Understanding the role of MasR in vascular pathology

By

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For

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Abstract

Background: Homocysteine was first suggested as a risk to CVD in 1969. Since then, elevated plasma homocysteine (derived from methionine) has remained a cardiovascular risk factor with no current treatment. Homocysteine is known to stimulate NADPH oxidase, and NADPH oxidase can be inhibited by stimulation of the Mas receptor (MasR) of the renin angiotensin system. Stimulation of MasR is known to reduce organ fibrosis through the production of NO. However, the role that MasR plays in homocysteine-induced vascular pathology, including endothelial dysfunction and organ fibrosis, is not known.

Aims: To determine if high methionine diet in $MasR^{-/-}$ mice will worsen cardiovascular pathology.

Methods: $MasR^{-/-}$ and C57BL/6 (control) mice were fed a 1% methionine or a control diet for 8 weeks (n=16 per group). Aortic endothelial function in response to acetylcholine was performed, fibrosis was semi-quantified in myocardium and kidney and aorta using fast green/sirius red staining. Immunohistochemical staining for Collagen I and III were performed.

Results: Aortic acetylcholine-induced relaxation was reduced by >50% in $MasR^{-/-}$ mice vs control (32.3±6.8% vs 66.1±7.7%, $p<0.001$), this dysfunction was worsened by methionine in $MasR^{-/-}$ but not in control (6.6±6.7% vs 74±7%, $p<0.0001$) mice. Methionine increased glomerular fibrosis by 22% in control ($p=0.02$), and 2.1 fold ($p<0.0001$) in $MasR^{-/-}$ mice whereas kidney interstitial/tubular fibrosis increased 5-fold ($p<0.0001$) only in $MasR^{-/-}$ mice. Methionine increased myocardial interstitial fibrosis by >3-fold ($p<0.0001$) only in $MasR^{-/-}$ mice and perivascular fibrosis by 31% in control ($p<0.001$) and 2.6-fold ($p<0.001$) in $MasR^{-/-}$ mice. However, aortic medial and adventitial fibrosis was not present in methionine fed $MasR^{-/-}$ mice. By contrast, aortae of methionine fed control mice ($p<0.01$) displayed a significant increase in fibrosis in both medial and adventitial tissue. Positive staining for collagen I and III was detected in heart, kidney and aorta.

Conclusion: Excess dietary methionine for 8 weeks worsens cardiovascular pathology in $MasR^{-/-}$ mice, suggesting that the MasR is protective against homocysteine-induced cardiovascular pathologies.

Student Declaration

Masters by Research Student Declaration

Master by Research Declaration

I, Sudarshan Rai, declare that the Master by Research thesis entitled “Understanding the role of MasR in vascular pathology” is no more than 60,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Tuesday, October 11, 16

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Introduction

Cardiovascular disease (CVD) is well documented as the number one cause of death worldwide. In 2005 alone, people who died from CVD accounted for 30% of all global deaths (World Health Organization 2008). It occurs almost equally in men as in women, and is predicted to be the most common cause of death worldwide by 2020 [(1)]. CVD encompasses ischemic heart disease, stroke and vascular diseases. CVD usually stems from vascular dysfunction, which leads to atherosclerosis. Atherosclerosis is an inflammatory disease involving the arterial endothelium [(2)]. The disease disrupts blood flow through the lumen due to the formation of plaques that may eventually lead to plaque rupture [(2)], which can lead to other complications such as myocardial and cerebral infarction [(3)].

There are several risk factors associated with CVD, the most common of which are dyslipidemia, hypertension, smoking, diabetes mellitus [(4-6)], and high plasma homocysteine, also known as hyperhomocysteinemia (>15μM) [(7)]. Current treatment for CVD can involve a combination of drugs such as, low-density lipoproteins (LDL) lowering statins, high blood pressure lowering aspirins or surgery to repair damaged blood vessels. Current methods of reducing cardiac and atherosclerotic events mainly include statin therapy, as this also reduces the occurrence of strokes and transient ischemic attacks [(8)]. However lipid-lowering therapy such as statins itself also carries a risk of muscular symptoms or myopathy with 7% of outpatients on statins reporting mild muscular pain [(9-11)].

Endothelial dysfunction and nitric oxide

It has been well established that endothelial dysfunction is in direct response to cardiovascular risk factors, and precedes the development of atherosclerosis. Endothelial dysfunction is characterized by a reduced bioavailability of vasodilators, in particular nitric oxide (NO), whilst endothelium-

derived vasoconstrictors are increased [12, 13]. The formation of lesions promoted by both early and late mechanisms of atherosclerosis, including increased chemokine secretion and leukocyte adherence, enhanced low-density lipoprotein oxidation, increased cell permeability, platelet activation, cytokine elaboration, up regulation of adhesion molecules, and vascular smooth muscle cell proliferation and migration, is a result of endothelial dysfunction [12, 14, 15]. A major vasodilator released by the endothelium is NO, as well as bradykinin and prostacyclin [14-16]. NO was indicated to be a vasodilator able to inhibit growth and inflammation[17, 18]. During diseased states, endothelial dysfunction can be characterized by the imbalance between vasodilators and vasoconstrictors, resulting in an impairment of endothelium-dependent vasodilation [12, 19]. Moreover, endothelial dysfunction has a chronic inflammatory, proliferative, and procoagulatory state that favors atherogenesis [19]. NO is produced when L-arginine is converted to L-citrulline by endothelial nitric oxide synthase (eNOS). It has been suggested that phosphorylation of eNOS at either and/or both serine1177 (ser1177) and serine633 (ser633) leads to eNOS activation in response to stimuli, with ser633 found to have higher efficacy in increasing the eNOS derived NO bioavailability [20-22].

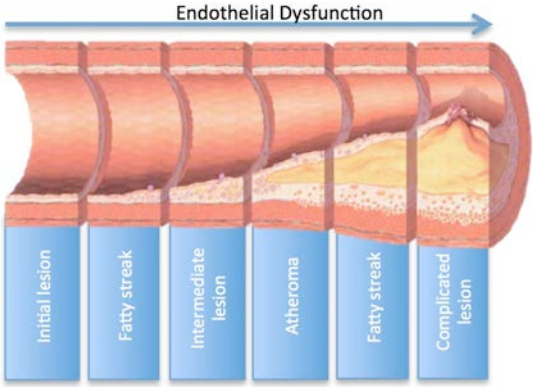


Figure 1. Evolustion of arterial wall changes in response to injury artery wall thickens as a result of invasion and accumulation of white blood cells (macrophages and foam cells) and proliferation of intimal-smooth-muscle cell progressing to atherosclerosis.

Previous studies have demonstrated that both NO production and endothelial function are impaired in diseased vascular tissue[23, 24], however recent results indicate that this may not be as a result of expression level of mRNA and the protein eNOS, as findings show their expression as either increased or not changed [25]. These findings suggest a reduced generation or bioavailability of NO, which may be regulated independently of eNOS activity [25, 26]. In support of this idea, a recent study suggested that an uncoupling of eNOS and decreased phosphorylation resulting in reduced NO synthesis was a major underlying cause of endothelial dysfunction [25, 27].

Risk factors for CVD and organ pathology

Methionine and Homocysteine:

Homocysteine, a metabolite of methionine found in meat, was proposed to be an independent atherogenic agent by Kilmer McCully more than 30 years ago [28]. McCully showed that an increase in plasma homocysteine affected all artery sizes, with the lumen of many large to medium arteries exhibiting narrowing due to focal intimal and medial fibrosis, which is often associated with fraying and discontinuity of internal elastic membrane[28]. Moderate thickening of the media and internal elastic membrane was also noted [28]. Additionally, focal proliferation of perivascular connective tissue of many small arteries, and an increase of collagen bundles, fibroblasts, and small elastic fibers was observed [28].

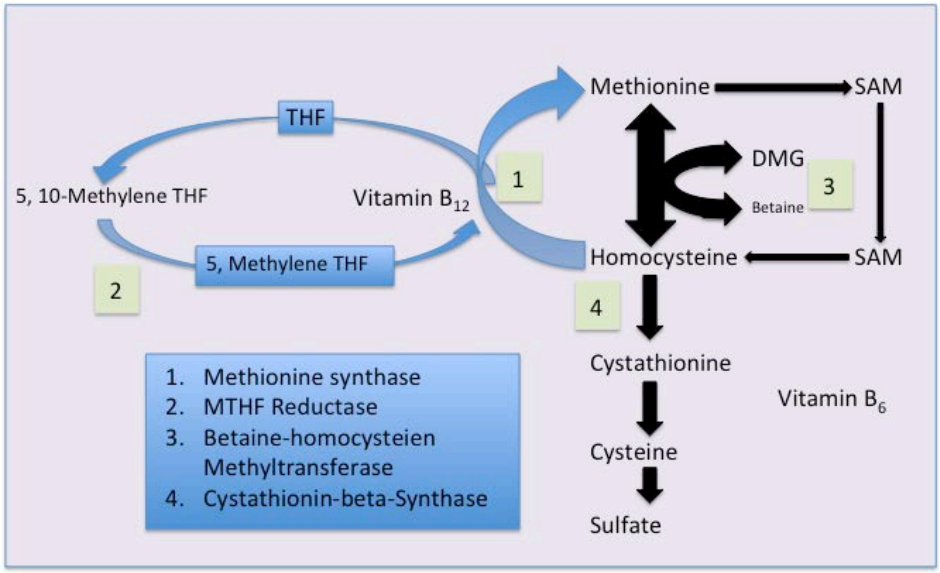


Figure 2. Schematic of homocysteine metabolism. Methionine is converted to homocysteine which forms cystathionine (in an irreversible reaction). Cystathionine is hydrolyzed to form cysteine, and excess cysteine is oxidized to inorganic sulfates and is excreted in urine.

Homocysteine is an endogenous amino acid, that once formed is catabolized to cysteine by transsulfuration or remethylated to methionine [29, 30]. Elevated homocysteine known as Hyperhomocysteinemia is widely considered to be an independent risk factor for various CVD [31, 32]. However, the precise mechanism in which homocysteine interferes with the cardiovascular system has not yet been elucidated.

Mild elevations in plasma homocysteine concentrations (10-15 µM) are frequently found in Western populations and are associated with an increase risk of CVD [33]. Humans synthesize between 15-20 mmol of homocysteine per day however the majority of this is converted to cysteine or to methionine [29]. Regulation of homocysteine levels sets the basal concentration between 5 to 15 µM [29, 34, 35]. Studies have suggested that elevations in plasma homocysteine are associated with an increased risk of occlusive artery disease, particularly in the brain, heart and kidney [36]. Hyperhomocysteinemia is described as moderate at 15-30 µM, intermediate between 30-100 µM or

severe measured at >100 μM [29, 34, 35], with 300μM being the highest recorded pathological level detected [12, 37, 38]. Additionally, impaired handling and metabolism of homocysteine has been exhibited after a methionine challenge in individuals with premature atherosclerosis. Essentially, hyperhomocysteinemia is a state where there is an increased risk of vascular constrictive affects, atherosclerotic, and atherothrombotic [39].

Reduced endothelial-dependent relaxation has also been observed in humans acutely affected by hyperhomocysteinemia[40, 41]. These observations have some to reason that hyperhomocysteinemia may not in fact be the cause of atherosclerosis intrinsically [41], but may have a role in altering vascular function so as to increase risk for complications of atherosclerosis [41]. Furthermore, endothelial-dependent flow-meditated dilation in brachial arteries was lower in hyperhomocysteinemia patients than in patients with normal homocysteine levels [42]. A study using healthy volunteers with oral methionine loading resulted in increased homocysteine levels and a reduced NO bioavailability by producing oxidative stress, causing decreased endothelial function [42-45].

Therefore, several large studies evaluating lowering total homocysteine have been performed to elucidate its affect on CVD [46]. Normalization of total homocysteine is typically done by supplementation with folic acid and vitamin B_{12} [33]. Consequently, clinical trials using dietary folate supplementation to efficiently lower total homocysteine in patients produce an array of effects. While treatment with folate did lower total homocysteine, this did not yield a protective effect on secondary adverse vascular events, leading investigators to conclude that hyperhomocysteinemia may be an early biomarker of cardiovascular risk rather than an independent risk factor [47, 48]. However, Weiss *et al.* 2002, demonstrated that normalizing homocysteine levels had in fact improved endothelial dysfunction, although it was unclear if this translated to a reduced risk of CVD [33, 45].

A Norwegian group reported in a prospective study that hyperhomocysteinemia was a strong predictor of mortality, although correlation between hyperhomocysteinemia and coronary artery atherosclerosis was not clear [41, 49]. Alternatively, studies investigating a moderate rise in plasma homocysteine found that it accelerated the formation of atherosclerotic lesions in ApoE knockout ($ApoE^{-/-}$) mice. $ApoE^{-/-}$ mice fed a hyperhomocysteinemic diet for up to 8 weeks developed atherosclerotic lesions in the aorta that were larger then mice fed on normal chow [41, 50]. The $ApoE^{-/-}$ mice were created to develop severe hypercholesterolemia in addition to lesions of atherosclerosis with similar characteristics to that those observed in humans [51]. The mice fed on the hyperhomocysteinemic diet had significantly larger mean aortic lesion when compared to control [52].

Studies such as the Heart Outcomes Prevention Evaluation (HOPE) 2 Investigators trial and the Norwegian Vitamin (NORVIT) trial have performed extensive investigation into the homocysteine lowering effects of folic acid and B vitamin treatments. While the trials concluded that treatment with folic acid and B vitamins did not reduce the risk major cardiovascular events in patients with vascular disease [53], or lower the risk of recurrent CVD after myocardial infraction [54], they did however acknowledge plasma total homocysteine levels were a predictor (or marker) of cardiovascular events [53, 54].

Studies show that oxidation and modification of low density lipoprotein (LDL) plays in important part in the initiation of atherosclerosis [55], where oxidized LDL cholesterol was able to impair endothelial-mediated relaxation [55]. Rabbits fed on a 0.5% cholesterol diet showed significantly less blood vessel relaxation then normal chow fed rabbits [56]. Our lab has also demonstrated that in addition to 0.5% cholesterol, when 1% methionine is added to the diet it intensifies the progression of atherosclerosis [57] and interestingly shows proliferation of mesenchymal stem cells and

endothelial hematopoietic stem cells which were identified using cell surface markers specific to the respective cell types (unpublished data).

Investigation into atherosclerosis, and endothelial integrity prior and during diseased states, shows the crucial role the endothelial plays; it acts as executive and sensory function, it can generate effector molecules that are able to regulate thrombosis, vascular tone, inflammation and vascular remodeling [58]. Injury or removal of the endothelial results in rapid migration and proliferation of smooth muscle cells which then subsides after the endothelium regenerates [58, 59]. Therefore, endothelial-dependent regulation of vascular tone and consequently endothelial dysfunction is indicative of a reduced bioavailability of endothelial-derived signaling molecule NO [60, 61]. Studies suggest that chronic hyperhomocysteinemic subjects that are free of any overt CVD exhibit reduced NO bioavailability [62-64]. This is supported in studies examining oral methionine administration to healthy subjects that also show reduced NO [43, 65-67]. Thus, a consensus can be drawn that lowering plasma homocysteine levels by supplementation with B vitamins, improves endothelial dysfunction by reducing the biochemical markers and improving overall endothelial-dependent vasodilatory function [45].

ROS:

Reactive oxygen species (ROS) when present at low concentrations at sites of injury and inflammation function as signaling molecules regulating cell activity and growth. Conversely, increased levels of ROS is believed to lead to cellular injury and death, often associated with microvascular pathology in age-related CVD [17, 68, 69]. Prolonged exposure to ROS and other cardiovascular risk factors can eventually fatigue the protective effect of endogenous anti-inflammatory systems within endothelial cells. As a consequence the endothelial becomes increasingly permeable and promotes leukocyte adhesion and alters endothelial signaling[12].

LDL modified via oxidation also plays a central role in the development of atherosclerosis, and maybe important in abnormal endothelial vasorelaxation[19, 70]. The nicotinamide adenine dinucleotide phosphate family of oxidases (NOX) is a crucial component of ROS production. NOX proteins are able to produce super oxide anions (O_2^-), which further react to form hydrogen peroxide (H_2O_2) [71, 72]. However, in the presence of NO, O_2^- combines to NO and forms peroxynitrite ($ONOO^-$) [71, 72]. The NOX family of NADPH oxidases has only one role, to generate ROS [71, 73]. There are seven members of the NADPH oxidase family, NOX1 through to NOX5 and Duox1 and Duox2. NOX1 is expressed in smooth muscle cells however in endothelial cells a lower level of expression was detected [71, 74, 75]. NOX2 is expressed in endothelial cells and adventitial fibroblasts [71, 76, 77]. NOX4 was found to be ubiquitously expressed, and thus was found in all the cell types: smooth muscle cells, endothelial cells and adventitial fibroblasts [71, 76, 78-85]. Our lab has previously studied the deleterious affects of homocysteine on endothelial response to acetylcholine with Nox inhibitor, however, only ML090 a Nox1 inhibitor was found to ameliorate homocysteine and its actions on the endothelial, which suggested a strong correlation between Nox1 and homocysteine-induced vascular damage [80]. In addition to our previous results, a study investigating salidroside (an antioxidant) demonstrated it protected against homocysteine-induced impairment of endothelial relaxation by inhibiting Nox2-dependent ROS overproduction and increasing NO bioavailability [84]. Other researchers have also shown that homocysteine inhibited endothelial-dependent relaxation in a concentration and time dependent manner [86]. These studies together indicate homocysteine causes endothelial dysfunction by way of Nox-dependent pathways.

RAS:

The renin-angiotensin system (RAS) plays a significant role in the regulation of blood pressure homeostasis, which includes body fluid control and electrolyte homeostasis [87, 88]. Consequently it has been a principal target for therapeutics for the treatment of hypertension and other such related

diseases. Extended stimulation of angiotensin type 1 receptor (AT_1R) by angiotensin II (AngII) signaling leads to fibrosis [89], inflammation, and cellular hypertrophy and cellular proliferation, which all contribute to disease pathophysiology [90, 91]. Studies have indicated that AngII is the primary vasoactive substance of the RAS and causes renal, arterial and cardiac lesions [91].

A recent discovery of an alternate pathway has expanded the classical view of RAS, where angiotensin peptide metabolism and signaling forms Ang (1-7) by degradation of AngII, by angiotensin converting enzyme 2 (ACE2) [90, 92, 93]. ACE2, a homologue of angiotensin converting enzyme (ACE) is highly expressed in the kidney, lungs, testis and heart [94, 95].

ACE2 catalyzes the conversion of Ang I to Ang (1-9) and Ang II to Ang (1-7) [96]. ACE2 facilitates Ang (1-7) production via two pathways, when Ang I is converted to Ang (1-9) allowing ACE to subsequently convert Ang (1-9) to Ang(1-7), and the direct AngII to Ang(1-7) hydrolysis [97]. Moreover it was discovered that ACE2 is able to catalyze Ang II substrate with a 400-fold efficiency when compared to the catalytic rate of Ang I, further suggesting that the ACE2/Ang(1-7) may counter regulatory to Ang I.

Ang (1-7), a heptapeptide that binds to and activates a unique receptor known as Mas [98], opposes many of the actions of the Ang I and AT_1R [87, 90, 92]. Therefore, MasR, activated by Ang (1-7) is observed as the protective arm of the RAS [99, 100] see Figure 2.

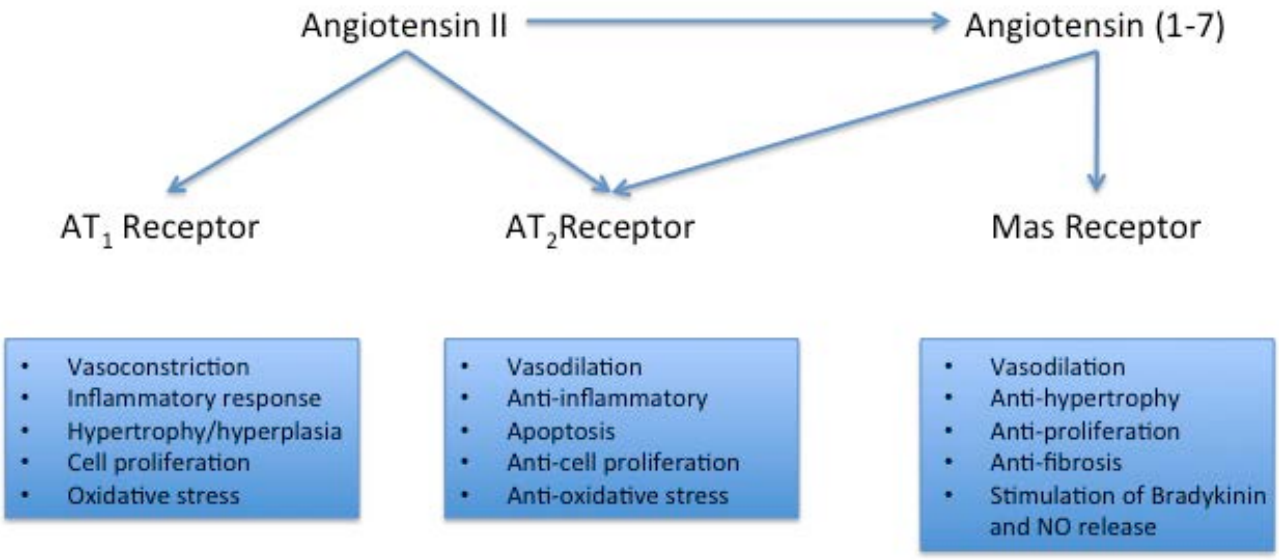


Figure 3. Ang II and Ang (1-7) and the respective pathways.

Both AT_1R and AT_2R have been identified in the vessel wall, although it is thought that AT_1R mediates most of the atherogenic actions of Ang II. Though both AT_1R and AT_2R belong to the same receptor family they differ greatly in their signaling cascades and biological activities [101]. AT_1R were most densely distributed in the vascular smooth muscle cells and endothelial cells [102, 103]. Activation of the AT_1R was shown to contribute independently to chronic CVD [101, 103]. The effects of Ang II through its AT_1R is thought to enhance oxidized LDL infiltration in the vessel wall [103].

Less is know about the AT_2R receptor signaling pathways. However recent evidence suggests that it may be involved in the control of cell proliferation, cell differentiation and development, angiogenesis, wound healing, tissue regeneration and generally counteracts the affects of AT_1R [101]. A well-established AT2R signaling mechanism is via NO/cGMP pathway, which is thought to be one of the vasoprotective actions of the AT2R. Expression of AT_2R is also observed to be up regulated during certain circumstances such as in heart failure, post-infarct repair, and skin and nervous system lesions [101].

Ang (1-7) and MasR:

The identification of the Mas receptor (MasR) in 1986 by Young et al. marked an important step in the understanding of the RAS [104]. The Mas proto-oncogene was originally detected due to its ability to render NIH 3T3 cells tumorigenic and was initially identified as a transforming protein when overexpressed in the NIH 3T3 cells [104, 105]. It is a G-protein coupled receptor, which includes a large family of cell surface receptors that mediate the actions of a diverse array of extracellular ligands [87, 104, 105], and is highly expressed within the cardiovascular system [106]. Transfection studies had proposed that the Mas gene encoded an AngII receptor [98], however several other studies concluded that AngII induced activation was only in cells endogenously expressing the AngII AT_1R [87, 107]. Furthermore, a radio-ligand-binding study with AngII in the kidney of wild type and MasR knockout ($^{-/-}$) [108] mice found that there is a clear interaction between MasR and Ang (1-7) [87]. Additionally the vasodilatory effects of Ang (1-7) were absent in the $MasR^{-/-}$ model [92, 109]. The anti-fibrotic effects of ACE2 were predominantly achieved through the MasR, as the $MasR^{-/-}$ mice displayed a predisposition to pro-fibrosis and mesenchymal stem cell proliferation in the cardiovascular system [110]. Therefore, based on these findings it was concluded that Ang (1-7) binding with MasR exerts a definite biological action, including prevention of organ fibrosis [87].

Long term infusion of Ang (1-7) was shown to have a vasoprotective and atheroprotective effect in ApoE knockout (ApoE $^{-/-}$) mice with increased eNOS expression and an overall improvement of endothelial function, along with reduced atherosclerosis development in both ApoE $^{-/-}$ and low-density lipoprotein receptor deficient mice fed on a high fat diet [106, 110-112]. Ligation studies where the left coronary artery is ligated and perfused with either Ang (1-7) or saline revealed that Ang (1-7) prevented the deterioration of cardiac function [113]. Furthermore AT_1R blockers and ACE inhibitors were administered to partially ligated abdominal aorta of rats to examine the affects on the expression levels of ACE2 and Mas receptor (MasR) [91]. AT_1R blocker caused an increase in

the expression of cardiac ACE2 along with an increased MasR level indicating an activation of the ACE2/Ang (1-7)/MasR axis [91]. However, the ACE inhibitor effects were shown elevate the ratio of cardiac ACE2/ACE since it was able to considerably reduce the level of ACE [91]. This suggests that an increased level of ACE2 and MasR may protect the heart against hypertension [91].

The potential for the ACE2/Ang (1-7)/MasR axis to be manipulated as a therapy for CVD, vascular occlusions that induce cardiac hypertrophy, hypertension and fibrosis has also been examined [90, 91], as it has anti-inflammatory, anti-proliferative and anti-oxidative stress properties [106]. The ACE2/Ang (1-7)/MasR axis has been demonstrated to counter the adverse effects of AngII and the AT_1R in a variety of inflammatory disease models, including hypertensive kidney disease, arthritis, asthma, acute respiratory disease and atherosclerosis [90, 111, 114, 115]. Treatment with Ang (1-7) on bleomycin lung fibrosis model, a model dependent on inflammation and oxidative stress was protected by the inhibition of ROS-meditated pathways [99, 116].

In recent studies the action of the ACE2/Ang (1-7)/MasR axis was shown to be regulated by changing the MasR expression in response to different pathophysiological conditions [92]. However, no significant changes were observed in MasR expression in normotensive rats undergoing physiological training, therefore supporting the view that physical training changes MasR expression primarily in diseased states [92]. Our lab has previously shown that $MasR^{-/-}$ mice model significantly increased intimal thickening by 4.5 fold when compared against a control group [117]. Furthermore, recent unpublished data has suggested that the cells that proliferate in an *in vitro* model in $MasR^{-/-}$ are mesenchymal stem cells. Our preliminary findings from unpublished data supports the idea that cellular migration and proliferation observed in diseased arteries could be caused by a lack of MasR regulatory anti-athero-protective properties [117].

Ang (1-7) increases NO production through the MasR and counters the effects of Ang II (e.g. stimulation of endothelial cells, cell proliferation, stimulation of NADPH in vascular smooth muscle cells), thus retaining endothelial function [111]. It has also been shown that stimulation of the MasR is able to inhibit the deleterious effects of Nox [118], as well as mediating fibrosis [116], cellular proliferation, and vascular remodeling [110, 117, 118]. Thus MasR$^{-/-}$ mice should exhibit a worsened disease stated when compared with the wild type mice; where Nox activity is elevated leading to increased fibrosis, cellular proliferation and vascular remodeling see Figure 3. Evidence from our lab had initially suggested that MasR ($^{-/-}$) did not effect cellular proliferation[117] however recent data in our laboratory (unpublished data) was able to show proliferation of mesenchymal stem cells, although we suggest that a different number of markers may be required to identify a possible second population of cells.

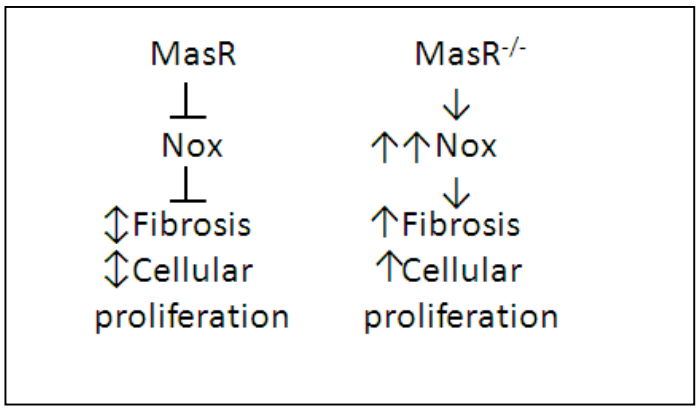


Figure 4. Overview of proposed rationale. A double-headed arrow signifies a balanced system. MasR$^{-/-}$ model increases Nox production leading to increased fibrosis and cellular proliferation. MasR can inhibit Nox thus, controlling fibrosis and cellular proliferation.

Aortic fibrosis and aortic compliance

It is accepted that CVD is associated with vascular fibrosis. Vascular fibrosis is understood to be an accumulation of extracellular matrix proteins, in particular collagen and fibronectin within the vascular media, which leads to structural remodeling and scar tissue formation [119]. Vascular

fibrosis is recognized as an important contributor to arterial stiffening and is also known to promote the development of atherosclerosis [120, 121]. Fibrosis or increased stiffening of the vascular walls can be attributed to a lack of elastin or excessive collagen [122].

Hyperhomocysteinemia has been reported in previous studies to increase collagen accumulation in the carotid artery in mice and rats [123]. Remodeling of vascular tissue contributes to an increased peripheral resistance, and is known to impact both development and complications of hypertension [124]. Biopsies of abdominal aortic aneurysms from patients revealed vascular remodeling and degeneration as well as a dilation of two to six times the normal aortic diameter [125]. Contrary to the belief that an enlarged aorta with the risk of rupture increasing, that the walls of the aorta may become thinner, it was discovered that the opposite was true, where the larger the aneurysm the thicker the walls and the smaller the aneurysm the thinner the walls [125]. Although the larger aneurysms present with thicker walls, they were thought to be weaker, thus leading to the much higher risk of rupture than smaller aneurysms [125].

Myocardial fibrosis in CVD

Long-term experimental models have established that hyperhomocysteinemia is a risk factor for systolic heart failure [126], and may also accelerate hyperhomocysteinemia-induced cardiac remodeling [127]. These hyperhomocysteinemic models have also suggested the presence of ventricular hypertrophy and heart fibrosis [126, 128, 129]. It was presented that homocysteine was able to induce an increase of collagen content and ventricular hypertrophy [130], which is in line with previous studies exhibiting a marked interstitial myocardial fibrosis in hypertensive and normal rats, as well as rabbits subjected to a diet supplemented with methionine and cholesterol enriched diet [126, 128, 129, 131]. Hyperhomocysteinemia also affects several other tissues, for example, along with myocardial fibrosis, liver fibrosis, lung fibrosis, and glomerulosclerosis; it also increases the collagen content of aortic atherosclerotic plaque in atherosclerosis mouse models [132].

Preclinical studies have further added to the understanding that hyperhomocysteinemia promotes myocardial fibrosis and dysfunction [126, 133, 134]. Remodeling of the cardiac tissue was also observed in rat models of mild hyperhomocysteinemia, which was associated with dysregulation of extracellular matrix degradation [130]. Although these collagen deposits can be attributed to an increase of collagen synthesis and/or decrease in collagen degradation, homocysteine was revealed to stimulate and elevate collagen, which was accompanied by cell proliferation[135-137].

All organs including cardiac tissue are composed of specialized parenchymal cells enclosed in stroma consisting of extracellular matrix, tissue fluid and undifferentiated, pluripotent mesenchymal cells [138]. Matrix integrity relies on synthesis and degradation of all its major components, including collagens. Extracellular matrix homeostasis is integral for cardiac function, as alterations can result in development of myocardial fibrosis leading to systolic and diastolic dysfunction [139]. Accumulation of collagen in response to injury further exacerbates the structural loss of myocyte due to ischemic necrosis, and progresses scar tissue formation [139]. This continued accumulation of collagen compromises and impairs contractile behavior leading to heart failure [138]. Myocardial fibrosis has also been linked with various other myocardial diseases, all of which fibrosis is observed to be increased, thus leading to a correspondingly increased susceptibility to arrhythmia [140]. Patchy fibrosis throughout the right ventricle was shown to increase susceptibility to arrhythmias and is thought to act as an anatomical substrate favoring arrhythmias [141].

The myocardial collagen networks consists of collagen I and III (85% and 10%, respectively), as observed in rodent and non-human heart tissue, with scar tissue consisting of 90% type I collagen [139, 142, 143]. Type I collagen provides rigidity and myocardial stiffness due to its tensile strength [144], while type III collagen contributes to elasticity as it consists of a fine reticular network [144, 145]. Alterations to the concentration of collagen and several other factors determine the development of

fibrosis [139, 143]. Cells capable of synthesizing collagen include endothelial cells, osteoblasts, and smooth muscle cells, with myofibroblasts being identified as the predominant cells responsible for the development of cardiac fibrosis [138, 139]. Studies have identified a correlation between cardiac function and myocardial remodeling with reduced ejection fraction related to worsening fibrosis and myocyte degeneration and elevated left ventricular end-diastolic pressure [146].

Renal disease in CVD:

Endothelial dysfunction is a well-accepted characteristic of atherosclerosis and is present within both large and small arteries in renal disease [147]. Incubation of rat small renal arteries with homocysteine reduces NO in renal artery endothelium. This decrease in NO not only results in endothelial dysfunction, but also in an increase in superoxide levels in the arterial endothelium [36, 39]. In normal kidney physiology, NO regulates vascular homeostasis, glomerular, and tubular function maintaining normal renal function, GFR and renal vascular resistance, vascular resistance [148, 149].

Biopsies taken from patients with severe kidney disease exhibit prominent fibrosis in the renal vasculature, glomeruli and tubuolinterstituim [95]. Studies have demonstrated that chronic activation of RAS can result in fibrosis and inflammation [150, 151] and production of Ang II (a potent vasoconstrictor) is a significant mediator of tubulointerstitial fibrosis and progression towards end-stage renal disease [95]. Moreover, evidence suggests hyperhomocysteinemia leads to renal oxidative stress and renal hemodynamic dysfunction due to altered L-Agr-NO pathway, and this may aggravate vascular disease as a result of renal failure [152].

Thus, the idea of the ACE2/Ang (1-7)/MasR axis, and its role in countering the effects of ACE/Ang II/AT_1R effects needs to be further investigated. It is known that local RAS is expressed in the kidneys and is able to function independently of the systemic RAS and is thought to contribute to

long-term blood pressure regulation and renal injury [153]. In order to elucidate the protective function of the ACE2/Ang (1-7)/MasR pathway, a study investigating the protective affects of $MasR^{-/-}$ mice with 1% methionine enriched diet was performed [154]. It was discovered that the diuresis effect produced by AVE 0991, a MasR nonpeptide agonist, was significantly reduced in $MasR^{-/-}$ mice [154]. The researchers concluding that MasR plays a role in kidney fluid homeostasis [154]. As the ACE2/Ang (1-7)/MasR axis are critically involved in kidney function, it remains unclear if this axis is protective against an unbalanced diet causing kidney disease, such as renal fibrosis.

Hypothesis

It is hypothesized that a high methionine diet will exacerbate vascular pathology in MasR($^{-/-}$) mice.

Aims

To determine if a high methionine diet in MasR($^{-/-}$) mice will worsen:

a) endothelial function

b) blood vessel collagen accumulation

c) myocardial interstitial and perivascular collagen accumulation

d) renal glomerular and tubule collagen accumulation

Materials and Method:

Animals:

Male MasR$^{-/-}$ (n=24) mice were be donated by Dr. Chandana Hearth, Austin Health. Wild type control (C57BL/6J), (n=16) were purchased from the Animal Research Centre, WA. The mice were divided into groups and fed their diet for 8 weeks:

1. MasR$^{-/-}$ - control diet (MasR-Control), n=12;
2. MasR$^{-/-}$ + 1% methionine (MasR+Meth) diet, n=12;
3. C57BL/6J – control diet (C57-Control), n=8
4. and C57BL/6J + 1% methionine diet (C57+Meth), n=8 [131, 155].

Mice were housed maximum 5 per cage and maintained on 12-h dark/12-h light cycle in an air-conditioned room (22.5±0.5°C, 50±5% humidity) with access to diet and water *ad libitum*. The mice were divided into 2 study groups, an acute organ bath and a fibrosis study group. Mice were culled and organs extracted (aorta, heart, and kidney). The experiments were approved by Victoria University, Animal Ethics Committee (approval # 14/005).

Organ bath – endothelial function:

The mice were anesthetized using oxygen and 4% isoflurane. A foot pinch test was performed to ensuring the mouse has been completely anesthetized before the mouse were then culled by cervical dislocation, and ensuring the mouse is unresponsive and not breathing. The aorta were taken from mice and cut into ~3mm rings. The rings were then mounted between two metal hooks in 5ml organ baths attached to force displacement transducers, which was in turn connected to a PC using MediDAQ software that allows the tension to be measured (OB8, Zultek, Engineering, VIC, Australia). The ring were placed in a normal physiological salt solution filled with Krebs solution [mmol/L: (NaCl 118mM, KCl 4.7mM, $NaHCO_3$ 25mM, $MgSO_47H_2O$ 1.2mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM, glucose 11.7mM)] and kept at a constant temperature of 37^{o}C and continuously

bubbled with 95% O_2 and 5% CO_2. The organ bath system is a traditional method allowing isometric or isotonic measurement [155, 156]. The rings were held to a basal tension of 0.5g and allowed to equilibrate for 30 minutes [155]. The rings were then washed once with fresh Krebs solution. The rings were then pre-constricted with U46619 (Sigma, #D8174-5MG) a thromboxane A_2 mimetic to approximately 30% - 50% of maximal contraction (where maximal range is between 0.9-1.2g total tension); after which a cumulative response curve to acetylcholine-induced relaxation was performed [155].

Fibrosis

These diets have previously been used in our lab to induce an atherosclerotic state in rabbits [57, 131, 155] and it was predicted to also occur in the current mice. The heart, kidney and aorta were removed and immediately placed in 4% PFA (Sigma, #158127-500G) for 24hrs, after which the 4% PFA was removed and replaced with 1 x PBS and refrigerated at 4^{o}C ready for processing. The tissue was processed by initially removing water from the tissue using a series of ethanol solutions of increase concentration until 100% water-free ethanol. Then a clearing process that involves using 3 different xylene immersions, which gradually replaces the ethanol with xylene. Finally the xylene is replaced by molten paraffin wax (SkinPath Pathology, Hawthorn East, VIC). Tissue was then embedded in paraffin and refrigerated at 4^{o}C until required. Sections were cut at 5 microns and dried over night at 37^{o}C on 26mm x 76mm glass slides.

Organ fibrosis was determined using a combination of Fast Green FCF (Sigma, #F7252-5G), Picric acid (Sigma, #197378) and Direct Red (sirius red) (Sigma,#365548-5G), which binds to collagen fibrils [131, 157]. Initial tests using fast green-picro-sirius red were performed to optimize the visual staining, as each tissue varied in colour with the same concentration. The following concentrations of fast green and Sirius red were dissolved in picric acid; 0.1% of both fast green and Sirius red, 0.01% fast green and 0.1% Sirius red, and 0.01% of both fast green and sirius red. The de-waxing

and dehydrating method is as follows; The picro-sirius red/fast green stain solution was prepared with the 3 different concentrations, **a)** 0.1% fast green and 0.1% sirius red in 100ml (saturated aqueous picric acid solution) in Coplin jar, **b)** 0.01% fast green and 0.1% sirius red in 100ml (saturated aqueous picric acid solution) in Coplin jar, **c)** 0.01% fast green and 0.01% sirius red in 100ml (saturated aqueous picric acid solution) in Coplin jar. Then the slides were run through 100% xylenes 2X 10min, 100% ethanol 2X 20dips, 90% ethanol 1X 20dips, ddH_2O 2X 2min, and placed in appropriate Coplin jars for 60 minutes. The slides were rinsed quickly in 10dips of ddH_2O and immediately rinsed in 90% ethanol 1X 20dips, 100% ethanol 2X 20dips, 100% xylenes 2X 20dips. The slide is then placed in a third 100% xylene, and finally a coverslip was placed on the slide with DPX mounting medium and left to dry overnight.

After initial analysis, the optimal concentration for heart was **c)** 0.01% fast green and 0.01% sirius red in 100ml (saturated aqueous picric acid solution), with kidney being **b)** 0.01% fast green and 0.1% sirius red in 100ml, and finally aorta with a concentration of **a)** 0.1% fast green and 0.1% sirius red in 100ml. These three concentrations were then used in the final analysis process as shown in Figures 4 - 12.

Immunohistochemistry:

Standard immunohistochemistry process was performed for collagen I (rabbit polyclonal anti-collagen I antibody, ab34710, abcam) and III (rabbit polyclonal anti-collagen III antibody , ab7778, abcam) with a dilution of 1:100 as follows; slides were run through 100% xylenes 2X 10min, 100% ethanol 2X 20dips, 90% ethanol 1X 20dips, ddH_2O 2X 2min, and 10mM Tris and left for 5mins. 200µl of blocking solution (ImmPRESS 2.5% normal goat serum blocking solution) was added onto tissue and placed into humidified sealed incubation camber for 20mins. The blocking solution was tipped off at 20mins and 200µl of primary antibody was added to the tissue and placed into humidified sealed incubation chamber overnight. The primary antibody was then tipped off and the

slide washed in Tris 10mM for 20dips and left for 5mins. 200µl of Secondary antibody (ImmPRESS HRP anti-rabbit IgG) was then added to the tissue and incubated for 1 hour. The slide was washed in Tris 10mM for 20dips and left for 5mins. DAB Chromogen was prepared by adding 1 drop of DAB chromogen per 1ml of substrate buffer. 200µl of DAB solution was added to tissue until browning was visible and no longer than 5mins to prevent over exposure. The DAB solution was tipped off and washed in ddH_2O 20dips. A 10ml syringe was used to draw up haematoxylin and a filter attached prior to applying to the slide for 1-2mins to ensure nuclei staining. Slides were washed once again in ddH_2O 20dips, and then placed in Scott's tap water (bluing solution) for 5-10mins. Slides were washed in ddH_2O 10dips and put through the dehydration process; 90% ethanol 20dips, 100% ethanol 2X 20dips, 100% xylene 2X 20dips, and finally into a third 100% xylene before mounting the coverslip with DPX mounting solution and left to dry overnight. Negative controls were performed using Rabbit IgG, polyclonal - Isotype Control (Abcam, #ab37415).

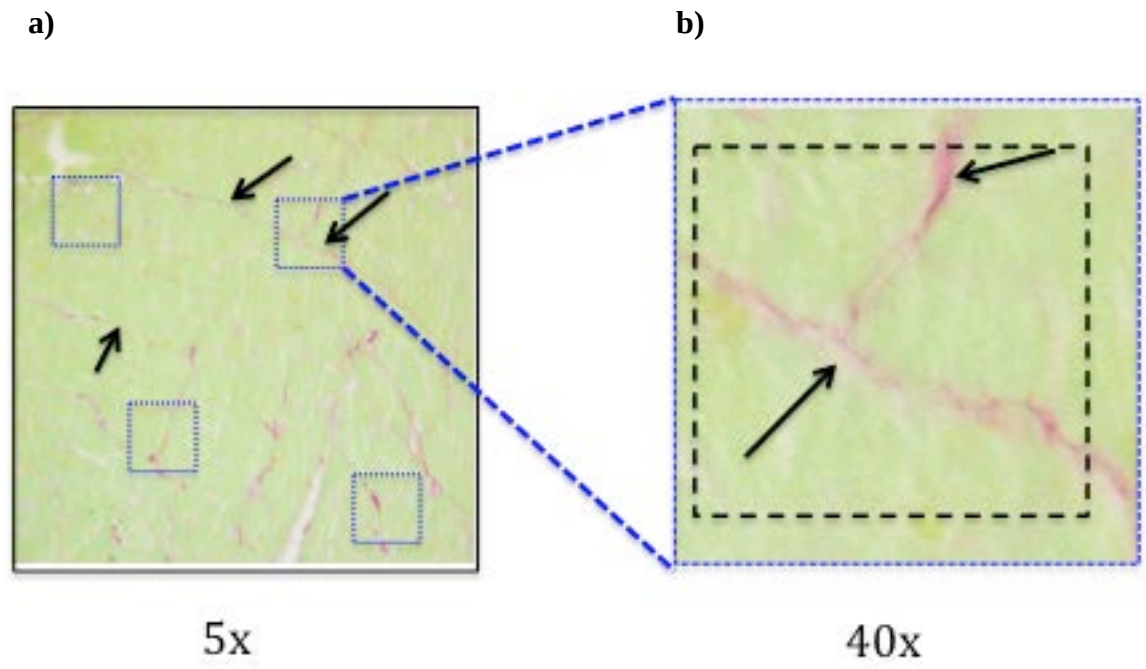


Figure 5.

Four sections of tissue were imaged, shown with the blue dotted line indicating the image observed on the Lecia software at 40X magnification. Black arrows indicate myocardial fibrosis detected by

fast green/sirus red staining. At 5x magnification **a)** blue doted squares were imaged at 40X. The **b)** tissue was traced using a square template, observed here as a black dashed line. Myocardial vessels were traced to analyze fibrosis.

Fibrosis and Immunohistochemistry data gathering and analysis

All pictures were taken using the Olympus BX53 upright microscope and the Leica DFC425 digital microscope camera, and the Leica Application Suite X Core software. For each heart tissue section, four randomised pictures, representative of the whole muscle tissue as well as any visible blood vessels were taken at 40X magnification see Figure 4. Approximately a ~75% proportion of the images were then analyzed using MCID Core 7.1. Heart perivascular tissue was analyzed for fibrosis see Figure 4, by using a tracing pointer to draw around the perivascular wall and surrounding fibrosis and subsequently allowing MCID Core 7.1 software to determine differences is colour changes via predetermined colour parameters. The blue dotted squares areas were chosen due to fibrosis. The glomeruli and aortic tissue was chosen according to relative size and tractability, thus ensuring uniform tissue analysis data.

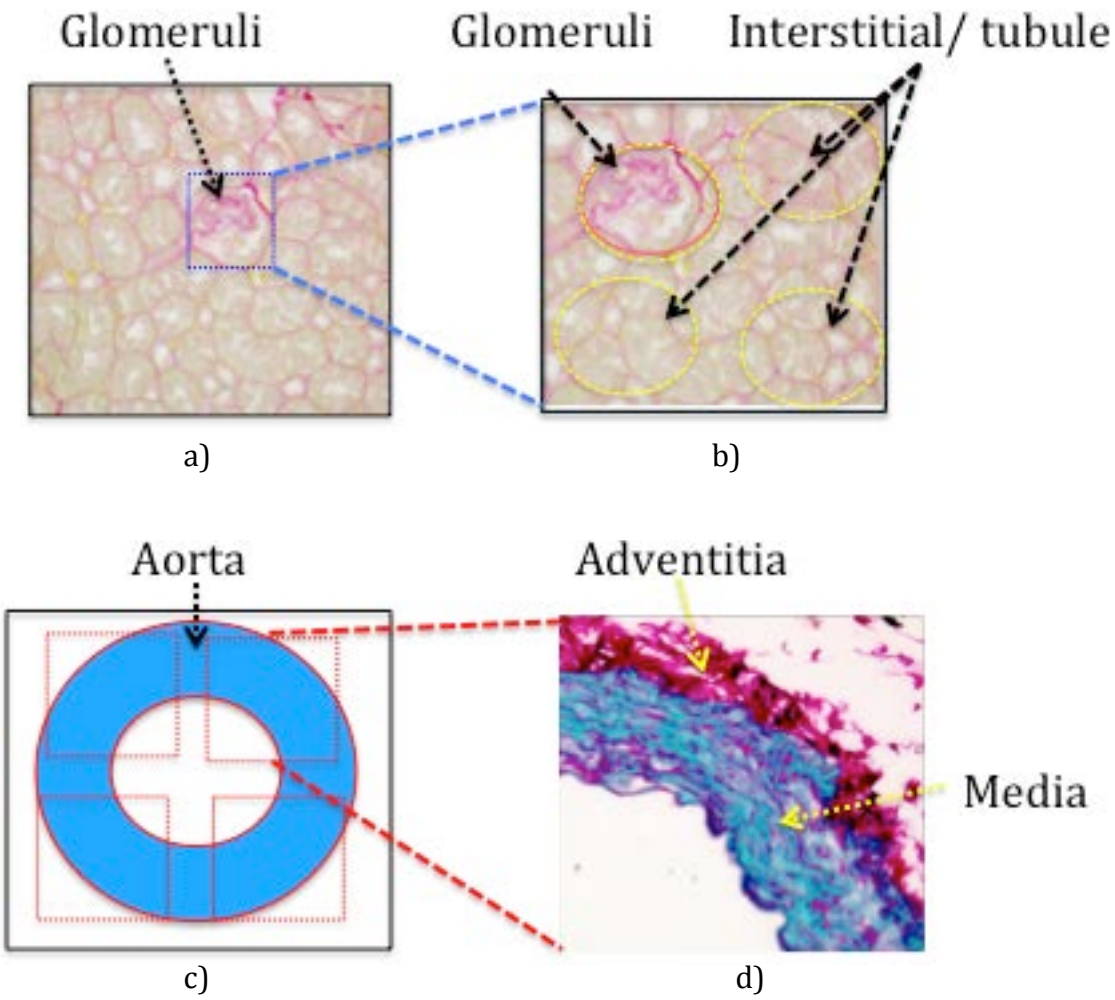


Figure 6.

In kidney **a)** black solid square represents the whole tissue, while the blue dotted square indicates the image observed on the Lecia software at 40X. The green circles with red fill represent glomeruli taken at 40X. **b)** Glomeruli and interstitial/tubule fibrosis were analyzed separately as shown by the yellow dashed oval. **c)** Aorta pictures taken are represented by the red dotted square at 40X. The **d)** adventitia and media were then traced (yellow dashed line) and analyzed separately.

Ten glomeruli images from each kidney section were taken at 40X magnification Figure 5. The glomeruli and interstitial/tubule images were separately analyzed, using MCID Core 7.1. Between two and eight images of the aorta were taken depending on the size of the aorta with larger aortas requiring greater amounts of images, following the contour of the blood vessel as observed in the Figure 5. The adventitia and media were traced and analyzed separately on MCID Core 7.1.

Results

To investigate the role of MasR in homocysteine induced vascular pathology, $MasR^{-/-}$ and C57BL/6 mice were fed a 1% methionine enriched or control diet. Endothelial function was determined in mouse aorta by organ bath methods and the response to acetylcholine was measured via signal transducers. Relaxation of the abdominal aorta is shown in Figure 6. Both C57BL/6 groups displayed a normal relaxation and there was no significant difference between the control and methionine diets. In contrast, the $MasR^{-/-}$ aorta displayed significantly less relaxation compared to the C57BL/6 mice (32.3±6.8% vs 66.1±7.7%, $p<0.001$). These results were further exacerbated in the MasR+Meth on the methionine diet, which displayed a further reduction in relaxation (6.6±6.7% vs 74±7%, $p<0.0001$).

In myocardial tissue analysis using immunohistochemistry (IHC), see Figures 9 and 10, 1% methionine feeding increased myocardial interstitial fibrosis by >3-fold ($p<0.0001$) in MasR+Meth mice. However there was no significant increase in fibrosis observed in C57BL/6 mice between on 1% methionine or normal chow diet. In contrast, myocardial perivascular fibrosis was present in C57BL/6 mice, displaying a 31% ($p<0.001$) increase while MasR+Meth mice displayed a 2.6-fold ($p<0.001$) in crease in fibrosis.

Organ fibrosis was investigated using IHC in the heart, kidney and aorta in order to investigate whether deletion of the MasR worsened the pathology caused by homocysteine. Renal fibrosis shown in figure 11 and 12, displayed a significant increase in glomerular and interstitial/tubular fibrosis. Methionine increased glomerular fibrosis by 22% in control mice ($p=0.02$), while a 2.1 fold ($p<0.0001$) increase was observed in MasR+Meth mice when compared to MasR+control mice. Renal interstitial/tubular tissue displayed the greatest level of fibrosis displaying a 5-fold ($p<0.0001$) increase however; this was only in MasR+Meth mice.

Interestingly, we found IHC analysis of our aortic medial and adventitial tissue did not present any significant increase in fibrosis, in MasR+Meth mice. By contrast, 1% methionine fed control mice ($p<0.01$) displayed significant aortic increase in fibrosis in both medial and adventitial tissue.

In addition, collagen I (Abcam, #ab34710) and III (Abcam, #ab7778) distribution was analyzed using IHC, see figures 13 – 18 for collagen I, and figures 19 – 24 for collagen III staining. Aorta, heart and renal tissues were analyzed, however no significant difference was detected between the animal groups; $MasR^{-/-}$ and C57BL/6 on either diets (1% methionine and normal chow). Furthermore, Rabbit IgG, polyclonal - Isotype Control (Abcam, #ab37415) used to analysis negative control displayed no positive staining.

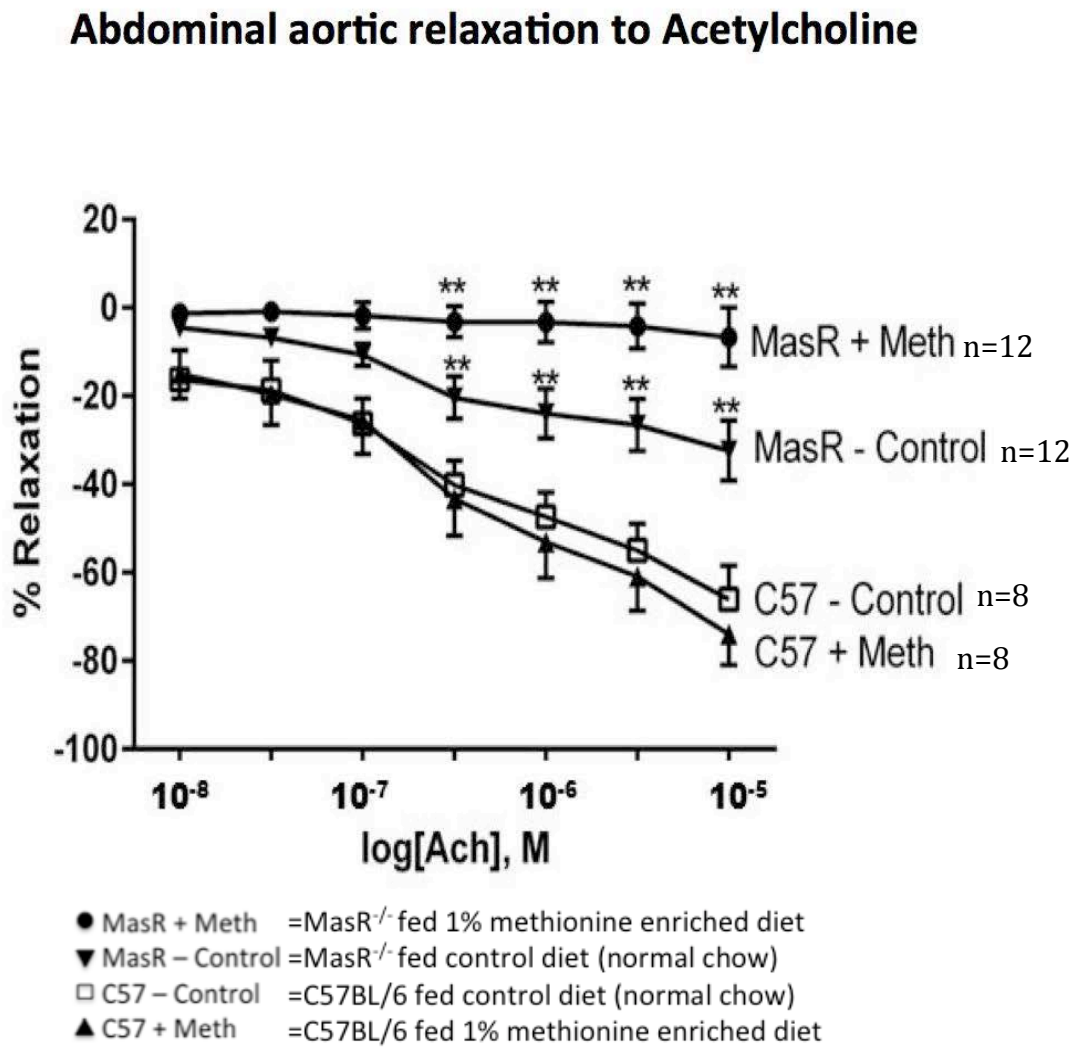


Figure 7.

Four groups were fed an 8-week normal mouse chow diet supplemented with 1% methionine as well as a control diet. MasR+Meth (n=12) (●), $MasR^{-/-}$ (n=12) and control (▼), C57 (n=8) and control (□), and C57 (n=8) + methionine (▲), were subjected to concentration response curves of endothelial dependent relaxation to acetylcholine in aortic rings. Preconstriction of (U46619, Sigma) aorta showed both C57 diet groups display relaxation, while a relaxation response was limited with $MasR^{-/-}$ + methionine group with the least relaxation present. Means ± SEM, **p<0.01

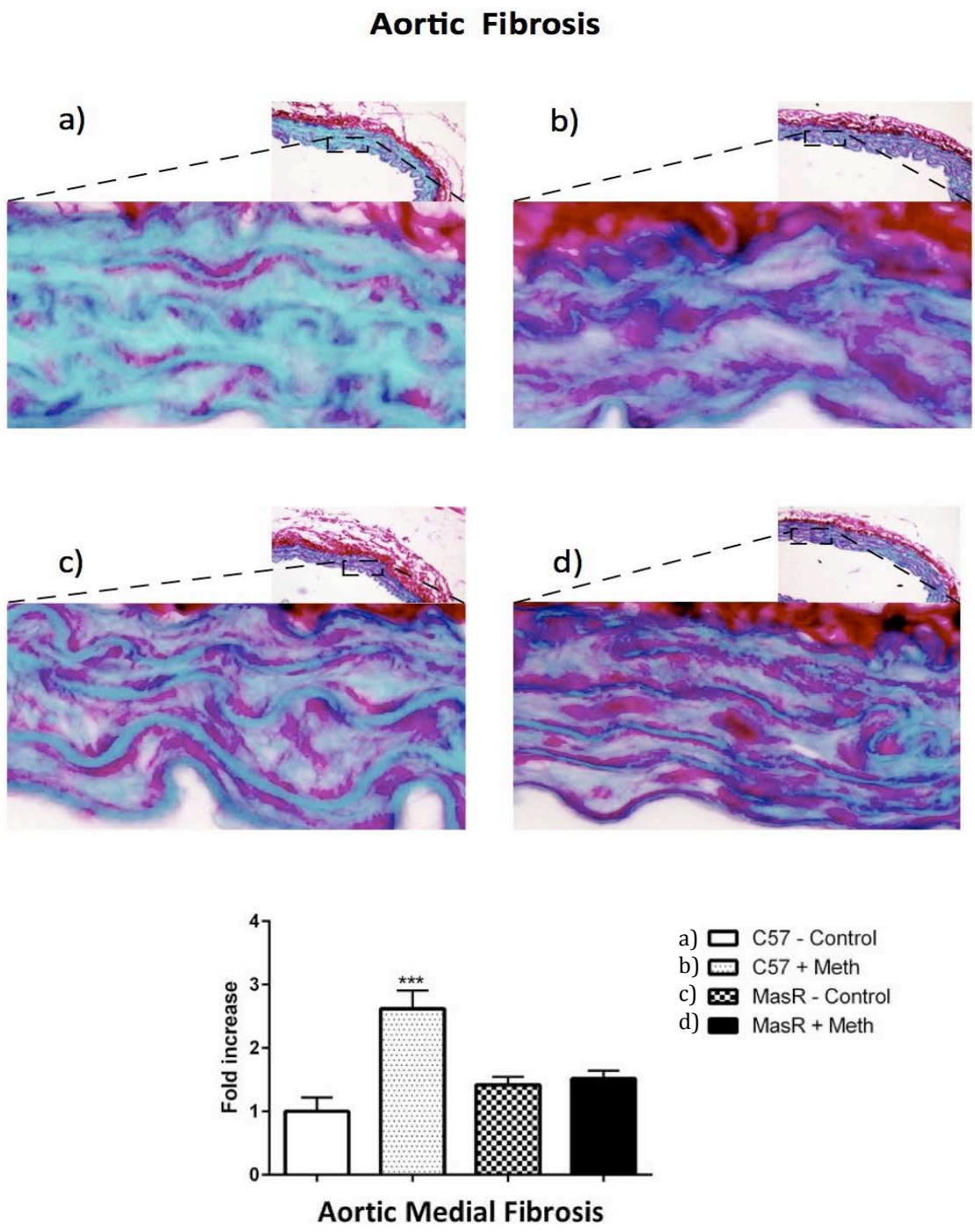


Figure 8.

Aortic medial fibrosis, analyzed using fast green and sirius red, where red is indicative of collagen, I and III. **a)** C57+control diet showing the least collagen content of all groups, **b)** C57+methionine diet appears to have to most collagen content with a higher distribution of red within the tissue sample, **c)** MasR$^{-/-}$ +control diet displays a higher collagen content (than C57 + control), **d)** MasR$^{-/-}$ +methionine only showed a slight increase in collagen content. Means ± SEM, ***p<0.001

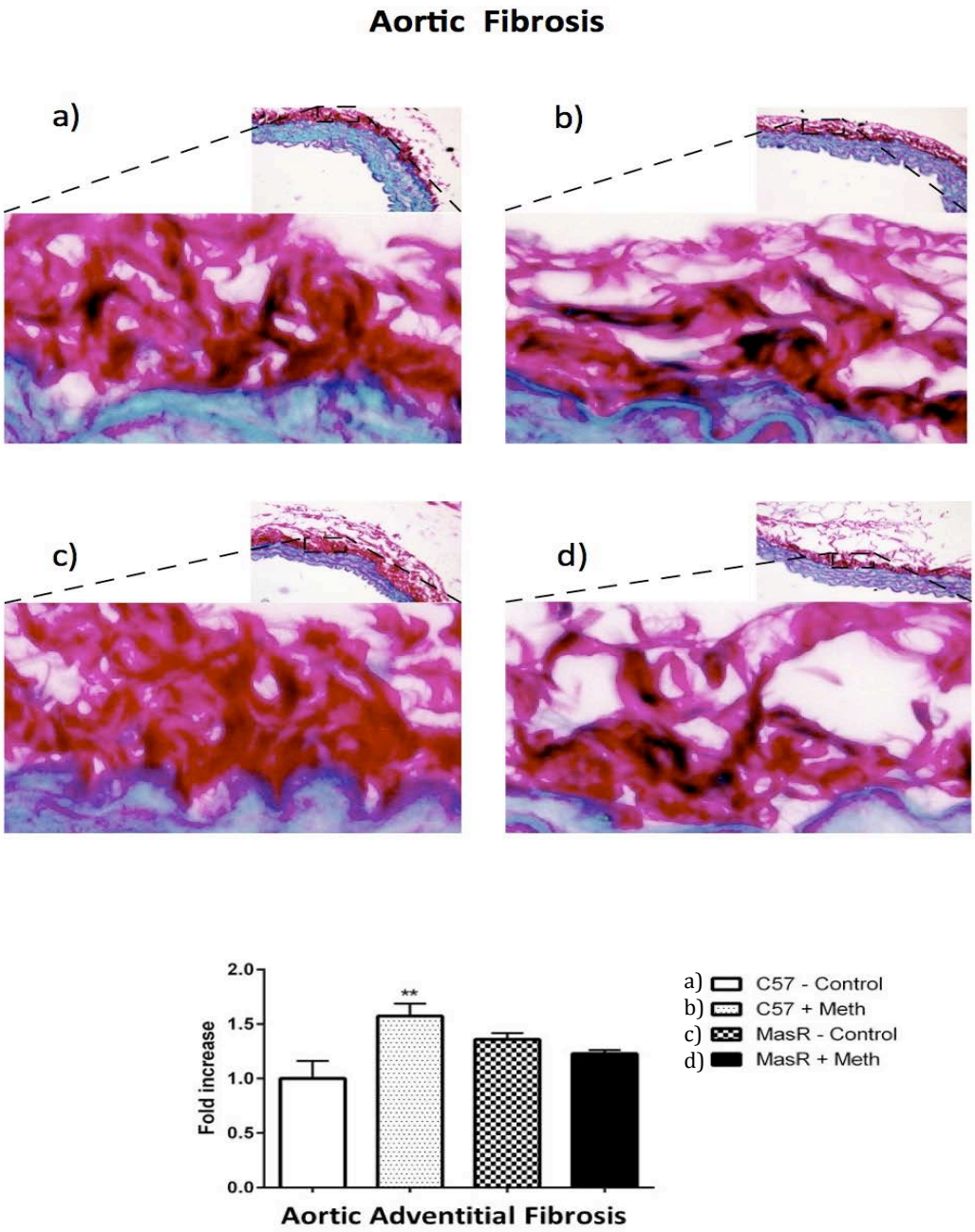


Figure 9.

Aortic adventitial fibrosis, analyzed using fast green and sirius red, where the colour red is indicative of collagen, I and III. **a)** C57 + control diet while not appearing to differ between the four samples displays the least collagen content post analysis, **b)** C57 + methionine diet was shown to have to most collagen content, **c)** MasR$^{-/-}$ + control diet had the second highest collagen content, with **d)** MasR $^{-/-}$ + methionine showing a slightly lower collagen content than the control. Means ± SEM, **p<0.01

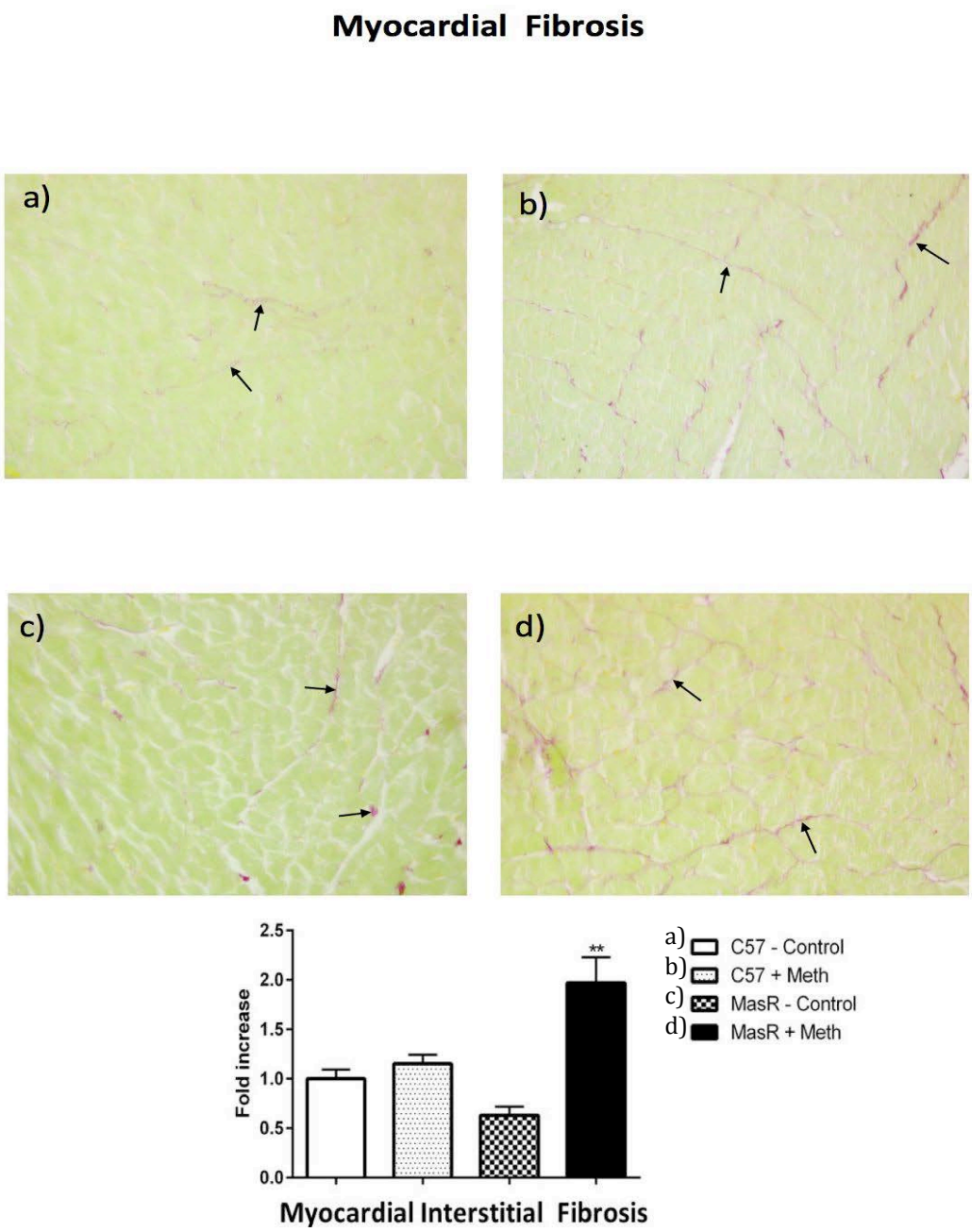


Figure 10.

Myocardial interstitial fibrosis was analyzed using fast green and sirius red, staining for protein and collagen respectively; arrows are used to denote collagen. **a)** C57 + control diet shows a sparse, though even distribution of collagen, while **b)** C57 + methionine indicates a similar distribution of collagen, however there seems to be a higher distribution of collagen in the myocardial tissue. **c)** $MasR^{-/-}$ + control shows the least collagen content, with very sparse distribution of collagen, and in contrast to the previous three groups, **d)** $MasR^{-/-}$ + methionine diet yielded the highest level (a 2 fold increase) of collagen content within myocardial tissue, with even and more visible distribution. Means ± SEM, **p<0.01

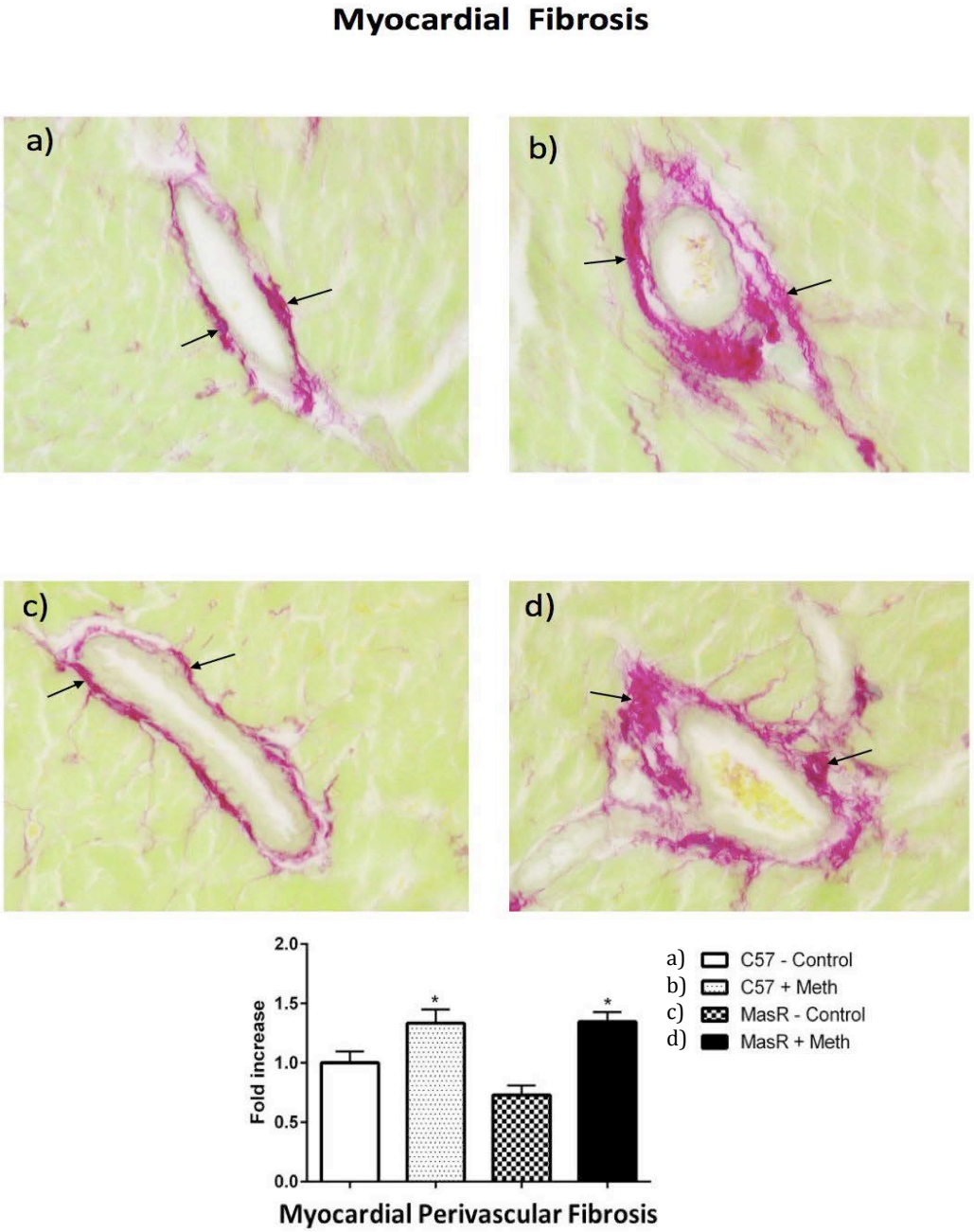


Figure 11.

Myocardial perivascular fibrosis is analyzed using fast green/sirus red staining for protein and collagen respectively. **a)** C57-Control diet shows a thin layer of perivascular collagen and has the second lowest content of collagen, however **b)** C57 + methionine had a more pronounced increase in perivascular collagen content which is also quite evident visually, while the **c)** $MasR^{-/-}$ + control has the lowest detected level of perivascular collagen distribution, and finally **d)** $MasR^{-/-}$ + Meth had a high level of perivascular collagen detected. Means ± SEM, *$p<0.05$

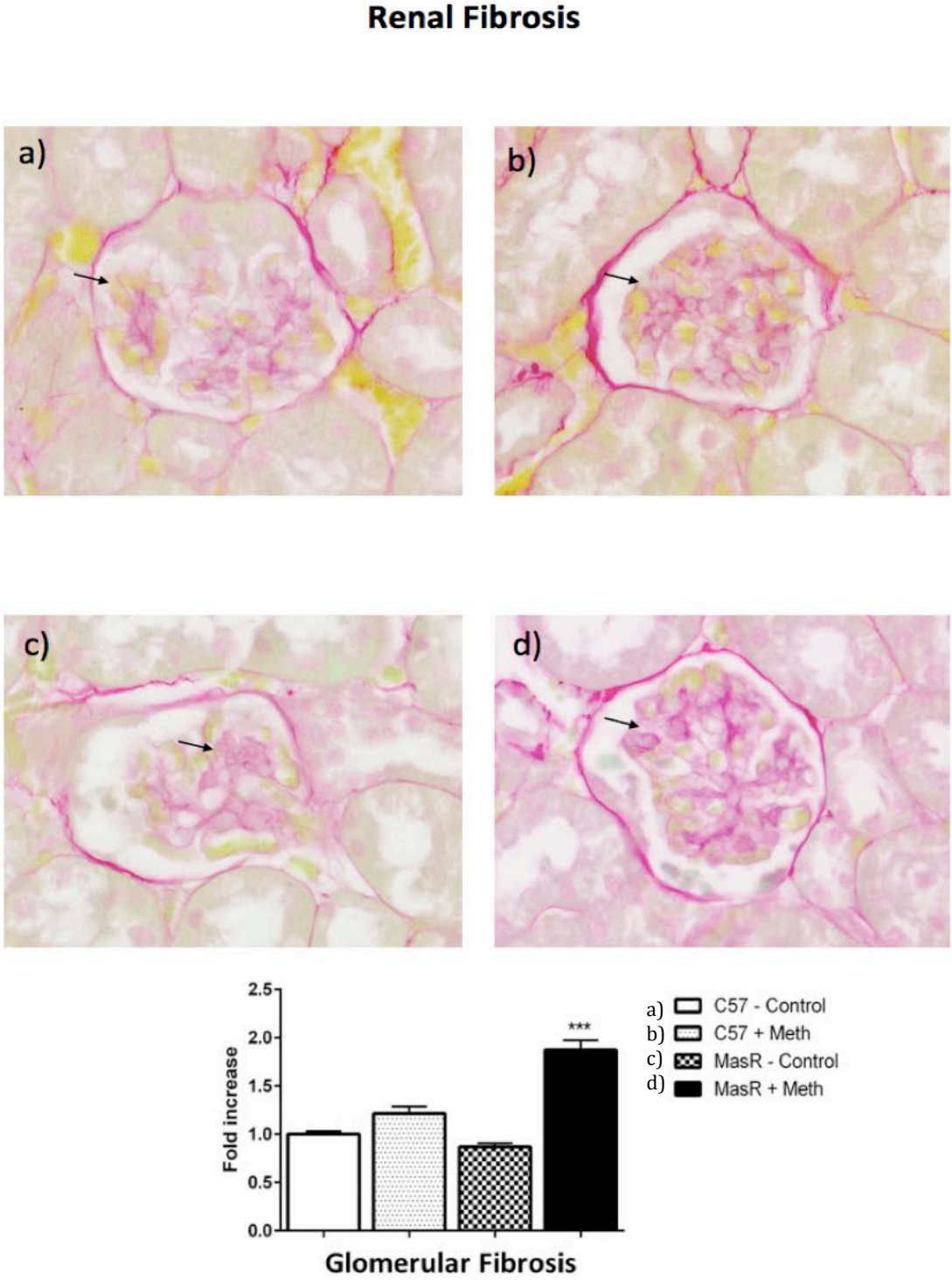


Figure 12.

Glomerular fibrosis is analyzed using fast green and sirius red staining for protein and collagen respectively. **a)** C57 + control, **b)** C57 + methionine, and **c)** $MasR^{-/-}$ + control all show similar levels of glomerular collagen content, with $MasR^{-/-}$ + methionine indicating the lowest collagen content, however **d)** $MasR^{-/-}$ + methionine has a significant (almost a 2 fold) increase in glomerular collagen content, which can visualized. Means ± SEM, ***$p<0.001$

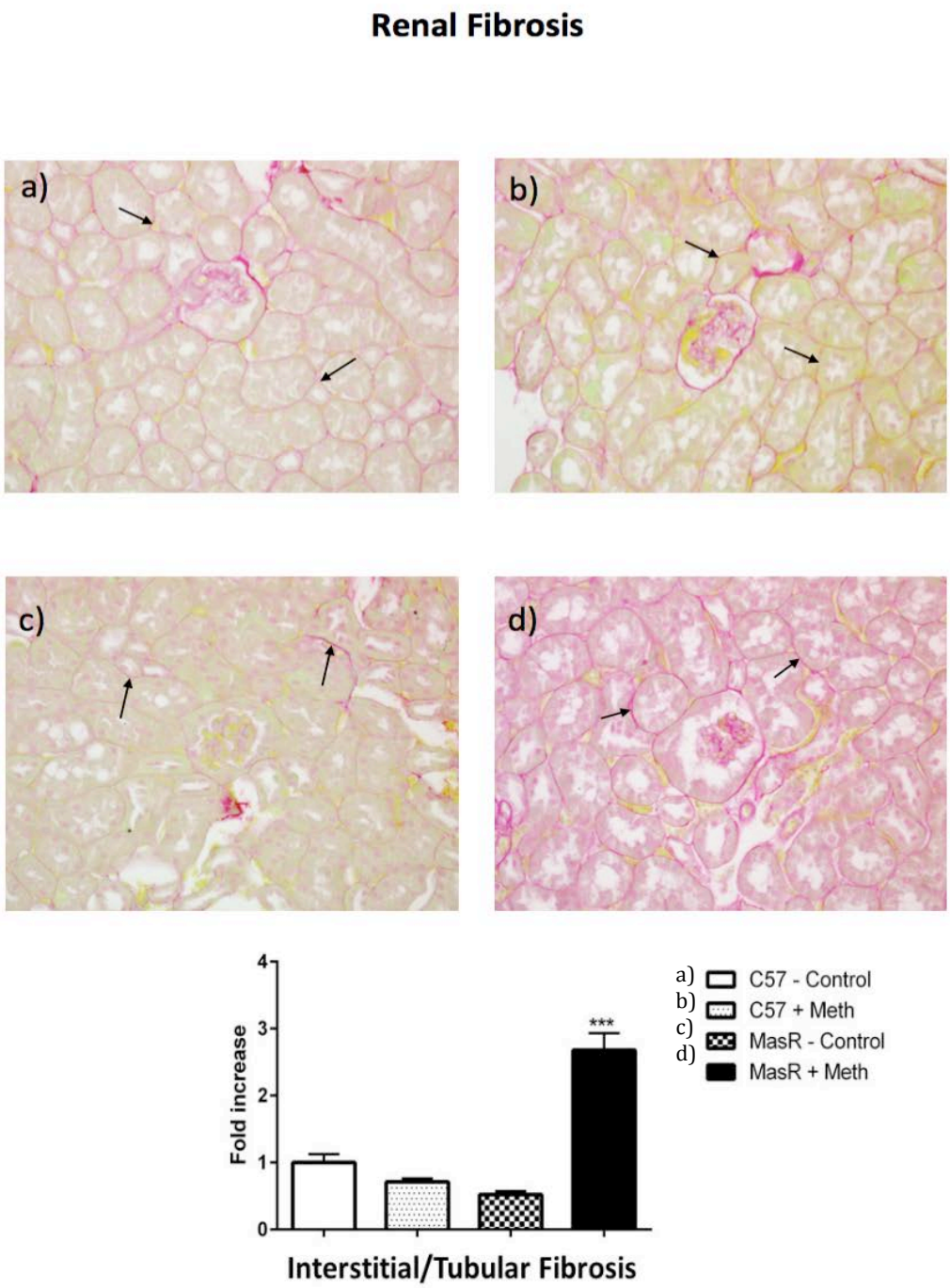


Figure 13.

Interstitial/tubular fibrosis is analyzed using fast green and sirius red staining for protein and collagen respectively. **a)** C57 + control, **b)** C57 + methionine, and **c)** $MasR^{-/-}$ + control all show similar levels of glomerular collagen content, interestingly the $MasR^{-/-}$ + methionine shows the lowest collagen content, however **d)** $MasR^{-/-}$ + methionine has a significant (more than a 2 fold) increase in glomerular collagen content, which can easily visualized by the deep red. Means ± SEM, ***p<0.0001

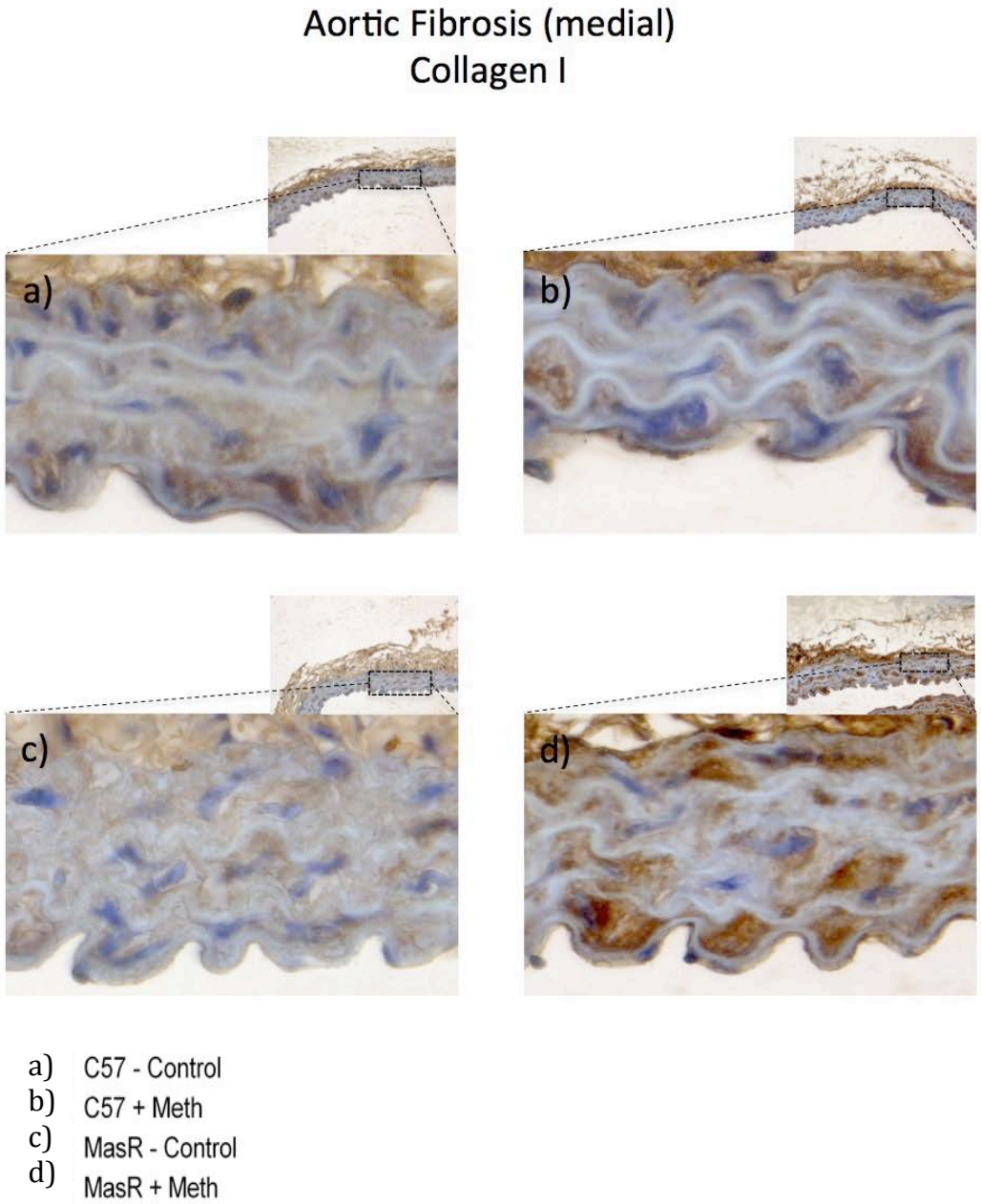


Figure 14.

Aortic medial fibrosis collagen I. Positive generalized non-specific brown staining is observed throughout the aortic media tissue. Positive adventitial staining is also displayed. No significant increase was observed between the groups.

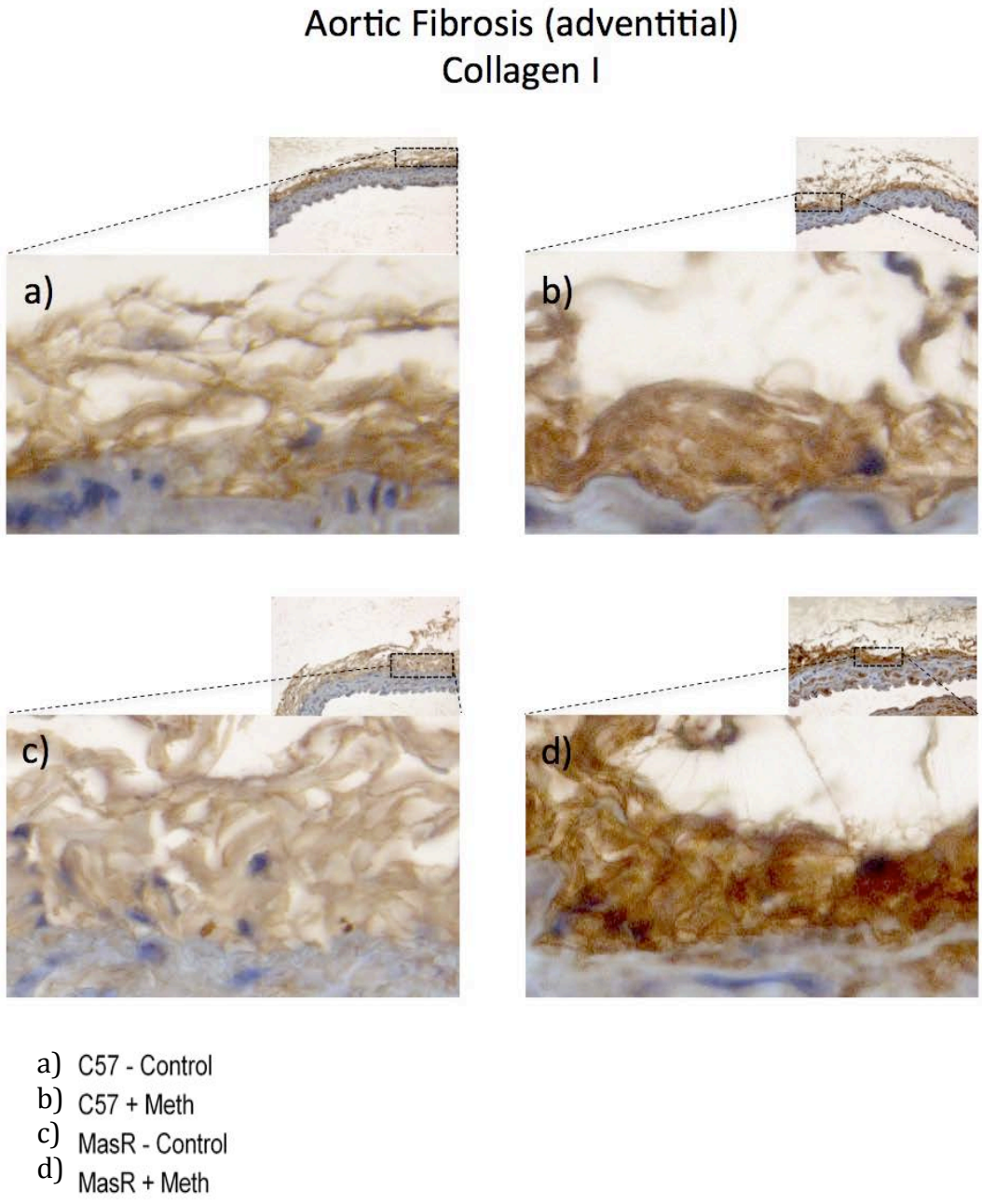


Figure 15.

Aortic adventitial fibrosis collagen I. Positive brown staining is present in aortic adventitial tissue, however no significant increase was identified between the groups.

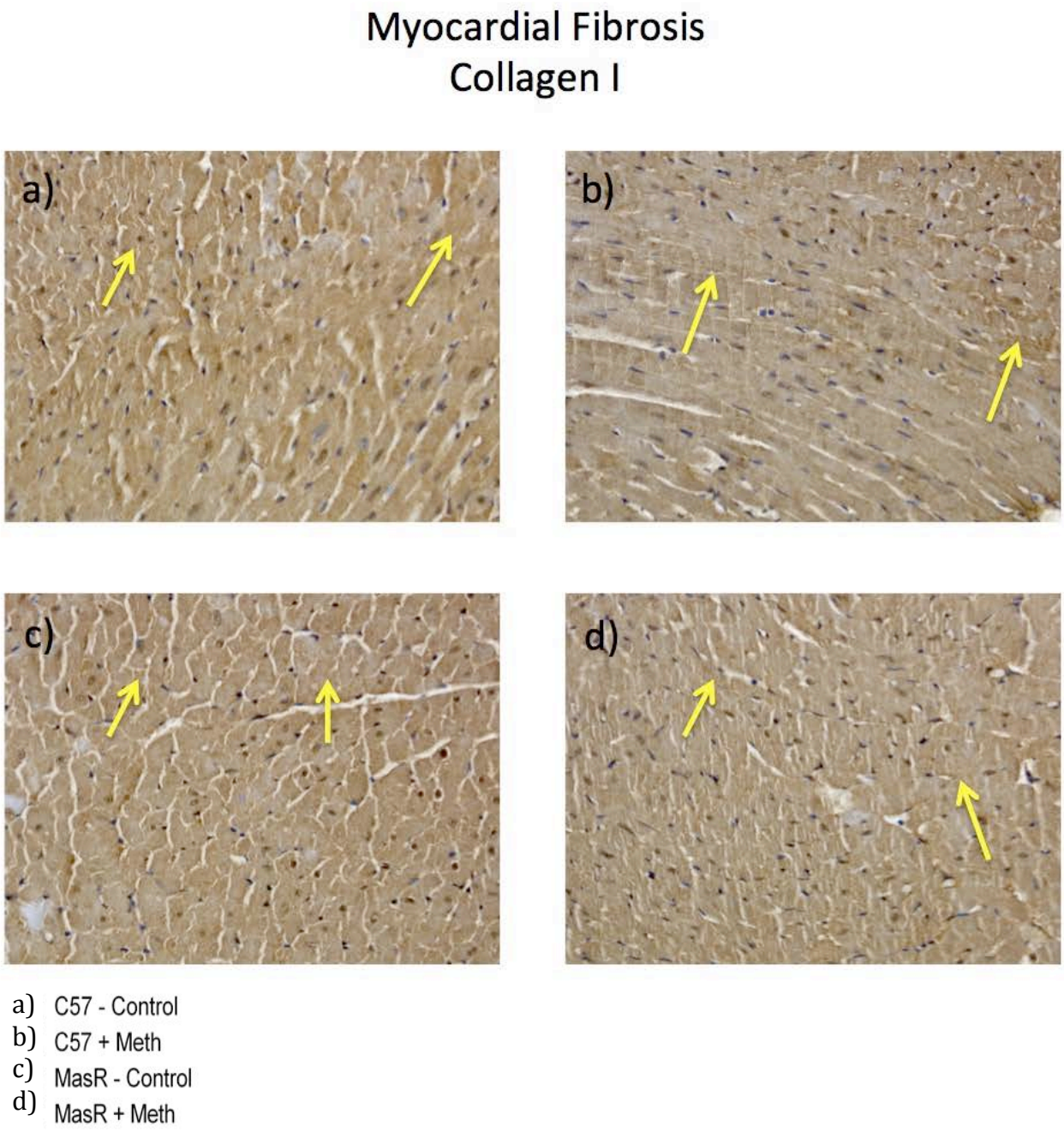


Figure 16.

Myocardial interstitial fibrosis collagen I staining. Yellow arrows denote positive brown staining in myocardial tissue. Generalized non-specific positive staining is observed within myocardial interstitial muscles. No significant increase was discovered between the groups.

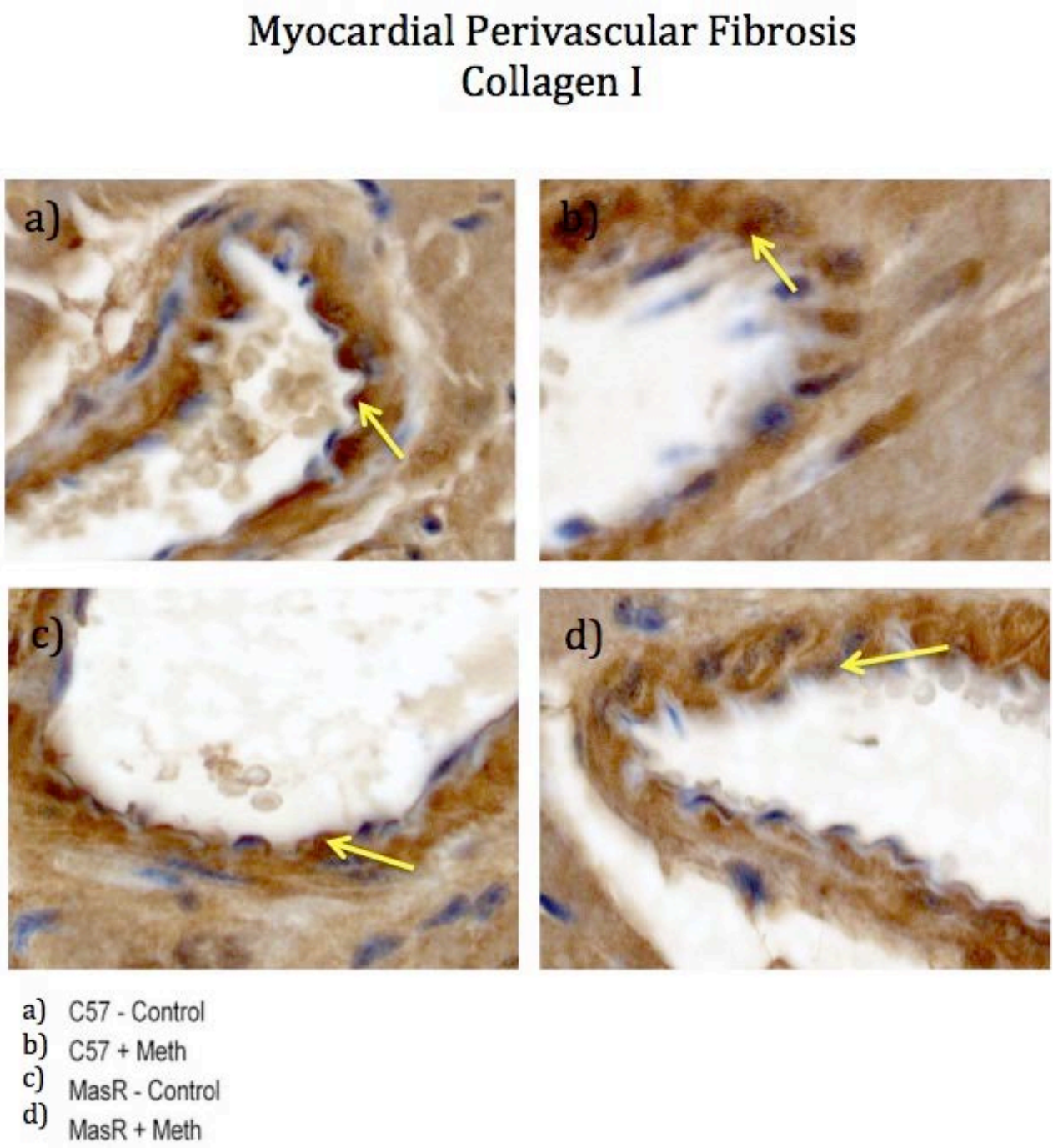


Figure 17.

Myocardial perivascular fibrosis collagen I. Yellow arrows denote positive brown staining in myocardial tissue. Generalized non-specific staining is positive for collagen I within vascular muscles as well as surrounding interstitial cardiomyocytes. No significant increase was detected between the groups.

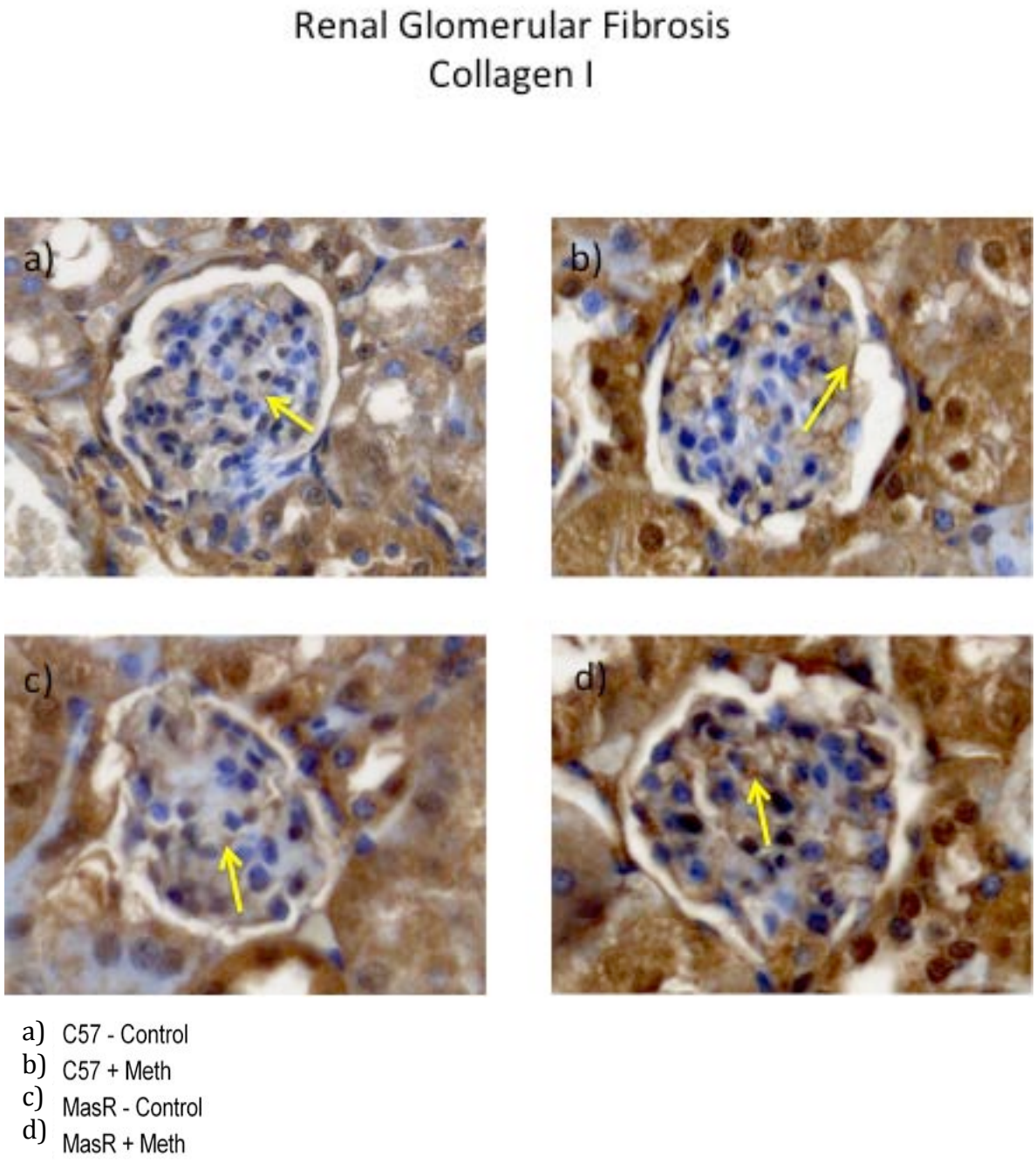


Figure 18.

Glomerular fibrosis staining for collagen I. Yellow arrows indicate positive brown staining observed in renal glomerular tissue. Generalized non-specific staining is observed in surrounding interstitial/tubular tissue. No significant increase was observed between the groups.

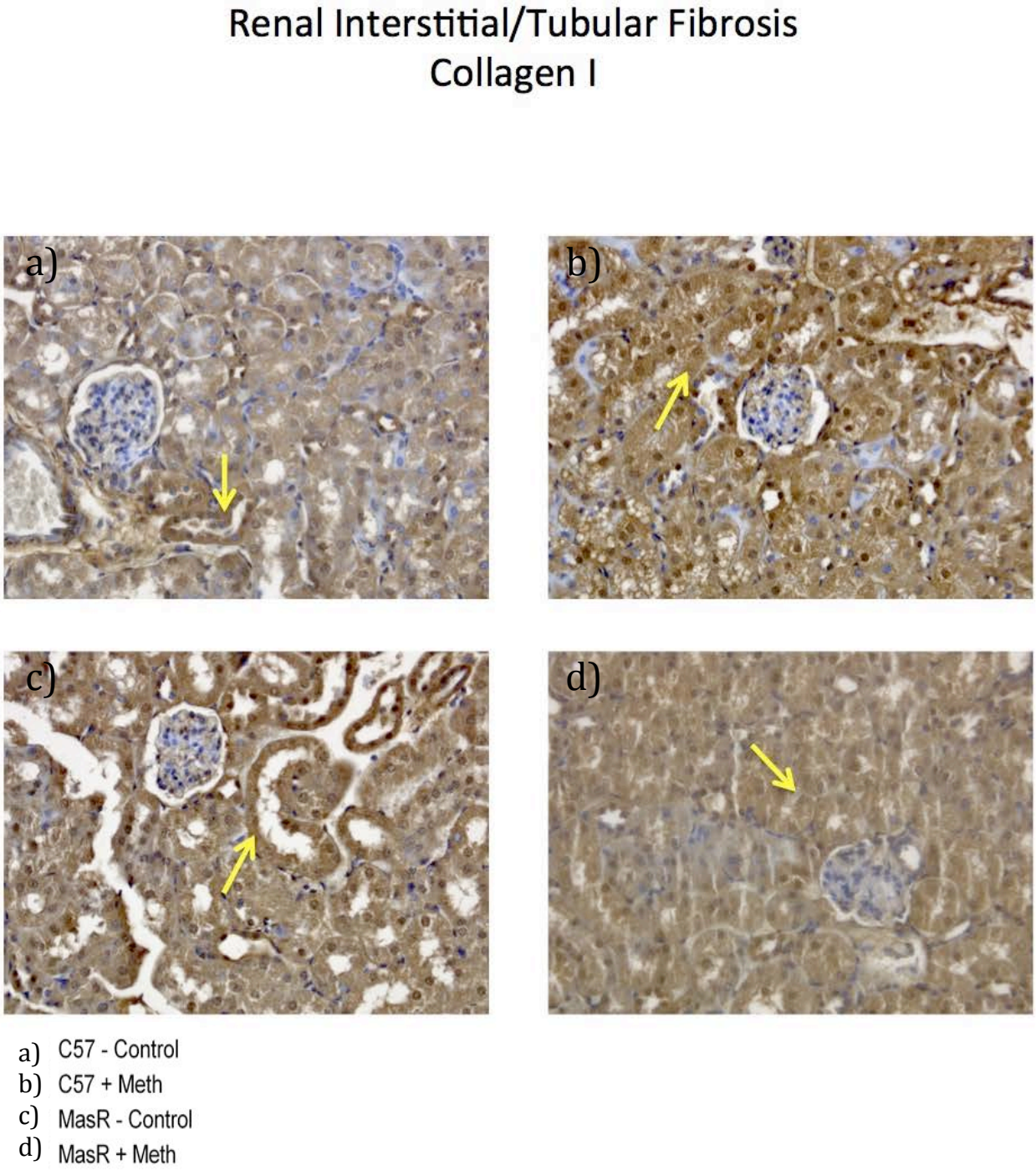


Figure 19.

Renal interstitial/tubular fibrosis staining for collagen I. Yellow arrows indicate positive brown staining detected throughout the renal interstitial/tubular tissue. Positive staining is detected in glomerular tissue. No significant increase was seen between the groups.

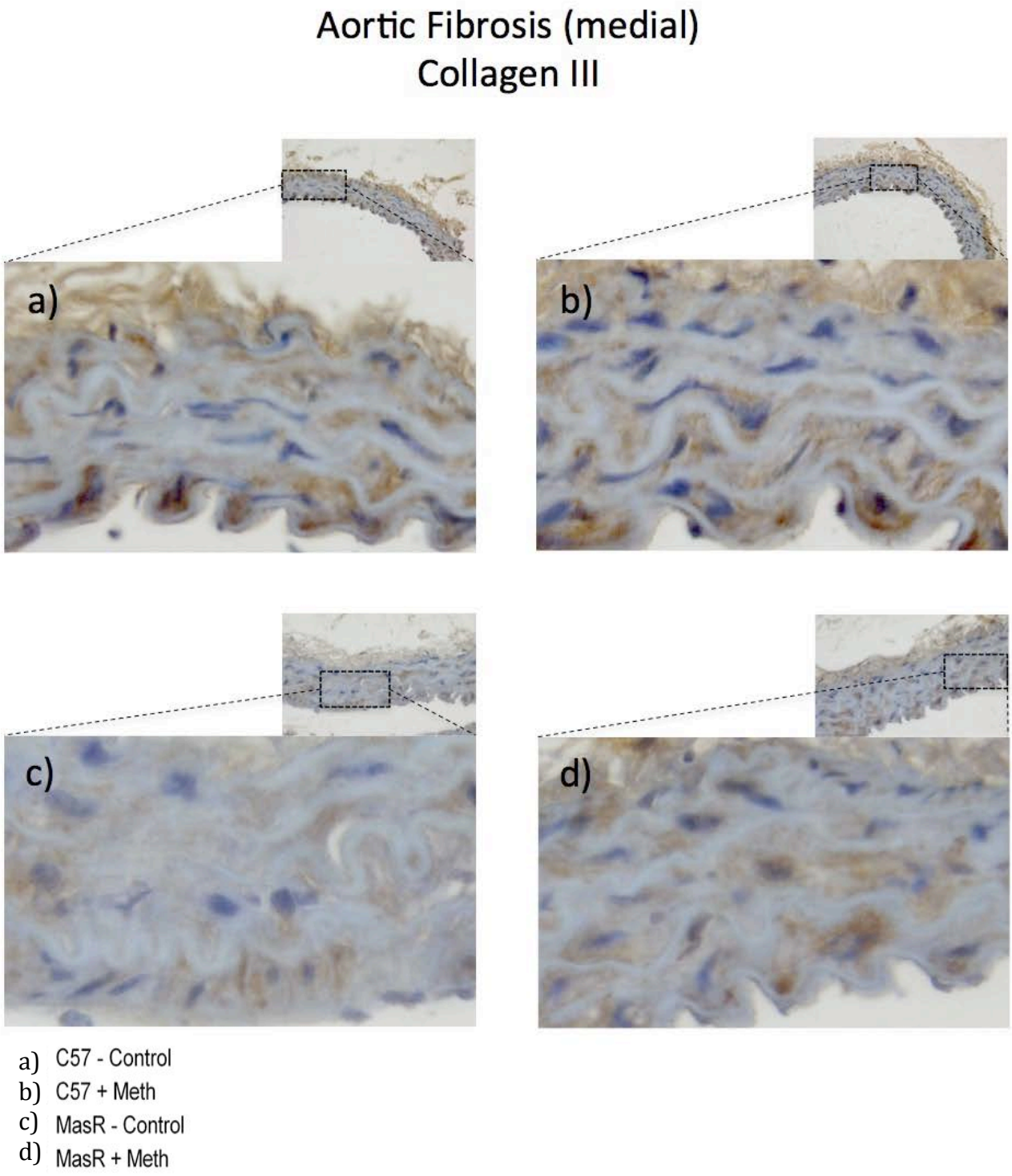


Figure 20.

Aortic medial fibrosis collagen III staining. Positive brown staining is observed in aortic medial tissue. This generalized non-specific staining is observed in the media in addition to positive staining observed in the adventitia. No significant increase was detected between the groups.

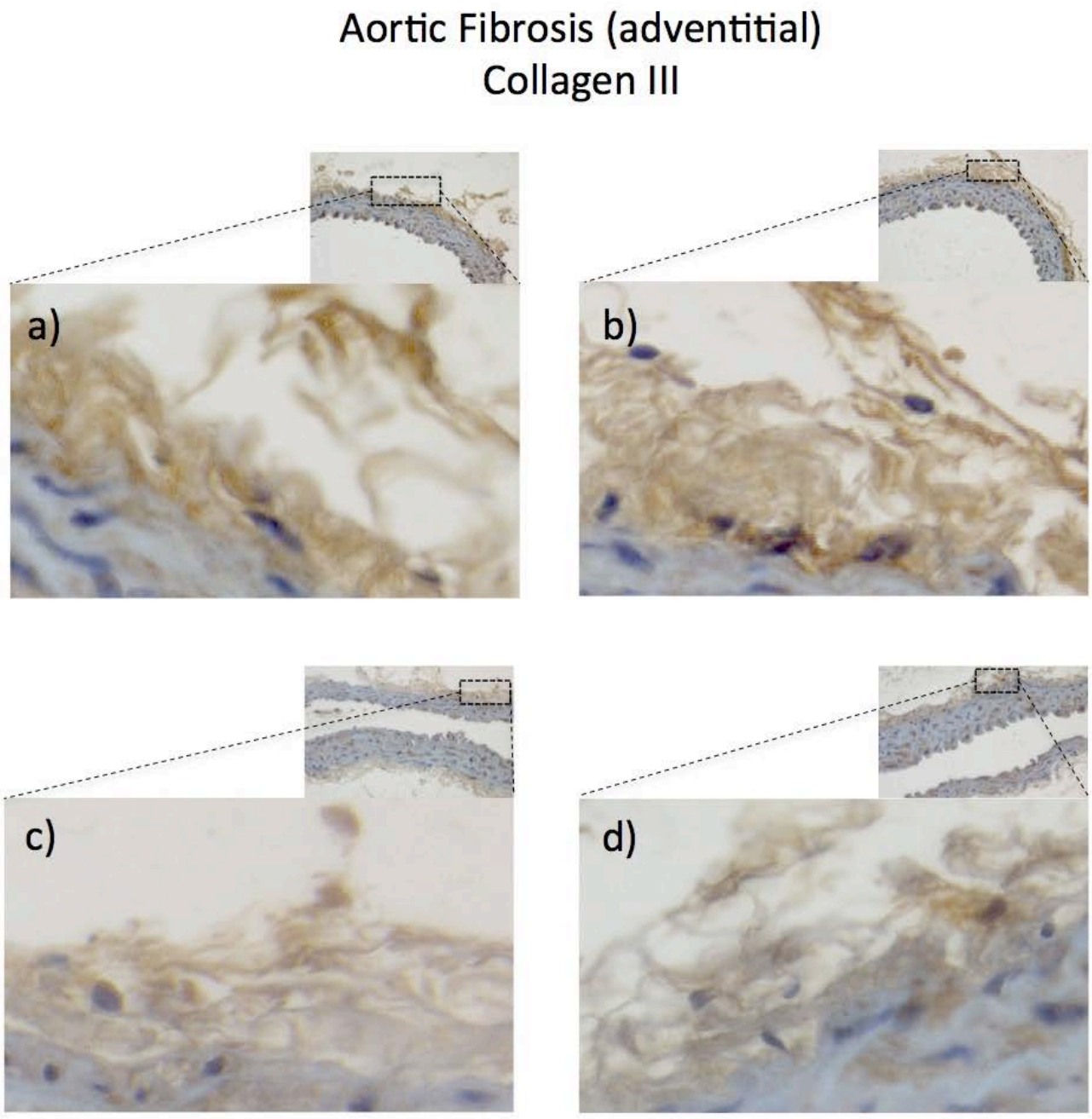


Figure 21.

Aortic adventitial fibrosis collagen III staining. Positive brown staining is observed in the aortic adventitial tissue. No significant increase was seen between the groups.

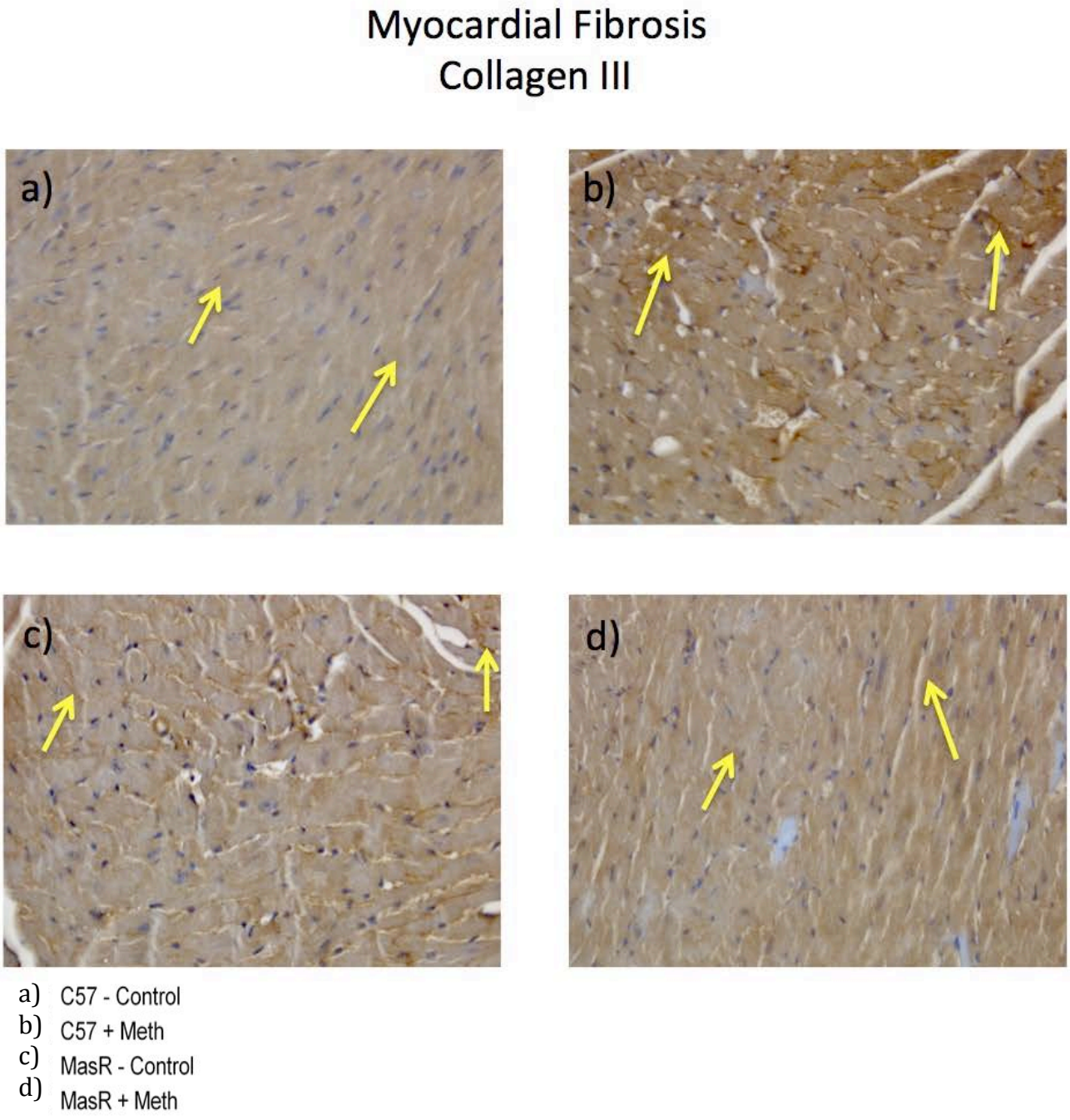


Figure 22.

Myocardial interstitial fibrosis collagen IIII staining. Yellow arrows indicate positive brown staining observed throughout the myocardial tissue. However, no significant increase was seen between the groups.

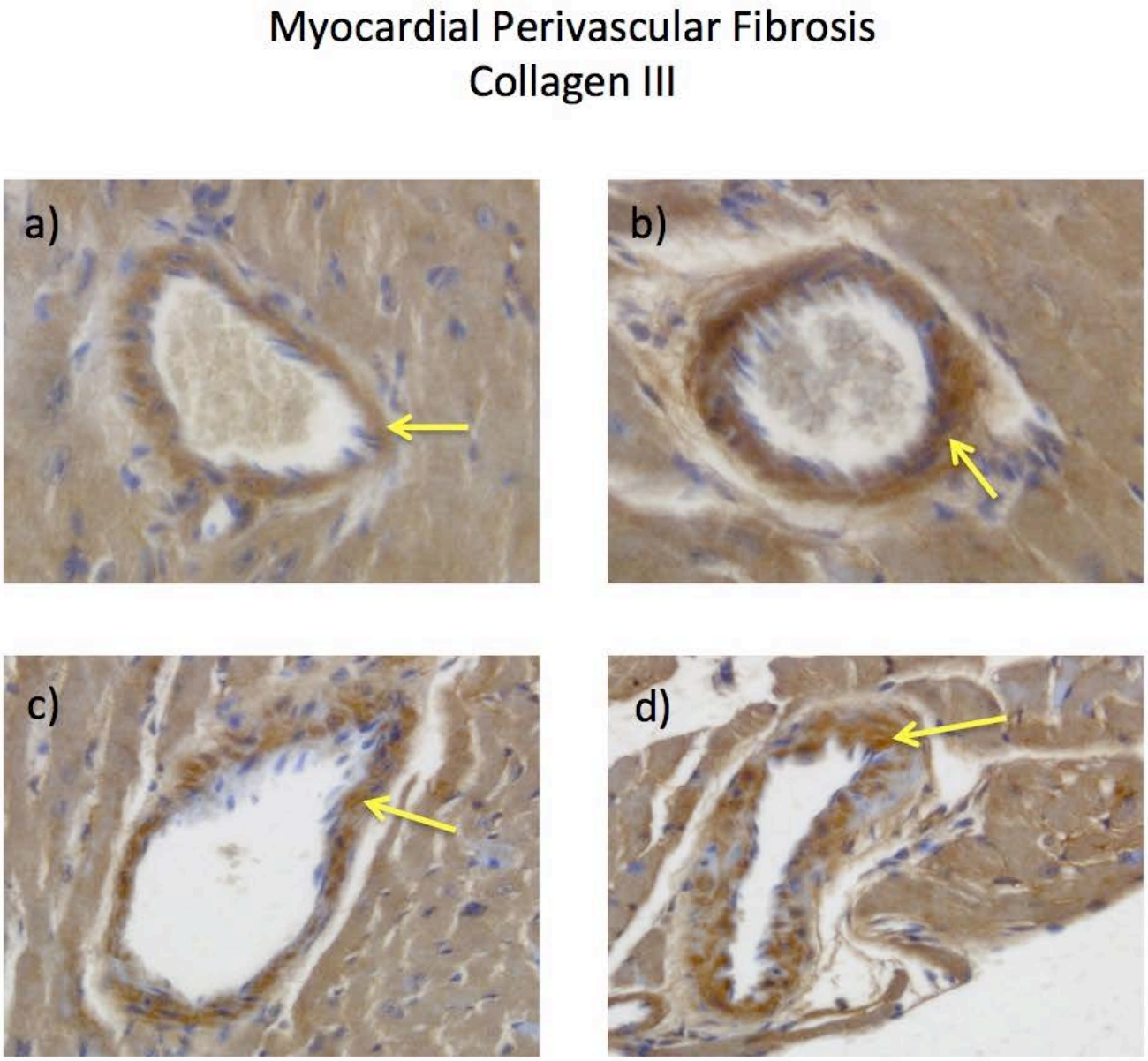


Figure 23.

Myocardial perivascular fibrosis collagen III staining. Yellow arrows denote positive brown staining observed throughout the myocardial tissue. This generalized non-specific staining is observed throughout vascular muscles, however post-analysis did not detect any significant increase in collagen III between the groups.

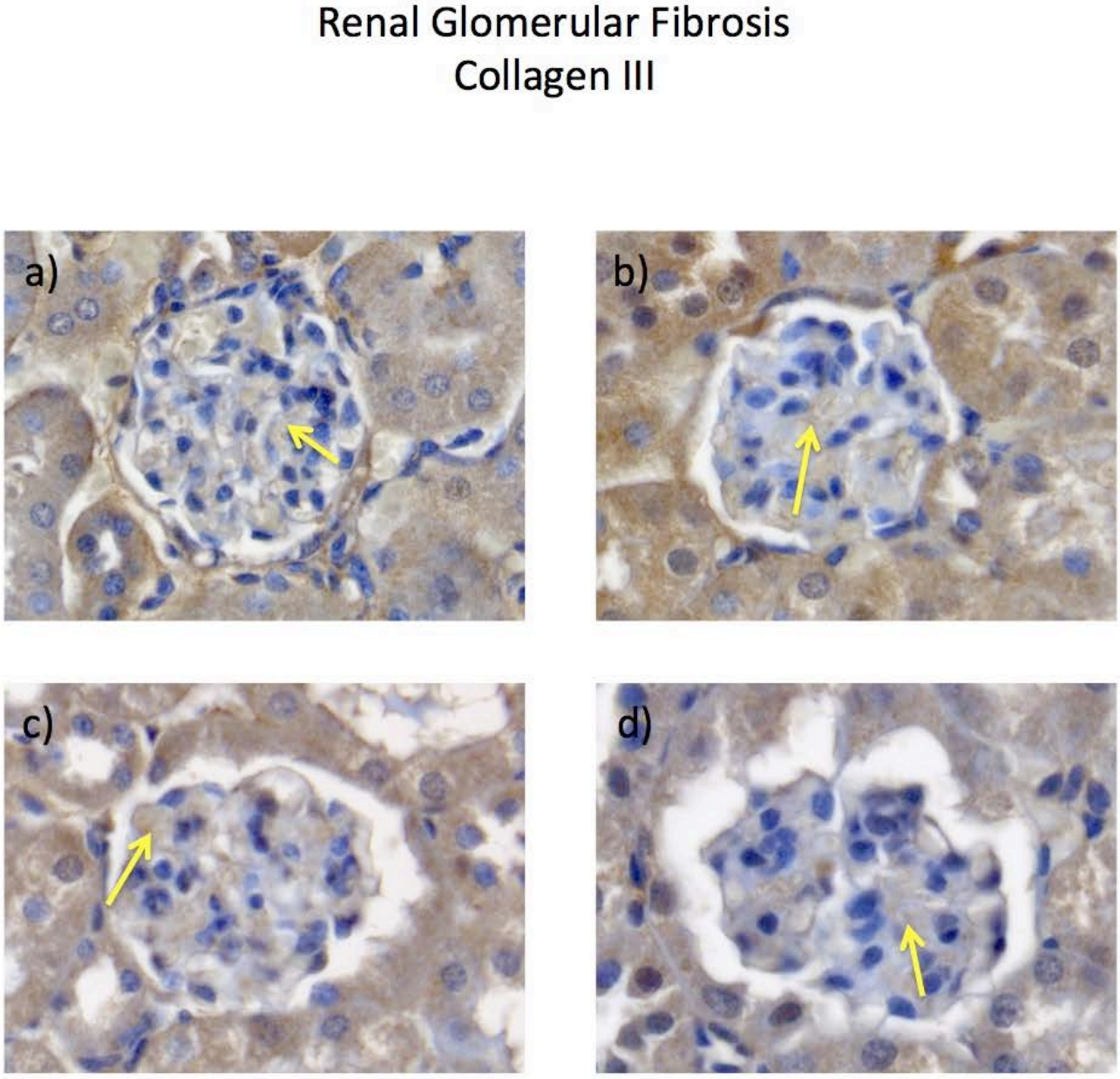


Figure 24.

Glomerular fibrosis staining for collagen III. Yellow arrows indicate positive brown staining observed throughout the renal glomerular tissue. This generalized non-specific staining is observed within the glomerular and surrounding interstitial/tubular tissue. No significant increase was observed between the groups.

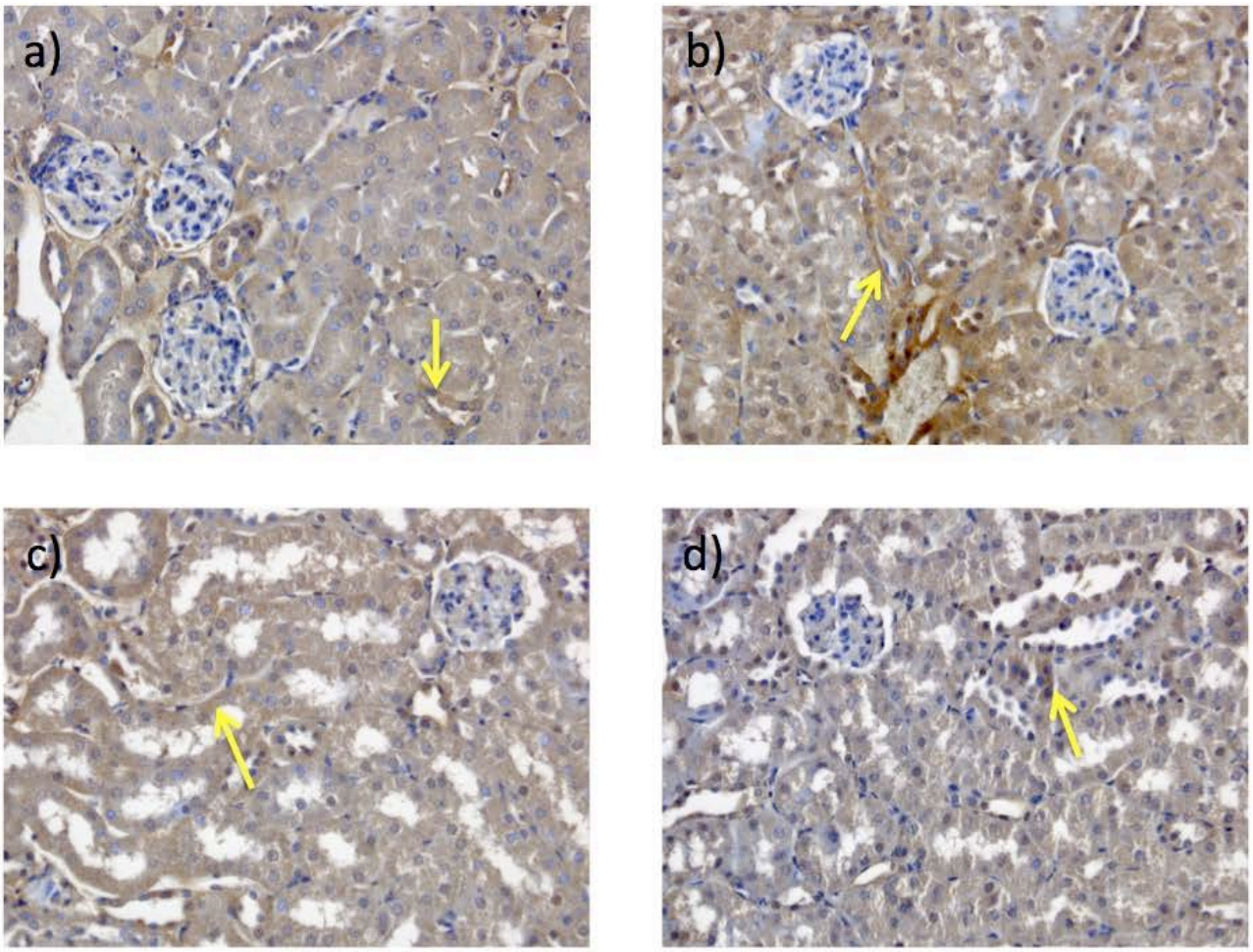


Figure 25.

Renal interstitial/tubular fibrosis staining for collagen III. Yellow arrows denote positive brown staining. Generalized brown non-specific staining is observed throughout renal interstitial/tubular tissue.

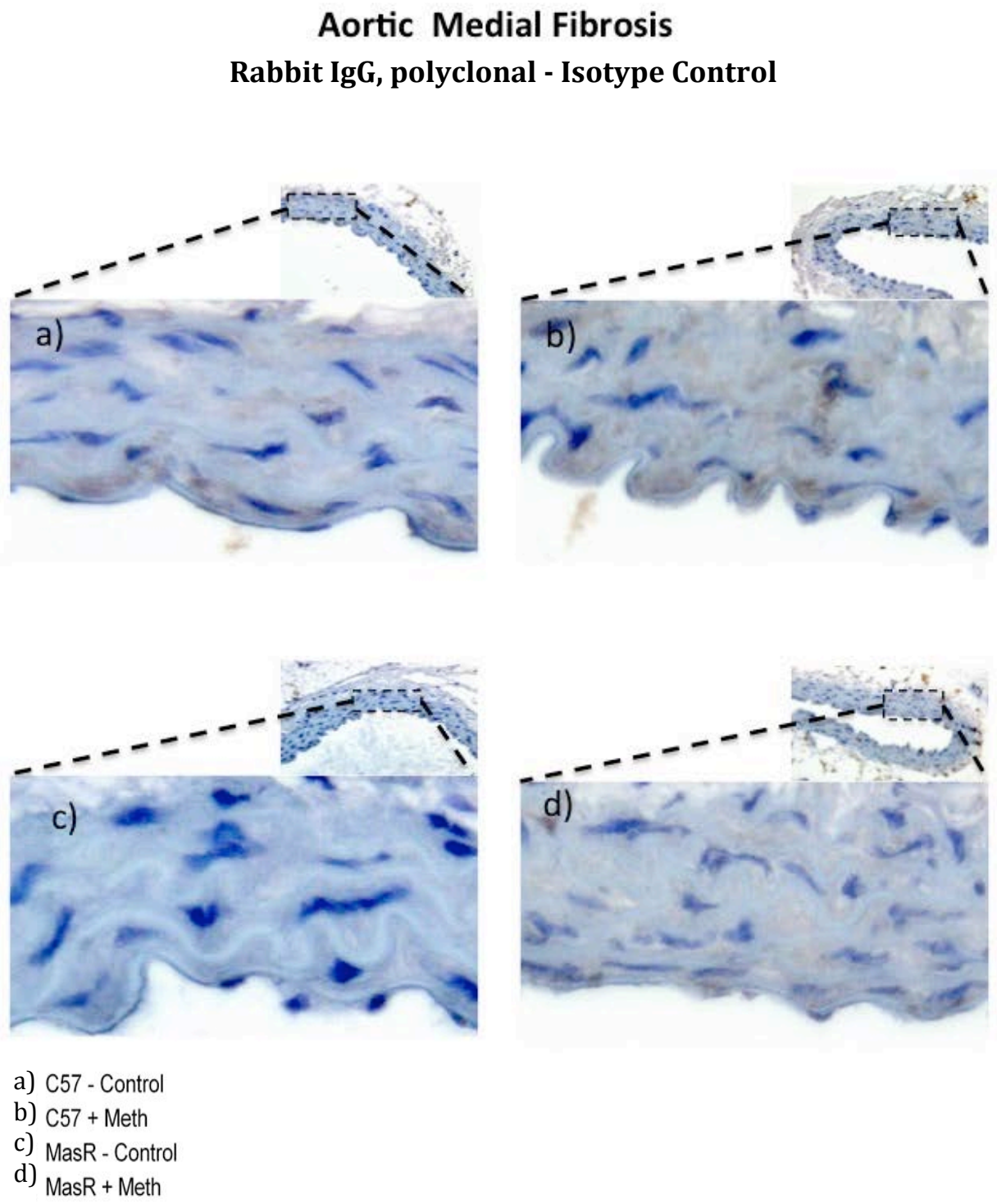


Figure 26. IHC analysis of aortic medial fibrosis using rabbit IgG, polyclonal - isotype control did not display positive staining.

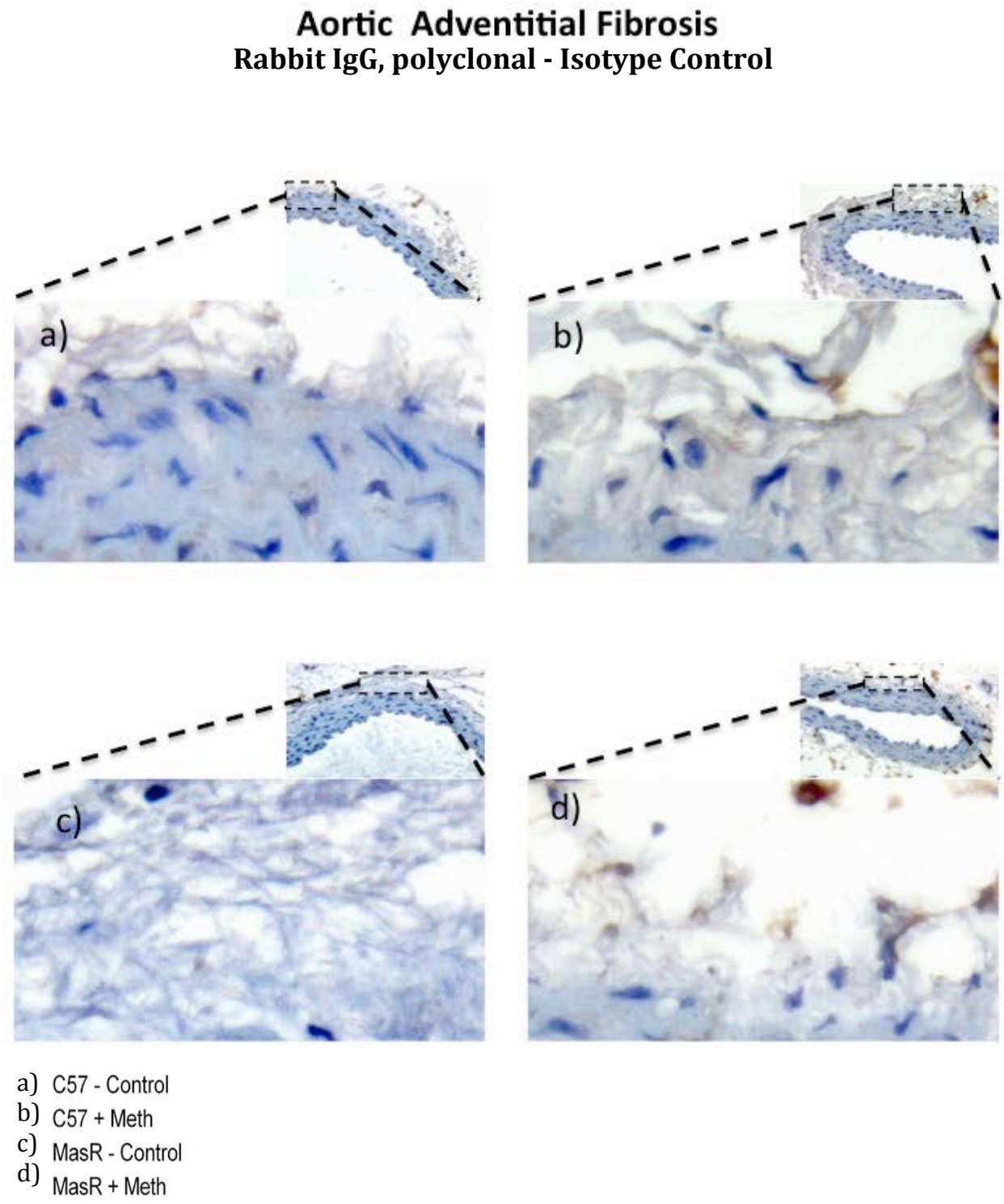


Figure 27. IHC analysis of aortic adventitial fibrosis using rabbit IgG, polyclonal - isotype control did not display positive staining.

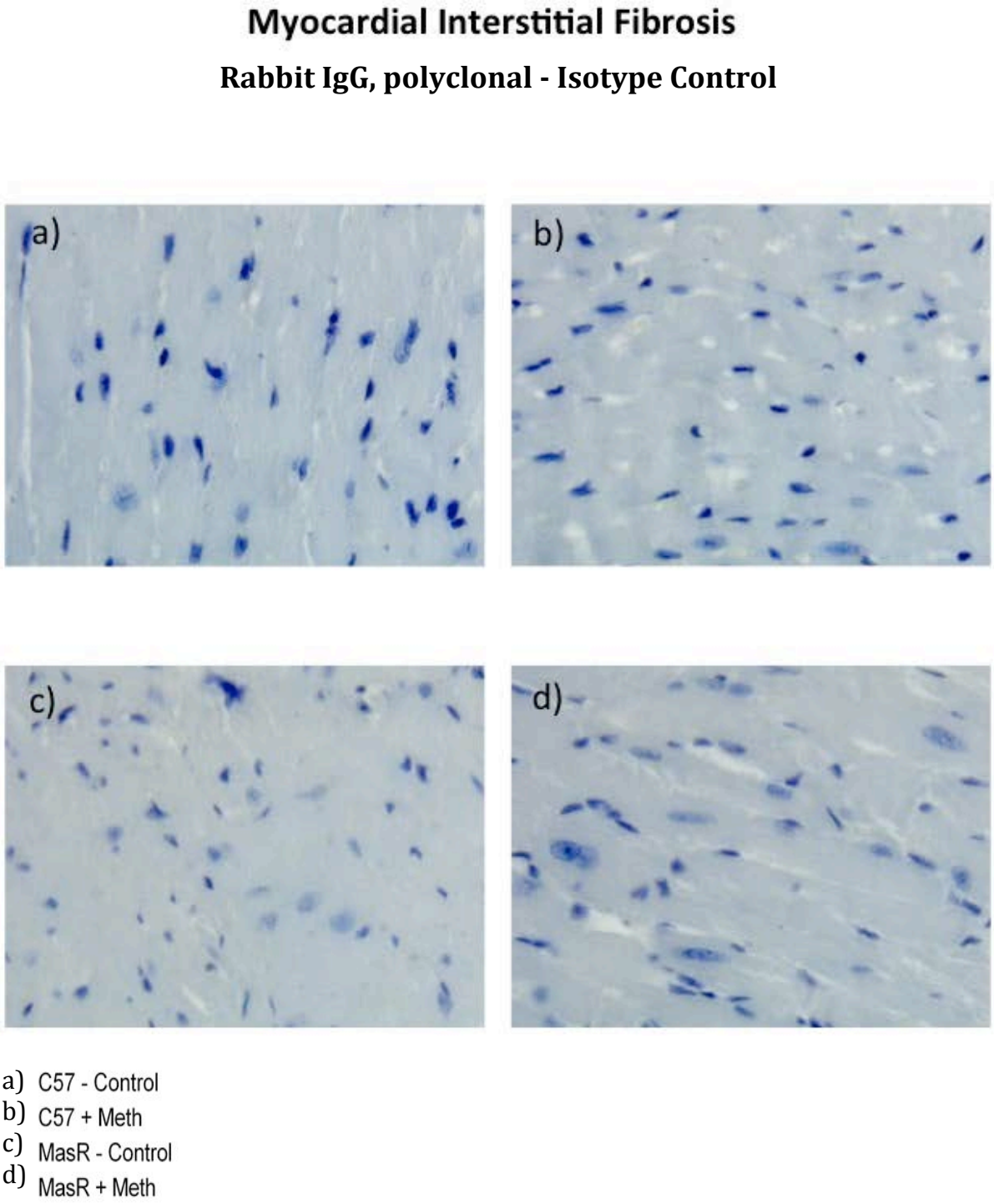
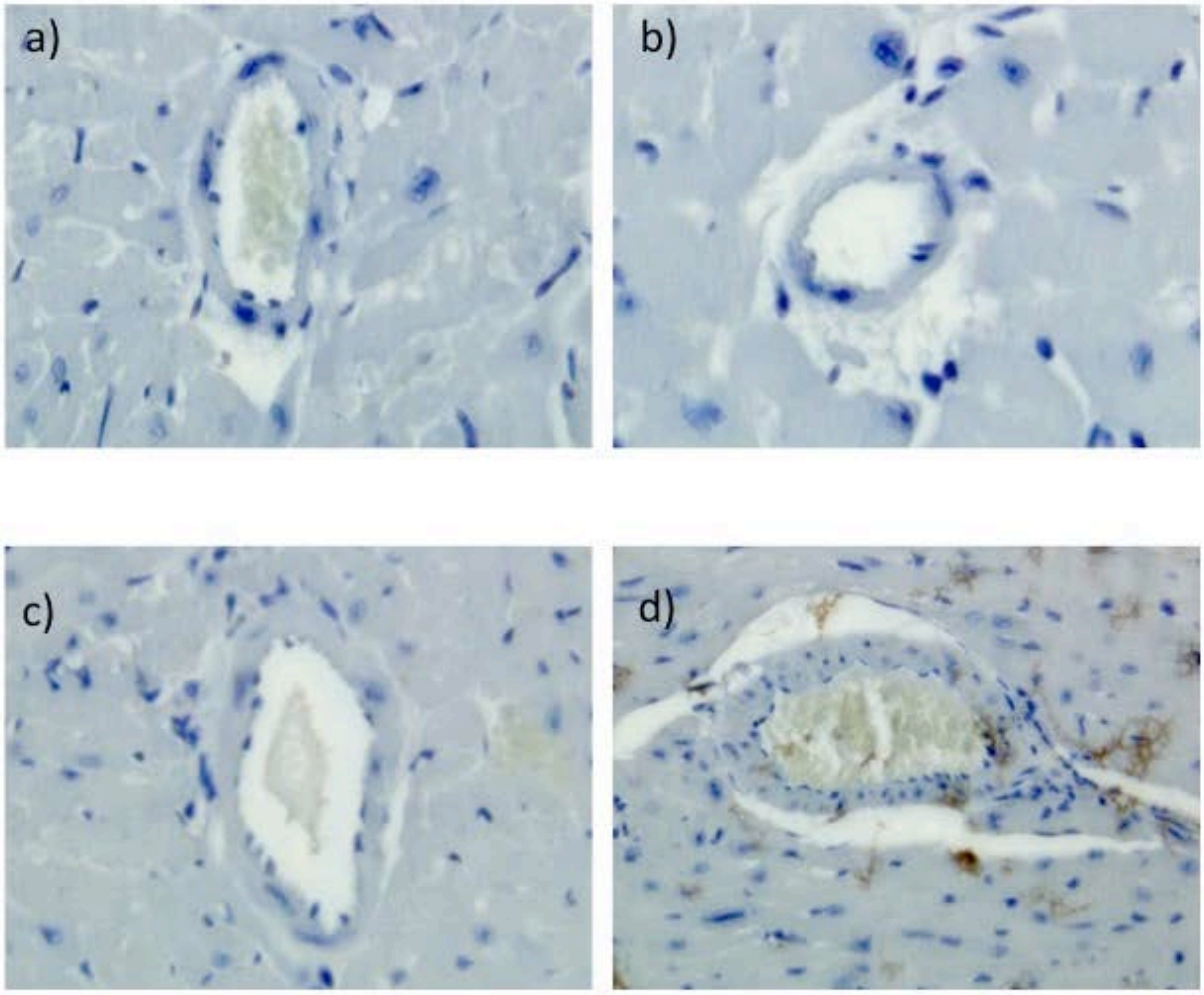


Figure 28. IHC analysis of myocardial interstitial fibrosis probing with rabbit IgG, polyclonal - isotype control did not display positive staining.

Myocardial Perivascular Fibrosis

Rabbit IgG, polyclonal - Isotype Control

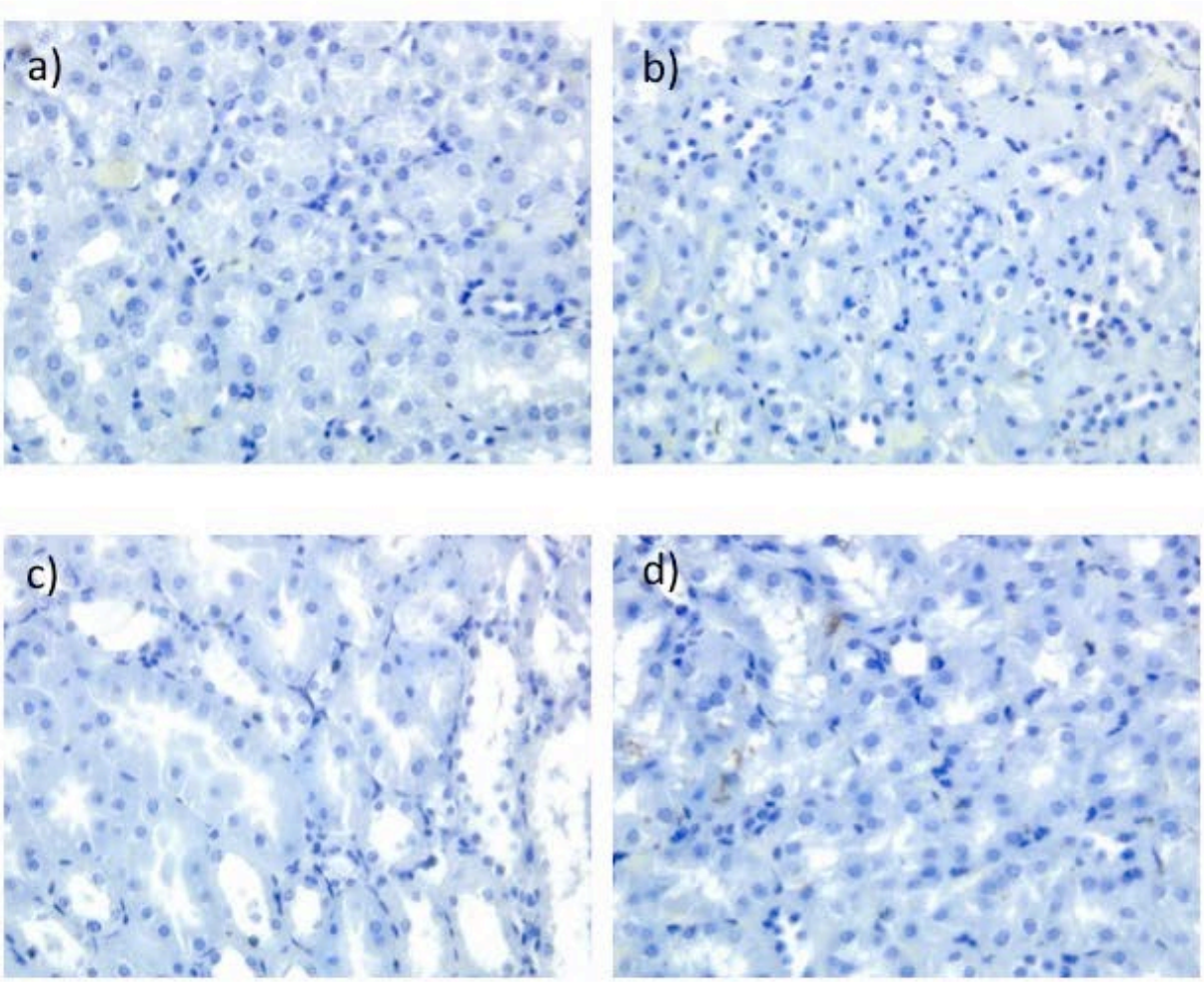


a) C57 - Control
b) C57 + Meth
c) MasR - Control
d) MasR + Meth

Figure 29. IHC analysis of myocardial perivascular fibrosis using rabbit IgG, polyclonal - isotype control did not display positive staining.

Renal Interstitial/Tubular Fibrosis

Rabbit IgG, polyclonal - Isotype Control

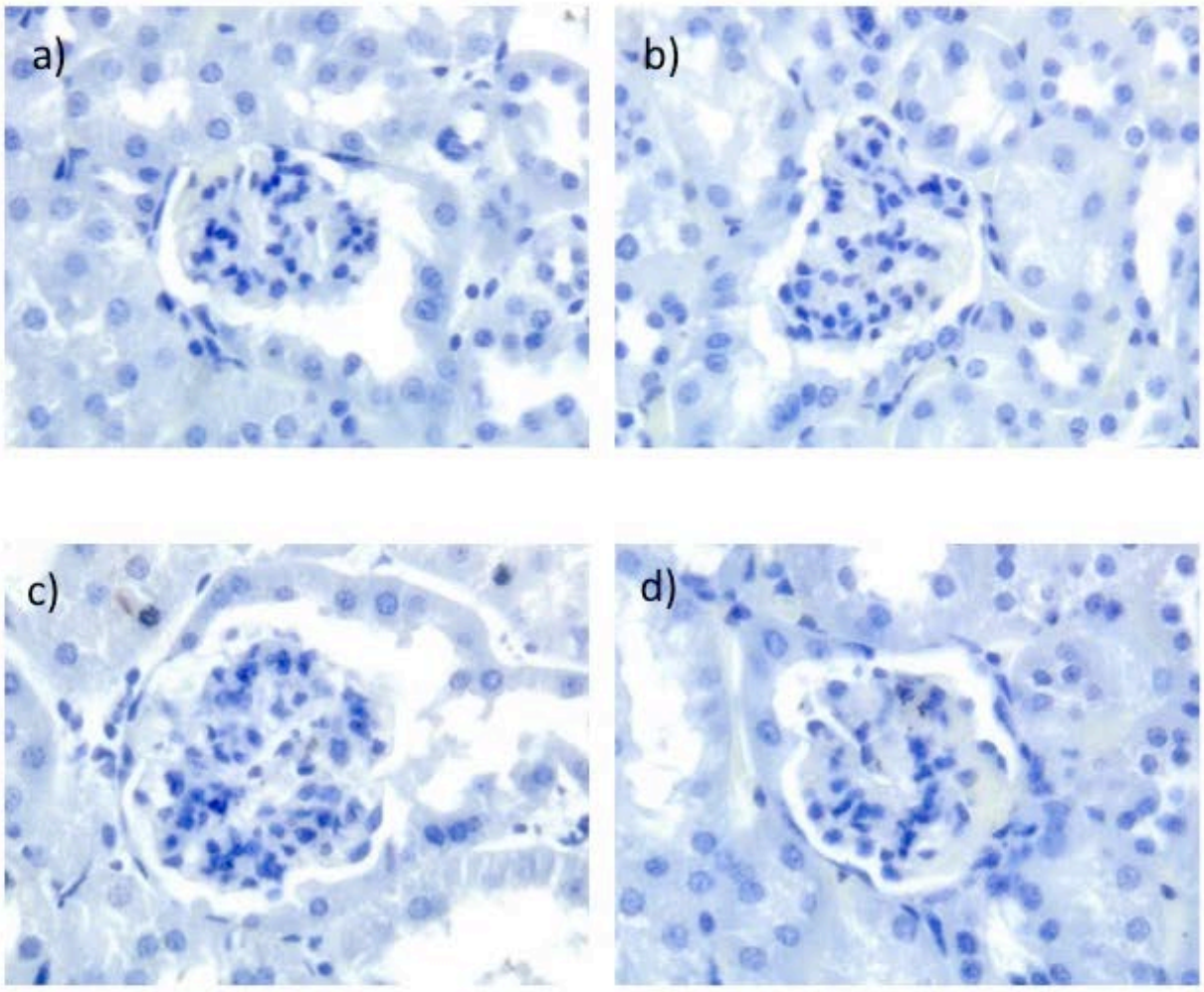


a) C57 - Control
b) C57 + Meth
c) MasR - Control
d) MasR + Meth

Figure 30. IHC analysis of renal interstitial/tubular fibrosis using rabbit IgG, polyclonal - isotype control did not display positive staining.

Renal Glomerular Fibrosis

Rabbit IgG, polyclonal - Isotype Control



a) C57 - Control
b) C57 + Meth
c) MasR - Control
d) MasR + Meth

Figure 31. IHC analysis of renal glomerular fibrosis using rabbit IgG, polyclonal - isotype control did not display positive staining.

Discussion:

In the present investigation, the major findings are that in both C57BL/6 and MasR$^{-/-}$ mice, a high methionine diet (1% methionine) caused an increase in collagen accumulation as detected by sirus red in the vasculature. This was worsened in MasR$^{-/-}$ mice, which also showed worsened endothelial function.

These studies suggest that the MasR may play a protective role against methionine-induced fibrosis within the myocardium (interstitially and perivascular), renal (glomeruli and interstitial/tubules), however, interestingly, this was not the case within aortic tissue, which showed an increase in fibrosis in C57BL/6 fed on 1% methionine but not in the MasR$^{-/-}$ mice. Aortic endothelial function was also negatively affected by 1% methionine diet in the MasR$^{-/-}$, however in the C57BL/6 mice methionine did not display any significant affects.

Endothelial Function

We investigated the affects of homocysteine on MasR$^{-/-}$ and C57BL/6 mice with regards to abdominal aorta relaxation to acetylcholine. Our study suggests that there is a significant reduction in endothelial relaxation in MasR$^{-/-}$ mice when compared to C57BL/6, and this is further exacerbated by the introduction of a methionine diet.

In this study we hypothesized to clarify the link between the role of the MasR, homocysteine and CVD. More recently the MasR and in particular the ACE2/Ang (1-7)/MasR axis has been identified as a possible treatment pathway in preventing the deleterious affects of the ACE/Ang II/AT$_1$R [(158)]. This current research determined that our endothelial function study exhibited a clear difference in the ability of the abdominal aorta to maximally relax in response to acetylcholine between the MasR$^{-/-}$ and the C57BL/6 mice. Thus, there was an indication that endothelial dysfunction had occurred and that this may directly relate to the lack of MasR. Our findings support the theory that

MasR plays a protective role in the normal homeostasis of blood vessels. MasR$^{-/-}$ mouse abdominal aorta had a reduced relaxation in response to acetylcholine compared to the control. Previous studies investigating the role of Ang (1-7) and its protective role on vascular beds was first demonstrated in MasR$^{-/-}$ mice, where MasR$^{-/-}$ mice exhibited impaired *in vivo* endothelial-dependent vasorelaxant response to acetylcholine [159, 160].

Studies have demonstrated the cardioprotective effects of the Ang (1-7)/MasR axis by the increased synthesis and phosphorylation of eNOS [161]. There is also evidence to suggest that defects that lead to a reduced production of NO may lead to endothelial dysfunction, which can also be predictive of future cardiovascular events [16]. It is well understood that the endothelium is vital in cardiovascular homeostasis while also providing protection against vascular disease. These cardioprotective effects are achieved through the production of NO, a free radical responsible for inhibition of vascular smooth muscle cells (VSMC) hypertrophy and relaxation of VSMC, platelet activation and adhesion, inflammatory molecule expression. It has been previously been reported that Ang (1-7) is an endogenous ligand for the MasR, thus the antidiuretic effects of Ang (1-7) on water-loaded mice and the vasodilator effects on aortic rings of wild type mice were eliminated in MasR$^{-/-}$ mice [87]. Other studies investigating Ang (1-7) agonists such as AVE0991 and CGEN-856S found that they were able to increase NO production, while the antagonist A-779 was able to inhibit the production of NO [109, 162]. Therefore the difference in relaxation in response to acetylcholine observed may be due to a reduced NO bioavailability. Our investigation is in line with results demonstrated in other studies suggesting that a reduced response to acetylcholine is indicative of a reduced production/bioavailability of NO in MasR$^{-/-}$ model.

While there is still no well-defined understanding of the direct or indirect affects of homocysteine on the vascular system, some have suggested that there is a risk associated with even a mild increase of plasma homocysteine levels, with studies suggesting that homocysteine is elevated in

cases of sudden death as a result of severe coronary artery disease [163]. Investigators have also argued that their data identified elevated homocysteine level as being casually associated with sudden death or the marker of a process related to sudden death [163]. However, other studies have revealed that lowering elevated homocysteine levels did not directly translate towards a healthy cardiovascular system, and in fact even after homocysteine levels were reduced, the risk of major cardiovascular events in patients with vascular disease did not significantly change [53, 54, 164]. We found that after an 8-week diet of 1% methionine, the response to acetylcholine was significantly reduced **only** in the $MasR^{-/-}$ mouse. Homocysteine is known to instigate the production of ROS via increased expression of NADPH oxidase leading to an alteration in tissue morphology. This increase in ROS production is also linked to increased generation of $ONOO^-$ and decreased NO bioavailability[165]. Additionally $MasR^{-/-}$ mice were shown to upregulate gene and protein expression of the NADPH oxidase catalytic subunit Nox2, while also reducing aortic SOD activity [159, 160], taken together, these findings suggest that the reduction in endothelial response to acetylcholine in $MasR^{-/-}$ could be due to upregulation of free radicals. The reduction of endothelial response to acetylcholine was further exacerbated by the introduction of a methionine-enriched diet. Other studies have also demonstrated that increased homocysteine levels induced higher O_2 production [152]. Xiao et al. demonstrated that MasR protects human brain microvascular endothelial cells against Ang II-induced oxidative stress and dysfunction and it has also been speculated that the underlying mechanism may rely on the ROS and NO signaling pathways[166]. It was also demonstrated that homocysteine reduced the resting cerebrovascular blood flow and additional, was able to attenuate the increase in blood flow produced by acetylcholine and S-nitroso-N-acetylpenicillamine (SNAP) [167]. Yet, our data suggests that there was no significant difference detected between the 1% methionine and control diet on endothelial function in C57BL/6 mice as observed in other studies, which is contrasting as others have previously determined an increment of 2 to 3mol/L in plasma homocysteine via oral methionine induced endothelial dysfunction in healthy volunteers (n=18) [168]. However, a 1% methionine diet was able to worsen endothelial

dysfunction in MasR$^{-/-}$ but no affect was observed in the C57BL/6 mice. 1% methionine diet and MasR$^{-/-}$ mouse and consequently higher levels of homocysteine, suggest that without the protective affects of the MasR, elevated plasma homocysteine levels are in part if not directly affecting vascular tissue function leading to endothelial dysfunction.

Fibrosis

Our investigation suggests that hyperhomocysteinemia, by the introduction of a 1% methionine diet, significantly increased fibrosis within myocardial and renal tissue in MasR$^{-/-}$ mice. However, there was no significant increase of fibrosis identified in C57BL/6 mice despite the 1% methionine diet. Interestingly however, with the same diet combination, the aortic tissue showed a significant increase in fibrosis, the C57BL/6 mouse and not in the MasR$^{-/-}$ mouse.

Our investigation of renal fibrosis within glomerular tissue showed a 2.1 fold significant increase of fibrosis in the MasR$^{-/-}$ mouse fed on 1% methionine diet over the control diet. However, there was only a 22% in control significant increase of fibrosis observed in the 1% methionine diet in the C57BL/6 mouse. Observations of fibrosis were even greater in interstitial/tubular tissue, with a greater than 5 fold increase in the MasR$^{-/-}$ on 1% methionine diet over the control diet. C57BL/6 showed no significant increase in fibrosis between the two diets in interstitial/tubular tissue. The Ang (1-7)/MasR axis is known to oppose the vasoconstrictor, profibrotic, and proliferative actions of Ang II [(158)], and is thought to play an important role in renal function. Furthermore, enzymes involved in the configuration of Ang (1-7) are abundantly found in the kidney [(158)] however, in MasR$^{-/-}$ mice the configuration and production of Ang (1-7) may have little to no affect due to its receptor binding site being absent. There are several other physiological ligands that also bind to and activate MasR such as the neuropeptide FF (NPFF) [(87)], Ang III and Ang IV [(169)] however, most effects of MasR were found to be mediated by Ang (1-7) [(170)]. In addition, a study investigating the distribution of enzymes involved in the metabolism of Ang II within the mouse renal tissue found

that Ang (1-7) was predominantly formed in the renal cortex [171], which suggested that ACE2 was mainly localized in the renal cortex[158]. Shi, Y. et al. 2015, demonstrated that Ang (1-7) treatment in Akita mice was able to effectively attenuate oxidative, normalise ACE2 and MasR expression, as well as suppressing expression of pro-fibrotic, pro-hypertensive and pro-apoptotic proteins, in renal proximal tubular cells [172]. These findings support our investigation as we demonstrated a clear protective role of MasR against the deleterious effects of the methionine diet.

Homocysteine is understood to cause oxidative stress via way of ROS production. It was reported that total homocysteine levels in patients with end stage renal failure was 3-5 times greater than normal, and the incidence of hyperhomocysteinemia in the patient group was at 85-100% [173]. Currently, elevated homocysteine is observed as having a significantly positive correlation with renal disease and is linked to kidney function however, it is debated as to whether elevated homocysteine is an independent risk factor for kidney disease. Interestingly, previous reports have suggested endothelial cells can detoxify homocysteine by stimulating the release of NO [174]. While there is limited literature on homocysteine and the role MasR plays in renal fibrosis, studies have demonstrated human endothelial cells treated with homocysteine for a 24h period at 100μM was able to reduce NO release, increase ROS and eventually lead to eNOS uncoupling by reducing intracellular BH4 availability [175]. This is reflective in part to our findings, where C57BL/6 mice on 1% methionine displayed an increase in glomerular but not interstitial/tubular fibrosis. However, our $MasR^{-/-}$ presented with the greatest increase in glomerular and interstitial/tubular fibrosis indicating that homocysteine in the absence of MasR produces an even greater increase of fibrotic tissue which, may of course be due to the reduced NO production leading to a reduced ability to detoxify ROS. In a 2004 study Ninomiya et al. reported that a 5-year investigation into total homocysteine levels in local Japanese community suggested that moderately elevated serum total homocysteine levels were a significant risk factor in the development of chronic kidney disease in the general population [176]. Interstitial fibrosis, vascular and glomerular sclerosis have been

identified as pathological abnormalities in chronic kidney disease [177]. Studies demonstrating that administration of diets that induced hyperhomocysteinemia for 12 weeks spontaneously provoked arterial and arteriolar wall thickening and renal tubulointerstitial fibrosis are supportive of our findings, in particular where we showed significant increase in fibrosis in our MasR$^{-/-}$ mice. Furthermore, the worsening of renal function and renal tubulointerstitial remodeling were closely correlated with the plasma total homocysteine levels [178]. Our MasR$^{-/-}$ mouse model, without the ability of protect against adverse affects of hyperhomocysteinemia and Ang II/AT$_1$R axis, presented with a significantly greater fibrosis level than that of the C57BL/6 mice. Interestingly, a study revealed that ACE2 was 20-fold more active in the mouse renal cortex than in cardiac tissue [179]. However, no obvious difference in terms of fold increase was detected.

Hyperhomocysteinemia has been linked with the burden of CVD causing adverse cardiac remodeling. Several experimental models of hyperhomocysteinemia have suggested heart fibrosis and ventricular hypertrophy as a result of methionine diet [126, 128, 129]. Investigators have also suggested that a methionine enriched diet increased plasma total homocysteine levels resulting in mild to moderate hyperhomocysteinemia [50, 126, 131]. However, currently there is little information in terms of literature as to whether the MasR functions to protect against hyperhomocysteinemia, therefore our aim was to elucidate to role of MasR in myocardial tissue. In our study we observed a more than 3-fold increase in myocardial interstitial fibrosis with the MasR$^{-/-}$ mice on the 1% methionine diet over the control diet. C57BL/6 mice did not present with any significant increase in collagen within the interstitial tissue. Previous reports have shown that hyperhomocysteinemia induces an increase of collagen content [130], with preclinical studies suggesting that hyperhomocysteinemia promotes myocardial fibrosis and tissue dysfunction [126, 133]. We showed that with our 8 weeks diet our study was in support of these previous findings although, the increase was only observed in MasR$^{-/-}$ mice. In addition, others have described a marked interstitial myocardial fibrosis in both hypertensive and normal rat subjects to homocysteine-supplemented

diet, as well as in rabbits on a methionine and cholesterol enriched diet [126, 131, 133]. The deposition of collagen may be a result of an increase of collagen synthesis and/or a decrease of its degradation [130]. Interestingly, while others have demonstrated an increase in fibrosis in control animals with methionine enriched diets [126, 131], we only had a marked increase in our MasR$^{-/-}$ mice which, suggests the within the myocardial interstitial tissue the MasR protects against homocysteine-induced remodeling and fibrosis. However, within the myocardium we observed an increase in perivascular fibrosis in both MasR$^{-/-}$ and C57BL/6 mice on the 1% methionine enriched diet. The locational variation observed in fibrosis accumulation between interstitial and perivascular may to NO availability. It is understood that homocysteine initiates matrix metalloproteinases (MMP) deterioration of elastic structures causing stiff collagen deposition resulting in vascular remodeling [180]. Furthermore, a reduced NO availability will also lead to increased MMPs activity responsible for extra cellular matrix degradation [181], with NO being shown to regulate MMP activity, vascular function and remodeling within the endothelium [165].

Interestingly, while the fold increase was not as pronounced as in the interstitial tissue, we identified a significant 2.6 fold increase in perivascular fibrosis in MasR$^{-/-}$, as well as a 31% increase in C57BL/6 mice fed on 1% methionine diet. Consideration must also be made that homocysteine may have a tissue specific response, hence the difference in fibrosis observed interstitially versus perivascularly. A 2002 study suggested that hyperhomocysteinemia was associated with a significant increase in perivascular collagen in spontaneously hypertensive mice hearts, additionally it was also suggested that while interstitial collagen level also increased in hyperhomocysteinemia groups relative to control, no statistical significance was evident [133]. The interstitial increase in collagen may be due to replacement fibrosis following myocyte loss [182]. Total collagen content was showing to increase proportionally to increasing levels of total plasma homocysteine [133].

Homocysteine is thought to promote oxidative stress via ROS[27], and this homocysteine-induced oxidative stress is thought to be dependent mainly on NOS activity [175]. Joseph, J. et al. in 2008 demonstrated that hyperhomocysteinemia acted through oxidative stress in order to promote myocardial fibrosis [129]. Additionally, oxidative stress is understood to be the most injurious stressor in the progression and pathogenesis of most diseases, with homocysteine itself having the ability to produce ROS when oxidized due to its reactive sulfhydryl group [181]. Homocysteine is also understood to indirectly decrease NO bioavailability via generation of superoxide to rapidly consume NO leading to generation of $ONOO^-$ [183]. In additional to the deleterious affects of hyperhomocysteinemia, our $MasR^{-/-}$ mice would also have a significantly reduced ability to produce NO via eNOS pathway, which has also been linked to myocardial fibrosis [184-186]. Studies have suggested that the increase in fibrosis due to hyperhomocysteinemia may be a result of excess accumulation of extracellular matrix factors such as collagen I, where, changes of histone modifications contributed to homocysteine-regulated gene expression of the collagen I gene [187]. Similar observations of tissue remodeling were demonstrated with long term administration of L-NAME resulting in decreased NO synthesis [186]. Our data suggests that 1% methionine diet was causing adverse remodeling by way of perivascular fibrosis. However we were unable to demonstrate a similar affect on interstitial tissue where only the $MasR^{-/-}$ mouse displayed an increase in fibrosis. This may be a result of actions by various chemokines understood to affect myocardial remodeling such as AngII and aldosterone, whose activities result in myocardial collagen deposition in the perivascular area trailed by interstitial distribution [182, 188].

Our aortic tissue fibrosis results are however different from the results obtained from myocardial and renal tissue. The significant increase was observed in the C57BL/6 mouse on the 1% methionine diet in both medial and adventitial tissue. Hyperhomocysteinemia has been recognized as an independent cardiovascular risk factor that may be involved in altering vascular structure [189], and has been linked in the development of several aortic diseases [190-192]. Furthermore, previous

studies involving vascular pathology associated with hyperhomocysteinemia have been characterized by excess extracellular matrix turnover, initiating increased deposition of collagen leading to endothelial dysfunction [193, 194]. We observed a significant increase in aortic fibrosis (medial and adventitial) in C57BL/6 mice fed on the 1% methionine diet. These results are in line with previous studies reporting an accumulation of collagen as well as injured vessels being susceptible to homocysteine-mediated vascular wall thickening [28, 120]. Homocysteine was also reported to increase collagen expression in smooth muscle cells in atherosclerotic plaques in ApoE knock out mice [195]. Interestingly, while our study is suggesting that homocysteine is causing aortic endothelial dysfunction in $MasR^{-/-}$ mice as measured by response to acetylcholine, we were not able discover any significant endothelial remodeling in either the adventitia or media of the aortic tissue.

MasR, activated by Ang (1-7) is understood to limit NADPH oxidase expression and activation. In addition $MasR^{-/-}$ mice exhibit an increased expression of Nox2 and elevated vascular ROS levels in comparison to control mice [160]. Furthermore, Ang (1-7)/MasR axis was found to protect human brain microvascular endothelial cells (HbmECs) against Ang II-induced dysfunction and oxidation, suggesting that the underlying mechanism may rely on modulation of ROS and NO signaling pathway [166]. Additionally, Ang (1-7) and the nonpeptide agonist AVE 0991 was observed to produce NO in wild-type mice however, this action was completed suppressed by blocking the Ang (1-7) receptor and also by deletion of the Mas gene [109]. We ascertained that hyperhomocysteinemia was responsible for endothelial dysfunction in our study, which is in line with previous studies. Therefore it was interesting to discover that our C57BL/6 mice presented with a higher level of fibrosis, when previous studies have suggested that $MasR^{-/-}$ should be more susceptible to fibrosis injury. With both adventitial and medial aortic tissue exhibiting no significant increase in fibrosis, our results suggest that a different pathway may be involved in the reduced fibrosis observed in $MasR^{-/-}$ mice fed on the 1% methionine and control diet. Apart form the MasR, it is known that other G-coupled protein receptor such as the AT_2R [196] and MrgD [197] also counter

the deleterious affects of AT_1R. The AT_2R receptor has shown to be upregulated in certain pathological conditions, such as heart failure, aging, vascular injury, hypertension, and atherosclerosis [131, 198-200], which may suggest a possible pathway in protection against fibrosis however, the role of the AT_2R is largely undefined. Additionally data suggests that Ang (1-7) may also be acting through the AT_2R[87, 201, 202] and not only the MasR. Furthermore, studies have demonstrated the heptapeptide alamandine (that only differs from Ang (1-7) by an N-terminal Ala^1 [Ala^1-Ang (1-7)]) functions in a similar manner to Ang (1-7), such as endothelial-induced vasodilation of aortic rings which, can be attenuated by NO synthase inhibitor N-nitro-l-arginine-methyl ester [203]. However, in our $MasR^{-/-}$ the functions of alamandine would becomes redundant without MasR, thus it is interesting to note that alamandine also functions through the MrgD receptor. A study demonstrated that using the MasR antagonist A-779 did not abolish alamandine-induced vasodilation and thus maintaining the vasodilatory affects of alamandine in $MasR^{-/-}$ mice [203]. However, the alamandine-induced vasodilation was blocked using the Ang (1-7) antagonist D-Pro^7-Ang(1-7) which suggests that alamandine may be exerting its actions via a different G-coupled protein receptor related to the MasR, the MrgD [169]. The low levels of fibrosis detected within the $MasR^{-/-}$ mouse suggests other protective, antifibrotic pathways are compensating for MasR, with the AT2R and MrgD two likely receptors playing a protective role in the absence of MasR. It will interesting to investigate whether the use of a combination of N-nitro-l-arginine- methyl ester, A-779 and D-Pro^7-Ang(1-7) on $MasR^{-/-}$ mice will be able to produce a fibrotic mouse however more studies need to be performed to elucidated MasR role in aortic fibrosis.

As well, we immunohistochemically examined collagen I and III distribution in the heart, kidney and aorta between $MasR^{-/-}$ and C57BL/6 mice. Positive staining of collagen I and III was detected in the heart, kidney and aorta in both $MasR^{-/-}$ and C57BL/6 diet groups. We detected presence of collagen I and III in myocardial interstitial and perivascular tissue which supports our fast green/Sirius red results found in MasR+Meth and other groups. A study determined that

homocysteine is associated with significant increases in perivascular collagen in hypertensive mice heart, with interstitial collagen also increasing [133], hence our results show positive generalized binding of collagen I and III. However, a study determined that treatment with AVE 0991 did significantly reduce collagen volume fraction as verified by reduced mRNA expression of collagen I and III [204]. This supports our results as the MasR+Meth mouse displayed a positive and possibly generalized non-specific binding to collagen I and III. Previously, collagen release measurements on human arterial smooth muscle cells by ELISA method had shown an increase of collagen I and III in response to homocysteine [195]. Collagen I was found to accumulate at a greater amount than collagen III even though numbers of cells (SMC) had not increased [195]. Collagen synthesis by SMC in response to homocysteine dramatically increased in a dose-dependent manner, with even the lowest concentration (50μM) significantly increasing collagen synthesis [136]. Our aorta media results indicated positive general non-specific Collagen I and III staining.

Conclusion/Future Directions

In conclusion, this study is the first to show a relationship between homocysteine and MasR, in that the MasR is protective against homocysteine-induced vascular pathologies. We have shown the presence of MasR is critical for protecting the cardiovascular system during CVD by maintaining endothelial function and preventing tissue fibrosis. Future studies should focus on western blot analysis of collagen I and III in heart, kidney, and aortic tissue to perform quantification of collagen types.

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