

Homocysteine and reactive oxygen species in metabolic syndrome, type 2 diabetes mellitus, and atherosclerosis: The pleiotropic effects of folate supplementation

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ABSTRACT

Homocysteine has emerged as a novel independent marker of risk for the development of cardiovascular disease over the past three decades. Additionally, there is a graded mortality risk associated with an elevated fasting plasma total homocysteine (tHcy).

Metabolic syndrome (MS) and type 2 diabetes mellitus (T2DM) are now considered to be a strong coronary heart disease (CHD) risk enhancer and a CHD risk equivalent respectively. Hyperhomocysteinemia (HHcy) in patients with MS and T2DM would be expected to share a similar prevalence to the general population of five to seven percent and of even greater importance is: Declining glomerular filtration and overt diabetic nephropathy is a major determinant of tHcy elevation in MS and T2DM.

There are multiple metabolic toxicities resulting in an excess of reactive oxygen species associated with MS, T2DM, and the accelerated atherosclerosis (atheroscleropathy).

HHcy is associated with an increased risk of cardiovascular disease, and its individual role and how it interacts with the other multiple toxicities are presented.

The water-soluble B vitamins (especially folate and cobalamin-vitamin B₁₂) have been shown to lower HHcy. The absence of the cystathionine beta synthase enzyme in human vascular cells contributes to the importance of a dual role of folic acid in lowering tHcy through remethylation, as well as, its action of being an electron and hydrogen donor to the essential cofactor tetrahydrobiopterin. This folate shuttle facilitates the important recoupling of the uncoupled endothelial nitric oxide synthase enzyme reaction and restores the synthesis of the omnipotent endothelial nitric oxide to the vasculature.

INTRODUCTION AND BACKGROUND

Homocysteine (Hcy) is a nonessential sulfur-containing amino acid and an intermediary metabolic product derived from the demethylated essential amino acid methionine (figure 1).

Since its discovery in 1932 by the 1955 Nobel Prize recipient Vincent DuVigneaud [1] and its association with premature arteriosclerotic (fibrotic) vascular disease described by Kilmer McCully in 1969 [2], Hcy has emerged as a novel marker of risk for cardiovascular disease.

In the past decade Hcy has become widely accepted as a novel risk marker associated with atherosclerotic cardiovascular disease (CVD) in the coronary, cerebral, and peripheral vascular beds. Additionally, it has been determined that hyperhomocysteinemia (HHcy) is an independent and a graded risk factor for the development of CVD [3-13]. The current role of Hcy as a causative factor still remains controversial and needs to be more fully elucidated.

The important role of oxidative - redox stress and HHcy is biologically plausible because Hcy promotes oxidant injury to vascular cells (particularly the endothelium and the eNOS enzyme reaction) through the auto-oxidation of Hcy, formation of Hcy mixed disulfides, interaction of Hcy thiolactones, and protein homocysteinylation [14-16].

Oxidation of two Hcy molecules yields the oxidized disulfide (homocystine), two protons (H^+), and two electrons (e^-), while promoting the formation of reactive oxygen species (ROS).

AUTO-OXIDATION OF HOMOCYSTEINE



Superoxide
Hydroxyl radical
Hydrogen peroxide

Also, formation of mixed disulfides contributes to the additional formation of ROS



R = any organic compound in the plasma with a thiol group (-SH) accessible to react with Hcy, such as proteins, cysteine, glutathione, gamma-glutamylcysteine, or cystinylglycine.

Additionally, Hcy may undergo complicated rearrangements to form Hcy thiolactone (a cyclic thioester), which is chemically reactive and acylates free amino groups such as the side-chain lysine groups in proteins. In the process of forming homocysteinylated proteins further oxidative stress develops and homocysteinylated proteins become damaged and may lose their biological activity. This results in the modification of proteins and in particular the modification of low-density lipoproteins (LDL-cholesterol), which contribute to their retention within the intima and subsequent inflammatory foam cell formation associated with atherogenesis.



**Aminoacyl-tRNA Synthetase
Bound Hcy adenylate (Hcy~AMP)**

Hcy thiolactone

Jakubowski has recently been able to demonstrate that Hcy is now considered to be a protein amino acid in humans. In areas of turbulent blood flow the formation of Hcy-N-protein mediated by S-nitroso-Hcy may account for the observation that atherosclerosis originates mostly at branch points and flow dividers in arteries [17].

Recent controversial findings have been published in regards to post percutaneous coronary angioplasty (PTCA) restenosis, one trial has shown a decrease in restenosis and major adverse events of death, nonfatal myocardial infarction, and need for repeat revascularization [18-20], while others have shown no significant effect on the occurrence of restenosis [21-23].

A recent secondary prevention trial by Liem *et al.* [24] has shown no effect in cardiovascular risk reduction within 2 and now 4 years treatment with low dose folic acid supplementation in patients previously on statin therapy and stable coronary artery disease.

Regardless of the current controversies regarding Hcy as being an independent risk factor or merely a risk marker, Hcy has definitely emerged as one of the novel-multifactorial substrates, which interacts with conventional CVD risk factors of the atherosclerotic process [25-26]

Numerous ongoing clinical trials have yet to determine if lowering Hcy by folic acid supplementation decrease the risk of cardiovascular events or cardiovascular mortality and these trials will be forthcoming within the next few years (table 1).

An attempt to shed some new light on the Hcy – HHcy story as it pertains to the metabolic syndrome (MS), prediabetes (PD), and overt type 2 diabetes mellitus (T2DM) and their association with the multiple metabolic toxicities resulting in an increase of

reactive oxygen species (ROS) will be discussed.

Furthermore, an attempt to develop a hypothesis that HHcy is a marker of oxidative and redox stress, as a result of an endogenous folate shuttle, which results in a relative endogenous endothelial folate deficiency due to a folate shuttle phenomenon to preferentially run the endothelial nitric oxide synthase (eNOS) reaction will be explored. The lack of Hcy catabolic transsulfuration in human vascular cells provides an even more important role for the folate shuttle phenomenon in vascular cells. This would result in a relative endogenous endothelial folate deficiency to operate the remethylation pathway via the methionine - folate cycle culminating in vascular cell HHcy.

This folate shuttle phenomenon could also be operative in hypertension and non-diabetic atherosclerosis due to an associated uncoupling of the eNOS enzyme reaction with decreased endothelial nitric oxide (eNO) production and resultant endothelial cell dysfunction.

There are multiple clinical factors associated with HHcy (table 2)), as well as, multiple damaging effects to the vascular system (table 3). In MS, PD, and T2DM there are multiple injurious stimuli to the endothelial cell (including HHcy), and these substrate interactions result in accelerated atherosclerosis → atheroscleropathy (figure 2).

While the liver normally contains the full complement of metabolic enzymes to handle HHcy through the remethylation and the transsulfuration catabolic metabolic pathway, it is limited in human vascular cells. Vascular cells in particular do not express cystathionine beta synthase (CBS), the first enzyme of the hepatic transsulfuration pathway; nor do they express the enzyme betaine homocysteine methyltransferase (BHMT), which catalyzes the alternate remethylation pathway in the liver using betaine

as a substrate [27-28].

Therefore in vascular cells, Hcy metabolism is limited to the B12 – folate dependent remethylation pathway catalyzed by methionine synthase of the methionine-folate cycle.

Thus, vascular cells and particularly endothelial cells may be especially vulnerable to the higher levels of circulating and endogenous Hcy found in patients with HHcy.

This makes the endothelial cell and smooth muscle cells quite vulnerable to the previously discussed oxidative stress of HHcy. In the MS, PD, and overt T2DM this would definitely result in an additive - synergistic effect to the associated multiple metabolic toxicities and the elevated substrates of the A-FLIGHT acronym (table 4).

These findings would help to explain the increased mortality and cardiovascular disease associated with HHcy, especially in T2DM [29-31]. In concluding this section it is important to note that:

HHcy is associated with an increased risk of cardiovascular disease, especially in non-insulin-dependent diabetes mellitus [8].

REACTIVE OXYGEN SPECIES: “TOXIC OXYGEN”

ROS consist primarily of the oxygen free radicals (superoxide [O_2^\bullet], hydroxyl radical [$-\text{OH}^\bullet$], peroxynitrite [ONOO^\bullet]), the potent oxidizing agents of the non-radical family (peroxide [H_2O_2] and hypochlorous acid [HClO]), and the organic analogues, which include reactive nitrogen species: specifically, peroxynitrite [ONOO^\bullet] (table 5).

It has been known for some time that ROS are detrimental and toxic to cells and tissues as a result of injury to lipids, nucleic acids, and proteins: (A). Lipid peroxidation of membranes (loss of membrane function and increased permeability) and generation of lipid autoperoxidation reactions. (B). DNA damage leading to mutation and death. (C).

Cross linking or vulcanization of sulfhydryl rich proteins (leading to stiff aged proteins specifically collagen of the extracellular matrix) [32].

Both intracellular and extracellular antioxidants play important roles in neutralizing these toxic oxygen molecules. The endogenous antioxidant enzyme family consists primarily of superoxide dismutase (SOD), catalase, glutathione peroxidase and the tri-peptide (gamma-L-glutamyl-L-cysteintyl-glycine) glutathione or (GSH), and free circulating thiols containing the sulfhydryl group (-SH) (table 6). These antioxidants are found to be systemically depleted in the clinical setting of T2DM and evidence is present for their depletion in atheromatous lesions [31-32].

There are multiple metabolic toxicities associated with MS, PD, T2DM, and their companion: accelerated atherosclerosis (atheroscleropathy). These multiple metabolic toxicities can be conveniently grouped in an acronym termed the A-FLIGHT toxicities (table 4). Each of these toxicity substrates result in the excessive production of ROS responsible for the damaging effects of oxidative - redox stress.

Not only are ROS involved in the development of autoimmune type 1 diabetes mellitus and T2DM but also play an important role in the long-term development of the associated complications: atheroscleropathy, diabetic cardiomyopathy, intimopathy, nephropathy, neuropathy, endotheliopathy, and retinopathy.

HYPERHOMOCYSTEINEMIA AND REACTIVE OXYGEN SPECIES IN INSULIN RESISTANCE, METABOLIC SYNDROME, TYPE 2 DIABETES MELLITUS, AND ATHEROSCLEROPATHY.

There are multiple metabolic toxicities associated with MS, PD, and overt T2DM, which are associated with the production of ROS. These toxic substrates and their associated ROS have been conveniently grouped together to form the acronym: A-FLIGHT

toxicities (table 4).

The general population of patients with MS, IR, PD, and overt T2DM will, in all probability, have a similar frequency of the common polymorphism affecting the folate-dependent, thermolabile gene coding for the 5,10-methylene tetrahydrofolate reductase MTHFR) (C677T, Ala → Val) is associated with a decreased activity of the enzyme with subsequent mild to moderate HHcy, which occurs in 10–15% of the general population [33-35].

This gene polymorphism is especially important in those individuals with a decrease in dietary folate. In these patients mild to moderate HHcy can be improved with dietary and folate supplementation, which improves endothelial-dependent endothelial cell dysfunction [36]. In the general population, mild to moderate HHcy occurs in approximately 5-7% [9].

HHcy is not usually present as a direct result of MS, PD, and T2DM unless there is an associated development of impaired renal function. As nephropathy develops, there is an associated elevation of total Hcy associated with a decline in glomerular filtration rate [37].

In the early latent Stage I and transitional Stage II of T2DM (table7) homocysteine may actually be lower than expected. Rosolova H et al. have found an unexpected inverse relationship between IR and serum Hcy in a population of healthy subjects with insulin resistance. They have attributed this finding to the associated increase in the glomerular hyperfiltration [38].

IR and PD (Stages I, II, and early Stage III) are associated for a period of time with hyperinsulinemia, hyperproinsulinemia, and hyperamylinemia and glomerular

hyperfiltration and hyperperfusion. Recently, the nitric oxide system has been shown to be involved in glomerular hyperfiltration in Japanese normo- and micro-albuminuric patients with T2DM and early stages of diabetic nephropathy [39]. Hiragushi K *et al.* were able to demonstrate an increase staining of eNOS enzyme (in glomerular endothelial cells) in microalbuminuric T2DM patients in comparison to non-diabetic control subjects. This adaptive early response results in glomerular enlargement and an increase in the glomerular filtration rate [40]. Additionally, Apakkan Aksun S *et al.* found that serum and urine nitric oxide levels were elevated, as well as, plasma malanodialdehyde (MDA), which points to the importance of oxidative stress in patients with T2DM. These authors feel that the high nitric oxide levels may lead to hyperfiltration and hyperperfusion, which in turn leads to an increase in urinary albumin excretion and thus causes progression of nephropathy in early T2DM [41].

Over time (Stages III-V, table 7) there is an increase in glucotoxicity (initially postprandial Stage III and fasting in Stage IV), as well as, an increase in redox and oxidative stress to the glomerular endothelium. This damage is associated with lipid peroxides, free fatty acids, and activation of Ang II as a result of hyperinsulinemia, hyperproinsulinemia, hyperamylinemia and the totality of reactive oxygen species associated with the A-FLIGHT toxicities.

The activation of Ang II would be associated with the activation of the membranous NAD(P)H oxidase enzyme and generation of superoxide with its effect on endothelial cell dysfunction and uncoupling of the eNOS reaction. Over time, this would result in decreases in eNO and loss of a natural occurring local antioxidant network within the glomerular endothelial microenvironment.

Glucotoxicity is associated with advanced glycosylation endproducts (AGEs) and their receptor (RAGE), activation of aldose reductase of the polyol-sorbitol pathway, activation of protein kinase C (PKC) isoforms, and transforming growth factor beta (TGFbeta). Once these glucotoxic mechanisms are in play and overt diabetic nephropathy with associated glomerulosclerosis and remodeling has developed, there will be a progressive decrease in glomerular filtration. These changes will result in Hcy elevation [37].

Thus, HHcy plays an extremely important role for additional oxidative-redox stress regarding the accelerated cardiovascular disease observed in diabetic patients on dialysis [42].

HHcy is thought to induce an oxidative inactivation of endothelial nitric oxide, in part by inhibiting or consuming the expression of cellular glutathione peroxidase (GPx). In heterozygous cystathionine beta-synthase deficient $-/+$ mice, Weiss et al. were able to restore endothelial cell function by increasing cellular thiols and reduced glutathione pools and increasing GPx activity with restoration of the endothelial function [43].

The ensuing cellular redox stress is magnified and total Hcy consumes NO by the indirect process of superoxide (O_2^-) converting NO to toxic peroxynitrite ($ONOO^-$).

In addition to $ONOO^-$ formation, NO in conjunction with thiols and oxygen radicals generate nitrotyrosine and nitroarginine. Nitroarginine, in turn, competes for the substrate L-arginine of the eNOS reaction in a feedback mechanism, limiting further NO generation [44-46]. As a result, there is endothelial cell dysfunction, endothelial cell toxicity and endothelial cell loss-apoptosis, increased ROS, increased $ONOO^-$ and decreased NO associated with HHcy [47]. HHcy is multiplicative in nature and even

though its effects may occur later in T2DM than the other associated A-FLIGHT substrate toxicities, it has a devastating - synergistic effect on endothelial cell function. Presently, we know there are other multiple metabolic toxicities (table 4) operating within the renal glomerulus producing microalbuminuria (reflecting endothelial cell dysfunction and damage) at a stage prior to the declining glomerular filtration rate responsible for HHcy (figure 2).

A recent clinical study by Maejima et al. [48] revealed significant elevated levels of ONOO⁻ peroxynitrite (by Griess method) in 129 T2DM patients as compared to 76 non-diabetic controls. While there was no difference in basal plasma NO(2)(-) levels between the T2DM and control patients, ONOO⁻ levels were related to the presence of hypertension and advanced microvascular complications. In addition, ONOO⁻ correlated positively with elevations in AGEs and serum lipid peroxide. These data support the hypothesis that decreased endothelium-dependent vasodilatation in diabetic subjects is associated with the impaired action of NO secondary to its consumption (quenching) from redox stress rather than decreased NO production from vascular endothelium. Clinically, abnormal NO metabolism is related to advanced diabetic microvascular complications.

Zhang et al. [49] were able to demonstrate that increased concentrations of Hcy resulted in a decreased NO response to bradykinin and L-arginine. Hcy stimulated the formation of superoxide anions and peroxynitrite with increased levels of nitrotyrosine. The addition of 5-methyltetrahydrofolate (folic acid) restored NO responses to bradykinin and L-arginine agonists. In addition, scavengers of peroxynitrite and SOD mimetics reversed the Hcy-induced suppression of NO production by endothelial cells. Concentrations of

Hcy (greater than 20 micromolar concentration) produced a significant indirect suppression of eNOS activity without any discernible effects on its expression.

Li et al. just published an article showing an unexpected effect of Hcy-induced oxidative stress resulting in an increase of 3-hydroxy-3-methylglutaryl coenzyme A reductase in vascular endothelial cells, as well as decreasing endothelial NO. They were also able to demonstrate that "statins" increased NO as well as decreasing cellular cholesterol [50]. Stuhlinger et al were able to demonstrate that Hcy impairs the nitric oxide synthase pathway. Homocysteine inhibits dimethylarginine dimethylaminohydrolase (DDAH), which is responsible for degrading ADMA. This effect of Hcy causes the endogenous inhibitor of nitric oxide synthase, ADMA, to accumulate and compete with L-arginine for nitric oxide production. This effect helps to explain the deleterious effect of Hcy on the endothelial cells ability to promote vasodilatation and associated endothelial cell dysfunction with decreased NO synthesis [51].

Previous publications have emphasized the importance of the eNOS enzyme and the important role of the Glu²⁹⁸→Asp gene polymorphism [32] [52]. Noiri and colleagues have recently demonstrated that the gene polymorphism (Glu²⁹⁸→Asp) affecting the eNOS enzyme is statistically and significantly associated with diabetic nephropathy in T2DM patients [53]. This finding points to the important role of the eNOS enzyme and eNO in controlling oxidative – redox stress and its relation to the development of diabetic nephropathy.

This same gene polymorphism (Glu²⁹⁸→Asp) of the eNOS enzyme (eNOS 894TT genotype) has also been recently demonstrated to be a risk factor for HHcy. These findings by Brown et al. indicate that the NOS3 894TT genotype (eNOS Glu²⁹⁸→Asp

gene polymorphism) is a risk factor for HHcy in healthy nonsmoking adults with low serum folate and supports the hypothesis that nitric oxide modulates Hcy through an effect on folate catabolism [54].

ACCELERATED ATHEROSCLEROSIS → ATHEROSCLEROPATHY

The above discussion also applies to the accelerated atherosclerosis (atheroscleropathy) and intimal redox stress and remodeling (intimopathy) associated with MS, PD, and overt T2DM [32].

Insulin resistance is central to the development of CHD and the accelerated atheroscleropathy associated with MS, PD, and overt T2DM. This being said, the ultimate manifestation of insulin resistance is T2DM [32] (figure 3).

Atherosclerosis, MS, PD, and T2DM present a pleiotropic mechanistic progression, in which, inflammation and upstream oxidative-redox stress and ROS appear to share a common - fertile soil background, and patients with diabetes experience both a preexisting and parallel-accelerated atherosclerosis, we have termed atheroscleropathy [32] [52] [55] (figure 4).

In regards to silent coronary artery disease (CAD), Gazzaruso C *et al.* were able to confirm that microalbuminuria and smoking may predict silent CAD in people with diabetes and additionally they were able to demonstrate an original finding of an independent association between HHcy and silent CAD [56].

In the Framingham offspring study [57], those with hyperinsulinemia had significantly higher Hcy levels than those without, and subjects with more than two MS phenotypes (hypertension, impaired glucose tolerance, or a central MS [consisting of two or more of the following: hyperinsulinemia, obesity, or dyslipidemia]) had significantly higher Hcy

levels compared to those with one or no MS phenotype.

Passaro and colleagues have been able to demonstrate in T2DM patients that Hcy levels are influenced by both the duration of the disease and metabolic control. Hcy levels improve with improvement of glycemic control of HbA1c. Hcy remained strongly associated to the presence of CHD independent from age, sex, body mass index, smoking status, hypertension and lipid patterns. Additionally, they conclude that Hcy can be seen as a major risk factor in T2DM patients with CHD [58-59].

The initial vascular lesions described by McCully in 1969 [2] described an arteriosclerotic fibrotic change within the intima, which is in contrast to the intimal fibrous-lipid laden lesions associated with atherosclerosis and the vulnerable thin-cap fibroatheroma prone to rupture.

Burke and colleagues have been the first to describe the plaque morphology and correlate these findings to total plasma homocysteine [60]. Their recent publication demonstrated that Hcy is elevated in sudden unexpected death in men as a result of severe coronary artery disease without acute or organized coronary thrombosis. Additionally, they were able to demonstrate that the above association seemed to be increased if there existed concomitant diabetes mellitus. HHcy was associated with an increase in fibrous plaques and a relative decrease in thin-cap atheromas. Their findings suggest a different mechanism of atherogenesis from that of hypercholesterolemia. Additionally, efforts to reduce Hcy could be complementary to lipid lowering therapy because plaque fibrosis may occur via a distinct mechanism. They suggest that one such mechanism could be endothelial dysfunction, which certainly points to the importance of the “Folate Shuttle phenomenon” within the endothelial cell and a relative endogenous endothelial folate

deficiency within the endothelial microenvironment milieu.

These morphological findings suggest the need for; and importance of; global risk reduction of the entire spectrum of elevated substrates within the A-FLIGHT multiple metabolic toxicities.

PPAR and Hcy

Peroxisome proliferator activated receptors (PPARs) are transcription factors belonging to the nuclear receptor super-family and the multiple roles of their effects are rapidly unfolding.

Clinically there are two classes of drugs currently in use: fibrates binding to PPAR alpha (gemfibrozil and fenofibrate) and thiazolidinediones (TZDs) binding primarily to PPAR gamma (rosiglitazone and pioglitazone). PPAR alpha agonists are responsible primarily for their hypolipidemic effect, while PPAR gamma agonists are antidiabetic agents – insulin sensitizers (enhancing insulin – mediated glucose uptake and lowering of HbA_{1C}), important in the regulation of the lipid metabolism and adipogenesis, and additionally expressed in the vasculature (endothelial cells, smooth muscle cells, and monocyte – macrophage cells). In vitro and clinical data show that TZDs can decrease thrombotic, inflammatory, and oxidative changes that contribute to endothelial dysfunction and their future vasculoprotective roles (table 8) in attenuating the progressive role of non-diabetic atherosclerosis and the accelerated atherosclerosis in MS, PD, and overt T2DM [61-67]. Current available information suggests that PPAR-gamma ligands may prevent the progression of insulin resistance (preserving islet Beta cell function) to diabetes and endothelial dysfunction to atherosclerosis [68].

Recently, Fonseca V. et al. were able to demonstrate that the PPAR agonist troglitazone

had a significant Hcy lowering effect in both the lean and fatty Zucker rat model of insulin resistance. This decline in Hcy was associated with significant increases in hepatic concentrations of S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) plus SAH. Additionally, there was a significant decline in the SAM/SAH ratio. The hepatic CBS was significantly higher in the troglitazone treated group as compared to the control group. The authors conclude that the Hcy lowering effect of troglitazone may be due to a shift in the concentrations of Hcy from the blood to the liver, as well as, a possible upregulation of hepatic CBS [69].

Tao et al. just published a timely article that ties together quite nicely the detrimental role of oxidative-redox stress and ROS and the antioxidant, antinflammatory, and vasculoprotective role of PPARs. They were able to demonstrate (in hypercholesterolemic rabbits) that treatment with the PPAR gamma agonist rosiglitazone enhanced PPARgamma expression, improved endothelium-dependent vasodilatation, preserved the phosphorylation of vasodilator-stimulated phosphoprotein (P-VASP), suppressed gp91(phox) of the NAD(P)H Oxidase enzyme and iNOS expression, reduced superoxide and total NOx production, and inhibited nitrotyrosine formation [70]. Even though Hcy levels were not part of this publication, a reasonable assumption would be that rosiglitazone could also lower Hcy levels.

Recently, we have been able to demonstrate an important finding regarding the interactions of Hcy with PPAR alpha and gamma receptors. In addition to demonstrating that HHcy decreases bioavailability of eNO, generates nitrotyrosine, and activates latent matrix metalloproteinase (which instigates endomyocardial endothelial fibrosis and dysfunction) in vivo, we have been able to demonstrate that Hcy acts in a competitive

manner with the PPAR receptor (in endomyocardial endothelial cells of mouse myocardium) in vitro. Specifically, Hcy interacts with the fibric acid PPAR alpha agonist ciprofibrate and the PPAR gamma agonist 15-deoxy-Delta(12,14)-prostaglandin J(2) (15d-PGJ₂) [71-74] .

This may have definite clinical applications regarding the actions of the PPAR agonist family of fibric acid, as well as the PPAR agonists: TDZs (rosiglitazone and pioglitazone currently in clinical use). If Hcy is elevated there may exist a competitive inhibition of the agonist effects of both of these clinically useful medications (figure 5).

These findings of the potential interaction of Hcy with the PPAR ligands may interfere with the positive role of glucose -lipid regulation and anti-inflammatory antiatherosclerotic actions of the fibric acid derivatives and thiazolidinediones.

HOMOCYSTEINE-FOLATE-COBALAMIN TRIAD AND ATHEROGENESIS

Flynn MA et al. have discussed atherogenesis and the Homocysteine-Folate-Cobalamin (HFC) Triad and it seems pertinent to expand each of their respective roles in this discussion [75].

The toxic role of Hcy on the vasculature has been extensively discussed previously, however, the important role of folic acid and cobalamin (vitamin B₁₂) needs further development.

FOLIC ACID AND THE FOLATE SHUTTLE

Folic acid, the water soluble B vitamin (Vitamin B₉), 5- methyltetrahydrofolate (5-MTHF), has been recently gaining considerable attention due to its positive role in cardiovascular disease, neurodegenerative disorders, neural tube defects, and cancer. Folic acid plays an important role in the remethylation (methionine – folate cycle) of

homocysteine and is thus capable of lowering elevated levels of homocysteine [76].

Deficiency or impairment of folate metabolism is associated with HHcy, hypomethylation (the decreased one carbon unit transfer to purines and pyrimidines for DNA repair and biosynthesis), DNA damage, and impaired cell proliferation, malignancies, and impaired eNO production [47] [77-80].

5-MTHF is an electron donor, a hydrogen donor, and a methyl donor [52] and this attribute confers a unique role to its function. In regards to the eNOS reaction, it supplies both hydrogen and electrons to the oxidatively modified tetrahydrobiopterin (BH4): BH2 and BH3. Once biopterin is completely reduced to BH4 (the necessary cofactor for the eNOS reaction), the eNOS reaction can re-couple and restore the capability of this reaction to be once again a net producer of eNO instead of superoxide. This unique function of folic acid can restore endothelial dependent vasodilatation in T2DM and hyperlipidemia [81].

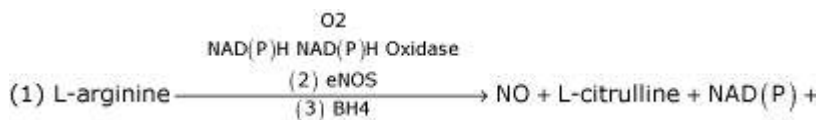
Folic acid supplementation would supply the methyl donating substrate to run the methionine-folate cycle, as well as, supply the hydrogen and electrons to stabilize the requisite BH4 cofactor to run the eNOS reaction resulting in the production of eNO by the endothelium. The restoration of the eNOS reaction, by folate supplementation, has been demonstrated acutely by flow mediated dilation studies in T2DM and coronary artery disease via mechanisms, which are largely independent of Hcy lowering [82-83].

We hypothesize that there may exist a folate shuttle, which is operative in the atheroscleropathy associated with MS, PD, and T2DM, as well as, non-diabetic atherosclerosis. This folate shuttle effect may operate as an effective mechanism in the endothelial microenvironment to recouple the oxidatively stressed, uncoupled, and

dysfunctional eNOS reaction (figures 1).

There are three critical arms associated with the eNOS reaction: the eNOS enzyme, the L-arginine substrate, and the BH₄ cofactor. Each of these three arms is crucial and has been discussed in previous publications [31-32] [52] [84]. The eNOS reaction may be uncoupled due to oxidative-redox stress associated with MS, PD, T2DM, atheroscleropathy, and non-diabetic atherosclerosis. When the eNOS reaction is uncoupled the reaction will become a net producer of superoxide (instead of eNO via the eNOS reaction) through the reaction of membranous NAD(P)H, oxygen, and the NAD(P)H Oxidase enzyme (figure 6).

The following eNOS reaction demonstrates the location of the vulnerable three arms of the eNOS reaction responsible for the generation of eNO (figure 6).



If the eNOS reaction is uncoupled: there is the net production of superoxide instead of eNO.



The healthy endothelium is a net producer of endothelial nitric oxide [eNO].

The activated – dysfunctional - uncoupled endothelium is a net producer of superoxide [O₂^{·-}] associated with MS, PD, T2DM, and atheroscleropathy.

The net production of superoxide by the uncoupled eNOS reaction in addition to the production of ROS via the A-FLIGHT metabolic toxicities results in an excessive oxidative-redox stress.

This oxidant stress may contribute to the oxidation of BH₄ to BH₂ and BH₃, which will be unable to run the eNOS reaction. Folate supplementation as a methyl donor, a hydrogen donor, and an electron donor may restore the oxidized BH₂ and BH₃ to the requisite completely reduced BH₄ by donating hydrogen and an electron to BH₂ and BH₃. With the restoration of BH₄, the eNOS reaction may recouple and once again run the eNOS reaction in order to be a net producer of eNO.

As folate is being shared, stolen, shunted, or shuttled from the remethylation methionine-folate cycle to the eNOS reaction there will develop a relative endogenous endothelial folate deficiency with ensuing intra endothelial Hcy accumulation.

The HHcy that results will then play its own additive auto-oxidative role in the continuing viscous cycle of oxidative stress to the endothelial microenvironment discussed previously.

If this endothelial folate shuttle does exist, then one might assume that Hcy could be a marker of underlying endothelial oxidative – redox stress in addition to being a risk factor. The importance of HHcy being a dual risk (a risk marker and a risk factor) in this patient population certainly points to its importance in being both a result of oxidative – redox stress and its important causative role in endothelial dysfunction.

The importance of the endothelial cell lacking the CBS enzyme only contributes to the hypothesis of an endothelial folate shuttle. Even if measured plasma folate and Hcy levels are normal there may exist a relative endogenous endothelial folate deficiency within the endothelial microenvironment due to the folate shuttle phenomenon.

COBALAMIN (VITAMIN B₁₂)

HHcy is a sensitive marker of clinical cobalamin (the water soluble vitamin B₁₂)

deficiency [85-86]. Folate fortification as well as a more widespread use of folate supplements will have an effect in reducing the frequency of folate deficiency and cobalamin deficiency may be ranked above folate deficiency as a primary modifiable cause of HHcy [87-89].

Common causes of cobalamin deficiency are malabsorption as a result of (i). lack or dysfunction of gastric intrinsic factor (ii). hypochlorhydria as a result of chronic gastritis, gastric atrophy, or the use of proton pump inhibitors (iii). the clinical syndromes of inflammatory bowel disease and celiac disease.

It is of interest to note that one of our newer medications in our treatment armamentarium for MS, PD, and T2DM is emerging as a common cause of cobalamin malabsorption.

Metformin use has recently skyrocketed as a popular treatment for the metabolic derangements associated with MS, PD, and T2DM. This more novel, yet increasingly, common cause of cobalamin deficiency in adults and teenager necessitates a careful approach for the clinician. Currently, in patients with vitamin B12 deficiency or evidence of peripheral neuropathy or macrocytic anemia the use of metformin should always be considered in the differential diagnosis of B12 malabsorption [90]. A strong consideration for patient screening of megaloblastic anemia and or B12 levels in patients on long-term use of metformin should be considered [91]

Folate fortification and supplementation may mask the megaloblastic anemia associated with cobalamin deficiency, while allowing the neurological manifestations of pernicious anemia (peripheral neuropathy, cognitive disturbances, dementia, and depression) to progress. Therefore it is imperative that cobalamin deficiency be aggressively sought out and treated, especially in the elderly. In the United States there are 37 million people

over the age of 65 with conservative estimates indicating a two to three percent (1.1 million) prevalence either having or will develop pernicious anemia.

Additionally, there are estimated to be 20-30 percent (7.4 – 11.1 million) of this population having subclinical cobalamin - B12 deficiency, which may lead to HHcy [87].

Flynn and colleagues [88] were able to demonstrate in their Longitudinal Aging Study [75] that B₁₂ supplementation (100 microgram by mouth daily) was effective (P value 0.0067) in alleviating HHcy in those patients with a baseline B12 level < 350.

In a meta-analysis of Hcy –lowering studies, folic acid by mouth (0.5-5 mg daily) resulted in a reduction of Hcy by 25% (P<0.001). The addition of 0.5 mg vitamin B₁₂ by mouth daily reduced the Hcy levels by another 3% to 10% over the reduction with folic acid alone. It was interesting to note that the addition of 16.5 mg vitamin B₆ daily produced no significant supplemental reducing effect on blood Hcy levels [89] [92-93].

CONCLUSION

The multiple metabolic A-FLIGHT toxicities (table 4) observed in MS, PD, T2DM, and atheroscleropathy are associated with an increase in ROS production. These ROS result in an increase in oxidative-redox stress and contribute to the uncoupling of the eNOS reaction and endothelial cell dysfunction.

When accessing the role of the dual risk of HHcy, one has to be aware of the synergistic effects of ROS produced by the other multiple toxicities of the A-FLIGHT acronym and their contributing role of oxidative-redox stress to the endothelium. This points to the important concept of global risk reduction when dealing with MS, PD, T2DM, and atheroscleropathy.

The current controversy of HHcy being a marker of risk or a risk factor (important in causation) is not as important as understanding its overall role in the association with endothelial cell damage, dysfunction, and a decrease in eNO bioavailability and how it interacts with other ROS.

The importance of a possible endothelial folate shuttle points to the important role of folate supplementation in these at risk patients in order to recouple the eNOS reaction and increase eNO production in addition to its remethylation properties in the methionine-folate cycle resulting in lowering of Hcy.

Recently we have become aware of the important role of highly sensitive C reactive protein (hsCRP) and the role of inflammation in the development and progression of cardiovascular disease, MS, PD, and T2DM [94-98]. It has been interesting to watch the hsCRP story unfold and since oxidative – redox stress occurs upstream from the inflammatory cycle via the activation of NFkappa B it seems so very important to better understand the role of HHcy and how it interacts with the ROS produced by the other metabolic toxicities of the A-FLIGHT acronym (figure 4).

Dietary folate supplementation is extremely important for proper endothelial cell health maintenance and function. A simplified overview of the positive roles of folate supplementation include the following [99]:

- I. Folate is a methyl donor. Important in lowering the toxic Hcy elevation. Hcy elevation may be a marker of folate deficiency or dysfunction
- II. Folate is a hydrogen donor. Important in maintaining and stabilizing the essential tetrahydrobiopterin (BH4) Cofactor of the eNOS enzyme reaction to prevent uncoupling.
- III. Folate is an electron donor. Important as an antioxidant to reduce oxidative – redox stress. Important as a chain breaking antioxidant. Stabilizing the eNOS reaction and protecting the generation of eNO.

Numbers II and III could be thought of as pleiotropic effects: The effects of folate

supplementation independent of Hcy lowering, which play a significant role in the stabilization of the eNOS reaction and production of eNO as discussed in the section regarding the FOLATE SHUTTLE phenomenon.

Therefore, in addition to nutritional – dietary folate intake [100], pharmacological folate supplementation should now be strongly considered as part of the clinical global risk reduction treatment paradigm for those at risk patients with MS, PD, T2DM, and atheroscleropathy.

Controversy exists as to who should be screened and who should be treated. The following questions are appropriate:

Should we do not test and do not treat?

Should we treat all patients because it makes good clinical sense and therapy is inexpensive?

Should we screen patients with established atherosclerotic and venous thromboembolic disease and high-risk patients with MS, T2DM, and atheroscleropathy and initiate therapy if Hcy is at or above the 9-15 micromol/L range?

The later is preferred and it may be suggested that the current treatment guidelines for the treatment of HHcy should be one of cardiovascular global risk reduction (table 9) of oxidative – redox stress and reactive oxygen species generated from the multiple metabolic toxicities (table 4). In addition to global risk reduction, current suggested guidelines for the treatment of HHcy as a result of this review are presented in (table 10) [101].

The significance of proper nutrition is of paramount importance; however, once moderate to severe HHcy is identified, existing concepts indicate that diet alone is insufficient to normalize Hcy levels in many patients.

This brief review has attempted to provide the reader with a database of knowledge regarding the current role of Hcy and how it interacts with the MS, T2DM, and atheroscleropathy. For those interested in a more in depth review consideration should be given to review the new Journal: *METABOLIC SYNDROME AND RELATED DISORDERS*. Volume 1 number 2 has been dedicated to homocysteine and the metabolic syndrome [102-110]

Soinio M *et al.* have just published an exciting article involving a large cohort of patients (462 men and 368 women in the age group of 45-64 years of age at baseline) with T2DM. They were able to demonstrate that plasma Hcy was a strong and independent risk factor for CHD events. Even though impaired renal function is associated with higher plasma Hcy concentrations, this group was able to show that the association between elevated plasma Hcy level and high incidence of CHD events did not depend on renal function [111].

The damaging – toxic role of Hcy in the vascular bed has been well documented in the medical literature and its diffuse role not only in MS, T2DM, and atheroscleropathy but also in multiorgan damage is emerging [80] (figure 7).

To summarize, HHcy in the general population is an independent risk factor for cardiovascular disease as occurs in thrombotic events (such as arterial and venous occlusion) and ischemic disease such as stroke and myocardial infarction (two of the most common causes of death and disability). With an aging population who are at greater risk of developing these morbid and mortal cardiovascular diseases associated with MS, T2DM, and atheroscleropathy, there should exist a dedicated consideration for folate and possible cobalamin supplementation, in addition to, global risk reduction.

LIST OF ABBREVIATIONS

Ang II	Angiotensin II.
RAAS	Renin angiotensin aldosterone system.
ROS	Reactive Oxygen Species ($O_2^{\cdot -}$, $\cdot OH$, H_2O_2 , $1O_2$).
AT-1	Angiotensin type one receptor.
PKC	Protein Kinase C.
IAPP	Islet Amyloid Polypeptide.
TGFbeta –1	Transforming Growth Factor beta- 1.
NAD(P)H oxidase	Nicotine Adenine Di nucleotide Phosphate reduced oxidase.
AGE	Advanced Glycation Endproducts.
AFE	Advanced Fructosylation Endproducts.
RAGE	Receptor for Advanced Glycosylation Endproducts.
ALE	Advanced Lipoxidation Endproducts.
eNOS	Endothelial Nitric Oxide Synthase.
NO	Nitric Oxide.
BH4	Tetra Hydro Biopterin.
FFA	Free Fatty Acids.
LC acyl –CoA’s	Long chain Acyl Co enzyme CoA.
VLDL	Very low density lipoprotein.
LDL	Low density lipoprotein.
HDL	High density lipoprotein.
MS	Metabolic Syndrome.
PD	Prediabetes.
T2DM	Type 2 Diabetes Mellitus.
PAI-1	Plasminogen Activator Inhibitor –1.
H₂O	Water.
Glut-4	Glucose Transporter-4.
PI3 Kinase	Phosotidyl inositol 3 Kinase.
Akt	Protein kinase B.
MAP Kinase	Mitogen Activated Protein Kinase.
MAP Kinase Shunt	MAP Kinase Shunt: The shunting away from the positive Glut 4 PI3 Kinase Akt pathway to the deleterious MAP Kinase pathway promoting remodeling due to an alteration in the NO redox sensitive PI3 Kinase /Akt pathway.
IL-6 IL-8	Interleukin-6 Interleukin-8.
TNF alpha	Tumor Necrosis Factor alpha.
MPO	Myeloperoxidase: Generation of Superoxide ($O_2^{\cdot -}$) via hypochlorous acid $HClO^{\cdot -}$
NF kappa B	Nuclear Factor kappa B.
ICAM	Inter Cellular Adhesion Molecule.
VCAM,	Vascular Cellular Adhesion Molecule.
MCP-1	Monocyte Chemoattractant Protein-1
NADH	Nicotinamide Adenine Dinucleotide reduced
NAD+	Nicotinamide Adenine Dinucleotide oxidized
DAG	Diacylglycerol.
GPx	Glutathione Peroxidase.
DDAH	Dimethylarginine dimethylaminohydrolase.
ADMA	Asymmetrical dimethyl arginine.
O₂^{·-} - ONOO^{·-}	Superoxide - Peroxynitrite.

COMPETING INTERESTS

None

AUTHORS CONTRIBUTIONS

M. R. Hayden and S.C. Tyagi contributed equally in the inception, writing, and editing of this manuscript.

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FIGURE LEGENDS:**Figure 1: HOMOCYSTEINE METABOLISM: METHIONINE – FOLATE CYCLE AND THE FOLATE SHUTTLE:**

Hcy is an intermediate metabolic product of methionine metabolism. Once methionine is demethylated, producing Hcy it can be further catabolized to cystathionine and cysteine via the transsulfuration pathway. This transsulfuration pathway is dependent on the cystathionine beta synthase enzyme (CBS) and vitamin B₆. Human vascular cells lack the CBS enzyme and therefore the transsulfuration pathway is not present. In the remethylation cycle termed the Methionine – Folate Cycle, folic acid (folate) serves as the methyl donor to convert Hcy to Methionine. This reaction is dependent on the Methionine Synthase (MS) enzyme and the cofactor vitamin B₁₂. Folate serves not only as a methyl donor but also as hydrogen and an electron donor. This hydrogen and electron donation capability renders folate as a dual source for stabilization of the tetrahydrobiopterin (BH₄) Cofactor of the endothelial nitric oxide synthase (eNOS) reaction producing the quintessential endothelial nitric oxide (eNO) and the additional function of being a local endothelial microenvironment antioxidant. These findings have led to the hypothesis of a Folate Shuttle phenomenon. As can be seen there may exist a relative endogenous endothelial folate deficiency resulting in a toxic effect of Hcy accumulation within endothelial cells as a result of this Folate Shuttle.

Figure 2: MULTIPLE INJURIOUS STIMULI TO THE ENDOTHELIUM, INTIMA, MEDIA, AND ADVENTITIA.

The endothelial cell is exposed to multiple injurious stimuli consisting of: modified LDL-cholesterol, various infection insults (viral and bacterial), angiotensin II, hemodynamic stress, LPa, glucose, homocysteine, and intimal redox stress or reactive oxygen species. As discussed in this review, the toxicity of homocysteine may act alone as well as in concert with the other multiple injurious stimuli to injure the endothelium resulting in endothelial cell dysfunction. Especially in the MS, PD, overt T2DM, and atheroscleropathy. It is of importance to note that native LDL-cholesterol is not atherogenic to the vascular intima. The process of oxidation, glycation, glycoxidation, or homocysteinylation must modify LDL-cholesterol in order to become atherogenic. Thus, the importance of the multiple injurious stimuli acting alone and synergistically to modify LDL-cholesterol and accelerate angiogenesis as seen in the accelerated atherosclerosis associated with MS, PD, and overt T2DM termed atheroscleropathy.

Figure 3: THE METABOLIC SYNDROME: SYNDROME X “RELOADED”

Metabolic syndrome (Syndrome X “reloaded”) is a unique clustering of clinical syndromes and metabolic derangements. Reaven initially described the MS in 1988. He initially discussed the four major determinants consisting of: I. Hypertension. II. Hyperinsulinemia. III. Hyperlipidemia (Dyslipidemia of elevated VLDL - triglycerides, decreased HDL-cholesterol, and elevated small dense atherogenic LDL-cholesterol). IV. Hyperglycemia or glucose intolerance. He also emphasized the importance of insulin resistance being central to the development of coronary artery disease or CHD. Since that time numerous papers and authors have added several associated findings to the MS and additionally several other names have been given to describe this clustering

phenomenon. The important association of polycystic ovary syndrome (PCOS), hyperuricemia, fibrinogen, hsCRP, microalbuminuria, PAI-1, and the more recently added asymmetrical dimethyl arginine (ADMA), reactive oxygen species (ROS) and now the damaging oxidative potential of Hcy and endothelial dysfunction have all contributed to a better understanding of this complicated clustering phenomenon. The red boxes (ROS, Hcy, ADMA, and hsCRP) indicate the newer additions giving rise to the new terminology: Metabolic Syndrome Reloaded.

Figure 4: THE REACTIVE OXYGEN SPECIES – INFLAMMATORY CYCLE: ROS ARE UPSTREAM FROM THE INFLAMMATORY CYCLE.

Inflammation in MS, PD, overt T2DM, and atheroscleropathy has recently emerged as an important factor. While it has been interesting to observe the development of this exciting story, it is important to remember that reactive oxygen species (ROS) cycle occurs upstream from the inflammatory cycle via the redox sensitive nuclear transcription factor: NF kappa B and the resulting inflammatory markers: Selectins, cellular adhesion molecules, monocyte chemoattractant protein – 1, and the cytokines: TNF alpha, IL-6, IL-8 and the newer clinical laboratory tool of the highly sensitive C-reactive protein (hsCRP).

Figure 5: HOMOCYSTEINE IS A COMPETITIVE INHIBITOR OF PPAR AGONISTS:

Hcy has recently been found to be a competitive inhibitor of the nuclear transcription factors: Peroxisome proliferator activated receptors (PPARs) alpha and gamma. Hcy acts as a ligand for the PPAR receptors alpha and gamma and therefore competes with the PPAR alpha ligand: ciprofibrate and the PPAR gamma ligand: 15-deoxy-Delta(12,14)-prostaglandin J(2) (15d-PGJ₂). These findings have definite clinical

applications, in that; if Hcy is not treated to optimal goals the thiazolidinediones (TZDs) may not have their maximal effect on the PPAR receptor.

Figure 6: UNCOUPLING OF THE eNOS REACTION

This figure demonstrates the three vulnerable arms of the plasma membrane bound eNOS reaction responsible for the production of the omnipotent endothelial nitric oxide.

Oxygen reacts with the eNOS enzyme in which the essential BH₄ cofactor has coupled NAD(P)H with L-arginine to be converted to eNO and L-citrulline. If uncoupled due to oxidative – redox stress with the essential BH₄ cofactor now in the BH₂ or BH₃ oxidized state the reaction will become one of superoxide production adding to the oxidative – redox stress within the endothelial microenvironment. The healthy endothelium is a net producer of eNO, whereas, the dysfunctional endothelium becomes a net producer of superoxide. It is in this situation that the stabilization of the essential BH₄ cofactor (preventing oxidation to BH₂) by adequate supplementation of folate becomes so very important.

Figure 7: THE HOMOCYSTEINE WHEEL: MULTIORGAN DAMAGE THROUGH REDOX STRESS

Homocysteine is capable of causing multi-organ damage through the process of oxidative - redox stress. The endothelium is very vulnerable to the effects of HHcy because the endothelium lacks the CBS enzyme, which results in the local loss of the important catabolic metabolism of Hcy. While this article has focused on the cardiovascular manifestations associated with MS, PD, and T2DM there exists numerous other organ systems and diseases associated with the damaging effects of HHcy.

TABLE 1**Homocysteine lowering intervention trials for the prevention of CVD.**

1. <i>SEARCH</i>: Study of the effectiveness of additional reduction in cholesterol and homocysteine. University of Oxford, England.
2. <i>PACIFIC</i>: Prevention with a combined inhibitor of folate in coronary heart disease. University of Sydney, Australia.
3. <i>WACS</i>: Women's antioxidant and cardiovascular disease study. Harvard Medical School, USA.
4. <i>CHAOS-2</i>: Cambridge heart antioxidant study. University of Cambridge, England.
5. <i>NORVITE</i>: Norwegian study of homocysteine lowering with B-vitamins in myocardial infarction. University of Tromso, Norway.
6. <i>BERGEN</i>: Bergen vitamin study. University of Bergen, Norway.
7. <i>HOPE-2</i>: Health outcome and prevention evaluation number 2. Canada.
8. <i>VISP</i>: Vitamin intervention for stroke prevention. Wake Forest University, USA.
9. <i>VITATOPS</i>: Vitamins to prevent strokes. Perth, Australia.
10. <i>IST-2</i>: International strokes trial number 2. Auckland, New Zealand.

TABLE 2
CLINICAL FACTORS ASSOCIATED WITH HHcy

1. Age and Gender:	HHcy is associated with aging and male gender. Females typically have Hcy levels 20% lower than men until menopause. After menopause fasting levels of Hcy rise to those of men of similar age.
2. Genetic: Not only deficiency but also dysfunction secondary to various gene polymorphisms.	<ol style="list-style-type: none"> 1. Cystathionine Beta Synthase deficiency. 2. 5,10-methylenetetrahydrofolate reductase deficiency. 3. Methionine synthase deficiency (including abnormalities in cobalamin metabolism). 4. Methylenetetrahydrofolate homocysteine methyltransferase deficiency.
3. Renal Function:	Hcy levels increase with elevations of creatinine levels. Due to impaired renal excretion (decreased glomerular filtration rate) and or impaired metabolism.
4. Nutritional Status:	Decreased vitamin cofactors (folate, vitamin B ₁₂ , and B ₆). Aging – Alterations in gastric mucosa affecting intrinsic factor, inflammatory bowel diseases such as Crohn’s disease and Ulcerative colitis, celiac disease, lymphomas and amyloidosis to name a few.
5. Disease States:	Hypothyroidism. Carcinomas (breast, ovary, and pancreas in particular). Chronic renal failure from any cause especially diabetic and hypertensive nephropathy. Systemic lupus erythematosus. Psoriasis. Solid organ transplantation. Malabsorption syndromes associated with nutritional status: Number 4.
6. Medications:	Anticonvulsants, such as phenytoin, Methotrexate, Theophylline and other bronchodilators of the phosphodiesterase inhibitor class, which interfere with pyridoxal phosphate synthesis. Nitrous oxide, L-dopa, Carbamazepine, Niacin, Fibrates, and Bile acid resins to name a few.
7. Endothelial Cell Dysfunction: Any cause for a decrease in eNOS enzyme or eNO bioavailability.	Any cause of endothelial cell dysfunction could result in HHcy if one accepts the endogenous endothelial folate shuttle HYPOTHESIS.

TABLE 3
DAMAGING EFFECTS OF HOMOCYSTEINE

1. Endothelial cell dysfunction. Decreased eNO bioavailability.
2. Endothelial cell toxicity - apoptosis
3. Smooth muscle cell proliferation – myointimal hyperplasia and hypertrophy. Hcy induces Ca⁺⁺ second messenger in vascular SMCs.
4. Extracellular matrix remodeling - activation of redox sensitive MMPs. Decreased bioavailability of eNO. Resulting in ECM fibrosis.
5. Promotes vasoconstriction
6. Promotes LDL-cholesterol modification: LDL-C – Homocysteine thiolactone aggregates
7. Pro-inflammatory: MCP-1 and IL-8
8. Promotes macrophage - foam cell formation via LDL-cholesterol modification
9. Prothrombotic: Hcy reduces thrombomodulin and heparan sulfate levels. Decreases protein C activity and inhibits binding of tPA to endothelial cells. Hcy activates factors V and XII and increases tissue factor expression. Hcy induces platelet adhesiveness and aggregation. *
10. Pro oxidative and redox stress via reactive oxygen species formation
11. Induces 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase in endothelial cells.
12. Promotes oxidation of BH4 to BH2 and BH3, which uncouples the eNOS reaction resulting in superoxide formation and a decrease in eNO.
13. Promotes atherogenesis, arteriosclerosis, atherosclerosis, and atheroscleropathy.

TABLE 4
MULTIPLE METABOLIC TOXICITIES IN MS AND T2DM:
THE A-FLIGHT ACRONYM

	<i>Initiator</i>	<i>Metabolic Defect</i>	<i>Metabolic mediator</i>	<i>Functional mediator</i>	<i>Consequence</i>
A	AMYLIN (Co-secreted – Co-packaged within the insulin secretory granule) by the islet Beta cell. Insulin’s “Fraternal Twin” Elevated in MS, PD, and Early T2DM)	Hyperamylinemia	Activation of ANG II	PKC Signal Transduction Islet Amyloid IAPP Islet aggregation and deposition. Beta cell apoptosis – Beta cell defect.	ROS IAPP Amyloid in islets contributing to Beta Cell defect. Possible deposition in the intima, mesangium, neuronal unit, and myocardial. REMODELING
	ANG II Via RAAS activation In MS, PD, and T2DM	Ang II Excess	Ang II Excess Most potent stimulus for: Activation of Vascular membrane bound NAD(P)H Oxidase Enzyme	PKC Signal Transduction. Superoxide production. Uncoupling of the eNOS reaction. TGF beta-1 activation	ROS NAD(P)H oxidase Derived Superoxide Myocardial, Renal, Intimal, Retinal, and Neuronal remodeling
	AGE Advanced Glycation Endproducts AFE Advanced fructosylation endproducts	AGE / AFE See Glucotoxicity (G) RAGE activation Receptor for AGE	Protein Cross – linking / Dysfunction RAGE Receptor for AGE	Matrix Defects Signal Transduction Matrix Defects Signal Transduction	ROS Myocardial, Renal, Intimal, Retinal, Neuronal- Endoneurial Fibrosis
	Advanced Lipoxidation Endproducts (ALE)	ALE	Protein Cross - linking	Matrix Defects Signal Transduction	ROS Matrix Remodeling
	Antioxidant Enzymes: Antioxidant reserve compromised	Reduced – Dysfunctional eNOS, SOD, GPx, GSH, Catalase, and Vit. C.	Decreased NO	Decreased NO REDOX STRESS	ROS REDOX STRESS
	Antioxidant Enzymes: Absence of antioxidant network	IMPAIRED eNOS L-arginine BH4	Decreased NO	Decreased NO	ROS Decreased NO
	AGING: Accumulation of multiple metabolic toxicities → ROS	Increased Ox-LDL-C, TNFalpha, Capase 3, Glomerulosclerosis.	Decreased NO:	Decreased NO	ROS Inflammation, Apoptosis
	Atherosclerotic Nephropathy	ROS beget ROS Atheroscleropathy	Decreased NO Self perpetuating Decreased NO	Decreased NO Athero – emboli Activated Platelets See Thrombotic Tox.	ROS beget ROS Decreased NO

F	Free fatty acid toxicity	Elevated FFA	LC acyl-CoA's	Mitochondrial Defects	ROS Cytotoxicity
L	Lipotoxicity Lipid Triad FFA ALE Long chain acyl-CoA's	Increased VLDL – VLDL Triglycerides and Small dense atherogenic LDL-Cholesterol with Decreased HDL-Cholesterol LIPID TRIAD	LC acyl-CoA's Fat Accumulation	Non Adipose Accumulation of Fat (LC acyl-CoA's) in Adipose and Non Adipose Tissue	ROS Accumulation of fat in non adipose tissues resulting in Ceramide induced: Cytotoxicity
I	Insulin toxicity ENDOGENOUS Insulin Resistance	Hyperinsulinemia Hyperamylinemia in : MS, PD, EARLY T2DM Glut 4 is NO dependent Redox sensitive pathway	Ang II Increase # AT-1 receptors Cross-talk with AT-1 Increase FFA Increase PAI-1 Increase Sympathetic tone and activity Increased Na+ and H2O reabsorption Increase Volume and Blood Pressure Hypertension	NAD(P)H REDOX STRESS <u>SIGNAL PATHWAYS</u> PI3 Kinase / Akt (Protein kinase B)→ MAP Kinase Shunt	ROS ROS ROS Extracellular Matrix Remodeling Islet, intimal, renal, myocardial, and neuronal.
	Inflammation toxicity. “Inflammatory Cycle” (figure 5)	Activation of the innate immune system: IL-6, IL-8, TNF alpha Macrophage (MPO) → Hypochlorous Acid Superoxide O ₂ ⁻	Acute Phase Reactants: C-Reactive Protein Serum Amyloid A Fibrinogen	NF kappa B Cellular Adhesion Molecules: ICAM, VCAM, and MCP-1	ROS Inflammation begets Inflammation “INFLAMMATORY CYCLE” (figure 5) ROS beget ROS
	Insulin deficiency	OVERT T2DM	GLUCOTOXICITY POLYOL SORBITOL PATHWAY	REDUCTIVE STRESS NADH > NAD+ PSEUDOHYPOXIA	ROS
G	Glucotoxicity	Glycation / AGE	See above	See above	See above
			Protein inactivation	Receptor-ligand defects	Dysfunctional Signal Transduction
			NO quenching	Vasoconstriction	Ischemia/Hypoxia ROS
			Macrophage Activation	Increased Cytokines, TGF-Beta	Cytotoxicity ROS
			Free Radical Formation	REDOX STRESS	Cytotoxicity ROS
		Auto-oxidation	Free Radical Formation	REDOX STRESS	Cytotoxicity ROS
	ORIGIN OF REDUCTIVE STRESS ! REDUCTIVE STRESS !	Polyol Sorbitol Pathway (eNO inhibits Aldose Reductase)	Increased NADH Lactate Reductive Stress	REDOX STRESS Decreased NO Pseudohypoxia	Cytotoxicity ROS Ischemia/ Hypoxia
			Decreased Taurine	REDOX STRESS	ROS Cytotoxicity
		Increased DAG	Increased PKC	Signal Transduction	Ischemia ROS
	Glucotoxicity	Glucotoxicity	Polyol – Sorbitol Pathway	PAS + material Interstitium, Basement Membrane	Remodeling – Cardiomyopathy CHF Diastolic Dysfunction

H	Hypertension Toxicity Homocysteine Toxicity	RAAS activation HHcy NO quenching and NEW: PPAR interaction.	Ang II Decreased GPx , DDAH with resultant ^ ADMA	NAD(P)H REDOX STRESS ^ ROS, O2', ONOO', nitrotyrosine	ROS Decreased NO, Endothelial Cell toxicity, dysfunction, and apoptosis
T	Triglyceride Toxicity Thrombotic Toxicity Taurine (antioxidant) depletion	Triglyceride – FFA exchange	See FFA – Lipotoxicity above eNOS uncoupling	REDOX STRESS Activated Platelets PAI-1 elevation Fibrinogen elevated. Decreased NO	ROS Athero-emboli ROS

TABLE 5

Origin, enzymatic pathways of reactive oxygen species, and their oxidized products.

Origin – Location Enzymatic Pathway	ROS Potent Oxidants	PRODUCTS Oxidized lipids and Proteins
Mitochondrial Respiratory Chain	O_2^{\cdot} $-OH^{\cdot}$	Oxidized lipids, proteins, nucleic acids, and autoxidation byproducts.
Inflammatory Macrophage Membraneous NAD(P)H Oxidase	O_2^{\cdot} OH^{\cdot} H_2O_2	Advanced Lipoxidation Endproducts (ALE) Ortho o-tyrosine Meta m- tyrosine
Granular Myeloperoxidase (MPO)	Hypochlorous Acid HOCL (bleach) $^{\cdot}Tyr$ NO_2^{\cdot}	3-Chlorotyrosine di-Tyrosine NO₂-Tyrosine (Nitrotyrosine)
Macrophage Nitric Oxide Synthase (NOS) Inducible (iNOS) Large bursts uncontrolled	$ONOO^{\cdot}$	NO₂-Tyr (Nitrotyrosine)
Endothelial Cell Nitric Oxide Synthase (NOS) Constitutive (cNOS) eNOS → NO nNOS → NO Small bursts (puffs) controlled	$NO + O_2^{\cdot} \rightarrow ONOO^{\cdot}$ $ONOO^{\cdot}$	NO₂-Tyr (Nitrotyrosine) NO₂-Tyr
eNOS derived NO	<u>NO</u>: the GOOD	Natural Occurring, Local Occurring, Chain Breaking Antioxidant
Superoxide	<u>O₂^{·-}</u>: the BAD	
Peroxynitrite	<u>ONOO[·]</u>: the UGLY	
Hypochlorous acid	<u>HClO</u>: the UGLY	
Restoration of endothelial derived NITRIC OXIDE Via the eNOS reaction..	ANTIOXIDANT ANTIREDOXANT → → →	Prevention of the devastating effects of ROS.

TABLE 6

Antioxidants: catalytic - enzymatic inactivation of free radicals.
Enzymatic Antioxidants
<p><u>SUPER OXIDE DISMUTASE (SOD)</u> $[O_2^- + SOD \rightarrow H_2O_2 + O_2]$ ecSOD (extracellular) MnSOD (mitochondrial) CuZnSOD (intracellular)</p>
<p><u>CATALASE</u> – Location: peroxisome $[2H_2O_2 + catalase \rightarrow 2 H_2O + O_2]$</p>
<p><u>GLUTATHIONE PEROXIDASE</u> – Location: mitochondrion – cytosol and systemic circulation. (Glutamyl-cysteinyl-glycine tripeptide) glutathione reduced –SH to the oxidized disulfide GSSG. (Glutathione peroxidase) $[GSH + 2H_2O_2 \rightarrow GSSG + H_2O + O_2]$ (Glutathione reductase) $[GSSG \rightarrow GSH]$ at the expense of $[NADH \rightarrow NAD^+]$ and/or $[NAD(P)H \rightarrow NAD(P)^+]$</p>
<p><u>NOS (nitric oxide synthase).</u> – Location: membrane Isoforms: (e)NOS (endothelial): good (n)NOS (neuronal): good (i)NOS (inducible-inflammatory): bad</p>
<p>O_2^- and nitric oxide (NO) are consumed in this process with the creation of reactive nitrogen species (RNS). $O_2^- + NO \rightarrow ONOO^-$ (peroxynitrite) + tyrosine \rightarrow nitrotyrosine. Nitrotyrosine reflects redox stress and leaves a measurable footprint. NO: the good; O_2^-: the bad; $ONOO^-$: the ugly [42]</p>
Nonenzymatic Antioxidants
<u>URIC ACID</u>
<u>VITAMIN A, VITAMIN C, and VITAMIN E</u>
<u>THIOLS:</u> Sulfhydryl (-SH) containing molecules.
<u>ALBUMIN:</u> Is an antioxidant due to thiol containing compounds.
<u>APOPROTEINS:</u> Ceruloplasmin and transferrin. Bind copper and iron in forms which cannot participate in the Fenton reaction

TABLE 7
The Five Stages of T2DM:

The Natural Progressive History of T2DM.	
I.	<u>LATENT STAGE: [EARLY]</u>
	Insulin Resistance:
	Genetic Component
	Environmental component. Modifiable: obesity/sedentary life style. Nonmodifiable: aging
	Beta Cell Defect: (Dysfunction)
	Genetic Abnormal processing, storage or secretion.
	Intracellular/extracellular oligomers of Islet Amyloid toxicity to the Beta Cell. Abnormal processing, storage or secretion
	Islet Amyloid: Diffusion Barrier: Secretory Defect: Intra Islet Absorptive Defect:
II.	<u>TRANSITION STAGE: [MIDDLE]</u>
	Persistent Hyperinsulinemia, Hyperproinsulinemia. → Ang II <u>Accelerated Atherosclerosis</u>
	Persistent Hyperamylanemia. → AngII <u>Accelerated Atherosclerosis</u>
	Continued remodeling of the endocrine pancreas (amyloid).
	Beta cell displacement, dysfunction, mass reduction due to the toxic effect of IAPP oligomers and the progressive developing diffusion barrier.
III.	<u>IGT STAGE (Impaired Glucose Tolerance): [LATE]</u> "Pre-diabetes" Human Health Services (HHS) and American Diabetes Association (ADA) term.
	[Start treatment at this time]
	Diagnose earlier: rejuvenation of the 2 hour glucose tolerance blood sugar 140-199 mg/dl
	Increased insulin resistance [Feeds forward] > Glucotoxicity [Feeds forward] > Insulin resistance [Feeds forward] > Glucotoxicity: creating a vicious cycle.
	Islet amyloid. Increasing beta cell defect. Loss of beta cell mass with displacement. (Remodeling of islet architecture including extracellular matrix). Beta cell loss centrally.
IV.	<u>IFG STAGE (Impaired Fasting Glucose): [LATER]</u> "Pre-diabetes" Human Health Services (HHS) and American Diabetes Association (ADA) term.
	Blood sugar ranging [110-125 mg/dL]
	*ADA new cut point IFG Nov 2003: [100 –125 mg/dl]
	Impaired hepatic glucose production: Increasing global insulin resistance (hepatic) with subsequent gluconeogenesis. Feeding forward in the vicious cycle to accelerate insulin resistance globally.
V.	<u>OVERT STAGE: [TOO LATE]</u> FBG 126 or greater: Random or 2hour OGTT 200 or >
	[50% loss of beta cell function at the time of clinical diagnosis]
	Use medications that do not increase endogenous insulin or amylin. Use combination therapy. Start treatment at stage III-IV (IGT-IFG).
•	<u>Paradigm Shift.</u> → Diagnose Early → Start treatment Early → Stage III: IGT.

TABLE 8
POSITIVE EFFECTS OF PPARalpha AND PPARgamma AGONISTS

<p><u>PPAR alpha: Lipid Effects</u></p> <p>a). Primarily a VLDL-Cholesterol (triglyceride) by increasing lipoprotein lipase and secondary LDL-Cholesterol lowering.</p> <p>b). HDL-cholesterol raising effect by increasing the expression of apo A-I and apo A-II. Positive outcomes of the Fibric acid (Gemfibrozil) (VA-HIT) and Fenofibrate (DAIS) studies.</p>
<p><u>PPAR gamma effects:</u></p>
<p>a). <u>Primary Clinical Action:</u> (Insulin Sensitizers). Improving insulin mediated glucose uptake.</p>
<p>b). Decrease thrombotic, inflammatory and oxidative changes that contribute to endothelial cell dysfunction.</p>
<p>c). Improve vascular reactivity.</p>
<p>d). Decrease plasminogen activator inhibitor-1 (PAI-1).</p>
<p>e). Decrease monocyte expression NFkappa-B.</p>
<p>f). <u>Anti-inflammatory effects:</u> Inhibition of macrophage activation and the production of inflammatory cytokines (TNFalpha, interleukin 6 and 1beta). <u>New findings:</u> May be through the effects of PPARdelta.</p>
<p>g). Decrease C-reactive protein and IL-6 (both markers of inflammation and cardiovascular risk). Dandonna P reference. Diabetes Technol Ther. 2002; 4(6): 809-15.</p>
<p>h). Inhibit vascular SMC migration and proliferation.</p>
<p>i). Lower blood pressure.</p>
<p>j). Attenuate myocardial hypertrophy and protect against ischemia-reperfusion injury.</p>
<p>k). Preserve pancreatic islet Beta cell function.</p>
<p>l). <u>Attenuate (specifically rosiglitazone) oxidative and nitrate stress:</u> enhanced PPAR gamma expression, improved endothelium – dependent vasodilation, preserved P-VASP, suppressed gp91(phox) of the membranous NAD(P)H Oxidase enzyme, reduced superoxide and total NOx (stable metabolic byproducts of NO) production, and inhibited nitrotyrosine formation.</p>
<p>m). <u>Increase levels of Adiponectin.</u></p>

TABLE 9

**THE RAAS ACRONYM:
GLOBAL RISK REDUCTION**

R Reductase inhibitors (HMG-CoA). Decreasing modified LDL-cholesterol, i.e. oxidized, acetylated LDL-cholesterol. Decreasing triglycerides and increasing HDL-cholesterol. Improving endothelial cell dysfunction. **Restoring** the abnormal Lipoprotein fractions. Thus, decreasing the redox and oxidative stress to the arterial vessel wall and myocardium.

Redox stress reduction.

A AngII inhibition or blockade:

ACEi-prils. **ARBs**-sartans. Both inhibiting the effect of angiotensin-II locally as well as systemically. Affecting hemodynamic stress through their antihypertensive effect as well as the deleterious effects of angiotensin II on cells at the local level - injurious stimuli -decreasing the stimulus for $O_2^{\cdot -}$ production. Decreasing the **A-FLIGHT** toxicities. Plus the direct-indirect antioxidant effect within the arterial vessel wall and capillary. **Antioxidant** effects.

Aspirin antiplatelet, anti-inflammatory effect.

Adrenergic (non-selective blockade) in addition to its blockade of Prorenin→Renin

Amlodipine with its calcium channel blocking antihypertensive effect, in addition to its direct antioxidant effects.

Redox stress reduction.

A Aggressive control of diabetes to HbA_{1c} of less than 7. (This usually requires combination therapy with the use of: Insulin secretagogues, insulin sensitizers (thiazolidinediones), biguanides, alpha-glucosidase inhibitors, and ultimately exogenous insulin.) [36-38]

Decreasing modified LDL cholesterol, i.e. glycated - glycoxidated LDL cholesterol. Improving endothelial cell dysfunction. Also decreasing glucotoxicity and the oxidative - redox stress to the intima and pancreatic islet.

Aggressive control of blood pressure, which usually requires combination therapy, including thiazide diuretics to attain JNC 7 guidelines.

Aggressive control of Hcy with folic acid and its associated pleiotropic positive effect on re-coupling the eNOS reaction by restoring the activity of the BH₄ cofactor to run the eNOS reaction and once again produce eNO, as well as, its direct antioxidant effects: BH₄ and eNOS stabilization

Redox stress reduction.

S Statins. Improving plaque stability (pleiotropic effects) independent of cholesterol lowering. Improving endothelial cell dysfunction. Plus, the direct - indirect antioxidant anti-inflammatory effects within the islet and the arterial vessel wall promoting stabilization of the unstable, vulnerable islet and the arterial vessel wall. **Style:** Lifestyle modification: lose weight, exercise, and change eating habits. **Stop Smoking**

Redox stress reduction

TABLE 10
SUGGESTED TREATMENT GUIDELINES FOR HHcy

Fasting plasma total Hcy (tHcy) levels:

The prevalence of HHcy in the general population is between five and ten percent (using a threshold set at the 90th to 95th percentile of 15 micromol / liter). Keep in mind that this figure may escalate to, as high as, 30% to 40 % in the elderly.

Normal:	5-15	micromol/L (Based on table below 5 – 9 micromol/L)
Moderate:	15-30	micromol/L
Intermediate:	31-100	micromol/L
Severe:	>100	micromol/L

Goal of Treatment: 9 micromol / Liter or less. (Based on table below)

HYPERHOMOCYSTEINEMIA



Check for secondary causes:

Renal dysfunction

Folate B₁₂ B₆ deficiency * Significant to exclude Pernicious Anemia

Hypothyroidism and others (table 2)

GLOBAL RISK REDUCTION ↓ (table 9)

1. Diet rich in B vitamins and folate [IF NOT TO GOAL]
2. Advance to multivitamin therapy 400 microgram folic acid, 2 mg B6, and 6 mg B12. [IF NOT TO GOAL]
3. Advance to prescription strength 1 mg folic acid, 25 mg B6, and 500 microgram B12. [IF NOT TO GOAL]
4. Advance to 2-5 mg folic acid, B₁₂ to 1,000 microgram, and B₆ 25 – 100 mg.
5. Sublingual and injectable B12 may be used if necessary, as well as, a trial of Betaine hydrochloride in intractable cases. Higher doses of up to 15 mg of folic acid may be required in hemodialysis patients.
6. Global Risk Reduction (table 9)

Consider: Total Hcy is associated with a graded mortality risk. Patients with known CAD have the following graded risk [95] [97]:

tHCY in micromol/liter	Relative risk of all cause death.	Relative risk of CAD death
< 9	1.0	1.0
9 - 14.9	1.9 (0.7 – 5.1)	2.3 (0.7 – 7.7)
15 - 19.9	2.8 (0.9 – 9.0)	2.5 (0.6 – 10.5)
> 20	4.5 (1.2 - 16.6)	7.8 (1.7 – 35.1)

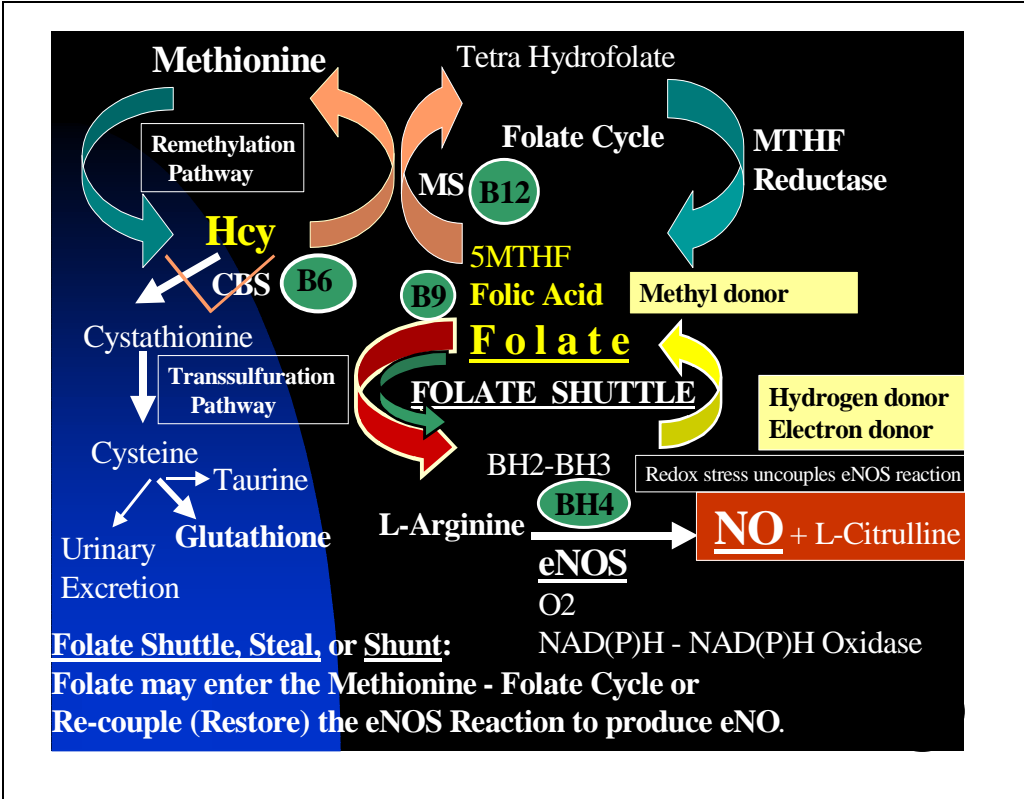


Figure 1

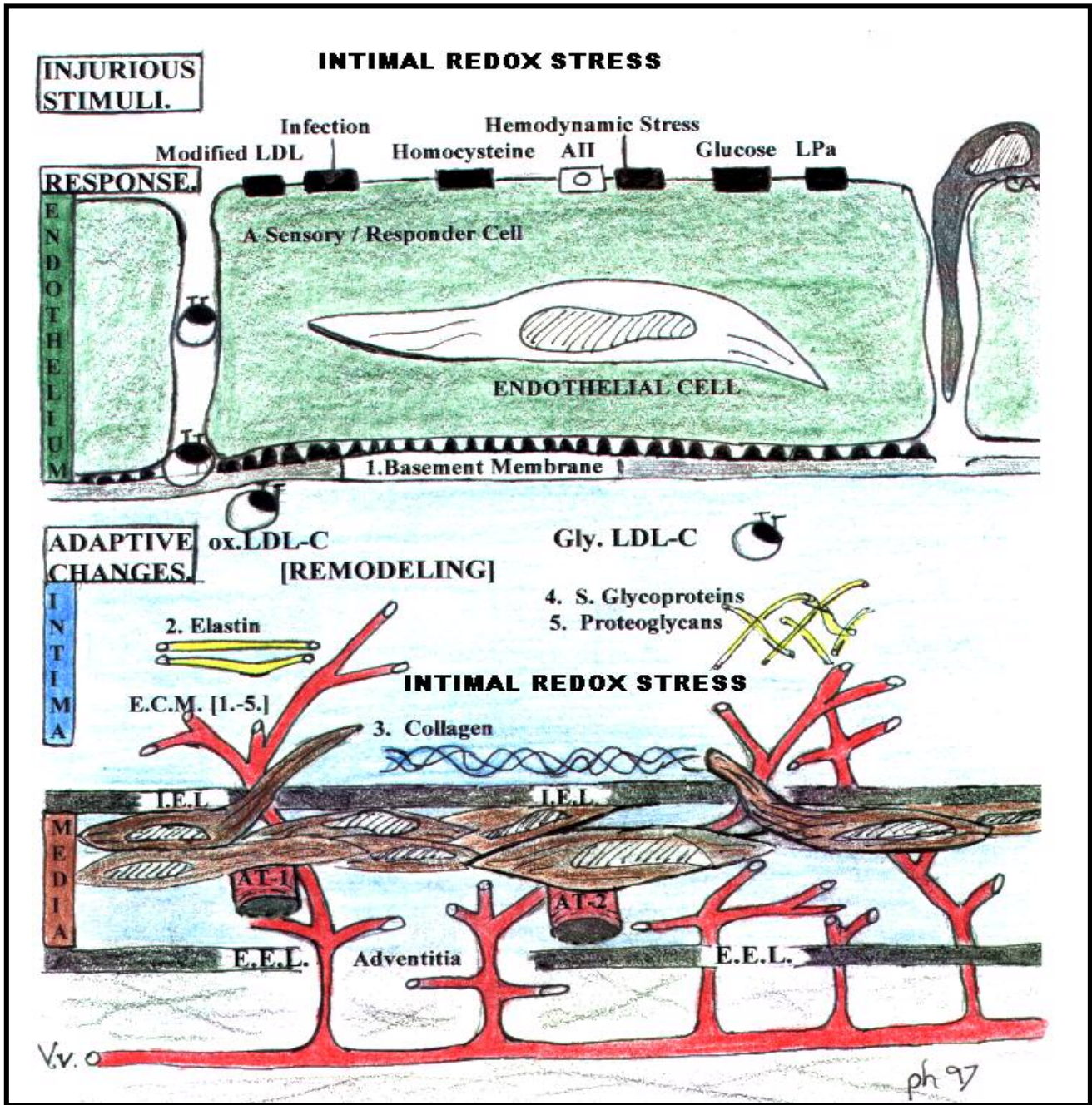


Figure 2

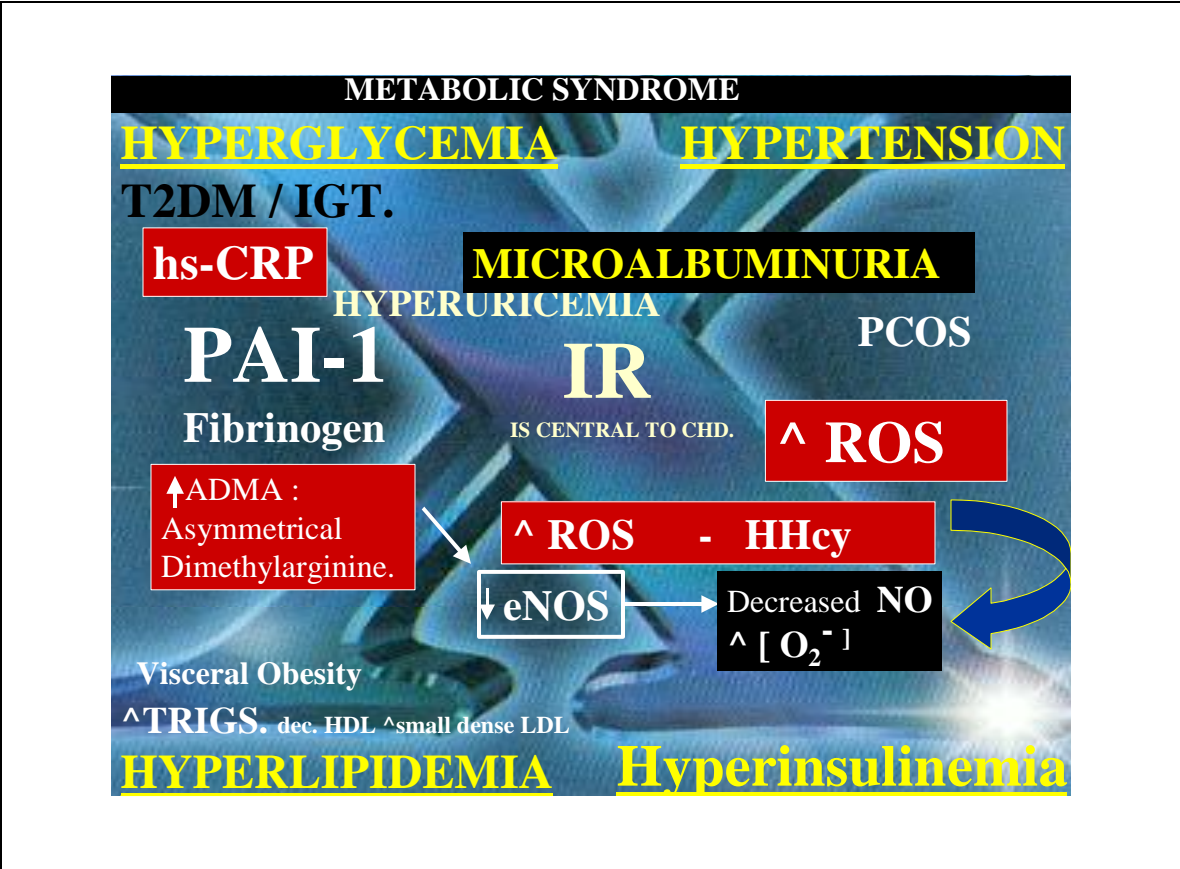


Figure 3

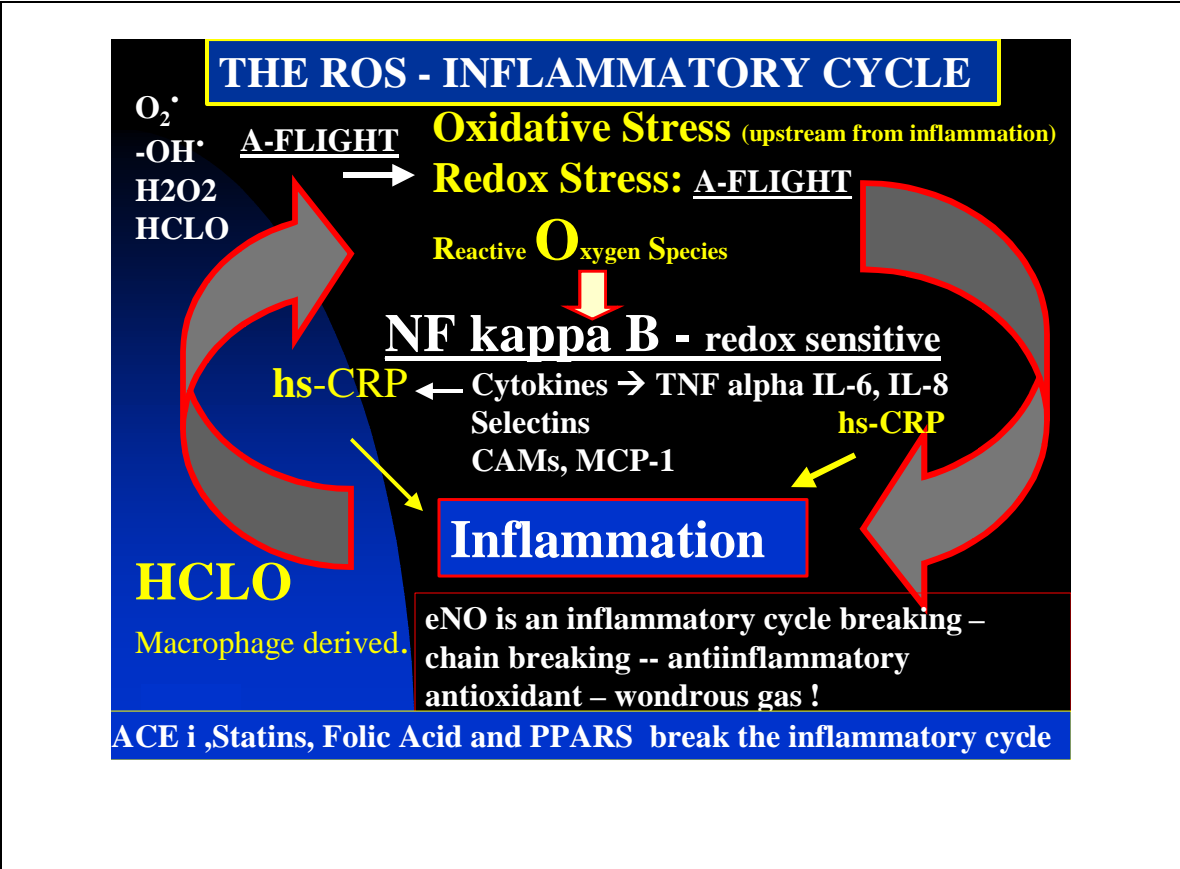


Figure 4

Hcy a competitive inhibitor of PPAR alpha
agonist: ciprofibrate and PPAR gamma
agonist: 15d-PGJ₂



Hcy
Hcy



TZDs:

Decreased effect of
PPAR alpha and gamma.



**All contributing to
Endothelial Cell
Dysfunction**

No CBS enzyme in Vascular
cells.
Relative Endothelial Folate
Deficiency.
ROS increasing Hcy.

12/14/03

100

Figure 5

Diabetes a net producer of: [O2'] Due to an uncoupling of the eNOS enzyme

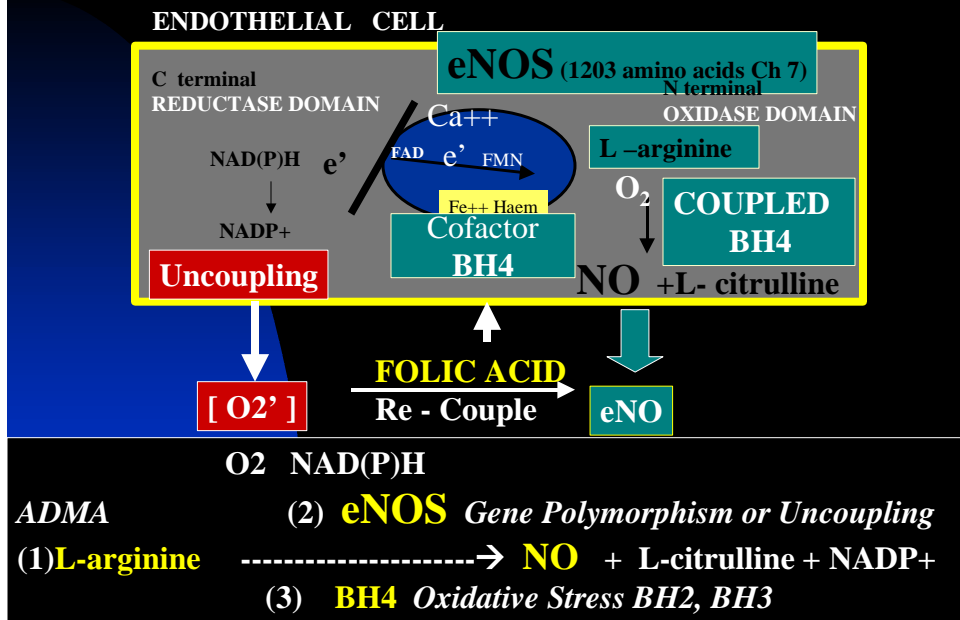


Figure 6

**HOMOCYSTEINE:
THE UNDERLYING CAUSE FOR MANY DISEASES?
MULTIORGAN DAMAGE THROUGH REDOX STRESS**

THE HOMOCYSTEINE WHEEL

HHCY

Decreases eNO.
Generates ONOO'.
Activates latent MMPs.
Competes with PPAR agonists.

Cardiovascular Disease.
Hypertension.
Heart: CHF Diastolic Dysfunction.
Vessel Wall.
Atherosclerosis.

Hypertension and Diabetic
Glomerulosclerosis

Renal Disease.

Cancer
HIV

Congenital Birth
Defects.
Neural Tube.
Heart Defects.

HOMOCYSTEINE.

Aging.
Progeria
AD

Obesity

MS PD
Overt T2DM

Endothelial Damage.
Endothelial Dysfunction.
Endothelial Apoptosis.
Prothrombotic aura.

Endogenous Endothelial Folate Deficiency: **THE FOLATE SHUTTLE**

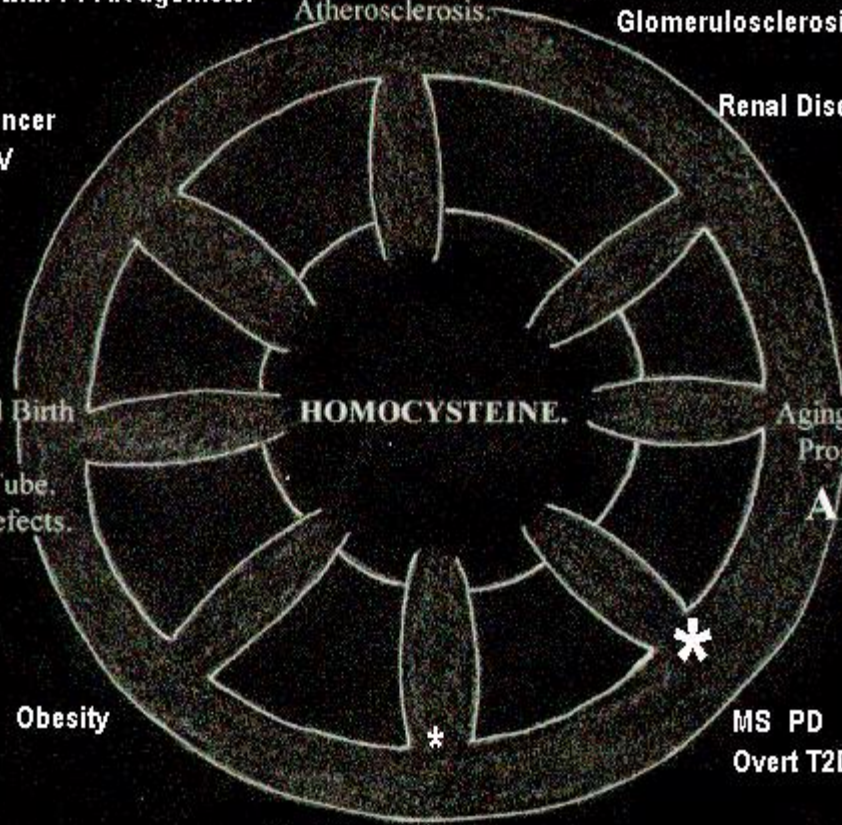


Figure 7