongated shape. The process of fusion is regulated by outer mitochondrial proteins: mitofusin 1 and 2 (Mfn-1, Mfn-2) and



inner mitochondrial protein: optic atrophy 1 (Opa-1) protein [134]. Studies have observed that Mfn-1 and Mfn-2 down-regulation promotes mitochondrial fission [135]. Cardiac specific Mfn-1 knockout mice expressed small round-shape fragmented mitochondria [136], however cardiac specific Mfn-2 knockout mice exhibited enlarged mitochondria in the heart [137]. Mfn-2 participates in mitophagy or the process of damaged mitochondria elimination, where Mfn-2 is phosphorylated by PTEN-induced kinase 1 (PINK 1) that attracts Parkin that further orchestrates mitochondria quality control process [138]. Overexpression of inner mitochondrial membrane protein Opa- 1 facilitates the formation of elongated mitochondria and enrichment of mitochondria network, however, Opa- 1 down-regulation promotes disintegration of cristae and excessive mitochondrial fragmentation [139].

Mitochondrial fission is the process of mitochondria disintegration with small spherical-shaped mitochondria formation. Upon activation mitochondrial fission protein Drp- 1 (dynamin related protein 1) shifts from cytosol to mitochondrial fission sites, where it recruits outer mitochondrial membrane protein- mitochondrial fission factor (Mff) to further facilitate fission process. Mitochondrial fission 1 (Fis- 1) is outer mitochondrial membrane protein that is highly required for the process of mitochondrial fragmentation. The initiation of mitochondrial fission starts from mitochondria constriction by endoplasmic reticulum tubules that orient and define the sites of division followed by Drp-1-mediated scission of mitochondria [140]. Drp-1 is an 80 kDa dynamin GTPase protein that is primarily located in cytosol as dimer/tetramer and upon activation

shifts to the sites of division on mitochondria for further mitochondria dissection. Studies suggest that due to the lack of mitochondrial targeting sequence, Drp-1 requires a receptor to be recruited to the outer mitochondrial membrane for mitochondrial fission. Fis-1 is a 17-kDa protein that is attached to the outer mitochondrial membrane and it was a potential candidate for the Drp-1 receptor [141]. Although, studies have confirmed that Fis-1 overexpression facilitates mitochondrial fission, Fis-1 protein knockdown did not affect recruitment of Drp-1 to mitochondria and mitochondrial fission respectively [142]. However, studies have shown that the other outer mitochondrial membrane protein Mff acts as Drp-1 receptor due to the fact that Mff knockdown suppressed Drp-1 translocation on mitochondria, but Mff overexpression was found to promote mitochondrial fission with excessive Drp-1 recruitment to mitochondria [142].

Both, mitochondrial fusion and fission are required for the normal function of mitochondria. Mitochondrial fusion allows maintenance of mitochondrial membrane potential; redistribution of protein components, mitochondrial DNA within mitochondrial network; mitochondrial DNA repair. During energy deprivation mitochondrial fusion helps to maintain adequate oxidative phosphorylation. On the other hand, mitochondrial fission is required for cell division, movement of mitochondria within cytoplasm, elimination of damaged/senescent mitochondria by mitophagy.

The impairments in mitochondrial dynamics have been associated with the development of several cardiovascular disease including heart failure, cardiac hypertrophy, atherosclerosis, diabetes, hypertension and ischemic heart disease

[143, 144]. In post- myocardial infarction there is excessive mitochondrial fragmentation in rat hearts, which was associated with Opa-1 reduction [145]. Loss of Mfn-2 has been shown to promote mitochondrial membrane permeabilization, followed by cytochrome c release that contributed to cardiomyocyte death [146]. It was found that cardiomyocytes exhibited mitochondrial fragmentation and dysfunction after 30 minutes of hypoxia followed by normoxia, however Mfn- 1 or Mfn- 2 overexpression inhibited mitochondrial membrane permeabilization and cell death. Treatment with Drp-1 inhibitor (Mdivi- 1) prevented mitochondrial dysfunction and cell death in vitro and decreased myocardial infarct size in vivo [147]. The other work described that treatment with  $\alpha$ - agonist phenylephrine reduced Mfn- 2 expression and elevated mitochondrial fission proteins including Drp- 1 and Fis- 1 [148]. Venous endothelial cells that were isolated from patients with diabetes mellitus expressed high levels of Fis- 1 protein suggesting the presence of mitochondrial fission. High glucose treatment of human aortic EC facilitated elevation of fission proteins Drp-1 and Fis- 1 that was accompanied by mitochondrial ROS production and reduction of eNOS. However, Fis- 1/Drp- 1 inhibition prevented impairments in mitochondrial dynamics followed by ROS reduction and eNOS activation [149]. Studies have reported that mitochondrial dynamics impairment contributes to pathogenesis of pulmonary arterial hypertension (PAH) [144, 150]. Marsboom et al. have shown that Drp- 1 elevation promotes pulmonary artery smooth muscle cell hyperproliferation that contributes to PAH, however Drp- 1 inhibition with Mdivi- 1 suppresses mitochondrial fission and smooth muscle cells hyperproliferation

preventing PAH. Mdivi- 1 treatment also improved exercise capacity, right ventricular function and hemodynamics in PAH [150].

### Mitochondrial Dynamics and Hyperhomocysteinemia

Several studies have highlighted the role of HHcy in mitochondrial dynamics alteration. Kumar et al. have observed altered mitochondrial membrane potential and fragmentation of the mitochondrial network in S- adenosylhomocysteine and Hcy- treated cells as compared to control cells [151]. In the same study, the elevation of Fis-1 and dynamin-1 proteins in S- adenosylhomocysteine and Hcy- treated cells was confirmed by PCR, suggesting that HHcy favors mitochondrial fission that could be a compensatory mechanism in response to mitochondrial stress allowing elimination of damaged mitochondria [151]. Kalani et al. have proposed the novel mechanism of bone remodeling in HHcy, where HHcy- mediated mitochondrial oxidative stress altered mitochondrial dynamics and mitochondrial epigenetics that contributed to increase in MMPs/TIMPs and collagen/elastin ratio promoting bone remodeling [152]. Ganapathy et al. for the first time described mitochondrial fission as a novel mechanism of Hcy toxicity to neurons [23]. In this study, it was found that there was an up-regulation of Fis-1, Opa-1 and cleaved caspase- 3 protein expressions in neural retinas of CBS+/- mouse model with mild HHcy. Mitochondria of CBS+/- mouse model in retina was fragmented, reduced in size, and increased in quantity as compared to control group [23].

## Mitochondria- Mediated Cell Death and Cardiovascular Diseases

Apoptosis is a highly regulated form of cellular suicide that is morphologically characterized as cell shrinkage, membrane blebbing, DNA fragmentation, chromatin condensation and cellular elimination via phagocytosis. Programmed cell death includes two pathways: the extrinsic and intrinsic pathways. The extrinsic pathway is triggered by ligand binding (FasL) to the cell surface TNF death- receptor, recruiting Fas- associated death domain protein (FADD) that activates caspase- 8 followed by stimulation of caspase- 3 [153]. The effector caspase- 3 promotes proteolytic cleavage of key structural and functional proteins, including DNA fragmentation factor (DFF) and poly ADP ribose polymerase (PARP) in the nucleus, facilitating DNA fragmentation [153]. The intrinsic cell death pathway is initiated in mitochondria and is triggered by ROS,  $Ca^{2+}$  overload, mitochondrial membrane depolarization. B- cell lymphoma 2 (Bcl-2) family proteins: Bax and Bak that are classified as pro- apoptotic proteins are highly required for the intrinsic apoptosis regulation [154]. Upon activation Bax is translocated from cytosol to mitochondria where it regulates mitochondrial outer membrane permeabilization (MOMP) followed by cytochrome c release that promotes apoptosome formation (cytochrome c - apoptotic protease- activating factor 1- procaspase- 9 complex). This, in turn activates caspase- 9, triggering caspase- 3 that contributes to DNA fragmentation.

A significant amount of studies have implicated that mitochondrial dynamics plays a unique role in mitochondria- mediated cell death. Mitochondrial fusion/fission imbalance has been shown to promote sustained mitochondrial

fragmentation leading to mitochondrial apoptosis initiation [155]. Several studies have described the mitochondrial fission protein interaction and co-localization (Drp-1) with pro-apoptotic proteins (Bax) in mitochondrial division foci suggesting that mitochondrial dynamics participates in mitochondria-mediated cell death [156].

Littlewood et al. have reported that apoptosis of endothelial cells, macrophages and vascular smooth muscle cells facilitates plaque growth and promote plaque rupture in atherosclerosis [157]. Mitochondria are known to be a primary intracellular source of ROS. Excessive mitochondrial ROS production promotes mitochondrial DNA damage, mitochondrial DNA mutation and initiation of mitochondrial apoptosis all contributing to cardiovascular diseases [158]. The increase of oxidized LDL induces mitochondria-mediated apoptosis in endothelial cells in diabetes type 2 [159]. Hyperglycemia in diabetes up-regulates BAX expression, promotes opening of mitochondrial transition pore followed by release of pro-apoptotic molecules that leads to endothelial cell apoptosis [21, 160, 161].

#### Mitochondrial Apoptosis and Hyperhomocysteinemia

A significant amount of studies have highlighted the role of HHcy in mitochondria-mediated cell death initiation in different cell types. Lee et al. have shown that Hcy treatment facilitated caspase-dependent apoptosis in human umbilical vein endothelial cells that was accompanied by ROS production, cytochrome c release, overexpression of pro-apoptotic molecules: p53 and Noxa [162]. In the

same study it was confirmed that the NO donor S- nitroso- N-acetylpenicillamine, adenoviral transfer of inducible NO synthase gene, and antioxidants ( $\alpha$ - tocopherol, superoxide dismutase) suppressed ROS production, Noxa expression and apoptosis in HHcy [162]. Sipkens et al. have reported that Hcy treatment of rat cardiomyoblast cells and adult rat cardiomyocytes induced apoptosis with ATP depletion and excessive NOX- 2 and ROS production in concentration- dependent manner [163]. Buemi et al. have observed that the addition of 10 mM/L of Hcy to the smooth muscle cell culture medium facilitated smooth muscle cell hyperproliferation and death through apoptosis [164]. There was a positive correlation between Hcy concentration and percentage of apoptotic cells and cells with necrotic morphology, however the addition of folic acid to the culture medium significantly reduced Hcy concentration in medium as well as apoptosis [164]. Tyagi N et al. have reported that Hcy treatment of rat heart microvascular endothelial cells (MVEC) induced ROS production leading to loss of mitochondrial membrane potential, decrease in Bcl<sub>2</sub>/Bax ratio, cytochrome c release, and caspase- 9, caspase-3 elevation that contributed to DNA fragmentation [165]. Treatment of MVEC with caspase- 9 small interfering RNA suppressed HHcy- mediated mitochondrial apoptosis in endothelial cells [165].

## CHAPTER III

### HYPOTHESIS AND SPECIFIC AIMS

#### Key Objective

The objective of this study was to define the mechanisms of homocysteine toxic effect on vascular wall that promote vascular remodeling and hypertension and explore the role of toll- like receptor 4 mutation in alleviation of homocysteine negative effects.

#### Hypothesis

HHcy-induced TLR-4 activation promotes vascular inflammation and mitochondrial dysfunction that lead to inward vascular remodeling inducing hypertension. TLR-4 mutation attenuates vascular inflammation and mitochondrial dysfunction that suppress hypertension (Figure 2.)

#### Specific Aims

**Specific Aims 1:** To determine whether HHcy impairs mitochondrial dynamics by increasing mitochondrial fission and reducing mitochondrial fusion that lead to vascular remodeling.

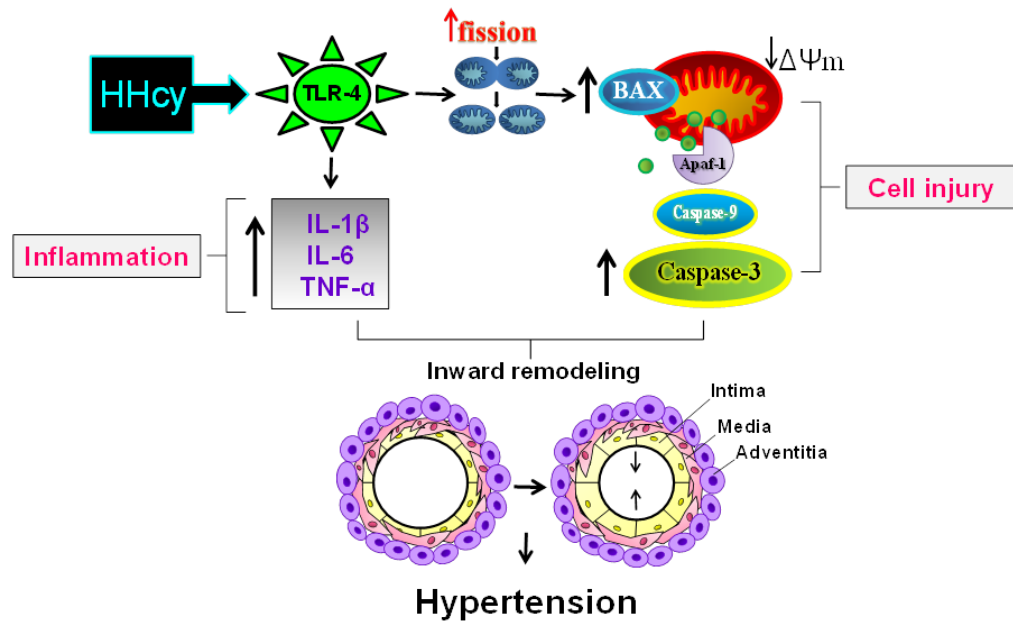


Specific aim 1 will be discussed in Chapter IV

**Specific Aims 2:** To determine whether HHcy activates TLR- 4 mediated inflammatory pathway with cytokine up- regulation (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) inducing vascular inflammation that contributes to hypertension.

**Specific Aims 3:** To determine whether HHcy- induced TLR-4 activation promotes mitochondria- mediated cell death followed by inward vascular remodeling facilitating hypertension.

Specific aims 2 and 3 will be discussed in Chapter V.



**Figure 2:** Schematic Representation of Overall Hypothesis

## CHAPTER IV

### MITOCHONDRIAL FRAGMENTATION FACILITATES MESENTERIC ARTERY REMODELING IN HYPERHOMOCYSTEINEMIA

#### **Introduction**

Clinical studies show that patients with hypertension and mesenteric artery occlusion are represented with high plasma homocysteine (Hcy) levels [7, 8, 166]. Based on the literature, Hcy elevation has been implicated in the development of endothelial dysfunction [167-169] and mesenteric artery remodeling, however the mechanisms are not well defined. The thiol group of Hcy is auto-oxidized to reactive oxygen species (ROS), amplifying the level of oxidative stress (OS) [170] and leading to inflammation. It has been reported that the levels of superoxide and peroxynitrite ( $\text{OONO}^-$  formed by the interaction of superoxide and nitric oxide) are increased in HHcy in rat aorta and are mediated by NADPH oxidase (NOX) [171]. Under pathological conditions, the activation of NOX in vascular cells leads to excessive superoxide ion production, contributing to vessel injury [172, 173]. The increase in reactive oxygen species (ROS) is accompanied by a compensatory increase in the antioxidant enzymes e.g. superoxide dismutase (SOD) [174]. Hence, the increased levels of superoxide

dismutase (SOD) and NOX are indicative of oxidative stress.

The other factor that contributes to endothelial dysfunction is decrease in endothelial nitric oxide synthase (eNOS- the enzyme catalyzing the production of NO from L- arginine) bioavailability that occurs in the presence of circulating ROS. Impaired endothelium with reduced eNOS is incapable to produce required vasodilators such as NO for the appropriate artery/arteriole vasodilation. Previous studies have reported that HHcy promotes endothelial dysfunction [167-169]. In addition to eNOS, connexins play an important role in the maintenance of the endothelium integrity and any disturbance in the levels of connexins is an indication of vasomotor tone disruption [175].

Despite abundant literature demonstrating that elevated homocysteine induces oxidative stress behind mesenteric artery remodeling, the role of mitochondrial dynamics has been explored only a little [23]. Mitochondria are the cell organelles whose primary functions are energy production, regulation of cell survival (apoptosis), ROS production and regulation of intracellular  $Ca^{2+}$  [133]. Mitochondria constantly undergo fission and fusion processes in response to physiological stimulus and stress [22]. Mitochondrial fission is the process of mitochondrial disintegration forming two or more separate mitochondrial compartments regulated by dynamin related protein 1(Drp1), fission-1(Fis-1) and mitochondrial fission factor (MFF). Mitochondrial fission process also contributes to quality control by enabling the removal of damaged mitochondria. Elimination of damaged mitochondria is also called mitophagy. On the other hand, mitochondrial fusion is union of two or more mitochondria within a cell to form

one, regulated by inner mitochondrial membrane protein optic atrophy-1 (OPA-1) and two outer mitochondrial membrane proteins: mitofusin- 1 and mitofusin- 2 (Mfn-1, Mfn-2). Excessive mitochondrial fission and decreased mitochondrial fusion leads to mitochondrial fragmentation. Accumulation of damaged mitochondria and sustained fission facilitate the release of pro-apoptotic molecules that initiate cell death. Impaired mitochondrial dynamics is also associated with cardiovascular diseases and diabetes [19-22]. Although HHcy causes mitochondrial fission [23], it is unclear whether it contributes to endothelial cell damage and peripheral vascular remodeling. Therefore, we hypothesize that HHcy impairs mitochondrial dynamics by increasing mitochondrial fission that promotes endothelial cell damage and endothelial dysfunction, facilitating peripheral vascular remodeling.

## **Materials and methods**

### **Animal models**

C57BL/6J (WT), cystathionine  $\beta$ - synthase deficient mice (CBS+/-) with genetic mild HHcy; C3H/HeJ (C3H) as per Jackson Laboratories (Bar Harbor, ME), these mice have a mutation in the TLR- 4 receptor and previous reports show that TLR- 4 mutants have reduced oxidative stress [176]; and mice with combined genetic HHcy and TLR-4 mutation (CBS+/-/C3H) were used in the present study. The age of the animals was 12 weeks and the body weight was 25-30 g. The mice were purchased from Jackson Laboratories (Bar Harbor, ME,

USA). All standard procedures and experiments with animals were followed in agreement with the National Institute of Health and approved by Institutional Animal Care and Use Committee (IACUC), University of Louisville.

## Genotyping

To confirm the background of CBS<sup>+/-</sup> heterozygous mice the protocol for genotyping were used as per Jackson Laboratories. Shortly, the samples of mice tails were collected for DNA extraction. DNA was used to amplify with cystathionine  $\beta$ - synthase primer sets by PCR. The PCR product samples were run on 1.2% agarose gel (prepared in TAE buffer, pH 8.4) with ethidium bromide. The images were recorded in gel documentation system (Bio- Rad, Hercules, CA, USA). To confirm TLR-4 mutation in C3H/HeJ mice the respective protocol of RFLP-PCR was used [176]. Shortly, DNA extracted from mice tails was amplified with specific TLR-4 set of primers (Table 2). PCR products were digested with NLa III restriction enzyme (RE) for overnight at 37<sup>0</sup>C. The RE digestion products were loaded on 10% PAGE gel and run at 80 Volts. The gel was incubated with 1X TAE buffer containing ethidium bromide. The images were made in gel documentation system (Bio- Rad, Hercules, CA, USA). Mutant TLR-4 gene includes CATG sequence that is cut by RE, yielding 96 and 108 bps bands, while absence of TLR-4 mutation is confirmed by undigested products at 204 bps. To confirm the genetic background of CBS<sup>+/-</sup>/C3H mice we first determined CBS gene deficiency in their DNA samples that were selected for further detection of TLR-4 mutation.

## Western blot analysis

The SMA protein content was extracted using mix of RIPA buffer (Boston BioProducts, MA, USA), PMSF (Calbiochem, La Jolla, CA, USA) and protease inhibitor (Sigma Aldrich, St. Louis, MO, USA). The protein extract was incubated for overnight at 4<sup>0</sup>C with shaking and centrifuged at 13 000 rpm for 20 min. The supernatant was collected in another tube for protein estimation using Bradford-dye method (Bio-Rad, CA, USA). The prepared protein lysate (60 ug) was heated at 95<sup>0</sup>C for 5 min and loaded on polyacrylamide gel with SDS in running buffer and run at constant current (100 Volts). For protein transferring to PVDF membrane electro transfer apparatus (Bio-Rad) was used. After transferring, the membranes were blocked in 5% nonfat milk for 1 hour at room temperature, followed by overnight incubation with primary antibodies (anti- Nox4, SOD-1, SOD-2, CBS, eNOS, Mfn-2, Drp-1; Santa Cruz Biotechnology, Dallas, TX, USA; Abcam, Cambridge, MA, USA.) at 4<sup>0</sup>C. After washing with TBS-T buffer, membranes were incubated with secondary antibodies (horse radish peroxidase-conjugated goat anti-mouse, goat anti- rabbit, and rabbit anti- goat IgG; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at RT with 1:5000 dilution followed by washing step. The membranes were developed with ECL Western blotting detection system (GE Healthcare, Piscataway, NJ, USA) and all images were recorded in the gel documentation system ChemiDoc XRS (Bio-Rad, Richmond, CA, USA). The membranes were stripped with stripping buffer (Boston BioProducts, Ashland, MA, USA) followed by blocking step with 5% milk for 1 hour at RT. After washing step membranes were reprobed with anti-

GAPDH antibody (Millipore, Billerica, MA, USA) as a loading control protein. The data were analyzed by Bio-Rad Image Lab densitometry software and normalized to GAPDH bands.

#### qPCR

To assess mRNA expression of different genes in the SMA, RNA was isolated with TRIzol<sup>®</sup> reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA quantification and purity was assessed by nanodrop-1000 (Thermo Scientific, Waltham, MA, USA). Aliquots (2µg) of total RNA were reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. The q-PCR was performed for different genes (Mfn- 2, Drp- 1), in a final reaction volume of 20 ul containing 10 ul PerfeCTa SYBR Green SuperMix, Low ROX (Quanta Biosciences, Gaithersburg, MD), 6 ul nuclease free water, 2ul cDNA, 40 picomoles of forward, and reverse primers. All sequence-specific oligonucleotide primers (Invitrogen, Carlsbad, CA, USA) are presented in (Table 2). The data was represented in fold expression, calculated as the cycle threshold difference between control and sample normalized with housekeeping gene Rn18s.



Table 2

Gene	Nucleotide sequence (forward)	Nucleotide sequence (reverse)
TLR-4	5'-TCAGAATGAGGACTGGGTGA	5'-CTCGGCAACCACATAGAAACT
IL-1 $\beta$	5'-CACCTCTCAAGCAGAGCACAG	5'-GGGTTCCATGGTGAAGTCAAC
IL-6	5'-TGGAGTCACAGAAGGAGTGGCTAAG	5'-TCTGACCACAGTGAGGAATGTCCAC
Mfn-2	5'-ACAGCCTCAGCCGACAGCAT	5'-TGCCGAAGGAGCAGACCTT
Drp-1	5'-GCGCTGATCCCGCGTCAT	5'-CCGCACCCACTGTGTTGA
BAX	5'-GGCTGGACACTGGACTTCT	5'-GGTGAGGACTCCAGCCACAA
Rn18S	5'-AAATCAGTTATGGTTCCTTGGTC	5'-GCTCTAGAATTACCACAGTTATCCAA

### Immunohistochemistry

The SMA tissue was immersed in tissue- freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) in disposable plastic tissue- embedding mold (Polysciences Inc., Warrington, PA, USA). The tissue blocks were kept at -80<sup>0</sup>C until use. To obtain 5 um thickness sections Cryocut (CM 1850; Leica Microsystems, Buffalo Grove, IL, USA) was used. Tissue sections were placed on polylysine coated slides (Polysciences, Inc., Warrington, PA, USA). Tissue sections were incubated with permeabilization solution (0.2 g Bovine Serum Albumin, 3 ul Triton X-100 in 10ml 1X PBS) for 1 hour at RT followed by washing step with 1X PBS. The sections were incubated with primary antibody (anti-CD31, connexin 40, Mfn-2, Drp-1) with 1: 250 dilution for overnight at 4<sup>0</sup>C. After washing with 1X PBS the slides were incubated with fluorescently labeled secondary antibodies (gout anti-mouse Alexa flour 488 and gout anti-rabbit Texas red; Invitrogen, Waltham, MA, USA) with 1: 500 dilution for 1 hour at RT

followed by DAPI staining with 1: 10,000 dilution for 20 min at RT. After washing step slides were mounted with mounting medium and visualized using laser scanning confocal microscope (Olympus Fluo View 1000; Center Valley, PA, USA). The images were analyzed by measuring fluorescence intensity with Image Pro-Plus software (Media Cybernetics, Rockville, MD, USA).

### Masson's trichrome staining

The same protocol as written above was used to get tissue frozen sections. The slides with tissue were hydrated with distilled water (100%, 90% and 70%) and placed in Bouin's Fluid at 56<sup>0</sup>C for 1 hour. After washing with tap water for 5 minutes sections were placed in Working Weigert's Iron Hematoxylin Stain (Weigert's Iron Hematoxylin A+ Weigert's Iron Hematoxylin B) for 10 minutes. After washing in tap water for 10 minutes tissue sections were stained in Biebrich Scarlet- Acid Fuchsin solution for 7minutes. After 30 seconds of washing in distilled water, slides were placed in Phosphotungstic-Phosphomolybdic Acid solution for 5 minutes and after that stained in Aniline Blue Stain solution for 7minutes. Then tissue sections were placed in 1% Acetic Acid solution for 1 minute, rinsed in distilled water for 30 seconds and dehydrated in anhydrous alcohol for 1min each (70%, 90% and 100%). After that slides were cleared in clearing reagent (Xyline) three times for 1min each and mounted. The images were obtained by using light microscopy (x20 objectives, QCapture Pro).

The images were analyzed by measuring the color intensity with image analysis software (Image Pro-Plus, Media Cybernetics).

## Statistical analysis

Statistical analysis was performed with Primer of Biostatistics 7.0 (McGraw-Hill, NY, USA). To determine the significance, we used one-way analysis of variance (ANOVA) followed by multiple comparison test (Holm test) between the groups. The difference was considered significant when  $p < 0.05$ . Values are presented as mean  $\pm$  SEM (n=4).

## Results

Genotyping for WT, CBS+/-, C3H and CBS+/-/C3H mice

For genotype analysis the Jackson Laboratory protocol was followed. CBS+/- and CBS+/-/C3H mice had two bands located at 450 and 308 bps while CBS+/+ mice had band located at 308 bps when primers specific for CBS gene were used (Fig.3A). TLR- 4 mutants (C3H and CBS+/-/C3H) had bands located at 96 and 108 bps, whereas non-mutants (WT, CBS+/-) had single band at 204 bps (Fig.3B) when PCR-RFLP was used with TLR-4 primers.

Western blotting for cystathionine  $\beta$ - synthase (CBS) protein expression

CBS+/- and CBS+/-/C3H exhibited reduced CBS protein expression in the SMA

tissue as compared to control group (Fig.4 A, B).

HHcy evoked oxidative stress in the mesenteric artery

We have found that Nox4 (oxidative stress marker), SOD-1 and SOD-2 (antioxidants) were up regulated in the SMA of CBS+/- mice as compared to WT mice. The protein expression of Nox4 and SOD-1 were decreased in C3H as in CBS+/-/C3H mouse models compared to CBS+/- mice. Interestingly, the expression of SOD-2 (mitochondrial antioxidant) was increased in C3H mouse model compared to control (Fig.5 A, B).

Altered mitochondrial dynamics in HHcy

To evaluate the effect of HHcy and oxidative stress on mitochondrial dynamics we analyzed two major proteins: Mfn-2 (regulates mitochondrial fusion) and Drp-1 (regulates mitochondrial fission) by western blot, qPCR and immunohistochemistry. Western blot data (Fig.6 A, B) showed that the protein expression of Mfn-2 was decreased in CBS+/- mouse model compared to WT mice. Drp-1 protein expression was significantly up regulated in CBS+/- mice as compared to WT mice. The fusion was increased and fission was down regulated in CBS+/-/C3H mice compared to CBS+/- mice. Using real time qPCR (Fig.7 A, B) we confirmed that Mfn-2 was also down regulated in CBS+/- mice as compared to WT and CBS+/-/C3H mice and Drp-1 was also up regulated in CBS+/- mice as compared to WT and CBS+/-/C3H mice. By Immunohistochemistry (Fig.8 A, B) we determined that the intensity of Mfn-2 was decreased in the mesenteric artery of CBS+/- mice as compared to WT, CBS+/-

/C3H mice. In addition, the intensity of Drp-1 in the mesenteric artery was increased in CBS+/- and CBS+/-/C3H mice as compared to WT mice. These results suggest HHcy- mediated prevalence of mitochondrial fission over mitochondrial fusion in CBS+/- mice that contributes to mitochondrial fragmentation.

#### HHcy induced endothelial cell layer damage

To determine whether elevated homocysteine contributes to endothelial cell layer damage, CD31 and connexin 40 intensity levels were observed (Fig.9 A, B). By immunohistochemistry we have determined that the intensity of CD31 was decreased in CBS+/- mice as compared to WT, C3H and CBS+/-/C3H mice. The intensity of connexin 40 (gap junction's protein in endothelial cells) was significantly reduced in CBS+/- and CBS+/-/C3H mice as compared to WT and C3H mice.

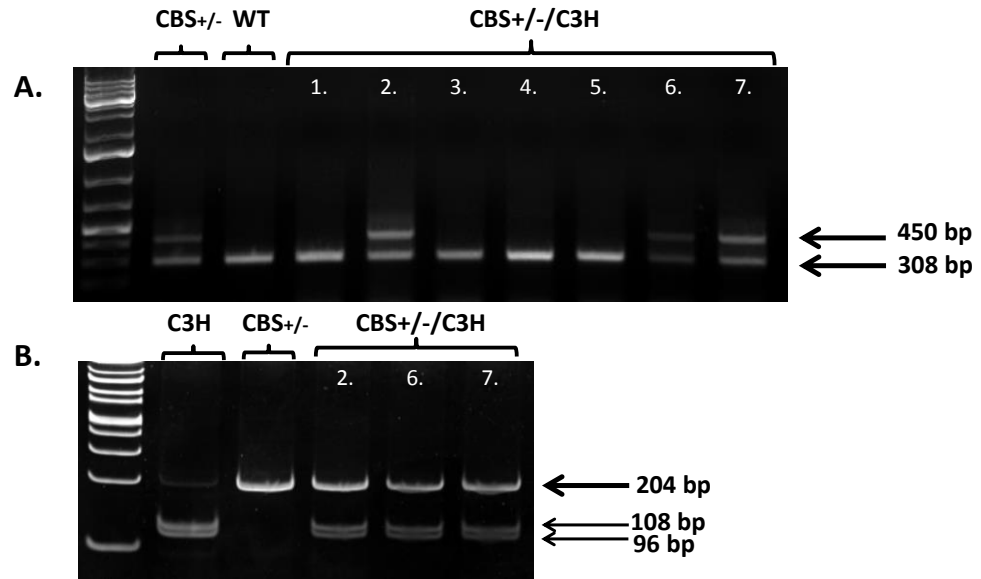
#### Western blotting for eNOS protein expression

We have observed eNOS down regulation in CBS+/- mice compared to WT mice (Fig.10 A, B). Moreover, the protein expression of eNOS was up regulated in the mesenteric artery of CBS+/-/C3H and C3H mice compared to CBS+/- mice.

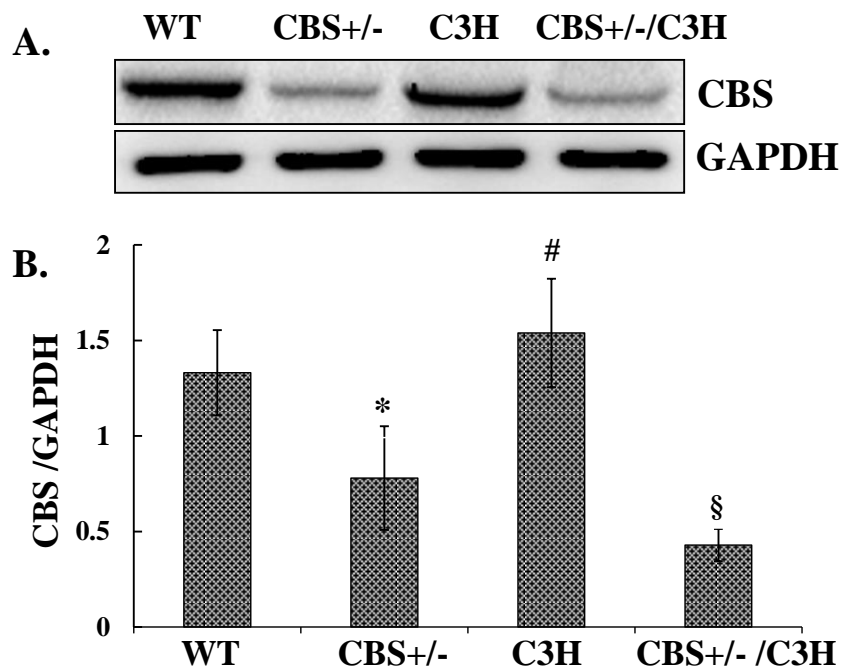
#### HHcy facilitated collagen accumulation in the mesenteric artery

To observe collagen deposition in the mesenteric artery, Masson's trichrome staining was performed (Fig.11 A, B). Collagen content was significantly up regulated in CBS+/- mice as compared to WT, C3H and CBS+/-/C3H mice. In

addition, it was found that collagen accumulation was reduced in CBS+/-/C3H mice as compared to CBS+/- mice.

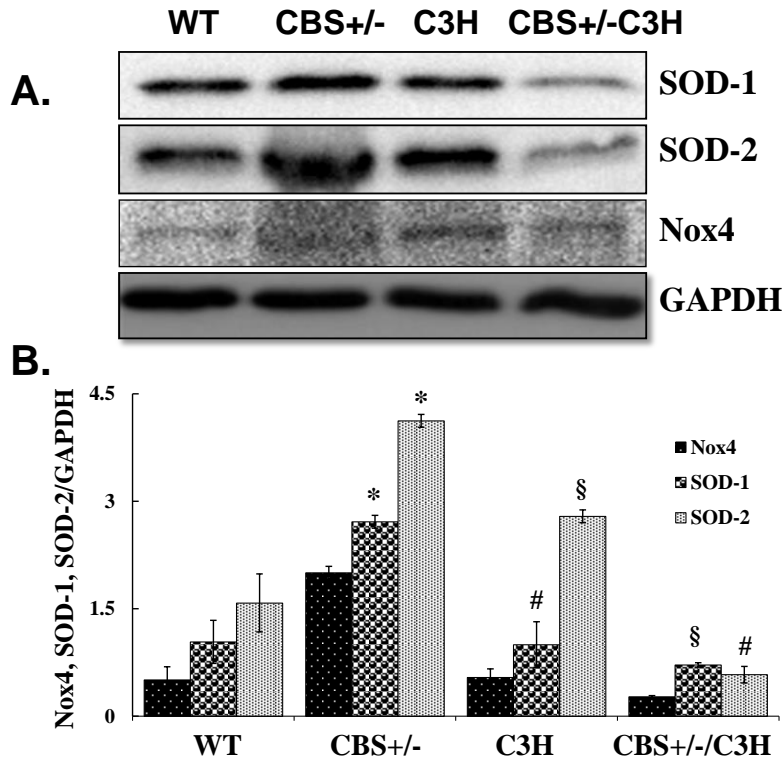


**Fig.3. Genotyping for CBS gene (A):** CBS+/- and CBS+/-/C3H mice had two bands located at 450 and 308 bp while CBS+/- mice had band located at 308 bp. PCR-RFLP for TLR-4 gene **(B):** TLR- 4 mutants (C3H and CBS+/-/C3H) had bands located at 96 and 108 bp, whereas non-mutants (WT, CBS+/-) had a single band at 204 bp.

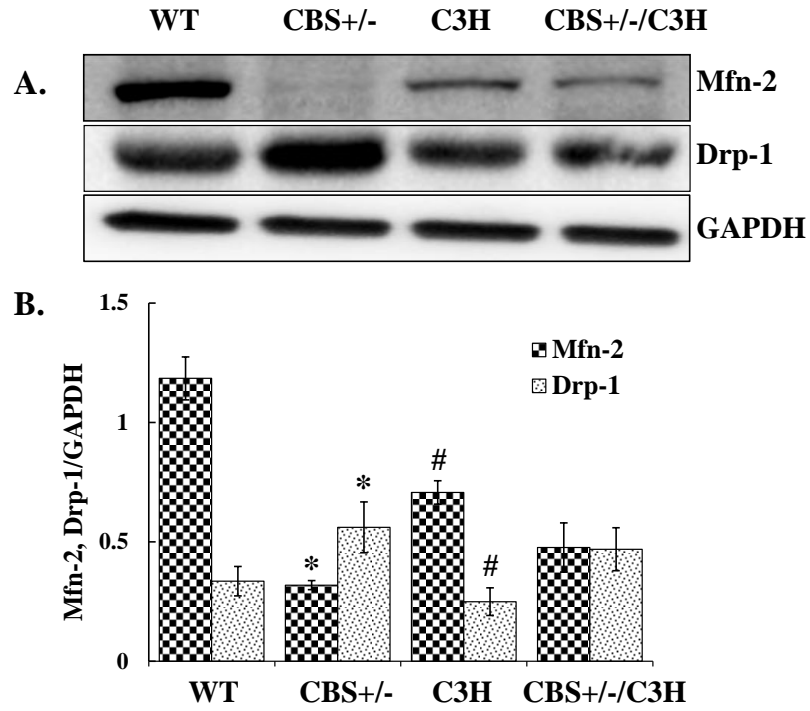


**Fig.4. A. Western blot for cystathionine  $\beta$ - synthase (CBS) protein expression, B. bar graphs for CBS protein expression, normalized with GAPDH, \*  $p < 0.05$  WT vs CBS+/-, #  $p < 0.05$  CBS+/- vs C3H, §  $p < 0.05$  WT vs CBS+/-/C3H,  $n = 4$ .**

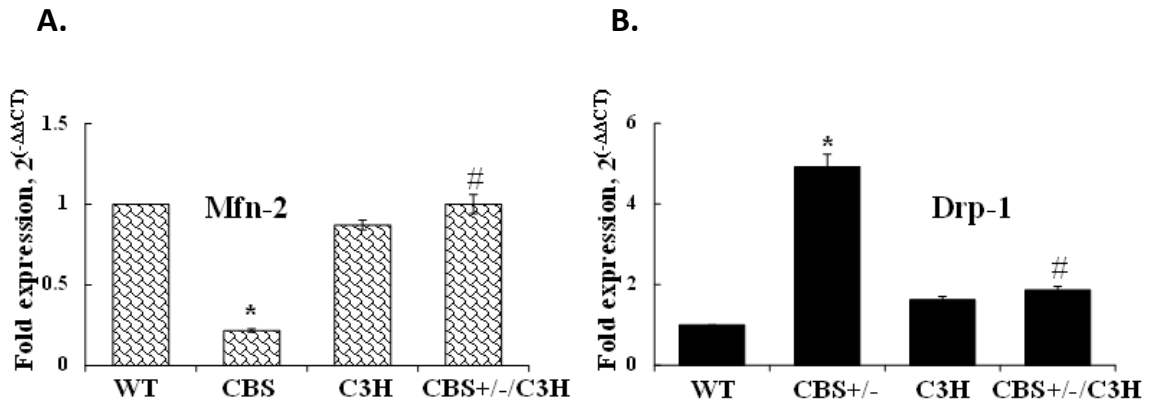




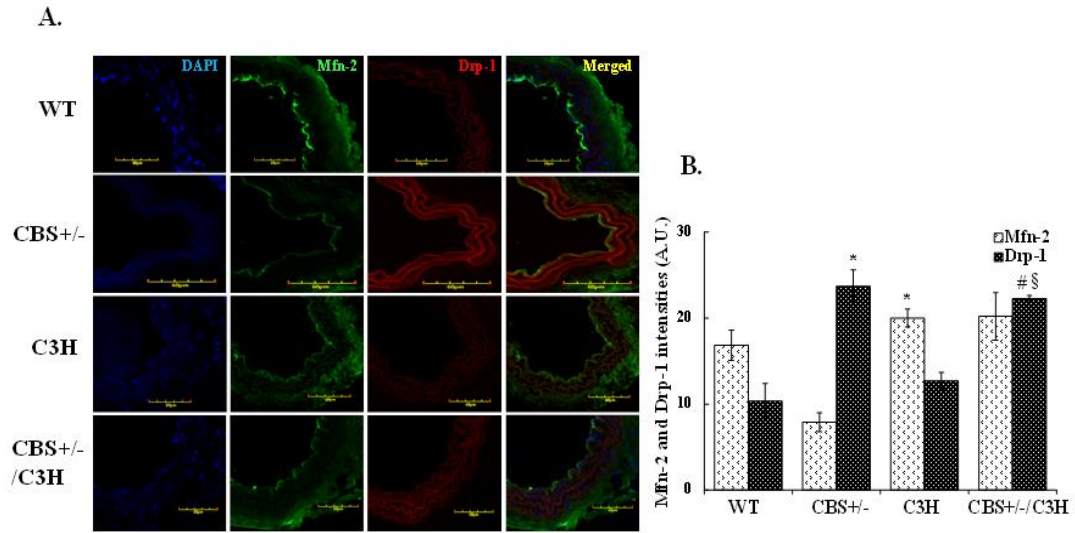
**Fig.5. Oxidative stress status in the mesenteric artery in HHcy: A.** Western blot analysis of Nox4, SOD-1 and SOD-2 protein expression **B.** Bar graph for respective protein in mesentery \* p<0.05 WT vs CBS+/-, # p<0.05 CBS+/- vs C3H, § p<0.05 CBS+/- vs CBS+/--C3H, n= 4 per group.



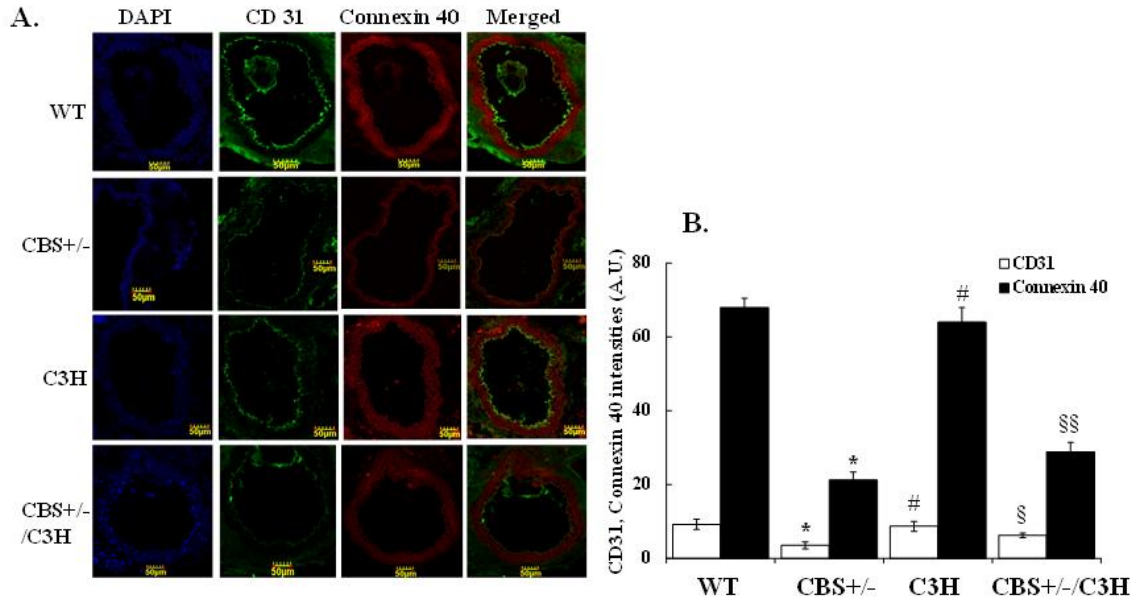
**Fig.6. Mitochondrial dynamics in the mesenteric artery in HHcy: A.** Western blot analysis of Mfn-2 and Drp-1 protein expressions in WT, CBS+/-, C3H and CBS+/-/C3H mice mesentery. **B.** Bar graph for Mfn-2 and Drp-1 protein expressions in mesentery, normalized with GAPDH, \* p<0.05 WT vs CBS+/-, # p<0.05 CBS+/- vs C3H, n=4



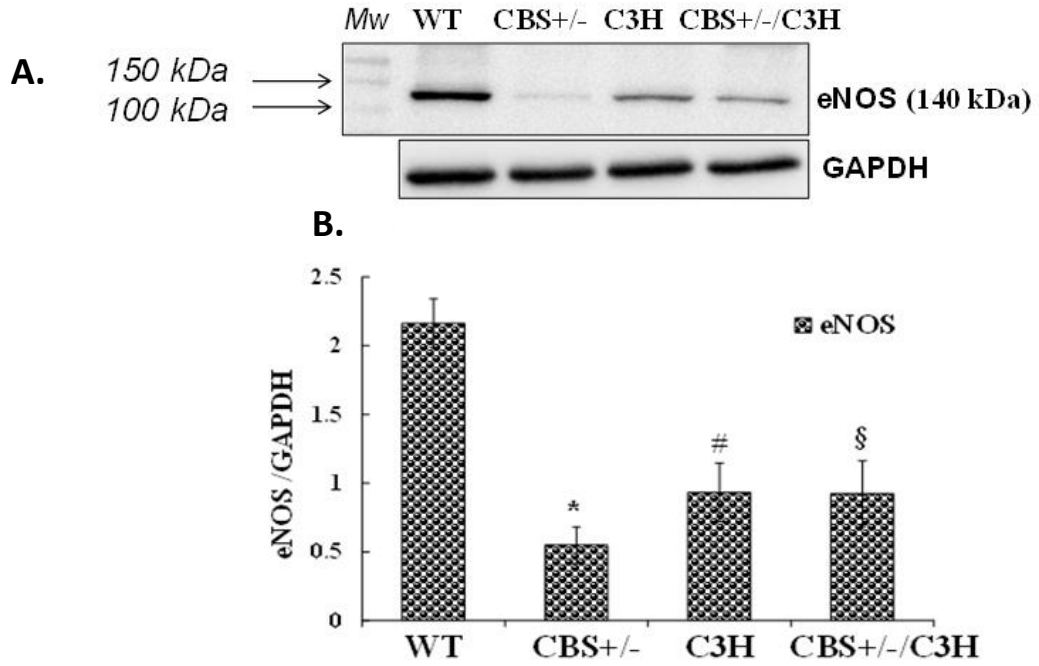
**Fig.7. Alteration of mitochondrial dynamics in the mesenteric artery in HHcy: A.** Real time expression of Mfn-2 mRNA in mesentery \*  $p < 0.05$  WT vs CBS+/-, #  $p < 0.05$  CBS+/- vs C3H; **B.** Real time expression of Drp-1 mRNA in mesentery \*  $p < 0.05$  WT vs CBS+/-, #  $p < 0.05$  CBS+/- vs C3H,  $n = 4$



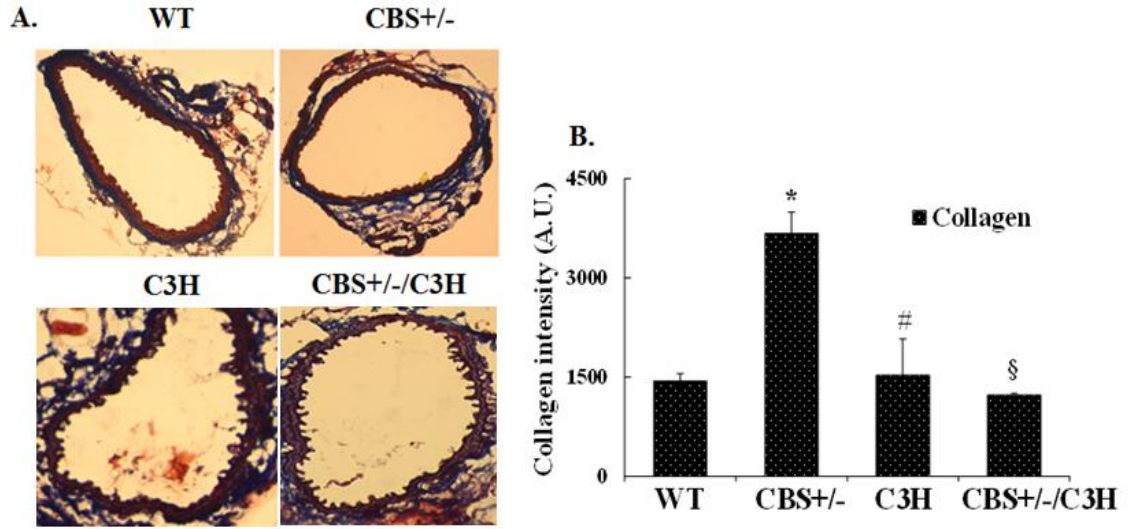
**Fig.8. Mitochondrial fragmentation in HHcy: A.** Mfn-2 and Drp-1 intensities in the mesenteric arteries of WT, CBS+/-, C3H and CBS+/-/C3H. **B.** Bar graph for Drp-1 expression in the mesenteric artery \*  $p < 0.05$  WT vs CBS+/-, #  $p < 0.05$  WT vs CBS+/-/C3H, §  $p < 0.05$  C3H vs CBS+/-/C3H; for Mfn-2 expression \*  $p < 0.05$  CBS+/- vs C3H,  $n = 4$



**Fig.9. Immunohistochemistry of the mesenteric artery in different mouse groups: A.** CD31 and Connexin 40 intensities in the mesenteric arteries of WT, CBS+/-, C3H and CBS+/-/C3H. **B.** Bar graph for respective proteins in the mesenteric artery \*  $p < 0.05$  WT vs CBS+/-, #  $p < 0.05$  CBS+/- vs C3H, §  $p < 0.05$  CBS+/- vs CBS+/-/C3H, §§  $p < 0.05$  WT vs CBS+/-/C3H and C3H vs CBS+/-/C3H,  $n = 4$



**Fig.10. Western blot analysis of eNOS protein expression.** **A.** Protein expression of eNOS in the mesenteric arteries of WT, CBS+/-, C3H and CBS+/-/C3H mice. **B.** Bar plot for eNOS protein expression normalized with GAPDH, \*  $p < 0.05$  WT vs CBS+/-, #  $p < 0.05$  CBS+/- vs C3H, §  $p < 0.05$  CBS+/- vs CBS+/-/C3H,  $n = 4$



**Fig.11. Masson's trichrome staining of the mesenteric artery in different mouse strains: A.** Collagen intensity in the mesenteric artery of WT, CBS+/-, C3H and CBS+/-/C3H mice, x20 magnification. **B.** Bar graph for collagen expression in the mesenteric arteries \* p<0.05 WT vs CBS+/-, # p<0.05 CBS+/- vs C3H, § p<0.05 CBS+/- vs CBS+/-/C3H, n=4

## Discussion

Oxidative stress is the main outcome and major player in HHcy pathogenesis. Many previous studies indicated the role of HHcy-mediated oxidative stress in cardiovascular, cerebrovascular and renovascular diseases [177-180]. In the present study we have observed the increase of Nox4 (oxidative stress marker), SOD-1 (antioxidant marker) and SOD-2 (mitochondrial antioxidant marker) in CBS<sup>+/-</sup> deficient mice as compared to WT, C3H and CBS<sup>+/-</sup>/C3H mice (fig.2A, 2B). With these findings we assume that in acute phase of oxidative stress in HHcy, the defensive mechanisms will be activated trying to alleviate oxidative damage and balance the biological system. However, in chronic stage of oxidative stress the defense mechanisms will be exhausted and it would lead to antioxidant reduction that was confirmed by previous reports [181]. In the meantime, studies have shown an increase of SOD-1 and SOD-2 in oxidative stress [182] or HHcy [174, 183]. Interestingly, SOD-2 was also up regulated in C3H mice, suggesting that this mouse strain has antioxidant properties and potentially more resistant to oxidative stress as compared to other strains, however the precise mechanism is not defined until now.

In the present study we have explored the effect of HHcy on mitochondrial dynamics and the role of it in the development of mesenteric artery remodeling. It has been reported that mitochondria constantly undergo fusion (regulated by OPA-1, Mfn-1 and Mfn-2) and fission (regulated by Drp-1, Fis-1 and MFF) in response to physiological stimuli or stress. It has been shown that excessive



mitochondrial fission and decreased mitochondrial fusion contributes to mitochondrial fragmentation and accumulation of damaged mitochondria, initiating mitophagy or cell apoptosis [22, 149, 184, 185]. Hyperhomocysteinemia promotes intense mitochondrial fission and decreases mitochondrial fusion facilitating cell death [23]. In the present study we have found a significant down regulation of Mfn-2 (mitochondrial fusion marker) in CBS+/- deficient mice as compared to WT and C3H mice. However, Drp-1 (mitochondrial fission marker) was significantly up regulated in CBS+/- mice as compared to WT and C3H mice. Interestingly, Mfn-2 was restored in CBS+/-/C3H mice as compared to CBS+/- mice and Drp-1 protein expression was reduced in CBS+/-/C3H mice as compared to CBS+/- mice.

The previous studies have reported that HHcy and oxidative stress mediate endothelial cell layer damage that leads to endothelial nitric oxide synthase loss and subsequent reduction in endothelium-dependent vasorelaxation [169, 186, 187]. Our findings have showed eNOS down regulation in CBS+/- mice as compared to WT, C3H and CBS+/-/C3H mice (fig.2C, 2D). In addition, we have observed CD31 (endothelial cell marker) and connexin 40 reduction in CBS+/- mice as compared to WT, C3H and CBS+/-/C3H mice (fig.6A, 6B). Connexin 40 is a gap junction protein that is highly expressed in the endothelial cells. Previous reports confirmed that connexin 40 deficient rodents develop arterial hypertension and exhibit impaired intercellular signaling with altered propagation of vasodilation [175, 188, 189]. On the contrary, other authors reported connexin 40 up regulation in endothelial cells during HHcy

[190].

Previous study from our lab have shown that HHcy induces hypertension and mesenteric artery remodeling through activation of MMP-9 followed by collagen deposition [190]. The present study also showed collagen accumulation in the mesenteric artery of CBS<sup>+/-</sup> mice as compared to WT, C3H and CBS<sup>+/-</sup>/C3H mice (fig.7A, 7B).

In summary, these findings indicate the prevalence of mitochondrial fission over mitochondrial fusion and oxidative stress in HHcy; which may explain endothelial cell loss and dysfunction followed by collagen deposition that contributes to mesenteric artery remodeling.

## CHAPTER V

### TOLL- LIKE RECEPTOR 4 MUTATION SUPPRESSES HYPERHOMOCYSTEINEMIA- INDUCED HYPERTENSION

#### **Introduction**

Primary or essential hypertension is the most common type of hypertension with unclarified etiology that affects 95% of all hypertensive patients. It has been reported that 75 million of adults in the United States and 1 billion people worldwide are affected by hypertension [191]. Persistent elevation of blood pressure has been known to be a risk factor for the development of stroke, myocardial infarction, chronic kidney and vascular diseases [192, 193]. A high level of homocysteine (Hcy) or hyperhomocysteinemia (HHcy) is an independent risk factor of hypertension [8, 194, 195]. Hcy is a product of methionine metabolism that is cleared in the body by re-methylation and transsulfuration pathways [1]. Hcy remethylation is mediated by methionine synthase (MS), where vitamin B<sub>12</sub> (cobalamin) is used as cofactor and 5-methyltetrahydrofolate (5-MTHF) is utilized as the methyl donor [2]. 5-MTHF or active form of folate (B<sub>9</sub>) is synthesized from 5, 10-methylenetetrahydrofolate by

methylenetetrahydrofolate reductase (MTHFR). In transsulfuration, that occurs only in the small intestine, liver, pancreas and kidney, a cofactor- vitamin B<sub>6</sub> (pyridoxal phosphate) is required to convert Hcy to cystathionine by cystathionine β- synthase (CBS) [3]. Cystathionine is hydrolyzed by vitamin B<sub>6</sub>- dependent cystathionine gamma- lyase (CSE) to cysteine that is used as a precursor for synthesis of antioxidant- glutathione or vasodilator-hydrogen sulfide [196]. Nutritional deficiencies in vitamin cofactors (B<sub>12</sub>, B<sub>9</sub>, and B<sub>6</sub>) and mutations in MTHFR, CBS and CSE enzymes are the common causes of HHcy [197-200].

HHcy- mediated vascular dysfunction and remodeling, as the hallmarks of hypertension, are developed due to complex of mechanisms, including instigation of oxidative stress, mitochondrial apoptosis and inflammation. Hcy undergoes auto-oxidation and has the ability to directly produce superoxide and hydrogen peroxide due to highly reactive thiol group [201]. The presence of NO in oxidative environment leads to peroxynitrite formation that further exacerbates oxidative stress reducing NO bioavailability [202]. In addition, our group and other studies have shown that in HHcy there is an increase in expression and activity of NADPH oxidase that mediates superoxide production [171, 203]. Elevation in Hcy level disrupts oxidant-antioxidant balance by diminishing the activity and expression of the antioxidant enzyme glutathione peroxidase [204]. Apart from oxidative stress, HHcy plays role in mitochondrial disorders instigation.

Mitochondrial dysfunction and mitochondria-dependent apoptosis have been shown to promote endothelial cell loss leading to endothelial dysfunction [15, 16] that contributes to pathogenesis of hypertension [17, 18]. The intrinsic

pathway or mitochondria- dependent apoptosis [205, 206] is triggered by various factors: ROS, DNA damage, Ca<sup>2+</sup> overload, hypoxia, oxLDL and is regulated by B- cell lymphoma- 2 (BCL -2) family of proteins that are classified into pro-apoptotic (BAX) and anti-apoptotic proteins (BCL-2). BAX is expressed in cytosol and is translocated to mitochondria upon activation where it initiates mitochondria outer membrane permeabilization followed by cytochrome c release and apoptosome (cytochrome c- APAF-1 complex) formation that plays role in caspase- 9 and caspase- 3 activation. The effector caspase-3 facilitates DNA and protein fragmentation that promote cell death. The third event mediated by HHcy in addition to oxidative stress and mitochondrial dysfunction is vascular inflammation.

Chronic vascular inflammation has been considered as one of the mechanisms of the initiation and exacerbation of hypertension [74-76]. Inflammation is the immediate response of the immune system to the presence of pathogens that is characterized by augmentation of pro- inflammatory cytokine (IL- 1 $\beta$ , IL- 6, TNF-  $\alpha$ ) and chemokine (MCP-1) secretion that provide the regulation of immune response and the migration of immune cells to target organs. Several in vitro studies have shown inflammatory markers up-regulation in HHcy, including activation of NF-kB that is inflammatory cytokine transcription factor [10, 126]. Zhang et al. have reported that plasma IL-6, TNF- $\alpha$  and MCP-1 levels are positively correlated with Hcy levels[11]. Scherer et al. have also observed mild HHcy- mediated augmentation of inflammatory cytokine production (IL- 1 $\beta$ , IL- 6, TNF-  $\alpha$ , MCP-1) in serum and different organs [12].

Although HHcy is known to promote pro-inflammatory cytokine production, the precise mechanism of inflammatory response initiation is still unclarified. Pathogen recognition receptors, and in particular TLRs are the antigen sensors that play role in innate immune system activation and have recently gained a significant attention in the field of hypertension. There are 13 TLRs that have been described in mammals (1-10 in humans and 11-13 in mice): cell surface TLRs that sense the presence of bacteria and fungus (TLR- 1, 2, 4, 5) and TLRs that localized to intracellular membranes and recognize viral or microbial nucleic acid (TLR- 3, 7, 8, 9) [74]. The role of TLR- 4 that is ubiquitously present within the vasculature (endothelial cells, VSMC) has been recently highlighted in sterile inflammation. TLR-4 activation with downstream NF-  $\kappa$ B- mediated cytokine up-regulation has been implicated in pathogenesis of hypertension [13, 14]. Hence, TLR-4 inhibition has been shown to reduce inflammatory cytokine elevation and attenuate hypertension [87, 89]. Therefore, we hypothesize that HHcy induces hypertension by TLR-4 activation that promotes inflammatory cytokine up-regulation (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) and initiation of mitochondria- dependent apoptosis leading to cell death and chronic vascular inflammation. In addition, we aimed to elucidate the role of TLR-4 mutation in attenuation of HHcy- mediated vascular inflammation and mitochondria- dependent cell death.

## Materials and methods

### Animal models

C57BL/6J (WT), cystathionine  $\beta$ - synthase deficient mice (CBS+/-) with genetic mild HHcy, TLR- 4 mutants (C3H/HeJ) and mice with combined genetic HHcy and TLR-4 mutation (CBS+/-/C3H) were used in the present study. The age of the animals was 13- 14 weeks and the body weight was 25-30 g. The mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All standard procedures and experiments with animals were followed in agreement with the National Institute of Health and approved by Institutional Animal Care and Use Committee (IACUC), University of Louisville.

### Blood pressure measurement

BP was recorded using noninvasive tail cuff method (CODA; Kent Scientific, Torrington, CT, USA). Prior to BP measurements the animals were placed into the restraining chambers on a warm platform for 30 min for a 3 consecutive days to ensure mice adaptation to the procedure. BP was recorded in a proper environment (room temperature, lightning and noise-free atmosphere) for 4 mouse groups, 10 animals per each group were used (n=10).

## Vascular ultrasonography

Ultrasound of the superior mesenteric artery (SMA) was performed with the Vevo 2100 system (Visual Sonics, Toronto, ON, Canada) under isoflurane anesthesia. Physiological parameters (heart rate and respiratory rate) were monitored during the procedure. The mouse was placed on a warmed platform (37°C) in supine position and the abdominal area was depilated. The imaging was performed using acoustic gel (Other-Sonic, Pharmaceutical innovations, Newark, NJ) that was applied on the skin in the abdominal area and a Vevo MS550D transducer (13- 24 MHz). To calculate wall-to-lumen ratio, wall thickness and lumen diameter were measured using SMA images in B mode. The SMA resistive index (RI) was calculated as  $RI = (PSV - EDV) / PSV$ , using Peak systolic velocities (PSVs) and end- diastolic velocities (EDVs) measured in pulsed- wave Doppler mode. The SMA pulsatility index (PI) was measured in pulsed- wave Doppler mode and calculated as  $PI = (PSV - EDV) / MV$ . Ultrasound of the SMA was performed for 4 groups, 5 animals per each group were used (n=5).

## qPCR

To assess mRNA expression of different genes in the SMA, RNA was isolated with TRIzol<sup>®</sup> reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA quantification and purity was assessed by nanodrop-1000 (Thermo Scientific, Waltham, MA, USA). Aliquots (2µg) of total



RNA were reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. The q-PCR was performed for different genes (BAX, IL-1 $\beta$ , IL-6), in a final reaction volume of 20  $\mu$ l containing 10  $\mu$ l PerfeCTa SYBR Green SuperMix, Low ROX (Quanta Biosciences, Gaithersburg, MD), 6  $\mu$ l nuclease free water, 2  $\mu$ l cDNA, 40 picomoles of forward, and reverse primers. All sequence-specific oligonucleotide primers (Invitrogen, Carlsbad, CA, USA) are presented in (Table 2). The data was represented in fold expression, calculated as the cycle threshold difference between control and sample normalized with housekeeping gene Rn18s.

#### Western blot analysis

The SMA protein content was extracted using mix of RIPA buffer (Boston BioProducts, MA, USA), PMSF (Calbiochem, La Jolla, CA, USA) and protease inhibitor (Sigma Aldrich, St. Louis, MO, USA). The protein extract was incubated for overnight at 4<sup>0</sup>C with shaking and centrifuged at 13 000 rpm for 20 min. The supernatant was collected in another tube for protein estimation using Bradford-dye method (Bio-Rad, CA, USA). The prepared protein lysate (60  $\mu$ g) was heated at 95<sup>0</sup>C for 5 min and loaded on polyacrylamide gel with SDS in running buffer and run at constant current (100 Volts). For protein transferring to PVDF membrane electro transfer apparatus (Bio-Rad) was used. After transferring, the membranes were blocked in 5% nonfat milk for 1 hour at room temperature,

followed by overnight incubation with primary antibodies (Table 3) at 4<sup>0</sup>C. After washing with TBS-T buffer, membranes were incubated with secondary antibodies for 1 hour at RT followed by washing step. The membranes were developed with ECL Western blotting detection system (GE Healthcare, Piscataway, NJ, USA) and all images were recorded in the gel documentation system ChemiDoc XRS (Bio-Rad, Richmond, CA, USA). The membranes were stripped with stripping buffer (Boston BioProducts, Ashland, MA, USA) followed by blocking step with 5% milk for 1 hour at RT. After washing step membranes were reprobred with anti-  $\beta$ - Actin antibody (Table 3) as a loading control protein. The data were analyzed by Bio-Rad Image Lab densitometry software and normalized to  $\beta$ - Actin bands.

Table 3

<b>Protein</b>	<b>Company</b>	<b>Catalog number</b>
Caspase- 9	Abcam	ab32539
Caspase-3 (cleaved)	Cell Signaling	9661
TLR- 4	Abcam	ab22048
TNF- $\alpha$	Abcam	ab6671
$\beta$ - Actin	Sigma Life Science	A2228

## Immunohistochemistry

The SMA tissue was immersed in tissue- freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) in disposable plastic tissue- embedding mold (Polysciences Inc., Warrington, PA, USA). The tissue blocks were kept at -80°C until use. To obtain 5 um thickness sections Cryocut (CM 1850; Leica Microsystems, Buffalo Grove, IL, USA) was used. Tissue sections were placed on polylysine coated slides (Polysciences, Inc., Warrington, PA, USA). Tissue sections were incubated with permeabilization solution (0.2 g Bovine Serum Albumin, 3 ul Triton X-100 in 10ml 1X PBS) for 1 hour at RT followed by washing step with 1X PBS. The sections were incubated with primary antibody (Table 3) with 1: 250 dilution for overnight at 4°C. After washing with 1X PBS the slides were incubated with fluorescently labeled secondary antibodies (gout anti-mouse Alexa flour 488 and gout anti-rabbit Texas red; Invitrogen, Waltham, MA, USA) with 1: 500 dilution for 1 hour at RT followed by DAPI staining with 1: 10,000 dilution for 20 min at RT. After washing step the slides were mounted with mounting medium and visualized using laser scanning confocal microscope (Olympus Fluo View 1000; Center Valley, PA, USA). The images were analyzed by measuring fluorescence intensity with Image Pro-Plus software (Media Cybernetics, Rockville, MD, USA).

## TUNEL assay

The DeadEnd Fluorometric TUNEL system measures nuclear DNA fragmentation that is an important biochemical hallmark of apoptosis. TUNEL System detects fragmented DNA by catalytically binding fluorescein-12-dUTP to 3'-OH DNA ends using Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT). The TUNEL assay was performed on frozen SMA tissue sections using commercially available kit (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI, USA). The assay was done according to manufacturer's protocol for tissue staining including positive control preparation and nuclear staining with DAPI. The slides were visualized with confocal microscope (Olympus Fluo View 1000; Center Valley, PA, USA) using green fluorescent filter to detect DNA fragmentation and blue DAPI filter for nucleus detection. The images were analyzed with Image Pro-Plus software (Media Cybernetics, Rockville, MD, USA).

## Statistical analysis

Statistical analysis was performed with Primer of Biostatistics 7.0 (McGraw-Hill, NY, USA). To determine the significance, we used one-way analysis of variance (ANOVA) followed by multiple comparison test (Holm test) between the groups. The difference was considered significant when  $p < 0.05$ . Values are presented as mean  $\pm$  SEM ( $n \geq 4$ ).

## Results

### TLR- 4 mutation suppresses HHcy- mediated hypertension

Hyperhomocysteinemic mice (CBS+/-) had significantly higher systolic, diastolic and mean BP than in the WT mice (Fig.12). TLR- 4 mutated mice (C3H) had significantly lower systolic, diastolic and mean BP compared to WT group (Fig.12). Mouse group with combined genetic HHcy and TLR- 4 mutation (CBS+/-/C3H) had significantly lower systolic, diastolic and mean BP than in the CBS+/- group (Fig.12).

### Wall-to-lumen ratio, RI and PI

To assess the structural changes in the SMA the wall thickness and the lumen diameter were measured and the SMA wall-to-lumen ratio was calculated accordingly. It was found that the SMA wall-to-lumen ratio was increased in the CBS+/- mice compared to WT and C3H groups (Fig. 13 A, B). The SMA wall-to-lumen ratio of the CBS+/-/C3H mice was similar to the control group (Fig. 13 A, B). The resistive index (RI) and pulsatility index (PI) were calculated from the blood flow velocities in the artery during cardiac cycle and were used to determine a peripheral resistance. The RI and PI of the SMA were increased in CBS+/- mice compared to WT and C3H groups (Fig.14 A, B). The SMA RI and PI were similar to the control in the mouse group with combined genetic HHcy and TLR-4 mutation (Fig.14 A, B).

## TLR-4 mutation reduces vascular inflammation

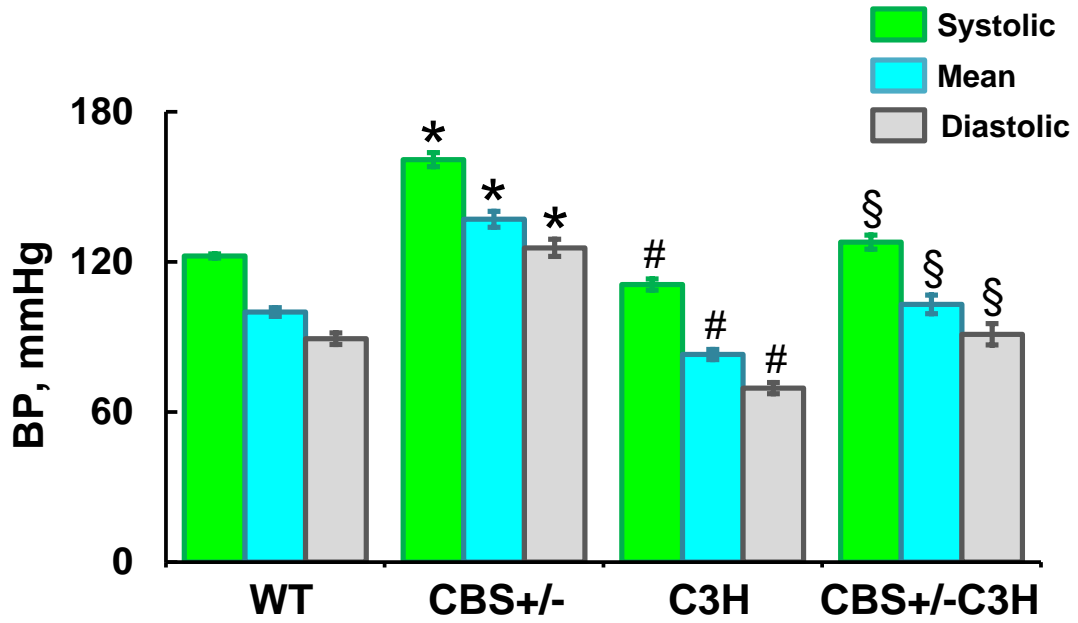
To examine inflammatory marker activation we analyzed TLR-4 and TNF- $\alpha$  expression by IHC (Fig.15 A, B); IL-1 $\beta$ , IL-6 mRNA expression by q-PCR (Fig.16 A, B). IHC showed increased intensity of TLR-4 and TNF- $\alpha$  in the SMA of CBS $^{+/-}$  mouse group. TLR-4 and TNF- $\alpha$  intensities were reduced in mouse group with combined genetic HHcy and TLR-4 mutation. IL-1 $\beta$ , IL-6 mRNA expression was elevated in the SMA of CBS $^{+/-}$  mouse group and reduced in the SMA of CBS $^{+/-}$ /C3H mice.

## TLR-4 mutation attenuates mitochondria-mediated cell death

To evaluate apoptotic marker activation we analyzed BAX gene expression by q-PCR (Fig.17), caspase-9 protein expression by western blotting (Fig.18) and cleaved caspase-3 expression by IHC (Fig.19). BAX mRNA expression was significantly up regulated in CBS $^{+/-}$  mice compared to other groups. CBS deficient mice with TLR-4 mutation (CBS $^{+/-}$ /C3H) had reduced BAX mRNA expression compared to CBS $^{+/-}$  mice. Caspase-9 protein expression was significantly increased in HHcy mice (CBS $^{+/-}$ ) and in CBS $^{+/-}$ /C3H group than in the WT and TLR-4 mutated (C3H) mice. In addition, the up regulation of cleaved caspase-3 expression in CBS $^{+/-}$  mice was confirmed by IHC. The intensity of cleaved caspase-3 was reduced in the SMA of CBS $^{+/-}$ /C3H mice compared to CBS $^{+/-}$  group. These results suggest the induction of mitochondria-dependent apoptosis in HHcy and that TLR-4 mutation mitigates mitochondria-mediated apoptosis.

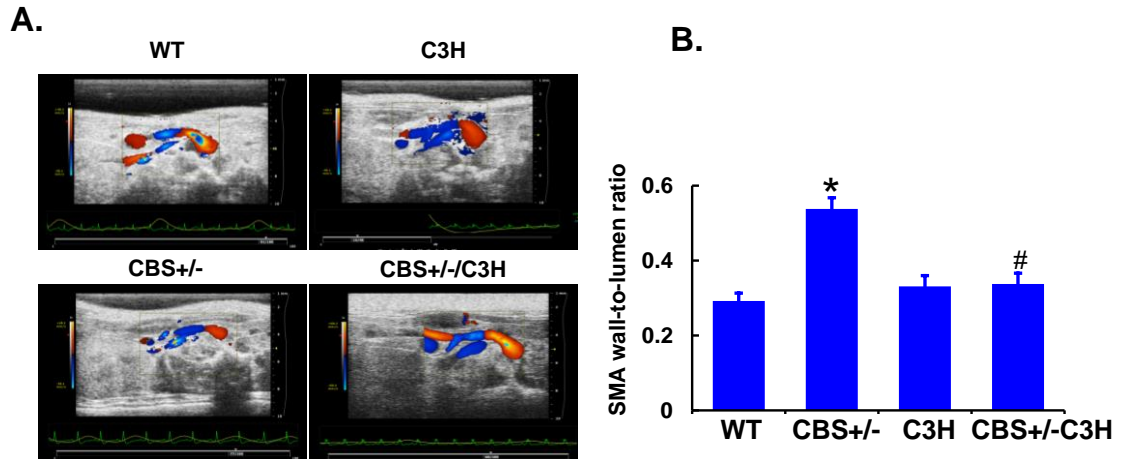
## TLR- 4 mutation mitigates HHcy- induced DNA fragmentation

TUNEL assay was used to evaluate DNA fragmentation in the SMA of different groups (Fig. 20). DNA fragmentation was significantly augmented in the SMA of CBS+/- mice compared to WT and C3H mice. TUNEL positive cell count was reduced in the SMA of CBS+/-/C3H mice as compared to CBS+/- group.

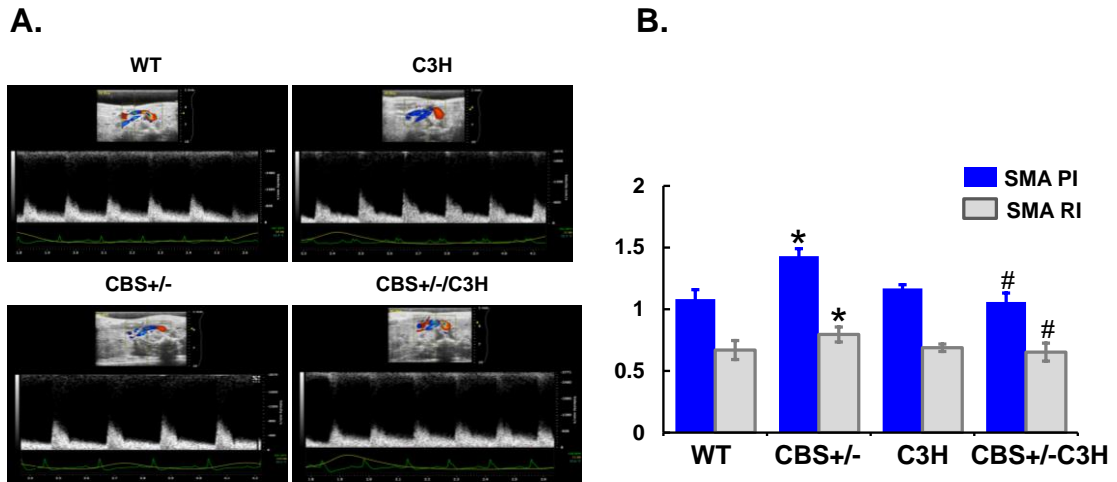


**Fig. 12.** Systolic, Mean and Diastolic blood pressure (BP) : HHcy mice had significantly higher systolic, mean and diastolic BP and mouse group with combined genetic HHcy and TLR- 4 mutation (CBS+/-/C3H) had significantly lower systolic, mean and diastolic BP. n=10; \*p< 0.05 WT vs CBS+/-; # p< 0.05 WT vs C3H; § p< 0.05 CBS+/- vs CBS+/-/C3H.

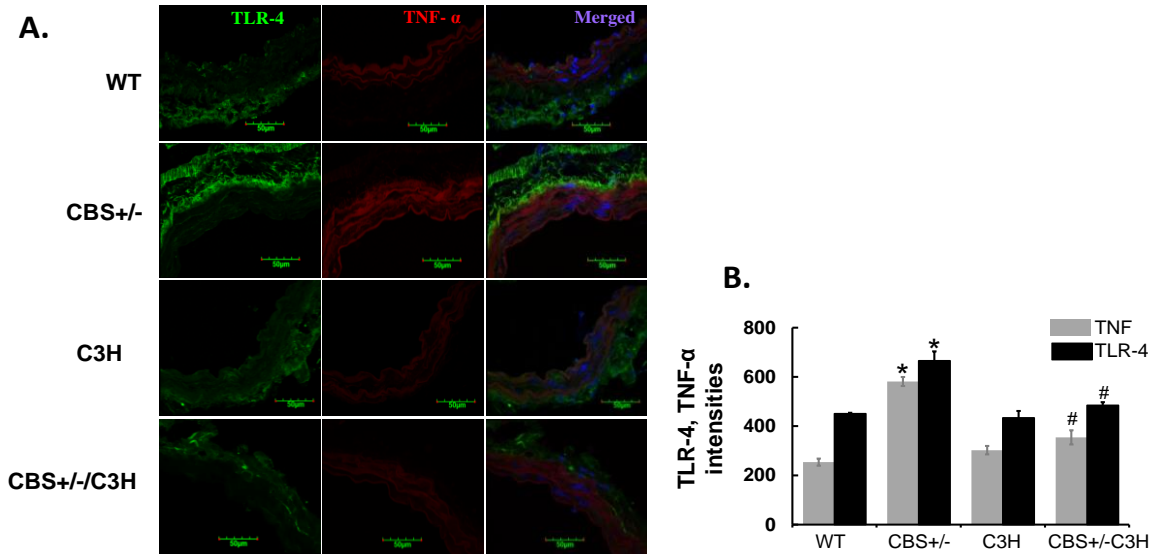




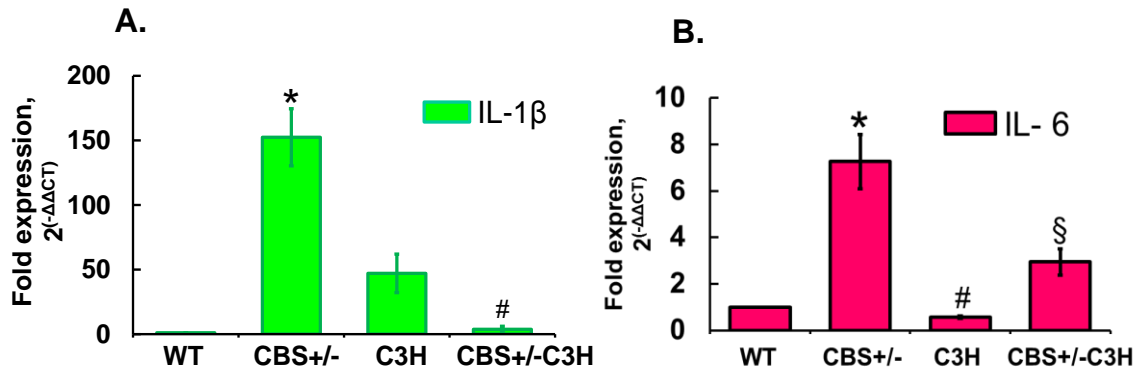
**Fig. 13.** Wall-to-lumen ratio **A.**: to assess the structural changes in the SMA, the wall thickness and the lumen diameter were measured and the SMA wall-to-lumen ratio was calculated accordingly. **B.** bar graph for wall-to-lumen ration values in different groups. It was found that the SMA wall-to-lumen ratio was increased in the CBS+/- mice compared to WT and C3H groups. The SMA wall-to-lumen ratio of the CBS+/-/C3H mice was similar to the control group ; n=5; \*p< 0.05 WT vs CBS+/- ; # p< 0.05 CBS+/- vs CBS+/-/C3H.



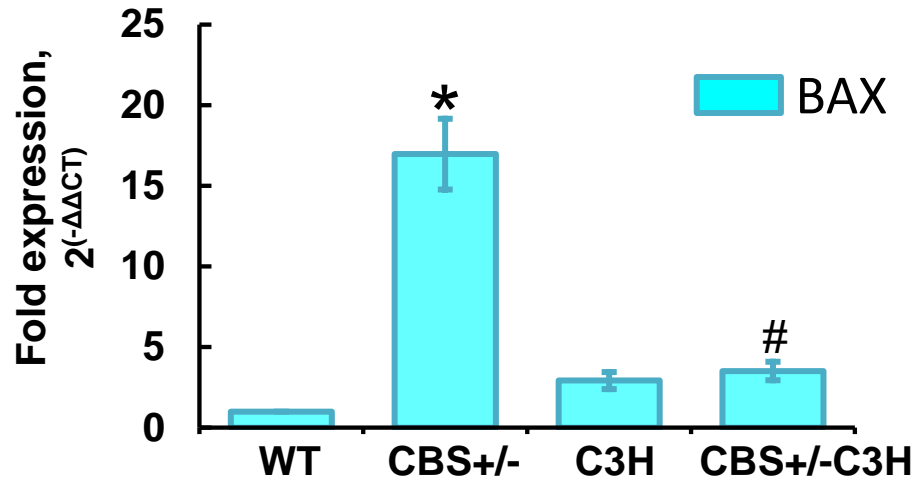
**Fig.14.** PI, RI of the SMA **A:** The pulsatility index (PI) and the resistive index (RI) are calculated from the blood flow velocities in the artery during cardiac cycle and are used to determine a peripheral resistance. **B.** bar graph for RI and PI values in different groups. The PI and RI of the SMA were increased in CBS+/- mice compared to WT and C3H groups. The SMA PI and RI were similar to the control in the mouse group with combined genetic HHcy and TLR-4 mutation; n=5; \*p< 0.05 WT vs CBS+/- ; # p< 0.05 CBS+/- vs CBS+/-C3H



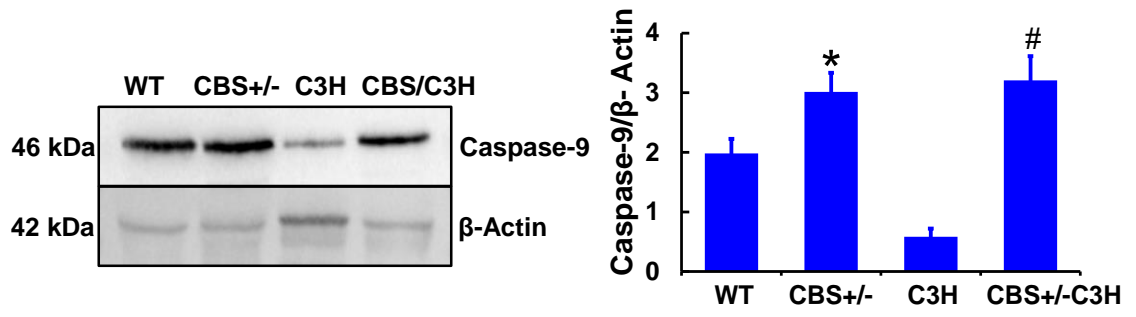
**Fig.15.** Immunohistochemistry for TLR-4 and TNF-  $\alpha$  (**A, B**): IHC showed increased intensity of TLR-4 and TNF-  $\alpha$  in the SMA of CBS+/- mouse group. TLR-4 and TNF-  $\alpha$  intensities were reduced in mouse group with combined genetic HHcy and TLR-4 mutation, n=4; \*p< 0.05 WT vs CBS+/- ; # p< 0.05 CBS+/- vs CBS+/-/C3H



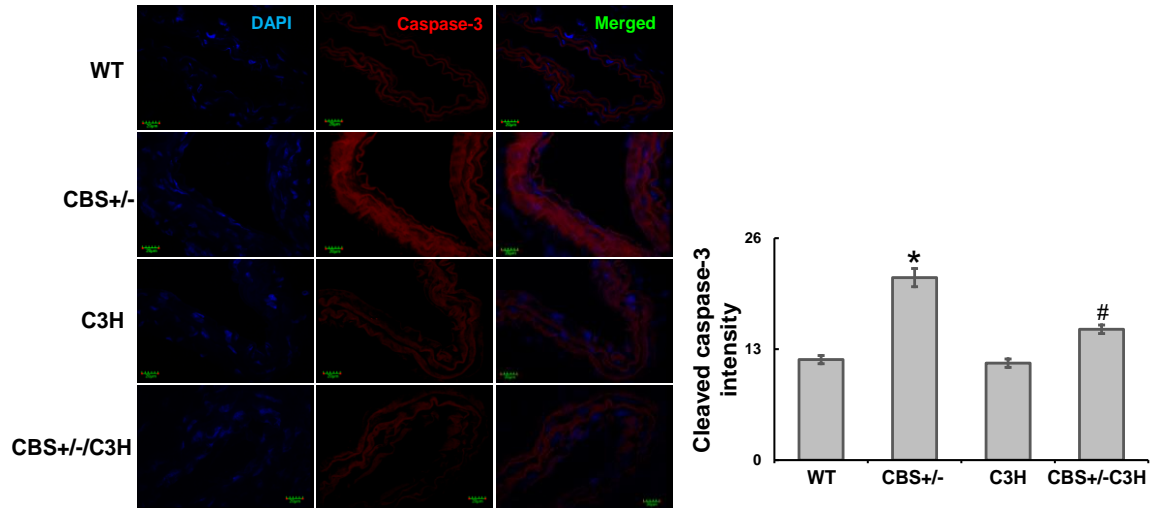
**Fig.16.** Quantitative RT-PCR for IL-1 $\beta$  gene (A) and IL-6 gene (B): IL-1 $\beta$ , IL-6 mRNA expression was elevated in the SMA of CBS+/- mouse group and reduced in the SMA of CBS+/-C3H mice ; (A): n=5; \*p< 0.05 WT vs CBS+/- ; # p< 0.05 CBS+/- vs CBS+/-C3H; (B): n=4; \*p< 0.05 WT vs CBS+/- ; # p< 0.05 CBS+/- vs C3H, §p< 0.05 CBS+/- vs CBS+/-C3H



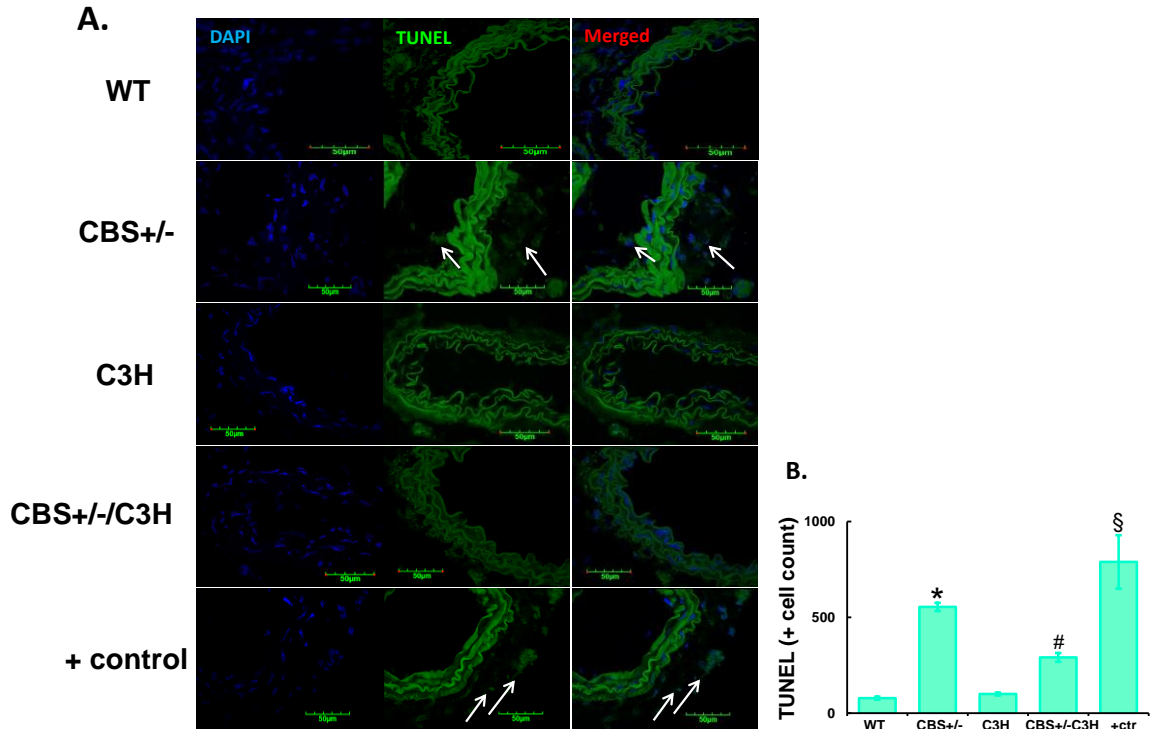
**Fig.17.** Quantitative RT-PCR for BAX gene: BAX mRNA expression was significantly up regulated in CBS+/- mice compared to other groups. CBS deficient mice with TLR- 4 mutation (CBS+/-/C3H) had reduced BAX mRNA expression compared to CBS+/- mice n=4; \*p< 0.05 WT vs CBS+/- ; # p< 0.05 CBS+/- vs CBS+/-/C3H



**Fig.18.** TLR- 4 mutation attenuates mitochondria- mediated cell death: Caspase- 9 protein expression was increased in HHcy mice (CBS+/-) and in CBS+/-/C3H mouse group compared to WT and TLR-4 mutated (C3H) mice; n=6; \*p< 0.05 CBS+/- vs C3H; # p< 0.05 C3H vs CBS+/-/C3H



**Fig.19.** Immunohistochemistry for cleaved caspase-3: IHC showed elevated levels of cleaved caspase-3 in SMA of CBS+/- mice. The intensity of cleaved caspase-3 was reduced in the SMA of CBS+/-C3H mice compared to CBS+/- group; n=4; \*p< 0.05 WT vs CBS+/- ; # p< 0.05 CBS+/- vs CBS+/-C3H



**Fig.20.** TLR- 4 mutation mitigates HHcy- induced DNA fragmentation: TUNEL assay were used to evaluate DNA fragmentation (indicated with arrows) in the SMA of different groups (A, B). DNA fragmentation was significantly augmented in the SMA of CBS+/- mice compared to WT and C3H mice. TUNEL positive cell count was reduced in the SMA of CBS+/-/C3H mice as compared to CBS+/- group, n=4.



## Discussion

Elevated plasma Hcy has been shown to be a risk factor for the peripheral arterial disease and hypertension [8, 197, 207, 208]. In our previous work we have shown that HHcy induced endothelial cell injury and peripheral vascular remodeling with collagen deposition in the SMA [203]. Consistent with our findings, several studies reported that HHcy promotes endothelial cell injury, vascular endothelial dysfunction and vascular remodeling that contribute to pathogenesis of hypertension [209-211]. The elevation of total peripheral resistance that is attributed to alterations in structural and physical properties of the resistance arteries is the hallmark of primary or essential hypertension. In the present study, using ultrasonography, the increased SMA wall-to-lumen ratio, RI and PI were detected in CBS+/- mice that is an indication of inward vascular remodeling and peripheral resistance elevation due to HHcy. The increase in peripheral vascular resistance has been associated with raised systolic, diastolic and mean arterial blood pressure values in CBS+/- mouse group. The RI, PI and wall-to-lumen ratio of the SMA in mouse group with combined genetic HHcy and TLR-4 mutation (CBS+/-/C3H) were similar to the control group that could explain attenuation of HHcy- mediated high blood pressure.

A significant number of studies have described inflammation as one of the toxic effects of Hcy. Zhang et al. reported that plasma Hcy was positively correlated with plasma pro-inflammatory cytokine and chemokine (IL-6, TNF-  $\alpha$  and MCP-1) levels and promoted inflammatory monocyte differentiation [11].

Zanin et al. have shown that HHcy is involved in the synthesis and secretion of IL-1 $\beta$  via NF- $\kappa$ B in murine macrophages [124]. Han et al. have observed endothelial cell inflammatory injury through activation of NF- $\kappa$ B and cytokine IL-6 up regulation in HHcy [125]. In agreement with previous findings, we have found an up-regulation of pro-inflammatory cytokine expression (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) in the SMA of CBS $\pm$  mouse group that confirmed chronic vascular inflammation induced by genetic mild HHcy. In addition, we observed that mice with combined genetic HHcy and TLR-4 mutation have less pro-inflammatory cytokine (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) expression in the SMA compared to CBS $\pm$  mouse group, indicating that TLR-4 mutation prevents HHcy-induced chronic vascular inflammation. The role of TLR-4-mediated signaling pathway has been recently highlighted in pathophysiology of several CVDs including hypertension [212-215]. Pryshchep et al. have observed the abundant expression of TLR-4 in all cell types of 6 different vascular beds (aorta, carotid, temporal, subclavian, iliac and mesenteric arteries). It was confirmed that TLR-4 is expressed in EC and VSMC in atherosclerosis while in normal, non-inflamed arteries adventitial DC are the major sensors of pathogen-related motifs [216]. McCarthy et al. have described the role of musculoskeletal injury-induced endothelial TLR-4 and damage-associated molecular pattern molecules (DAMPs: HMGB<sub>1</sub>, mtDNA) interaction in the development of hypertension in football players [13]. Dange et al. reported increased blood and brain TLR-4, TNF- $\alpha$  and IL-1 $\beta$  expressions in Angiotensin II-induced hypertension and have also shown that central blockade of TLR-4 delayed the progression of

hypertension and improved cardiac function in hypertensive rats [217]. Bobek et al. have observed that TNF- $\alpha$ -infused mice developed proteinuric hypertension similar to human preeclampsia that was accompanied by TLR-4 protein expression up-regulation in placenta [218]. Li et al. have reported TLR-4-induced MMP-9 elevation in human aortic smooth muscle cells and confirmed that TLR-4 siRNA silencing regulated the MMP-9 expression, indicating the role of TLR-4-mediated signaling pathway in vascular remodeling [219]. Balistrelli et al. for the first time described rs4986790 TLR-4 polymorphism that confers a higher susceptibility for sporadic thoracic aorta aneurism (TAA) and together with rs1799752 ACE, rs3918242 MMP-9, and rs2285053 MMP-2 SNPs is an independent sporadic TAA risk factor. In addition, the authors observed that the cases with combined risk genotype showed higher levels of inflammatory mediators and plasma MMP-9, MMP-2 levels that was accompanied by elastic fragmentation in tissue aorta samples [220, 221]. In previous work we have reported TLR-4, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  up-regulation in heart tissues of CBS+/- mice with genetic mild HHcy. The inflammatory markers were further increased when mice were fed with high methionine diet that exacerbated HHcy [222]. In agreement with previous studies, we have found TLR-4 expression up-regulation in the SMA of CBS+/- mice that connects the downstream inflammatory cytokine signaling pathway activation in mild HHcy. The mouse group with combined genetic HHcy and TLR-4 mutation exhibits less TLR-4 expression compared to CBS+/- mouse group preventing downstream inflammatory cytokine elevation.

Mitochondria-dependent apoptosis has been implicated in the induction of

vascular remodeling that contributes to pathogenesis of hypertension [223-225]. A significant number of studies have described the role of HHcy in the initiation of mitochondria- dependent apoptosis through activation of ROS. Kim et al. have shown that Hcy treatment of primary human bone marrow stromal cells led to activation of ROS, caspase-9, 3 and cytochrome c release into the cytosol from mitochondria, suggesting that mitochondria- initiated cell death pathway is a predominant mechanism of apoptosis in HHcy [226]. Sipkens et al. have observed elevation of cellular NOX2, NOX4 and Hcy treatment concentration dependent increase of caspase- 3 in human umbilical vein endothelial cells [227]. Moreira et al. reported that Hcy increases superoxide levels and cell death in human aortic endothelial cells, but vitamin B<sub>12</sub> supplementation prevents oxidative stress- induced cellular death [228]. However, there are studies that have been stressing on inflammatory component as the critical mechanism of mitochondria-mediated cell death. Pan et al. reported that NF-kB is a transcription factor that regulates the expression of both pro-inflammatory genes and genes that contribute to mitochondria-dependent apoptosis (BCL-2 gene family) [229]. It has been implicated that NF-kB activation happens prior to DNA fragmentation and is accompanied by up-regulation of pro-apoptotic proteins (BAX) [230, 231]. Aoki et al. have reported that NF-kB promotes endothelial cell death through translocation of BAX to mitochondria and down- regulation of Bcl-2 [232]. In the present study, we have found an up-regulation of BAX, caspase-9, caspase-3 expression in the SMA of CBS+/- mice with mild HHcy compared to control group. The mouse group with TLR-4 mutation alone expressed less BAX,

caspase-9, caspase-3 in the SMA tissue. The group with combined genetic HHcy and TLR-4 mutation exhibited down regulation of BAX, caspase-3 expression in the SMA. In addition, DNA fragmentation in the SMA was increased in the mouse model of mild HHcy, but groups with TLR-4 mutation (C3H, CBS+/-/C3H) showed decreased DNA fragmentation in the SMA tissue. These findings suggest that TLR-4- driven inflammatory signaling pathway contributes to initiation of mitochondria- mediated cell death and TLR-4 mutation prevents the onset of mitochondrial apoptosis.

To summarize, our study for the first time has illustrated that Hcy acts through TLR-4 receptors that are highly expressed in all cell types of the vascular bed HHcy- mediated TLR-4 activation promotes chronic vascular inflammation with pro- inflammatory cytokine elevation, facilitating mitochondria- mediated cell death that all favor inward vascular remodeling. In our work we have also demonstrated that TLR-4 mutation attenuated chronic vascular inflammation and mitochondria- induced cell injury that prevented peripheral vascular remodeling and suppressed hypertension.

## CHAPTER VI

### SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

Experimental studies have demonstrated that elevated plasma Hcy is a risk factor for the peripheral arterial disease and hypertension. However, the precise mechanism of detrimental Hcy interaction with vascular wall is incompletely understood. In the present study we have described different aspects of Hcy action in the development of peripheral vascular remodeling that is a hallmark of hypertension. Our study for the first time has illustrated that Hcy acts through TLR-4 receptors that are highly expressed in all cell types of the vascular bed. HHcy-mediated TLR-4 activation promotes chronic vascular inflammation with pro-inflammatory cytokine elevation, facilitating mitochondria-mediated cell death that all favor inward vascular remodeling. In our work we have also demonstrated that TLR-4 mutation attenuated chronic vascular inflammation and mitochondria-induced cell injury that prevented peripheral vascular remodeling and suppressed hypertension.

### **Future Directions:**

Several experimental studies have illustrated the promising effect of TLR-4-targeted therapy in prevention of hypertension. However, further studies are required for detailed investigation of the role of TLR-4-mediated signaling pathway in pathogenesis of hypertension and other CVDs. Future studies that will elucidate the mechanisms that underlie TLR-4-mediated inflammatory pathway may offer novel concepts for hypertension therapy.

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## CURRICULUM VITA

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### **Education**

University of Louisville- Louisville, KY

Doctor of Phylosophy June 2016

Physiology and Biophysics

Proposed Graduation Date: June 2016

3.88/4.0 Current Gradate GPA

University of Louisville- Louisville, KY

Masters of Science Physiology and Biophysics

Omsk State Medical Academy- Omsk, Russia

Medical Doctor June 2010

Pediatrics

**Related Skills and Techniques:** Real Time PCR, RFLP- PCR, RNA Isolation and Purification, Immunohistochemistry, Immunocytochemistry, Masson's trichrome Staining for collagen, Flow cytometry, Semiquantitative PCR, Western blotting, Cell culture, Transfection with siRNA, Animal handling and surgical procedures, Blood pressure measurement by tail- cuff technique, TUNEL assay, Laser doppler imaging, Ultrasonography, Exosome isolation, PKH67 exosome labeling, Western blotting to analyze exosome proteins, siRNA transfection into exosomes, familiar with statistical softwares.

### **Honors**

2004-2010 Omsk State Governmental Scholarship

2013-2015 IPIBS scholarship

2015 Grant Pierce Young Investigator Competition Finalist in Cardiovascular Sciences

2016 Robert Gunn Student Award Finalist, The American Physiological Society Cell and Molecular Physiology Section.

### **Professional membership**

American Physiological Society

### **Contribution to scientific meetings**

1. **Oral Presentation in the session titled Grant Pierce Young Investigator Competition in Cardiovascular Sciences. Mitochondrial-**

Dependent Apoptosis in Arterial Remodeling: Link to Hypertension.  
Annual Meeting of the International Academy of Cardiovascular Sciences  
(IACS): North American Section, Omaha, NE, USA, 2015

2. **Poster Presentation: Robert Gunn Student Award Finalist, The American Physiological Society Cell and Molecular Physiology Section.** Toll-like Receptor 4 Mutation Moderates Hyperhomocysteinemia-Induced Hypertension. Experimental Biology Meeting 2016, San Diego, CA, USA
3. **Poster Presentation in the special session for poster competition:** Mitochondrial- Dependent Apoptosis in Arterial Remodeling: Link to Hypertension. Council on Hyertension 2015 Scientific Sessions, AHA, ASA, Washington, DC, USA
4. Poster Presentation: Link between Mitophagy and Apoptosis in Endothelial Cells: Exosomal Delivery of Mfn-2 siRNA. Experimental Biology Meeting 2015, Boston, MA, USA
5. Poster Presentation: Mitochondrial Dynamics and Mitochondrial Dysfunction in Mesenteric Artery: Role of Homocysteine. Experimental Biology Meeting 2014, Boston, MA, USA

#### **Selected Peer-reviewed Publications**

1. **Familtseva A, Kalani A, Chaturvedi P, Tyagi N, Metreveli N, and Tyagi SC.** Mitochondrial mitophagy in mesenteric artery remodeling in hyperhomocysteinemia. *Physiological reports* 2: e00283, 2014.



2. **Chaturvedi P, Kamat PK, Kalani A, Familtseva A, Tyagi SC.** High Methionine Diet Poses Cardiac Threat: A Molecular Insight. *J Cell Physiol.* 2015.
3. **Chaturvedi P, Kalani A, Medina I, Familtseva A, and Tyagi SC.** Cardiosome mediated regulation of MMP9 in diabetic heart: Role of mir29b and mir455 in exercise. *J Cell Mol Med* 2015.
4. **Kalani A, Kamat PK, Familtseva A, Chaturvedi P, Muradashvili N, Narayanan N, Tyagi SC, and Tyagi N.** Role of microRNA29b in blood-brain barrier dysfunction during hyperhomocysteinemia: an epigenetic mechanism. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 34: 1212-1222, 2014.
5. **Chaturvedi P, Kalani A, Familtseva A, Kamat PK, Metreveli N, and Tyagi SC.** Cardiac tissue inhibitor of matrix metalloprotease 4 dictates cardiomyocyte contractility and differentiation of embryonic stem cells into cardiomyocytes: Road to therapy. *International journal of cardiology* 184: 350-363, 2015.
6. **Chaturvedi P, Kalani A, Givvimani S, Kamat PK, Familtseva A, and Tyagi SC.** Differential regulation of DNA methylation versus histone acetylation in cardiomyocytes during HHcy in vitro and in vivo: an epigenetic mechanism. *Physiological genomics* 46: 245-255, 2014.
7. Manuscript in preparation: Anastasia Familtseva, P. Chaturvedi, A. Kalani, N. Jeremic, N. Metreveli, G.H. Kunkel, Suresh C Tyagi. Toll-like Receptor

4 Mutation suppresses Hyperhomocysteinemia- Induced Hypertension.  
Submitted to *American Journal of Physiology, Cell Physiology*, 2016.

### **Abstracts published**

1. **Familtseva A, Chaturvedi P, Kalani A, Metreveli N, Kunkel G. H., Tyagi S.C.** Toll-Like Receptor 4 Mutation Moderates Hyperhomocysteinemia- Induced Hypertension. *FASEB Journal* vol.30 969.13, 2016
2. **Chaturvedi P, Kunkel G. H., Familtseva A, Kalani A., Tyagi S.C.** Exercise Mitigates Calpain 1 Induced Cardiac Dysfunction Through PCP4 in Diabetes. *FASEB Journal* vol.30 954.1, 2016
3. **Kunkel G. H., Chaturvedi P, Familtseva A, Tyagi S.C.** Mitochondrial Transcription Factor: The Root to Cardiomyocyte Survival. *FASEB Journal* vol.30 1224.10, 2016
4. **Familtseva A, Kalani A, Chaturvedi P, Metreveli N, Tyagi S.C.** Mitochondrial- Dependent Apoptosis in Arterial Remodeling: Link to Hypertension. *Hypertension Journal*, 2015; 66: AP133
5. **Familtseva A, Chaturvedi P, Kalani A, Tyagi N, Tyagi S.C.** A Link between Mitophagy and Apoptosis in Endothelial Cells: Exosomal Delivery of Mfn-2 siRNA. *FASEB Journal* vol.29 974.13, 2015
6. **Familtseva A, Kalani A, Chaturvedi P, Kamat P, Tyagi N, Tyagi S.C.** Mitochondrial Dynamics and Mitochondrial Dysfunction in Mesenteric Artery: Role of Homocysteine. *FASEB Journal* vol. 28 867.11, 2014