The Effects of Hyperhomocysteinemia on Vascular Disease

Philip M. Kyles
Eastern Kentucky University, philip_kyles@mymail.eku.edu

EASTERN KENTUCKY UNIVERSITY

The Effects of Hyperhomocysteinemia on Vascular Disease

Honors Thesis
Submitted
in Partial Fulfillment
of the
Requirements of HON 420
Fall 2013

By

Philip M. Kyles

Mentors

Dr. Martin Brock

Eastern Kentucky University Department of Chemistry

&

Dr. Pradip Kamat

University of Louisville School of Medicine Department of Physiology

Abstract:

Homocysteine, a metabolite from methionine breakdown, can have detrimental effects to multiple systems in the human body. Methionine is naturally acquired from diet and if too much is consumed, as in diets with high red meats, the metabolic system becomes overloaded leading to elevated blood levels of homocysteine. This condition is called hyperhomocysteinemia (Hcy). Hyperhomocysteinemia is also related to aging and several genetic factors which ensure its proper regulation. More importantly, hyperhomocysteinemia has been shown as a risk factor to numerous disease states including cardiovascular disease and in neuropsychiatric illness. With respect to cardiovascular pathology, it has been found from the results of 80 clinical studies which sampled around 10,000 patients that elevated levels of homocysteine is linked with increased cardiovascular mortality rates. Along with Hcy's effect on the cardiovascular system it has also been shown to induce neurological disorders in the form of neural tube defects, mental disorders, and cognitive impairment. From the literature I will explain plausible routes that Hcy affects vascular function and from data acquired during research conducted at the University of Louisville I will show it's implication in memory impairment. The data also includes the possible treatment of sodium hydrogen sulfide to alleviate Hcy's neurological effects.

Key Words: homocysteine, hyperhomocysteinemia, thiolactone, endothelium, oxidative stress, neurotoxicity, mitochondria, cognitive impairment, thrombosis, arteriosclerotic lesions, blood pressure

Table of Contents

List of Figures and Tables

Acknowledgements

The author would like to thank his mentor, Dr. Martin Brock, for his help and support in this project and his carefulness to teach instead of tell.

Thank you also to the friendly staff and research personnel at the University of Louisville Preston Street Research Campus, they have taught different cultures as well as different techniques of scientific investigation.

And lastly, he would like to the directors of Eastern Kentucky University's Honors programs during his thesis process, Dr. Linda Frost and Dr. David Coleman, for their support as well as organization of several research conferences and trips.

I: What is Homocysteine?:

Neurovascular and cardiovascular diseases are becoming increasingly prevalent as the population ages. Many of these diseases, including Alzheimer's, stroke, Parkinson's, depression, and dementia, have a link to elevated levels of homocysteine in the blood [1,2,3]. From the prevalence of these diseases, homocysteine's negative effects need to be examined further. Homocysteine itself is a sulfur-containing, non-proteinogenic amino acid that is a by-product of normal methionine metabolism [4].

Methionine is one of two sulfur containing essential amino acids and plays an important role in the proper function of many proteins. Methionine is normally consumed in high levels by eating red meats (0.843g/100g) and eggs (2.48g/100g), as well as a variety of nuts, fish, and poultry [5]. Because methionine contains sulfur it is prone to oxidation, but generally it is stabilized by hydrophobic interactions and is not highly nucleophilic like its sister molecule cysteine. Methionine is normally metabolized properly in most people, but the pathway seems to be less effective with aging and also with epigenetic factors such as poor diet and exercise [6]. The pathway and subsequent enzymes involved are shown below [Figure 1].

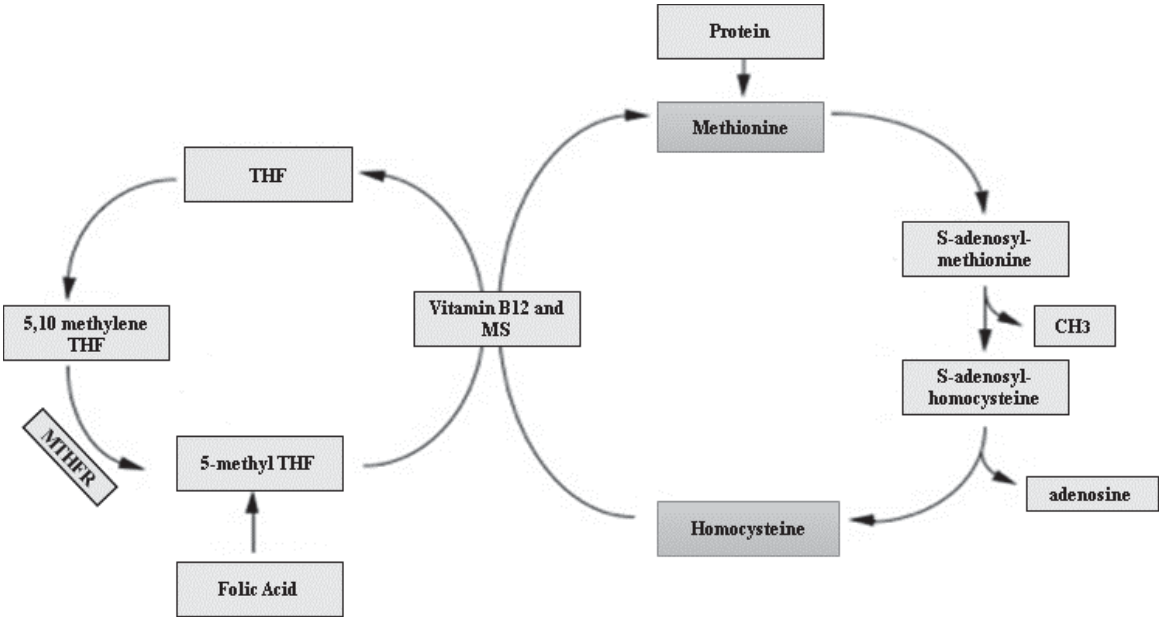


Figure 1: Shown above is the basic methionine metabolic pathway. B12 (1) and folic acid (2) play key roles in proper breakdown. Under proper conditions homocysteine is converted back to methionine due to its essential nature.

The alteration of the methionine pathway can lead to a buildup of homocysteine within the blood above normal concentrations, which is termed hyperhomocysteinemia. Normally, homocysteine is metabolized into two primary fates: it is either transmethylated back to methionine to assist the folate cycle or is transulfanated into cystathionine by CBS.

The transmethylation pathway requires cyanocobalamin (B12), folate and the enzyme 5,10- methylenetetrahydrofolate reductase (MTHFR). Homocysteine acquires a methyl group from N-5-methyltetrahydrofolate (5-methyl THF) which is an intermediary in the folate cycle that occurs in all tissue and is dependent on cyanocobalamin (B12) [29]. After acquiring a methyl group homocysteine is converted into tetrahydrofolate (THF) and methionine by methionine synthase (MS) and cyanocobalamin (B12) [29]. There is another way homocysteine is remethylated but this primarily occurs in the liver

and kidneys with betaine donating a methyl group by ways of betaine homocysteine S-methyltransferase into dimethylglycine [30].

The transulfanation pathway entails the enzyme cystathionine beta-synthase (CBS) and pyridoxine (B6). The initial step in this pathway is the union of homocysteine and serine, catalyzed by cystathionine beta synthase, creating cystathionine [30]. For CBS, pyridoxine (B6) is an essential cofactor explaining increase of homocysteine with certain diets. Abnormalities of this pathway can also influence remethylation by stressing its capacity. When the folate cycle is overwhelmed it uses S-Adenosylmethioine (SAM) to re-convert back to methionine. In cases where methionine is concentrated, CBS and MTHFR are unavailable or ineffective, the system is overloaded leading to an accumulation of homocysteine. The basic metabolism of homocysteine is shown below [Figure 2].

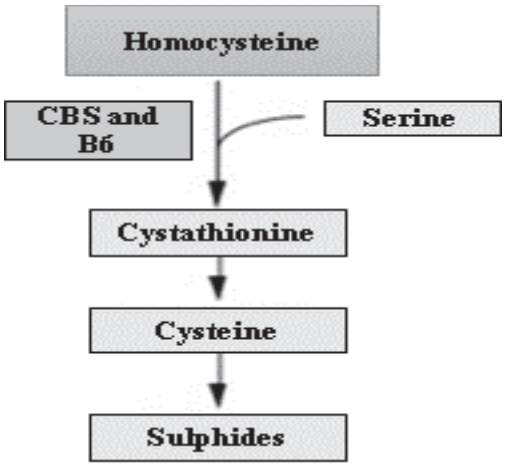


Figure 2

II: Common Causes of Alternating Homocysteine Levels:

The progressions of homocysteine concentrations in human blood are as follows- normal: 8-15 μmol/L, moderate: 15-30 μmol/L, elevated: 30-100 μmol/L, and high: 100>

μmol/L [7]. As previously stated these levels can vary upon several factors including: genetic factors, diet and exercise, age, gender, and smoking.

Genetic factors can lead to the elevation of plasma homocysteine through the mutation or knockout of several enzymes. Children whose parents have had a history of cardiovascular disease tend to have higher levels of plasma homocysteine levels leading to the conclusion of inheritance. This increased risk is attributed to two main genetic factors: the alteration of the CBS and MTHFR genes. CBS, as previously mentioned, is essential for proper homocysteine metabolism. Homozygosity of for CBS deficiency is rare (1/300,000 births) and similarly, heterozygosity for CBS occurs in less than 1% of the population [11]. Homozygosity of CBS causes severe hyperhomocystemia and CBS heterozygosity causes mild hyperhomocystemia and is why CBS +/- knockout mice are common experimental models.

Methyltetrahydrofolate reductase (MTHR) is also important to proper re-conversion of homocysteine to methionine and its modification can cause increased homocysteine levels as well. The C677T mutation of the MTHFR gene is more common than CBS issues and occurs in 35-39% of the Caucasian North American population and leads to a predisposition towards vitamin deficiencies and elevated homocysteine levels [12]. The C677T homozygous mutation occurs in 10-15% of the population and leads to a threefold increase in cardiovascular risk. The heterozygous mutation of C677T is more prevalent at 22-25% of the population, but only leads to moderate increases in plasma homocysteine levels in individuals lacking proper diet [12, 13].

Diet and exercise also play a role in elevated homocysteine levels. Diet seems to play a significant role in managing homocysteine levels while the impact of exercise is

less clear. Increased intake of foods containing higher levels of folic acid, cyanocobalamin (B12), and pyridoxine (B6) will reduce blood levels of homocysteine regardless of pre-diet levels [11, 14].

Dr. Kilmer McCully MD in his book, *The Homocysteine Revolution,* presents a strong argument for proper diet in reducing both homocysteine and prominence of vascular issues. He argues that in developed countries diets are full of processed foods which knowingly are short in pyridoxine (B6) and folic acid due to their sensitively towards destruction in food processing; for example in the refining and preservation of wheat into white flour, 50-90% of pyridoxine (B6) is destroyed, and in the canning of meats and fish the loss ranges from 40-50% [60]. On the opposite end however, fruits and vegetables are common choices that contain high folate levels and have been shown to offer protection against the onset of cardiovascular disease [15].

For cyanocobalamin (B12) and pyridoxine (B6), it has been observed that elderly populations are usually lacking in these vitamins due to poor diets and failure to absorb them properly which adds to their apparent heightened age specific homocysteine levels [16]. The combination of these three nutrients is essential for proper managing of homocysteine levels [17]. In fact, cyanocobalamin (B12) is required for methionine synthase to function properly. However it is important to note that the decrease in homocysteine levels does not repair the damage caused, nor does the decrease prevent recurrence of cardiovascular or neurovascular events- its control simply seems to prevent issues from arising [19,60]. On the contrary to the effects of proper folate and pyridoxine (B6) and cyanocobalamin (B12) consumption, some certain dietary foods can lead to a marked accumulation of homocysteine. Increased consumption of coffee, ethanol, red

meats, fish, chicken, cheese, and eggs as opposed to plant protein sources can cause increased homocysteine levels [18].

The relationship between increased exercise and homocysteine levels is widely debated, but important to mention when researching homocysteine and its effects. The results of numerous exercise studies seems to indicate that chronic, higher intensity exercise speeds up the metabolism of methionine to homocysteine leading to increased levels in individuals. Similarly individuals who had sedimentary lifestyles before beginning a moderate exercise regimen composed of walking for approximately 30 minutes, several times a week, reported having lower homocysteine levels as compared to the beginning of the trial [18]. These general studies however should be taken lightly in that they fail to encompass a number of factors that could influence homocysteine levels such as: stress, general health to begin with, accuracy of exercise reporting, creatinine levels, and many more. Besides the inconsistency in exercise effectiveness in moderating homocysteine levels, numerous reports support the idea that exercise improves both cardiovascular and nervous system function as a whole.

Gender also plays a role in blood concentrations of homocysteine. From several studies, premenopausal women are known to be protected from coronary disease compared to postmenopausal women suggesting the role of estrogen in vascular health [24]. Estrogen and homocysteine studies suggest that higher estrogen status “is associated with lower homocysteine levels independent of nutrition and muscle mass and may explain the reported male-female difference in total homocysteine concentration” [20,21]. Estrogen levels also seem to play a role in increased folate utilization, which would assist the remethylation of homocysteine [22]. From the differing hormone levels, the 5-8 fold

risk of coronary artery disease of males aged 25-55 years compared to females may be explained by the fluctuating homocysteine levels associated with estrogen. The difference in levels may also be associated with higher muscle mass in men. The formation of muscles is associated with increased creatine/ creatinine synthesis which runs simultaneously to homocysteine formation [33].

The last major contributor to homocysteine levels is smoking. Smoking exacerbates the effects of homocysteine in that it also acts independently from other vascular risk factors. It is a well-known fact that people who smoke have a higher occurrence of a number of cardiovascular and neurovascular issues and it comes to no surprise that this risk is parallel to their overall homocysteine levels. Smoking has also been shown to change plasma chemistry affecting the thiol redox balance and also may inhibit methionine synthase [34]. A comparison study's results between smokers and non- smokers is shown below [23] [Table 1]. However it is important to note that this study did not base line for diet, but the p-values for the results suggest significance outside of this influence.

Parameters	Smokers, n=162	Non-Smokers, n=138	p-Value
Homocysteine, µmol/L	18.46	12.75	0.04
Folic acid, nmol/L	4.30	4.45	0.56
Vitamin B12, pmol/L	363.94	407.45	$<10^{-7}$

Table 1*:* Comparison between non-smokers and smokers with levels of homocysteine, folic acid, and vitamin B12 represented. The results were obtained from *Mohamed Fadhel Najjar, et al. "Effect of Cigarette Smoking on Plasma Homocysteine Concentrations."*

III: Important Forms of Homocysteine in the Blood:

The sources listed above all provide for increases in homocysteine levels. Once concentrations of homocysteine overwhelm the mechanisms meant to keep it in check, homocysteine can take several damaging forms. These main forms are protein bound, free oxidized, and free reduced, and together encompass total homocysteine levels [Table 2].

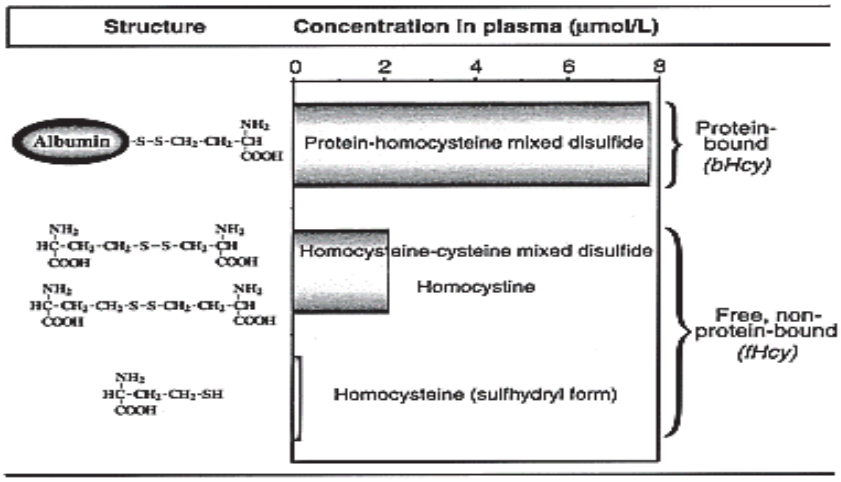


Table 2*:* The three main classes of plasma homocysteine. Relative to 12 µmol/L obtained from Mudd SH, Finkelstein JD, Refsum H, Ueland PM, Malinow MR, Lentz SR, et al. Homocysteine and its disulfide derivatives: a suggested consensus terminology. Arterioscler Thromb Vasc Biol 2000;20: 1704-1706.

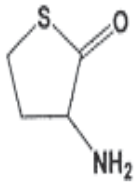
The most common form of homocysteine found in plasma is when it is bound to proteins such as albumin. It also can bind to other proteins such as ɣ-globulin, α-glycoprotein, transthyretin, and high-density lipoproteins, but in lessening levels. The combination of these constitutes approximately 80% of total plasma levels [27, 28]. Albumin is particularly prone to homocysteine due to a free cysteine residue at an N-terminal position where oxidation with homocysteine's sulphur can occur. These protein-homocysteine compounds are termed S- Homocysteinyl-Protein and are generally considered to be biologically unreactive [27]; however this process of homocysteinylation can alter metabolizing agents such as homocysteine's own

endogenous metabolizing enzyme cystathionine γ-lyase which is responsible, among other things, for the catalysis of L-cystine to hydrogen sulfide [42]. The alteration and substrate inhibition of this enzyme may limit the supply of the potent anti-oxidizing agent and vaso-relaxing agent hydrogen sulfide, a subject we discuss in section V [44].

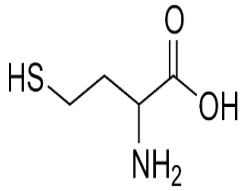
The next form is free oxidized homocysteine which occurs when two homocysteine molecules interact forming a new homocystine compound or when homocysteine interacts with cysteine forming a homocysteine-cysteine molecule. These compounds are formed due the chemical reactivity of homocysteine at physiological pH causing the thiols to form a bridge creating the homodimer homocystine or a homocysteine bound to a cysteine [28]. The reaction is shown in the following generalized formula: $2\ R\text{-}SH = R\text{-}SS\text{-}R + 2H^{+} + 2e^{-}$. These free electrons can then react singly or in pairs in reducing oxygen; the product is superoxide if one electron reacts and a peroxide if 2 elections react. Oxidized homocysteine is relatively reactive due to oxidative stress, but the free reduced form of homocysteine is the most reactive of them all.

The last major form of homocysteine in the plasma is free (reduced) homocysteine and it exists in less than 2% of total levels [28]. There is evidence suggesting that the free thiol form of homocysteine is more harmful than the protein bound and the disulfide species described earlier [28]. Normally free homocysteine binds to proteins or interacts with itself or cysteine but some is left over. The left over is termed reduced homocysteine and has a highly reactive free thiol group that can participate in redox reactions [28,29]. Several studies have shown that this free thiol is associated with endothelial dysfunction in humans while the protein and disulfide forms did not

contribute significantly [31, 32]. The free reduced form is mainly oxidized to the disulfide forms. However some of this free form is converted into homocysteine's thiolactone [32].



Homocysteine thiolactone



Homocysteine

Homocysteine's thiolactone is related to serum levels of free reduced homocysteine; homocysteine's thiolactone is synthesized by methionyl-tRNA synthetase (MetRS) in human endothelial cells, as well as several others such as fibroblasts and breast cancer cells [37]. It is important to note that homocysteine's thiolactone level of 0.2 nM in normal individuals increases to 14.4 nM and 11.8 nM in CBS and MTHFR mutated patients respectively [36]. Although, as previously mentioned, patients with CBS/ MTHFR issues show elevated total homocysteine levels, the increase of thiolactone occurs significantly outside of the total increase showing its importance in vascular issues associated with hyperhomocysteinemia [36]. Because free reduced homocysteine and its thiolactone are related, thiolactone proves to be highly reactive and may provide a mechanism of hyperhomocysteinemia's vascular effects and will be discussed later [36,37].

As presented, numerous meta-analysis and cohort studies have shown a link between homocysteine and increased vascular risks. There are many sources of increased homocysteine levels, ranging from diet to genetics, and it is clear that these increased concentrations summate towards the deregulation of proper vascular and nervous

function. In this study we aim to show the negative effects of homocysteine through the vascular system first and then in its apparent implication in memory by experiment.

IV: Homocysteine and Endothelial Dysfunction:

The first reports linking plasma levels of homocysteine to cardiovascular risks were published on mentally retarded children in 1962. Physicians initially noticed high concentrations of homocysteine in the children's urine, a condition called homocystinuria, and in later years found the gene coding for cysteine-beta synthase (CBS) to be lacking in many cases [8]. Mudd and colleagues described four major hallmarks of the CBS deficient patients: ocular lens dislocation, skeletal abnormalities, differing degrees of mental retardation, and thromboembolic disease which was often the cause of their premature deaths [50]. Also Mudd, and later McCully documented widespread vascular atherosclerotic lesions in autopsies of hyperhomocysteinemic patients [50]. However, at the time many believed that hyperhomocysteinemia simply blended in with other risk factors such as HDL and LDL in causing vascular issues; but we know now this is not entirely the case.

Upon further investigation it was found that children with defects in CBS, and subsequently high levels of circulating homocysteine, frequently had thromboembolic events. This finding was later re-confirmed by Wilcken and Wilcken in 1976 when they linked altered homocysteine metabolism to coronary artery issues [9]. In a landmark study in 1985, Mudd and colleagues studied 600 patients and their response to high doses to pyridoxine (B6) to test if the reduction of homocysteine was parallel to reduced vascular problems; they found risk was significantly reduced in the treatment population

[51]. Also the previously noted concern for homocysteine masking by other risk factors was disproven by the meta-analysis of Boushey et al in 1995, and in later studies of the 2000s which concluded that the association between plasma homocysteine levels and cardiovascular problems remains strong even after adjustment for other risk factors [10,67].

From the compiled information linking homocysteine to cardiovascular issues the question of how does homocysteine specifically act on the cardiovascular system arises. To understand the routes of action a brief review of cardiovascular components is needed. The cardiovascular system is essentially composed of the heart, the arteries and veins, and the blood. All of these parts work in cooperation to create circulation. The functional unit of each of these components is primarily the vascular endothelial cell. Vascular endothelial cells provide lining for capillaries, veins and arteries, and the thick walls of the heart and their upkeep is essential to a healthy cardiovascular system. Congruently the majority of cardiovascular problems arise from issues in endothelial lining. For the purpose of this report we will limit the discussion to how homocysteine affects the endothelial lining in explaining its negative impacts.

The major route of homocysteine acting on endothelial cells is widely contested, but recently increased evidence has confirmed its importance. In this section it is important to note that many of homocysteine's effects, especially in the formation of atherosclerosis, can be activated by other means besides a lining breech [69]. In this article we will entertain that homocysteine effects the endothelial vessel lining in four major ways: by increasing blood pressure, by activating platelet aggregation, by oxidative

stress release and MMP activation by monocyte adhesion, and by inducing smooth muscle proliferation.

The first route homocysteine can act on the endothelial lining is by increasing blood pressure. In the cardiovascular system pressures differ greatly with each heartbeat as expressed by the systolic (maximum) and diastolic (minimum) readings of a blood pressure cuff. The endothelial lining is expected to undergo functional adaptations to accompany the pressure load. The development of blood pressure issues arises when this adaptation is abused and elasticity is lost or structural components of the endothelium are compromised providing resistance. These developments occur 95% of the time from idiopathic or multifactorial sources [43]. Because blood pressure can compromise and activate a plethora of endothelial issues, it is important to note how homocysteine may play a role in its increase.

Homocysteine has been shown to increase blood pressure by the "Hordaland Study" in which 16,000 people aging from 40-67 years old with no history of hypertension were tested and homocysteine levels were found to be positively correlated with pressure levels [45]. Several mechanisms have been proposed to explain this find including: increased vascular hypertrophy (enlargement of endothelial cells), reduced availability of endothelial nitrous oxide and H_2S (vaso-dilating agents), increased vascular smooth muscle cell proliferation (leading to tightness), antagonizing the smooth muscle cells (thickness/ vasoconstriction from angiotensin), increased intimal-medial thickness by amplified levels of collagen impairing vascular compliance (less elasticity), and increased intracellular calcium levels affecting vascular muscle cell tension (muscles squeeze vessels) [42]. Much research needs to be done elucidating the exact mechanisms

of each. The rise in tension and narrowing of the vessels caused by homocysteine and increased blood pressure can lead to an increase in thrombotic events such as stroke and heart attack.

The second route homocysteine can act on the cardiovascular system is through increased platelet aggregation. This theory of homocysteine's influence on clot formation was first proposed by McDonald et al. in 1964 when he noticed an increased adhesion of homocysteine's platelets on a glass surface [51, pg 415]. As previously mentioned homocysteine can become autoxidized in the blood forming dimers and also can be bonded to proteins. It can also be converted into a thiolactone [25]. This molecule will become important with its interactions with platelets.

Platelets, also known as thrombocytes, are involved in the clotting mechanism. Individually platelets contain integrin molecules, as well as many other receptors, on their surfaces. One of these integrin surface proteins is glycoprotein IIb/IIIa (integrin αIIbβ3) [38]. This integrin is the most abundant platelet adhesion molecule and is found in a density of approximately 80,000 receptors per platelet [52]. During vessel injury and clot formation this receptor's job is to show affinity and bind to fibrinogen and also to activate von Willebrand factors which exponentially increase the "stickiness" of a platelet aggregate [46]. These factors also increase the platelets affinity to exposed collagen on the endothelium which rapidly occurs in injuries where the basal collagenous membrane of the vessel is exposed.

In patients with hyperhomocysteinemia, it has been shown that homocysteine and its thiolactone both antagonize integrin αIIbβ3 changing its confirmation permitting binding of its ligand, fibrinogen [38]. This primarily occurs by homocysteine's

thiolactone interacting with a cysteine group within integrin inadvertently activating it [52]. It is interesting to note that integrin αIIbβ3 has been shown to have thiol isomerase activity, which in normal processes acts in appropriately changing confirmation to an active state, so it is plausible that thiolactone could interact to inadvertently activate this adhesion receptor [52,53]. The binding of integrin αIIbβ3 to fibrinogen creates a cross bridge to another platelet causing the beginnings of a clot [39]. This ligand binding in turn activates alpha granules inside each platelet, which release p-selectins that signal other platelets to become more "sticky" [38, 39, 40]. Several studies show that increased levels of homocysteine and its thiolactone augment platelet activation causing the platelets to form clots more readily [38]. Not surprisingly in these studies thiolactone was found to be more effective in modulating platelet adhesion [38]. If these clots reach sizes of arterial blockage, heart attacks and strokes can occur by putting stress on weak spots in the endothelial lining of vessels, especially in a capillary/ micro vessel setting, as we will see in the microvasculature of the brain later on. This stress is a topic that is essential in understanding homocysteine's negative vascular effects and also in its neuronal pathology.

As previously mentioned, blood pressure is markedly increased in hyperhomocysteinemic patients which is caused by an increase in collagen deposition in the extra-cellular matrix among other factors [47]. This increase in endothelial tension can cause atherosclerotic injuries at bifurcated vessels where pressure is the highest. These micro lesions on the endothelium lining then expose collagen and subsequently create a "rough" patch on the otherwise smooth lining. This rough spot creates a perfect place for the platelet's increased affinity for other platelets, collagen, and stickiness to

combine. This complex of platelet's can then aggregate creating thrombotic and atherosclerotic events leading to increased risks of heart attacks and stroke.

The third route to be discussed on homocysteine's interaction with endothelial cells is autoxidation and MMP activation. Homocysteine's autoxidation at physiological pH was previously explained through its homodimer formation and interactions with cysteine to form a homocysteine- cysteine molecule [28]. This creates either a superoxide or peroxide depending on how many electrons react with oxygen. The increased oxidative stress on endothelial cells induces the expression of several matrix metalloproteinases by mimicking an injurious environment.

Matrix metalloproteinases are a family of endopeptidases that break down amino acids within proteins and can be thought of as protein "scissors". Their primary job is to regulate the extracellular matrix, which in terms of endothelial cells, is the structural support that keeps vessels healthy. In events where these proteases are overly activated there is a marked decrease in vascular wall integrity [61]. Within the endothelial extracellular matrix two MMPs are of importance; these are MMP-2 and MMP-9. The reason that these two MMPs are of high importance is because they are the only ones capable of degrading collagen type IV and are classified as gelitanases which allow for easier determination of their activity in vitro by zymography. This interaction with collagen is especially important in capillaries and micro-vessels, a subject we will discuss later in relation to neuron microvascular health.

In hyperhomocysteinemia these two MMPs have been shown to interact with endothelial tight junction proteins in capillary settings and increase permeability and leakage [48]. Also in knockout studies MMP-2 and MMP-9 have been implemented in

cardiac rupture after myocardial infarctions which suggests their role in major vessel structure and the creation of new vessels as well [49]. However, much about MMP activation in hyperhomocysteinemic events is not fully understood. There is some evidence supporting a hypothesis of MMP activation besides just saying, “reactive oxidative species activate MMPs,” which is a common phrase in oxidative studies of hyperhomocysteinemia. The activation of these MMPs may be generated by vascular cell adhesion protein (VCAM-1) and intercellular adhesion molecule (ICAM-1) being up-regulated in hyperhomocysteinemia leading to increased monocyte and leukocyte binding [54, 55].

In normal individuals the site of endothelial damage is quickly relieved and suppressed to prevent vascular issues from arising. One step in this process is to help reduce the chance of infection by increasing the injured area’s affinity for monocytes. As previously mentioned, the blood is always circulating with each heartbeat and the injured site needs some way to stop these monocytes from simply rolling away- the answer is up regulation of the VCAM and ICAM receptors [39, 56]. In hyperhomocysteinemia, the vessels are stressed from increased pressure, reactive oxidative species, reduction of bioavailable NO, and increased cytokine concentrations which can mimic an inflamed injurious environment [54, 56, 62]. Due to this increased stress several studies have reported a drastic up-regulation in VCAM and ICAM and subsequently an increased binding of monocytes [54, 55, 57].

Upon binding to the up-regulated VCAM and ICAM receptors, the monocyte undergoes diapedesis (intake during inflammation) into the sub-endothelial space where they convert into macrophages. During this process there is evidence that the binding of

monocytes to VCAM and ICAM stimulates their NAPDH oxidases to undergo a "respiratory burst" which is intended to destroy any pathogens around [56, 58, 59]. Normally this "burst" of reactive peroxidases and superoxides are kept in check by powerful antioxidants, but in a non-injured vessel who's antioxidant systems are already compromised by the unavailability of NO (nitrous oxide) and possibly GSH (gluthionine), along with the increased affinity and occurrence of monocyte adhesion could lead to a situation where reactive oxidative species are increasingly prevalent. Also the activation of these cells leads to a release cytokines, chemokines, and growth factors which gives rise to an inflammatory environment [62]. This increase of reactive species would cause the activation of MMP-9 and MMP-2 to begin remodeling the endothelial extracellular matrix, basal membranes, and tight junction proteins which can cause increased permeability of LDL cholesterol to enter the sub-endothelial level forming a "bubble" in the intima as shown below [Figure 3].

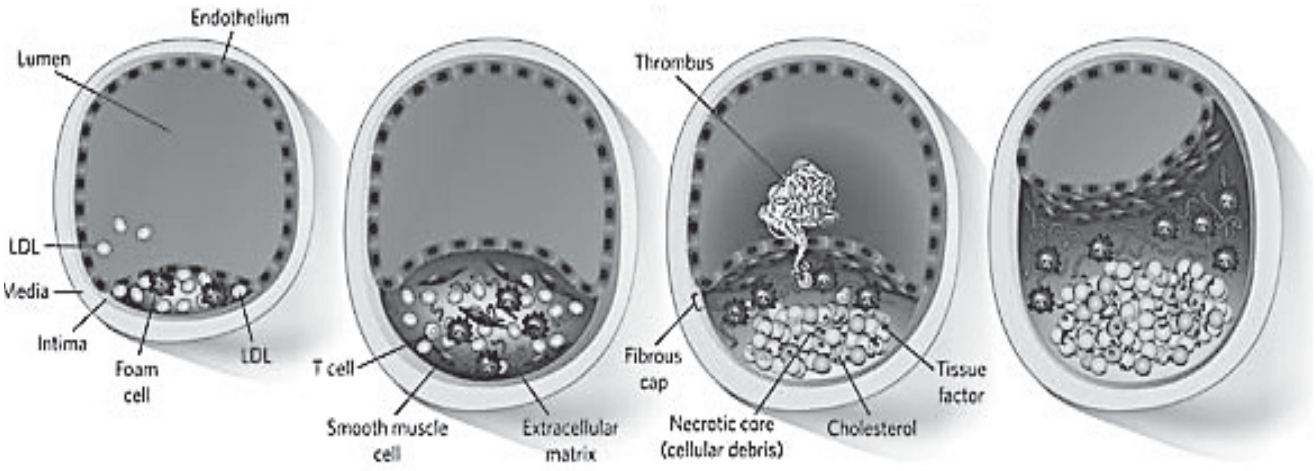


Figure 3: Formation of arterial blockage and thrombotic events. The endothelial is compromised leading to monocyte diapedesis and macrophage conversion by LDL into foam cells freeing cholesterol and increasing fatty buildup. Daniel J. Rader & Alan Daugherty Nature 451, 904-913(21 February 2008)

The fourth major route homocysteine interacts with the endothelial lining to be discussed in this report deals with smooth muscle cell proliferation. From studies of atherosclerosis it was found that often smooth muscle cells migrate to the sub-endothelium in regions of vascular injury [62]. Normally these cells circulate as progenitor cells and are activated by platelet derived growth factor which can be found in alpha granules distributed by activated platelets, which as explained earlier, can be induced by homocysteine's interactions with integrin [38, 65]. This migration is also followed by a marked increase in their proliferation in hyperhomocysteinemic conditions [64]. This increased proliferation creates increased amounts of layers thus decreasing the size of the vascular lumen creating an environment ripe for atheroma, which is the swelling of artery walls and accumulation of plaque [60].

The route of this activation is still currently being investigated and involves many key players. However it is clear that homocysteine can induce connective tissue growth factor (CTGF), a potent stimulator of smooth muscle proliferation in the vascular intima. Several pathways in hyperhomocysteinemic conditions lead to the up-regulation of CTGF. These up-regulations seem to be caused by a possible stimulation of PKC by homocysteine and also by the release of ROS, especially H_2O_2 and its apparent direct activation of Janus Kinase 2 and 3 (JAK2/JAK3) which leads to CTGF and cytokine release [66,68]. Both of these pathways are shown to be up-regulated in hyperhomocysteinemic conditions and cause an increase in the "layers" of muscle cells and collagen deposition which affect proper vessel structure as shown below in figure 4. The image below represents smooth muscle proliferation after injury and shows the result of "increased layers."

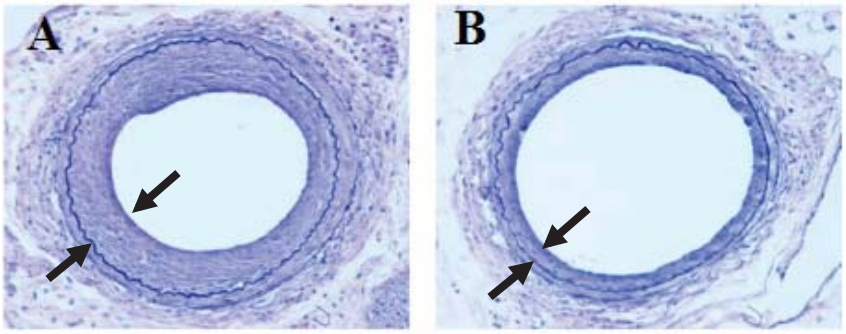


Figure 4: Pictures of mice arteries comparing smooth muscle proliferation after a denudation injury. [A] is the injured vessel [B] normal. These images are to show vessel narrowing by SMC proliferation. Findeisen, Hannes M, et al. "Epigenetic Regulation of Vascular Smooth Muscle Cell Proliferation."

The four routes of homocysteine's interactions with the endothelial cell can affect both its proper function and structure. In summary these were discussed in four routes: increased blood pressure, activated platelet aggregation, oxidative stress release and MMP activation by monocyte adhesion, and smooth muscle proliferation. However when dealing with homocysteine in the neuron and memory impairment the vessels are merely half the picture, the other half is the neuron itself and through research conducted at the University of Louisville the intricate relationship between micro-vessels, the neuron, and their role in memory was explored.

V: Homocysteine, the Blood Brain Barrier, and Memory Impairment- University of Louisville Summer Research Program:

Homocysteine and its effect on vascular function are not limited to the endothelial cells, but can also be implicated in proper neuronal function. In a review of neurological diseases it was found that homocysteine was elevated when cognitive decline occurred [70]. Initially an increase of homocysteine levels was reported by Regland and colleagues in patients with dementia in 1990 [70, 71]; since then, the link has become clearer with an increased interest in the research of elderly conditions such as Alzheimer's and Parkinson's [72]. In one study a cohort of 321 men were assessed for diet, folate, B

vitamins, and homocysteine over the course of 3 years with increments of examination through the Mini-Mental State Examination; the group found that there was a strong link between a decreased cognitive score and increased homocysteine levels and interestingly, they also found that folate intake seemed to offset this decline [73]. Along with a plethora of other studies, the link between homocysteine and its metabolism has been connected to cognitive impairment. The mechanism of this supposed decline is more appropriately addressed through the memory center of the brain.

The role of the hippocampus was discovered by the famous case of Henry Molaison and his surgical removal of the region to alleviate seizures. His surgery occurred in 1953 and removed most of his hippocampus and surrounding tissues. After his surgery scientists found he suffered anterograde amnesia, which is the inability to form new memories, and moderate retrograde amnesia, which deals with past memories [74]. This finding started an intense rise of memory related research and also serves as a prime starting point in investigating homocysteine's apparent impact on memory formation.

During research conducted at the University of Louisville's Cardiovascular and Physiology Summer Program for Undergraduate Students 2013, the role of homocysteine in cognitive decline and the possible treatment with hydrogen sulfide was investigated. This paper will give a brief explanation of some of the findings during the three month experiment, but in order to understand the experiment more fully a brief summary of what was done is in order.

In the experiment there were six treatment groups (mice) that were injected with the following respective compounds intracranially at the start of the trial: artificial

cerebrospinal fluid (aCSF), homocysteine (Hcy), homocysteine+ NaHS, homocysteine+ MK801, MK801 per se, and NaHS per se. The NaHS (sodium hydrogen sulfide) and MK801 treatments were continued intraperitoneally for a 7 day period. After the 7 day period was finished, the animals were removed and subjected to novel object tests for memory, depression tests, tail cuff blood pressure tests, Doppler fluorometry tests, and finally sacrificed for immunohistochemistry tests such as western blots, semi-quantitative PcR, zymography, and slide tissue staining.

In our particular project we set out to confirm the reported effects homocysteine had on the hippocampus as well as to determine if hydrogen sulfide could attenuate the negative effects through mitigation of the NMDA receptor as well as reduce the oxidative stress and tension on the micro-vessels through its strong antioxidant properties and by doing so, hopefully improve memory [81]. Also we wanted to investigate homocysteine's role in altering the relationship between the neuron and the blood brain barrier and to see if hydrogen sulfide could prevent this alternation from occurring. The reason MK801 was used was to help determine whether the NMDA receptor was indeed the receptor orchestrating homocysteine's negative effects on the neuron because MK801 is a known antagonist to the NMDA receptor [82].

As aforementioned, homocysteine has been linked numerous times to a decline in cognitive functions both in human and animal studies. In our cognitive tests, our results were congruent with the studies mentioned above by showing an increase in cognitive impairment with the rise of homocysteine levels [Table 3].

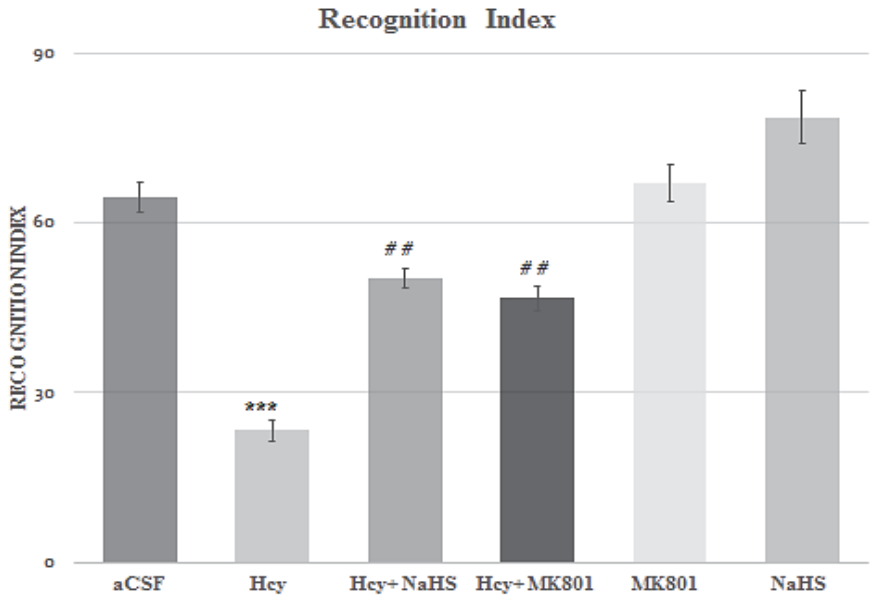


Table 3: Novel object test results in terms of recognition index which is the total time spent exploring the novel object as compared to the old one. It discerns how well the mice can determine the difference between an old memory and a new one. Significance of Hcy was compared to the aCSF control and the treatment NaHS and MK801 are compared to the Hcy group.

Alongside this supposed alteration of cognitive function is a marked increase of depression which suggests a further role of homocysteine in normal brain function. Several studies have shown correlation between elevated homocysteine and increased occurrence of depression [77-78]. One study sampled 3,752 men aged 73 years or older and found that higher levels of homocysteine increased the risk of depression and lowering their homocysteine levels within normal range reduced the risk by 20% [79]. This relationship can be attributed to the anxiety of stroke and cardiovascular events which have been known to induce depression in patients, but there seems to be a

biochemical role outside of these psychological affects that is attributed to neurotoxicity [79, 80]. In our mouse models we found similar results through a depression swim test [Table 4].

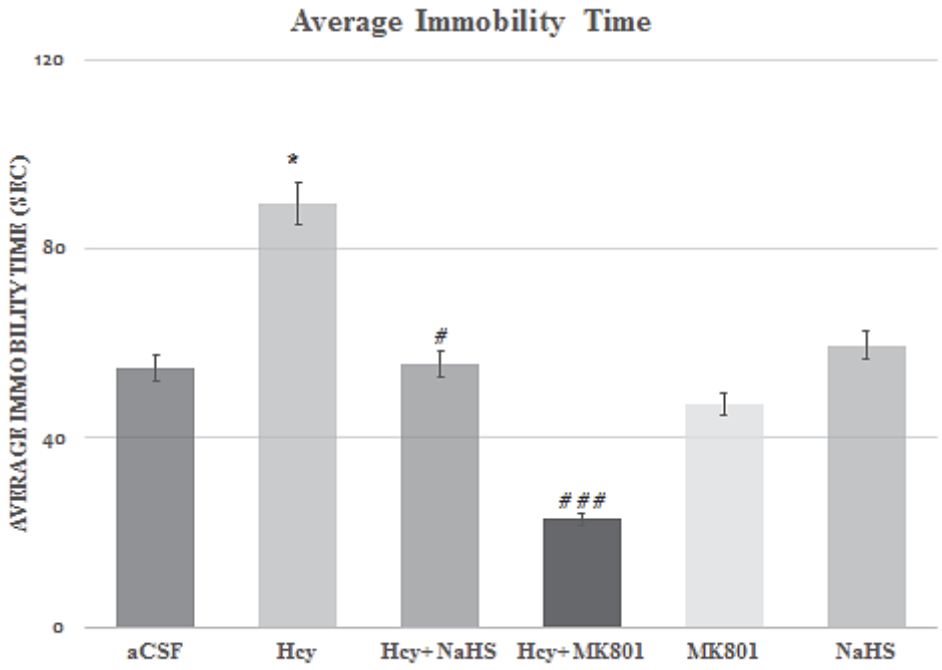


Table 4: Results of the depression swim test are expressed in average seconds "floating" which is representative of depression in mice. Significance of Hcy was compared to the aCSF control and the treatment NaHS and MK801 are compared to the Hcy group.

The hypothesis explaining this cognitive impairment and depression by homocysteine was based upon two routes that both summated to neurotoxicity. The first route was through homocysteine's effect on the blood brain barrier and its micro-vessels. The second route homocysteine was through the neuron by its ability to induce an excitatory ion influx by antagonizing the NMDA receptor and overexciting the mitochondria. Our central hypothesis is shown below [Figure 5].

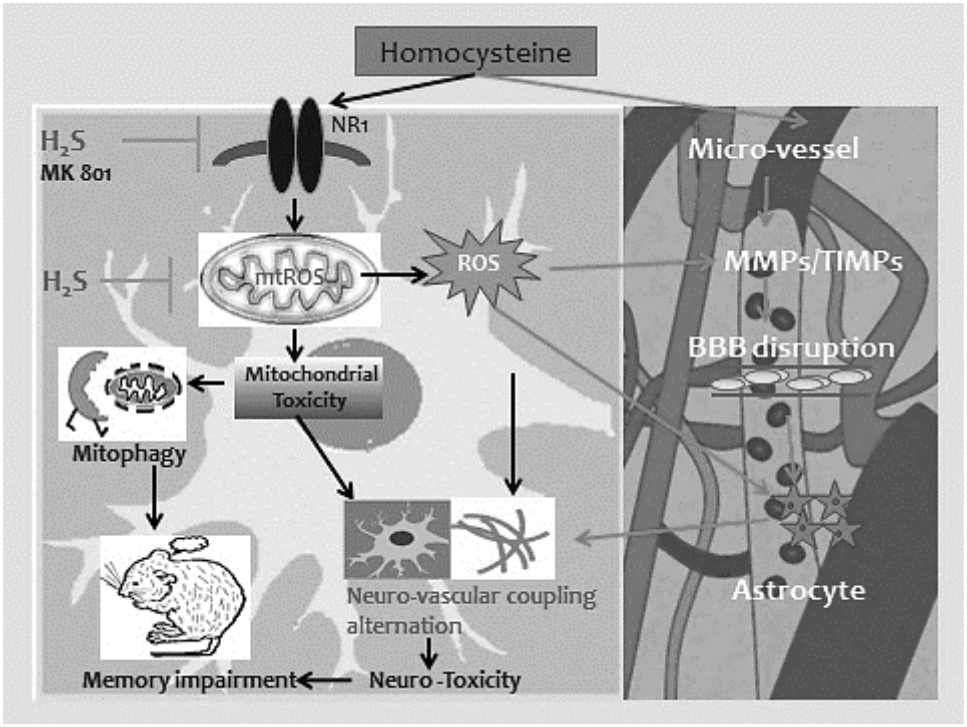


Figure 5: Hypothesis for homocysteine's interaction with the neuron ultimately leading to cognitive impairment.

The first route we examined was how homocysteine interacted with the blood brain barrier. The blood brain barrier is a delicate system of capillaries that supplies nutrients and proteins to the neuron. One hallmark of capillary structure is a series of tight junctions that provide strict control of cellular transport by forming an almost impenetrable, closely regulated zone of transport [75]. The relationship between the capillary and the neuron is bridged by astrocytes whose primary job is to ensure the proper delivery of nutrients and signals, as well as the expulsion of waste products [76]. These astrocytes sit just behind the endothelial lining in the basil lamina where they branch out with end feet to create more surface area. Astrocyte end feet, termed glia limitans, are often associated with the formation of tight junctions between the endothelial cells and are often found in close contact [75].

In order to understand how homocysteine was inducing its effects on micro vessels and the blood brain barrier (BBB) a series of stress tests, slide staining, western blots, and PcR were used to test our hypothesis. The first of these tests was a fluorometric experiment to investigate the integrity of the BBB after the 7th day of the trial. The results of the test confirm our hypothesis by showing an increase in permeability of the vessels in the homocysteine group and the reduction of permeability by the NaHS group. The results are shown below in Table 5.

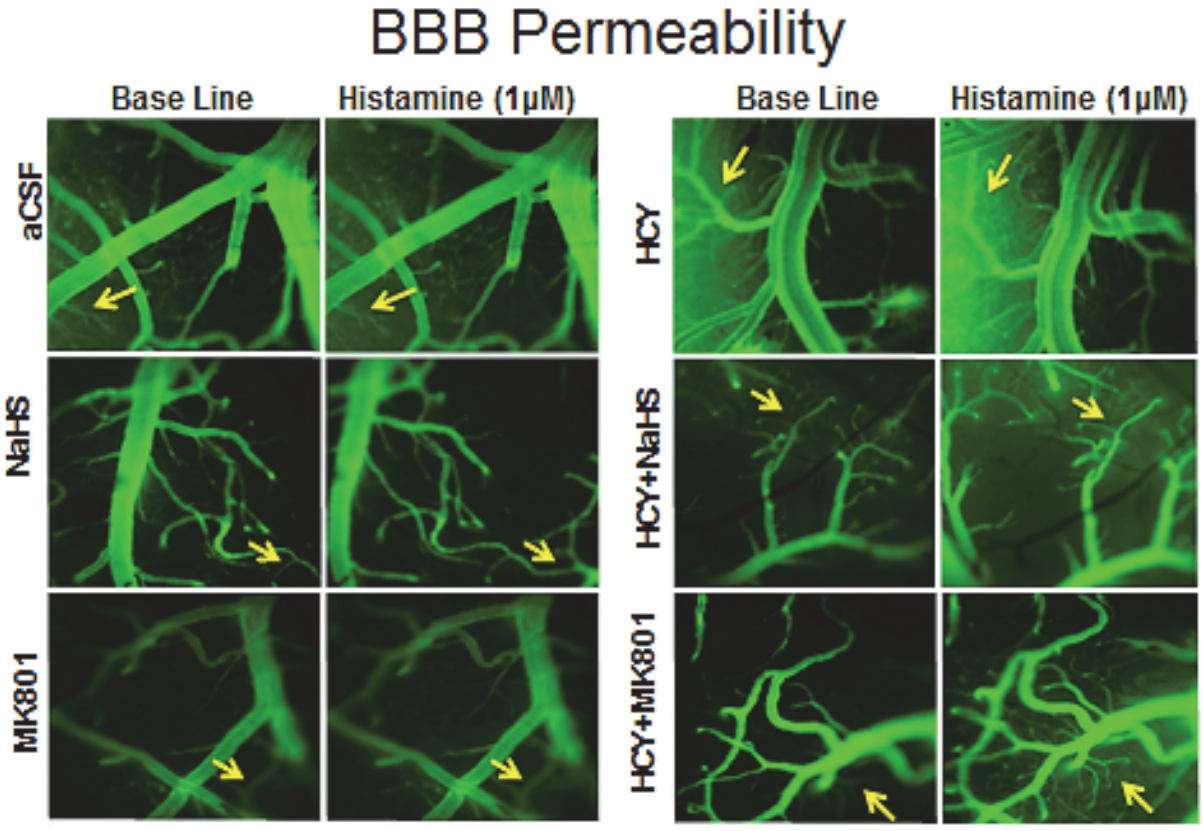
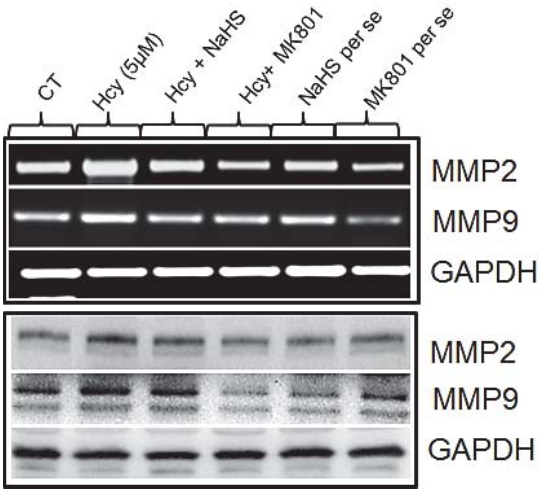


Table 5: Results of blood brain barrier stress test induced by histamine vasodilation. Notice the increased permeability of the Hcy group and the subsequent normality of the NaHS group. The increased "fuzziness" and brighter areas are the dye leaking out of the vessels showing permeability issues.

To explain this increase of leakage we wanted to see if MMP activation played a role in reducing vascular integrity by compromising both the basal lamina and the tight

junctions of the micro-vessels to which the blood brain barrier is known for. To test this we used PcR and western blotting to examine if up regulation and increased expression of MMP occurred along with zymography, to test its activity. Also this increased leakage was thought to interfere with the neurocoupling between the neuron and the vessels by way of astrocyte retraction. To test this we slide stained portions of the hippocampus for VE-Cadherin and GFAP and viewed them under a fluorescent microscope. VE-Cadherin is used to show vessel strength and healthiness of vessel junctions (red) [83]. GFAP stands for glial fibrillary acidic protein and is used as a marker for astrocytes (green) [84]. The results for these prospective studies are shown below in Tables 6, 7, and 8.



	CT	Hcy (5μM)	Hcy + NaHS	Hcy+ MK801	NaHS per se	MK801 per se
MMP2	---	↑	↓	↓	↓	↓
MMP9	---	↑	↓	↓	↓	↓
GAPDH	---	---	---	---	---	---

Table 6: The results of MMP PcR (top) and western blotting (bottom). Right is the relative increase or decrease in expression. GFAP is shown as an indicator of proper concentrations. Note the decrease hydrogen sulfide has on MMP expression.

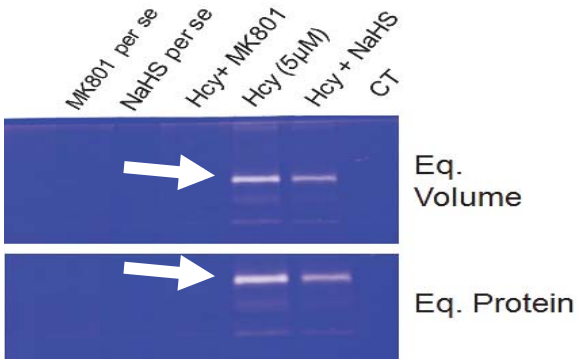


Table 7: The white arrows point to the active form of MMP-9, and the dash below signifies the activity of MMP-2. Both activities are increased in Hcy and reduced in NaHS treatment. The MMP break down the dye and reveal light below showing if the protein is active.

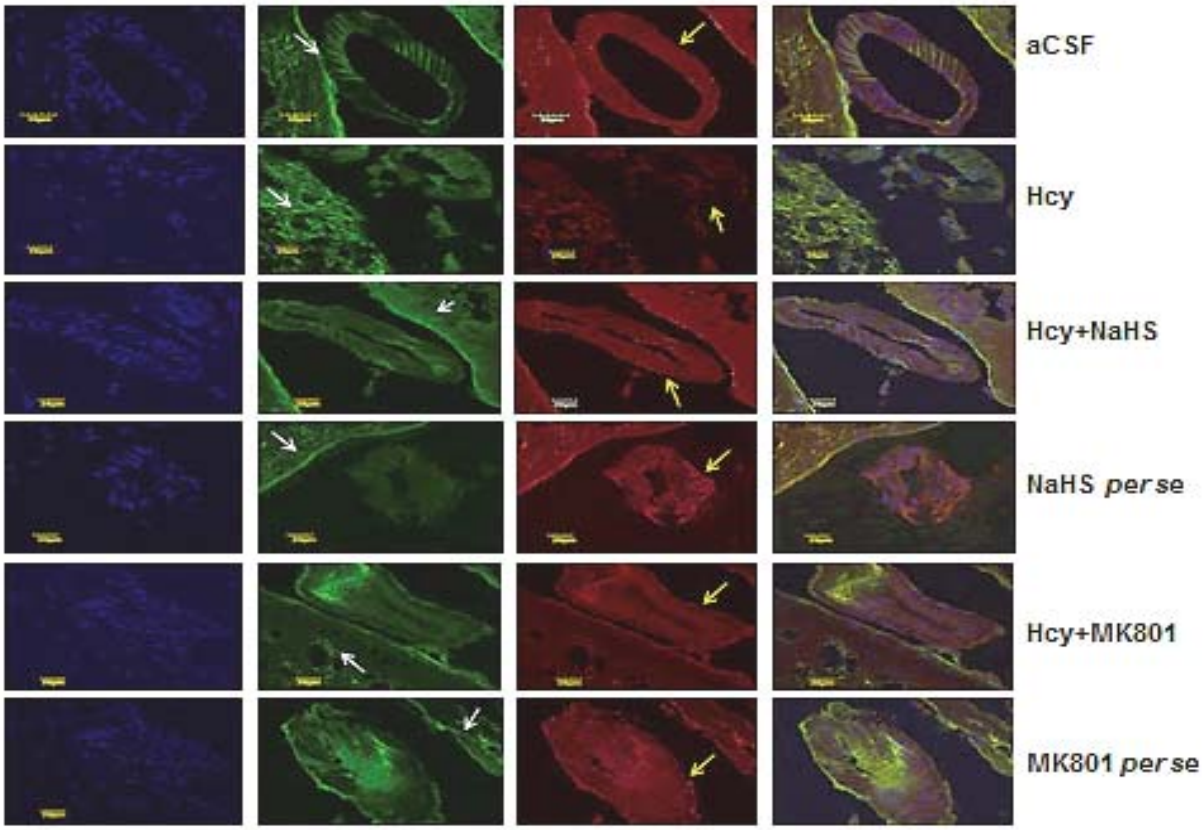


Table 8: The slide stains are of vessels in the hippocampal region. The first blue stain is DAPI for nucleus staining. The green second column is GFAP. Take note of the astrocyte retreat into the tissue in Hcy. The red third column is VE-Cadherin showing structural integrity of vessels and TJP. Notice Hcy's effect on vessel integrity and NaHS revival. The last column is a merge of the results to help show location of vessels.

These results suggest that homocysteine is activating MMPs, which then degrade the tight junction proteins and basal lamina of the blood brain barrier, causing a retraction of astrocytes and an increase in permeability, thus disrupting neurocoupling causing the link between the neuron and the vessel to be broken which agrees with our proposed mechanism. This subsequent breaking can have negative effects for the health of the neuron because it is starved for nutrients and believes the disruption of the blood vessels to be an injurious event, possibly creating a neurotoxic environment where cellular waste can compile. However the route of homocysteine through the vessels is not the only way homocysteine can induce neurotoxicity in hippocampal neurons [85].

The second route that homocysteine can take in decreasing cognitive ability by neurotoxicity is by acting directly on the neuron itself. This process occurs by its interaction with the NMDA receptor [86, 87]. The NMDA receptor is a glutamate receptor heavily involved in proper ion movement, especially in the context of calcium importation [90]. Because of this the NMDA plays a major role in memory and learning by long and short-term potentiation of nerve signals, it is widely considered one of the most important receptors in the brain [90]. Homocysteine has been known to antagonize the NMDA receptor located on the external membrane of the neuron. The opening and increased expression of this receptor causes a massive importation of calcium into the neuron and induces several downstream signals that affect the mitochondria. The increased intracellular calcium excites the mitochondria causing it to release reactive oxidative species and cytokines leading to a neurotoxic environment and possibly cell death [88].

To combat this excitatory effect the neuron implements mitophagy, the process of removing mitochondria, to remove the defective "power factory" and reduce the threat of total cell destruction [89]. One major event in our hypothesis is the stimulation of the NMDA receptor and is why we chose MK801 to act as the negative control. Also mitophagy and the cytotoxic release of the mitochondria induced by excitatory calcium were important to our hypothesis [91, 92, 93]. The result of an intracellular calcium test is shown in Table 9. The results of our PcR and western blot test to show mitophagy/ autophagy, increased release of ROS, and NMDA activation are shown in Tables 10 and 11.

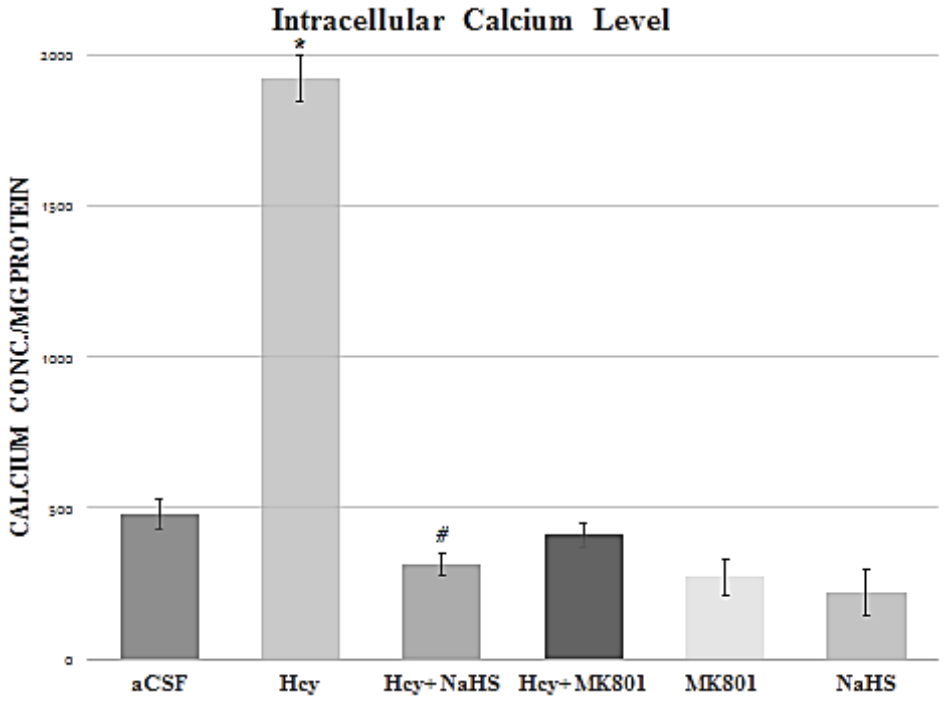
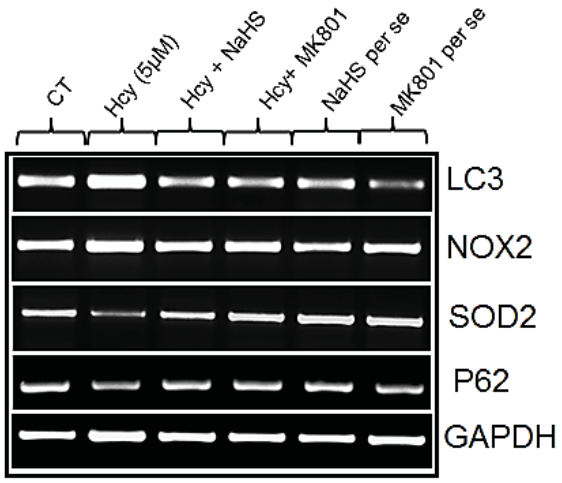
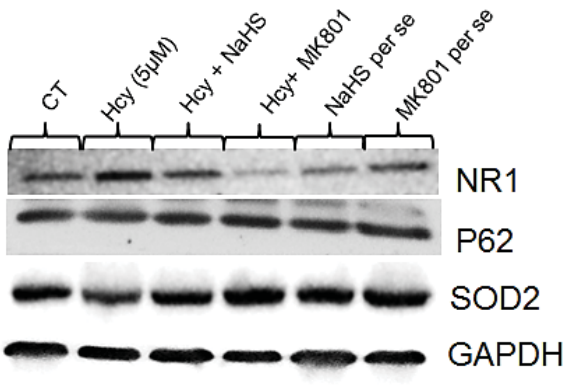


Table 9: Intracellular Calcium levels measured in hippocampal regions. Notice Hcy massive increase through NMDA antagonizing and NaHS's apparent mitigation. Significance of Hcy related to aCSF control and Hcy+ NaHS related to Hcy.



LC3	---	↑	↓	↓	↓	↓
NOX2	---	↑	↓	↓	↓	↓
SOD2	---	↓	↑	↑	↑	↑
P62	---	↓	↑	↑	↑	---
GAPDH	---	---	---	---	---	---

Table 10: Semi Quantitative PcR results: LC3 is an autophagy marker and its up-regulation is indicative of neuronal mitochondrial destruction. NOX2 marks for NADPH oxidase and reactive oxidative species. SOD2 marks mitochondrial superoxide dismutase which is active in removing reactive oxidative species. P62 is an autophagy marker whose decrease is indicative of autophagic occurrences. GAPDH is baseline for loading.



NR1	---	↑	↓	↓	↓	↓
P62	---	↓	↑	↑	↑	↑
SOD2	---	↓	↑	↑	↑	↑
GAPDH	---	---	---	---	---	---

Table 11: Western Blot Results: NR1 is a subunit of the NMDA receptor whose increase in Hcy in concurrent with its excitatory effects; NaHS treatment reduces increase. P62 is an autophagy marker whose decrease is indicative of autophagic occurrences. SOD2 marks mitochondrial superoxide dismutase which is active in removing reactive oxidative species. GAPDH is loading concentrations for baseline.

These results help to confirm our hypothesis. The increased level of intracellular calcium is a hallmark of antagonizing the NMDA receptor. This increase of calcium can alter synaptic function and lead to improper signaling which ultimately affects the proper formation of memories [93]. We confirm the antagonization with NR1 which is a subunit

of the NMDA receptor. NR1's increase in expression is indicative of its excitation [91]. The increased expression of autophagy and mitochondrial dismutase markers is a sign of neurotoxicity as well as mitochondrial damage [91, 92]. Also in our experiment we found that ATP concentrations in the hippocampal regions were significantly decreased in the homocysteine treatment group as compared to the control. This decrease in ATP is yet another indicator that the neuron's internal environment is being affected by the influx of calcium as well as the broken neurocoupling between the vessels.

VI: Conclusion:

Homocysteine's wide range of negative effects gives rise to its involvement in the possible pathologies of several systems. Ever increasing research on this molecule is finding implications in numerous diseased states and the mechanisms controlling them. In terms of the vascular system however, homocysteine's effects on endothelial cells seems to be quite detrimental by simulating an injurious environment, but a great deal of research still needs to be done to elucidate the key molecular players and major routes of action. This increase in vascular stress can induce a myriad of complications ranging from stroke and atherosclerosis to coronary heart failure and degradation of the neuronal microvessels. In the nervous system, specifically the neurons of the hippocampus, homocysteine's effects follow the route of excitatory theory by antagonizing the NMDA receptor causing an increased occurrence of mitophagy and autophagy by way of calcium importation. The degradation of mitochondria and generation of reactive oxidative species paired with the altercation of the delicate astrocyte connection to the microvessels provides a suitable mechanism of cognitive decline by means of neurotoxicity.

All in all the control of homocysteine levels in one's life can be easily accomplished by practicing a balanced diet with regular exercise. The simple addition of B vitamins and folic acid to diets have been shown to significantly reduce the onset of several vascular issues as well as to lower blood concentrations of homocysteine. Garlic and its stimulation of hydrogen sulfide production has also been shown to mediate homocysteine's negative effects within the brain by inducing glutathione production and mediating the NMDA receptor [94]. From the broad range and sharp increase of homocysteine research, it seems as if it will play a much bigger role in the future of medicine, and may someday pass cholesterol as a biomarker for general vascular health. Keeping an eye on homocysteine would be a wise decision for students entering the medical profession and the continuation of its research is sure to produce some fascinating results.

References

1. Hooshmand, Babak, et al. "Plasma Homocysteine, Alzheimer and Cerebrovascular Pathology: A Population-Based Autopsy Study." *Brain: A Journal of Neurology* 136.9 (2013): 2707-2716.

2. Song, In-Uk, et al. "Clinical Significance of Homocysteine (Hcy) On Dementia in Parkinson's Disease (PD)." *Archives of Gerontology & Geriatrics* 57.3 (2013): 288-291.

3. Bottiglieri T, Laundy M, Crellin R, Toone BK, Carney MW, Reynolds EH. “Homocysteine, Folate, Methylation, and Monoamine Metabolism in Depression.” *J. Neurol Neurosurg Psychiatry*. (2000) 69:228–232

4. Medina M, Urdiales J, Amores-Sánchez M. “Roles of Homocysteine in Cell Metabolism.” *European Journal of Biochemistry* (2001): 268(14):3871-3882.

7. Kang SS, Wong PW, Malinow MR. “Hyperhomocyst(e)inemia as a Risk Factor for Occlusive Vascular Disease.” *Annual Review Nutrition.* (1992): 12: 279–298.

8. Mudd SH, Finkelstein JD, Irreverre F, et al. “Homocystinuria: an Enzymatic Defect.” *Science* (1964)143:1443–45

9. Wilcken DEL, Wilcken B. “The Pathogenesis of Coronary Artery Disease. A possible Role for Methionine Metabolism. *J. Clin. Investigation*. (1976). 57:1079–82

10. Boushey CJ, Beresford SAA, Omenn GS, et al. “A Quantitative Assessment of Plasma Homocysteine as a Risk Factor for Vascular Disease: Probable Benefits of Increasing Folic Acid Intakes.” *JAMA* (1995). 274:1049–57

11. Mudd SH, Levy HL, Skovby F. “Disorders of Transsulfuration. in the Metabolic and Molecular Basis of Inherited Disease,” ed. CR Scriver, AL Beaudet, WS Sly, et al. New York:McGraw-Hill (1995) pp. 1279–327.

12. Deloughery TG, Evans A, Sadeghi A, et al. “Common Mutation in Methylenetetrahydrofolate Reductase: Correlation with Homocysteine Metabolism and Late-onset Vascular Disease. *Circulation* (1996) 94: 3074-3078

13. Frosst P, Blom HJ, Milos R. et al. “A Candidate Genetic Risk Factor for Vascular Disease: A Common Mutation in Methylenetetrahydrofolate Reductase.” *Nature Genetics*. (1995) 10:111–113

14. Brattström L. “Vitamins as Homocysteine-Lowering Agents.” *Journal of Nutrition.* (1996) 126:1276S–80S (Suppl.)

15. .Ness AR, Powles JW. “Fruit and Vegetables, and Cardiovascular Disease: a Review.” *International Journal Epidemiology*. (1997) 26:1–13

16. Lindenbaum J, Rosenberg IH, Wilson PW, et al. “Prevalence of Cobalamin Deficiency in the Framingham Elderly Population. *American J. Clinical Nutrition.* (1994) 60:2–11

17. Spence, John David. "B Vitamin Therapy For Homocysteine: Renal Function And Vitamin B12 Determine Cardiovascular Outcomes." *Clinical Chemistry & Laboratory Medicine* (2013) 51:3 633-637

18. Lanae, J. Manore, M. "Exercise, Nutrition, and Homocysteine." *International Journal of Sport Nutrition & Exercise Metabolism.* (2006)16.4: 341-361

19. Knut Rasmussen, et al. "Homocysteine Lowering and Cardiovascular Events after Acute Myocardial Infarction." *New England Journal of Medicine.* (2006) 354:15 1578-1588

20. Morris M., Jacques P., Selhub J., Rosenberg I. “Total Homocysteine and Estrogen Status Indicators in the Third National Health and Nutritional Examination Survey.” *American Journal of Epidemiology.* (2000) 152:140-148

21. Van baal M., Smolders R., Van der Mooren M., et al. “Hormone Replacement Therapy and Plasma Homocysteine Levels.” *Obstetric Gynecology.* (1999) 94:485-491.

22. Dimitrova KR, DeGroot K, Myers AK, Kim YD. “Estrogen and homocysteine.” *Cardiovascular Research.* (2002) 53: 577–588

23. Mohamed Fadhel Najjar, et al. "Effect of Cigarette Smoking on Plasma Homocysteine Concentrations." *Clinical Chemistry & Laboratory Medicine.* (2011) 49.3: 479-483

24. Mukadder Serter, et al. "Effects of Early Initiated Estrogen Replacement Therapy on Plasma Homocysteine Levels in Women in Surgically Induced Menopause." *Gynecological Endocrinology*. (2011) 27.2: 101-106

25. Olas, Beata, Joanna Malinowska, and Joanna Rywaniak. "Homocysteine and its Thiolactone may Promote Apoptotic Events in Blood Platelets in Vitro." *Platelets.* (2010) 21.7: 533-540

26. Jakubowski, H. "Molecular Basis of Homocysteine Toxicity in Humans." *Cellular & Molecular Life Sciences.* (2004) 61.4: 470-487

27. Malinowska, Joanna, Paweł Nowak, and Beata Olas. "Comparison of the Effect of Homocysteine in the Reduced Form, Its Thiolactone and Protein Homocysteinylation on

Hemostatic Properties of Plasma." *Thrombosis Research.* (2011) 127.3: 214-219

28. Jakubowski, Hieronim. “Homocysteine in Protein Structure/Function and Human Disease Chemical Biology of Homocysteine Containing Proteins.” (2013) Dordrecht: Springer, Web. <http://ekulib.kyvl.org/vwebv/search?searchArg=Homocysteine in Protein Structure/Function and Human Disease >.

29. Eldibany, Mohamed M., and Joseph A. Caprini. "Hyperhomocysteinemia and Thrombosis: An Overview." *Archives of Pathology & Laboratory Medicine.* (2007)131.6: 872-884

30. Pellanda, Hélène. "Betaine Homocysteine Methyltransferase (BHMT)-Dependent Remethylation Pathway in Human Healthy and Tumoral Liver." *Clinical Chemistry & Laboratory Medicine.* (2013) 51.3: 617-621

31. Chambers JC, Obeid OA, Kooner JS. “Physiological Increments in Plasma Homocysteine Induce Vascular Endothelial Dysfunction in Normal Human Subjects.” *Arteriosclerosis Thromb. Vasc. Biol*. (1999) 19(12):2922–2927

32. Chambers JC, Ueland PM, Wright M, Dore CJ, Refsum H, and Kooner JS
Investigation of Relationship between Reduced, Oxidized and Protein-Bound Homo-
Cysteine and Vascular Endothelial Function in Healthy Human Subjects.”
Circulation Research. (2001) 89:187–192

33. Norlund L, Grubb A, Fex G, Leksell H, Nilsson JE, Schenck H, and Hultberg B. “The Increase of Plasma Homocysteine Concentrations with Age is Partly Due to the Deterioration of Renal Function as Determined by Plasma Cystatin C.” *Clinical Chem Lab Med.* (1998) 36:175–178

34. Blom HJ. “Determinants of plasma homocysteine.” *American Journal Clinical Nutrition.* (1998) 67:188–189.

35. Jakebowski H. “The Determination of Homocysteine-Thiolactone in Biological Samples.” *Analytical Biochem.* (2002) 308(1): 112

36. Jakubowski, Hieronim. “Homocysteine in Protein Structure/Function and Human Disease Chemical Biology of Homocysteine Containing Proteins.” (2013) Dordrecht: Springer, Web. <http://ekulib.kyvl.org/vwebv/search?searchArg=Homocysteine in Protein Structure/Function and Human Disease >.

37. Jakubowski, Hieronim. "Homocysteine Thiolactone: Metabolic Origin and Protein Homocysteinylation in Humans." *Journal of Nutrition.* (2000) 130.2: 377S-381S

38. Malinowska, Joanna, and Beata Olas. "Homocysteine and its Thiolactone-Mediated Modification of Fibrinogen Affect Blood Platelet Adhesion." *Platelets.* (2012) 23.5: 409-412

39. Biochemistry 7th ed., by Berg, Tymoczko, and Stryer (2011), Freeman Press, New York.

40. Tailor, Anitaben, Dianne Cooper, and D. Neil Granger. "Platelet–Vessel Wall Interactions in the Microcirculation." *Microcirculation.* (2005) 12.3: 275-285

41. McGarrigle SA, O'Neill S, Walsh GM, Moran N, Groham IM, Cooney MT, et al. “IntegrinαIIbβ3 Exists in an Activated State in Subjects with Elevated Plasma Homo-cysteine Levels. *Platelets.* (2011) 22: 63–71

42. Sen U, Mishra PK, Tyagi N, Tyagi SC. “Homocysteine to Hydrogen Sulfide or Hypertension.” *Cellular Biochem Biophysics* (2010) 57: 49–58

43. Oparil, Suzanne, M. Amin Zaman, and David A. Calhoun. "Pathogenesis of Hypertension." *Annals of Internal Medicine.* (2003) 139.9: 761-776

44 Lin, Chang, et al. "Hydrogen Sulfide Inhibits Myocardial Injury Induced By Homocysteine in Rats." *Amino Acids* 34.4 (2008): 573-585. *Academic Search Premier*. Web. 14 Nov. 2013.

45 Refsum, Helga, and Ottar Nygard. "The Hordaland Homocysteine Study: The Opposite Tails Odds." *Journal of Nutrition.* (1996) 126.4: 1244S-1248S

46 Nurden AT, Poujol C, Durrieu-Jais C, et al. “Platelet Glycoprotein IIb/IIIa Inhibitors: Basic and Clinical Aspects.” *Arterioscler Thromb Vasc Biol*. (1999) 19: 2835–2840

47. Lentz, S R, et al. "Vascular Dysfunction In Monkeys With Diet-Induced Hyperhomocyst(E)Inemia." *The Journal of Clinical Investigation.* (1996) 98.1: 24-29

48. Genersch, E., Hayess, K., Neuenfeld, Y.,and Haller, H.. “Sustained ERK Phosphorylation is Necessary but not sufficient for MMP-9 Regulation in Endothelial Cells.” *J. Cell Sci. (*2000) 113: 4319–4330

49. H. Nagase, R. Visse, G. Murphy. “Structure and Function of Matrix Metalloproteinases and TIMPs.” *Cardiovasc. Res.* (2006) 69:562–573

50. Mudd, SH, Finkelstein, JD, Irreverre, F, Laster, L. “Homocystinuria: An Enzyme Defect.” *Science*. (1964) 143:1443-45

51. Carmel, Ralph, comp. “Homocysteine in Health and Disease.” 1st. Cambridge, MA: Cambridge University Press, (2001). Print. Pg 2-6

52. McGarrigle, Sarah A., et al. "Integrin AIIb B3 Exists In an Activated State In Subjects With Elevated Plasma Homocysteine Levels." *Platelets* (2011) 22.1: 63-71

53. O’Neill S, Robinson A, Deering A, Ryan M, Fitzgerald DJ, Moran N. “The Platelet Integrin AlphaIIb Beta3 has an Endogenous Thiol Isomerase Activity. *J Biol Chem* (2000) 275:36984–36990.

54. Silverman MD, Ramapogal JT, Davis M, Lopez G, Rosenbaum JT, Lelkes PI. “Homocysteine Upregulates Vascular Cell Adhesion Molecule-1 Expression in Cultured Human Aortic Endothelial Cells and Enhances Monocyte Adhesion.” *Arteriosclerosis Thromb Vasc Biol.* (2002) 22:587–*592*

55. Deem TL**,** Cook-Mills JM. “Vascular Cell Adhesion Molecule-1 (VCAM-1) Activation of Endothelial Cell Matrix Metalloproteinases.” *Blood* (2004)104*:* 2385–*2393*

56. Carmel, Ralph, comp. “Homocysteine in Health and Disease.” 1st. Cambridge, MA: Cambridge University Press, (2001). Print. pg 431-438.

57. Kim, Cuk-Seong, et al. "Homocysteine Promotes Human Endothelial Cell Dysfunction Via Site-Specific Epigenetic Regulation Of P66shc." *Cardiovascular Research.* (2011) 92.3: 466-475

58. Pankhurst, Tanya, Caroline O. S. Savage, and Mark A Little. "Review Article: Leukocyte-Endothelial Dysregulation in Systemic Small Vessel Vasculitis." *Nephrology* (2009) 14.1: 3-10

59. Brock, Tom. "The NADPH Oxidase Complex: A Super Oxide Generator." *Caymen Chemical.* (2013). <https://www.caymanchem.com/app/template/Article.vm/article/2126>.

60. McCully, Kilmer. “The Homocysteine Revolution.” 1st ed. New Canaan, Connecticut: Keats Publishing, 1998. 60-65. Print.

61. Whatling C, McPheat W, Hurt-Camejo E. “Matrix Management: Assigning Different Roles for MMP-2 and MMP-9 in Vascular Remodeling.” *Arterioscler Thromb Vasc Biol*. (2004) 24: 10–11

62. Ross, Russell. "Atherosclerosis: An Inflammatory Disease." *New England Journal of Medicine* (1999): 340.2 115-126

64. Changtao, Jiang, et al. "Homocysteine Promotes Vascular Smooth Muscle Cell Migration by Induction of the Adipokine Resistin." *American Journal of Physiology* (2009) 66.6: C1466-C1476.

65. Nishio E *&* Watanabe Y. “Homocysteine as a Modulator of Platelet-Derived Growth Factor Action in Vascular Smooth Muscle Cells: a possible role for hydrogen peroxide.*” J Pharmacol.* (1997) 122*:* 269 74.

66. LIU, X., et al. "Homocysteine Induces Connective Tissue Growth Factor Expression in Vascular Smooth Muscle Cells." *Journal of Thrombosis & Haemostasis* (2008) 6.1: 184-192

67. Wierzbicki, Anthony S. "Homocysteine and Cardiovascular Disease: A Review of the Evidence." *Diabetes & Vascular Disease Research: Official Journal of the International Society of Diabetes and Vascular Disease.* (2007) 4.2: 143-150

68. Majors AK, Sengupta S, Jacobsen DW, Pyeritz RE. “Upregulation of Smooth muscle cell collagen production by homocysteine-insight into the pathogenesis of Homocystinuria.” *Mol Genet Metab*. (2002)76:92–9.

69. Dalton, M L, et al. "Homocysteine Signal Cascade: Production of Phospholipids, Activation of Protein Kinase C, and the Induction of C-Fos and C-Myb in Smooth Muscle Cells." *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* (1997) 11.8: 703-711.

70. Regland B, Abrahamsson L, Gottfries CG, Magnus E. “Vitamin B12 Analogues, Homocysteine, Methylmalonic Acid, and Transcobalamins in the Study of Vitamin B12 Deficiency in Primary Degenerative Dementia.” *Dementia.* (1990) 1:272–7

71. Teunissen, Charlotte E., et al. "Homocysteine In Relation To Cognitive Performance in Pathological and Non-Pathological Conditions." *Clinical Chemistry & Laboratory Medicine* (2005) 43.10:1089-1095

73. Tucker, Katherine L, et al. "High Homocysteine and Low B Vitamins Predict Cognitive Decline in Aging Men: The Veterans Affairs Normative Aging Study." *American Journal of Clinical Nutrition.* (2005) 82.3: 627-635

74. Sweaney, Katherine W. "The Most Famous Brain In The World": Performance And Pedagogy on an Amnesiac's Brain." *Review of Education, Pedagogy & Cultural Studies.* (2012) 34.3/4: 184-196

75. Maeda, Toshihiko, and Takashi Kanda. "Blood-Brain Barrier and Blood-Nerve Barrier in Neuroinflammatory Diseases." *Nihon Rinsho Japanese Journal of Clinical Medicine.* (2013) 71.5: 789-794

76. Abbott, N. Joan, Lars Rönnbäck, and Elisabeth Hansson. "Astrocyte–Endothelial Interactions at the Blood–Brain Barrier." *Nature Reviews Neuroscience*. (2006) 7.1: 41-53

77. Bottiglieri T, Laundy M, Crellin R, Toone BK, Carney MW, Reynolds EH. “Homocysteine, Folate, Methylation, and Monoamine Metabolism in Depression. *J Neurol Neurosurg Psychiatry*. (2000) 69:228–232

78. Bell IR, Edman JS, Morrow FD, Marby DW, Mirages S, Perrone G, Kayne HL, Cole JO: B “Complex Vitamin Patterns in Geriatric and Young Adult Inpatients with Major Depression.” *Journal of American Geriac Soc*. (1991) 39:252–257

79. Almeida, Osvaldo P, et al. "Homocysteine and Depression in Later Life." *Archives of General Psychiatry.* (2008) 65.11:1286-1294

80. Folstein, Marshal, et al. "The Homocysteine Hypothesis of Depression." *American Journal of Psychiatry.* (2007) 164.6: 861-867

81. Szabó, Csaba. "Hydrogen Sulphide and Its Therapeutic Potential." *Nature Reviews Drug Discovery*. (2007) 6.11: 917-935

82. Wong, E H, et al. "The Anticonvulsant MK-801 Is a Potent N-Methyl-D-Aspartate Antagonist." *Proceedings of the National Academy of Sciences of the United States of America*. (1986) 83.18: 7104-7108

83. Vestweber, Dietmar. "VE-Cadherin: The Major Endothelial Adhesion Molecule Controlling Cellular Junctions and Blood Vessel Formation." *Arteriosclerosis, Thrombosis, and Vascular Biology*. (2008) 28.2: 223-232

84. Glial fibrillary acidic protein, Uniprot Antibodies. http://www.uniprot.org/uniprot/P14136

85. Obeid, Rima, and Wolfgang Herrmann. "Mechanisms of Homocysteine Neurotoxicity in Neurodegenerative Diseases with Special Reference to Dementia." *Febs Letters.* (2006) 580.13 2994-3005

86. Ganapathy, Preethi S, et al. "The Role of N-Methyl-D-Aspartate Receptor Activation in Homocysteine-Induced Death of Retinal Ganglion Cells." *Investigative Ophthalmology & Visual Science.* (2011) 52.8: 5515-5524

87. Kamat, P.K., et al. "Hydrogen Sulfide Attenuates Neurodegeneration and Neurovascular Dysfunction Induced By Intracerebral-Administered Homocysteine in Mice." *Neuroscience*. (2013): 252: 302-319

88. Qiu, Jing, et al. "Mitochondrial Calcium Uniporter Mcu Controls Excitotoxicity and is Transcriptionally Repressed by Neuroprotective Nuclear Calcium Signals." *Nature Communications*. (2013) 4:2034

89. Szydlowska, Kinga, and Michael Tymianski. "Calcium, Ischemia and Excitotoxicity." *Cell Calcium* (2010) 47.2:122-129

90. Zitto, K. "NMDA Receptor Function and Physiological Modulation." *Neurobiology*. (2009) 1157-1165. Web. <http://brain.phgy.queensu.ca/pare/assets/Neurobiology2.pdf>

91. Adam-Vizi V, Starkov A. “Calcium and Mitochondrial Reactive Oxygen Species Generation: How to Read the Facts.” *Journal of Alzheimer's Disease*. (2010) 20:413-426

92. Brennan A, Sang Won S, Swanson R, et al. “NADPH Oxidase is the Primary Source of Superoxide Induced by NMDA Receptor Activation. *Nature Neuroscience*. (2009) 12(7):857-863

93. MacDermott A.B, Mayer, L. M, Westbrook, L. G. “NMDA-receptor Activation Increases Cytoplasmic Calcium Concentration in Cultured Spinal Cord Neurons.” *Nature*. (1986) 321:519-522

94. Kashfi, Khosrow, and Kenneth R. Olson. "Biology And Therapeutic Potential Of Hydrogen Sulfide And Hydrogen Sulfide-Releasing Chimeras." *Biochemical Pharmacology* (2013) 85.5: 689-703