Homocysteine in Neurology: A Risk Factor or Something Different in Small Vessel Disease
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Homocysteine (Hcy) is a sulfur-containing amino acid that is generated during methionine metabolism. Hyperhomocysteinemia (HHcy) is typically defined as levels >15 micro mols/L. Elevated plasma levels of Hcy can be caused by the deficiency of either vitamin B12 or folate. The active role of homocysteine is quite ambivalent: many studies detected its potential impact on neurological events; others try to identify it as one of the possible risk factors of cardiovascular events, but with a complementary and secondary role. HHcy has been reported in many neurologic disorders, including cognitive impairment and stroke, independent of long-recognized factors such as hyperlipidemia, hypertension, diabetes mellitus, and smoking. Nowadays, homocysteine could be considered as a possible link between a common vascular risk factor and potential alterations in degenerative neuronal disorders. HHcy-induced oxidative stress, endothelial dysfunction, inflammation, smooth muscle cell proliferation, and endoplasmic reticulum stress; all these aspects have been considered to play an essential role in the pathogenesis of several diseases, including atherosclerosis, major stroke, and vascular dementia. Specific models of astocytes impairment in HHcy-mice, which mimic small vessel disease, have been developed with a three-step investigation (at 6, 10, 14 weeks of B6, B9, and B12 detrimental diet in wild type HHcy mouse). These studies found out that after ten weeks on a diet (at the most after 14 weeks), end-feet disruption occurs. This phenomenon is concomitant to reduced vascular labeling for aquaporin -4-water channels, lower protein/mRNA levels for Kir4.1, and BK potassium channels, associated with a higher expression of MMP-9. The most exciting finding is that microglial activation in this mice model was evident since the precocious time of observation (6-week time) and precedes astrocitic changes. Our research aims to review the possible role of HHcy in neurodegenerative disease and small-vessel disease and to understand its pathogenic impact.
Cerebral small vessel disease (SVD) primarily distresses the small perforating arteries, defined as vessels with less than 50 μm diameters, supplying the deep brain structures and the leptomeningeal space [1,2]. It is tightly related to general increased arterial stiffness and is the most important and common cause of vascular dementia, leading to 45% of dementia, and accounts for about 20-30% of all stroke worldwide, 25% of ischemic (or lacunar strokes). Moreover, it significantly increases the risk of future stroke [3, 4, 5].

Moreover, there is a consensus in the extension of the pathological target from small perforating arteriole towards the perivascular spaces (PVS) (usually a virtual space, where the pericyte membrane fuse together)[6,7], that act as a canal for the exchange between cerebrospinal fluid (CSF), and interstitial fluid (ISF) and for the clearance of catabolites [6], a so-called 'paravascular' or 'glymphatic' space [7,8, 9]. The significant consequences of their enlargement and disruption are the accumulation of catabolites and toxic substances, together with a pronounced neural starvation [9, 10]. The immobility of the fluid drainage can support PVS's role in different diseases: the possible explanation of the PVS involvement in SVD is the argued relationship demonstrated between an altered cerebrovascular reactivity, which is the change in cerebral blood flow in response to a vaso-active stimulus in the so-called neurovascular coupling [11, 12]. In SVD, we observe too the occlusion of deep periventricular-draining veins [13], associated with the disruption of the blood-brain barrier (BBB). All these facts together lead a consequent leakage of fluid and plasma cells, which eventually might potentiating the perivascular inflammation, and all the cascades of the inflammatory/obstructive/stagnation-induced process [11,14, 15, 16].

Above all these new aspects, the pathological expression of SVD is the arteriolosclerosis process [8, 17, 18, 19]. The loss of arterial elasticity and the consequent reduction of arterial compliance [20] are the primary determinant of the altered autoregulation capacities, leading to the brain of SVD patients' profound sensitiveness decreases of blood pressure [21, 22, 23]. The primary site of pathological localization is the subcortical infarcts, white matter hyperintensities, lacunes, prominent perivascular spaces, and cerebral microbleeds (visible in a conventional MRI) [24, 25], localized in the deep white matter capsule, along with the frontal and prefrontal-thalamus-basal forebrain networks, [26, 27], the caudate nucleus (the most precociously affected region), the putamen, insula, precentral gyrus, inferior frontal gyrus, and middle frontal gyrus.

Moreover, a low-level functioning of the autonomic nervous system has been observed, with direct and endothelium-mediated altered baroreflex activity [28,29,30,31]. Finally, SVD could affect the medial cholinergic pathway's integrity, for the hypoperfusion preferred localization, in the deep white matter capsule [32], or due to the multiple lacunar infarcts, the basal forebrain cholinergic bundle could be deafferentated from the tubero-mamillary tracts [33, 34]. These aspects affect the normally-accurate cerebral flow regulation and can further disturb the "retrograde vasodilatation system" with necessary consequences in neurovascular coupling [35]. Moreover, SVD is characterized by an associated hypoperfusion progression, causing incomplete ischemia of the deep white matter [36, 37, 38, 39] accompanied by inflammation, diffuse rarefaction of myelin sheaths, axonal disruption, and astrocyte gliosis [8]. SVD could affect the integrity of the medial cholinergic pathway, for the hypoperfusion preferred localization, in the deep white matter capsule, [32], or due to the multiple lacunar infarcts, the basal forebrain cholinergic bundle could be deafferentated from the tubero-mamillary tracts [33, 34]. These aspects affect the normally-accurate cerebral flow regulation and can further disturb the "retrograde vasodilatation system" with necessary consequences in neurovascular coupling [8].

Moreover, SVD is characterized by an associated hypoperfusion progression, causing incomplete ischemia of the deep white matter [36, 37, 38, 39] accompanied by inflammation, diffuse rarefaction of myelin sheaths, axonal disruption, and astrocyte gliosis [35]. In small vessel disease, occlusion of deep periventricular-draining veins is also evident [13], together with a disruption of the blood-brain barrier; the two factors are together causing a severe leakage of fluid and plasma cells to potentiate the inflammatory cascade, which seems to happen in the course of chronic hypoperfusion, collecting multifactorial causes for white matter alterations [14,15, 16]. Cerebral small vessel disease is what has been described as "a progressive disease" [8]. Lesions progress over time, and the long-term outcome and impact on brain damage vary, even not knowing why or how; reasonably, it should be said that the most rapid and confluent progression of the isolated
white matter hyperintensities could be considered as the most relevant predictor, at the best of knowledge, of the fatal progression of SVD [40, 41, 42, 43]. Of course, the total amount of lacunes and the profound white matter alterations relate to the degree of cognitive impairment [44,45,46]. The lesions' preferred location is placed along with the frontal and prefrontal-thalamus-basal forebrain networks [26, 27], directly implying the so-called cortical-deafferentation.

Additionally, lesions due to SVD are specific to the caudate nucleus (the most precociously affected region), the putamen, insula, precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. The pathology has been fully explained by the higher metabolic request of these regions (more than 20%) at steady state than other brain areas [47,48, 49, 50, 51,52,53,54]. Arteriolosclerosis occurs in two primary histological forms: hyperplastic and the hyaline arteriolosclerosis [55, 56]. The hyperplastic is the most common lesion, principally due to the chronic state of hypertension. It begins with the hypertrophy of the smooth muscle in the media, and it is accompanied by the reduplication of elastic laminae, the growth of new cells in the intima, and the deposition of collagen, which progressively substitutes the muscle cells (onion skin arteries), and severely obliterate the lumen [55]. Hyaline sclerosis is another change in hypertensive patients' vessels: the vessel wall becomes thickened with collagen [57, 58]. The hyaline material is a consequence of the leakage of the plasma proteins, mainly the inactive form of complement (C3b) through the endothelium, and also by an increment of the basement membrane components by the smooth muscle cells [58]. Healthy aging implies the loss of the Windkessel effect, the loss of arterial elasticity, revealing an anticipated and precocious return of the so-called wave reflection. Healthy aging also determines an increase of the systolic and a decrease of diastolic pressure, with a loss of resting flow effect through the Willis, which decrements the usual high perfusion pressure towards the most profound small arteries of the brain [59, 60, 61], provoking a loss of brain flow autoregulation. This aging effect is amplified in SVD.

Arteriolosclerosis perpetuates the hypo-perfusion in the profound territories irrigated by penetrating arteries. Chronic ischemia determines a severe oligodendrocyte degeneration; soon after, it causes microglial activation and is further associated with an increase of apoptosis processes associated with an elevation of caspase 3 RNA and matrix- metalloprotease 2 (MMP-2) expression [62, 63]. Astrocytes react to the chronic ischemic condition as a result of the length and severity of the insult. In the early ischemic period, the astrocytes respond with a remarkable proliferation. In persistent hypoperfusion, with their degeneration and death [64, 65,66, 67, 68]. Astrocytic death, due to chronic hypoperfusion, leads to an expanding and auto-potentiating system of neuronal death, due to a misleading neurovascular coupling.

Moreover, small arteries undergo a systemic poorness of cholinergic network regulation. Many hypothesis have been raised for a possible explanation, starting from an altered cholinergic response to inflammation, which is a constant in chronic ischemic condition [69, 70, 71, 72], up to a disruption of the cholinergic networks, which subcortically approaches the basal forebrain, since this is a preferential location of lacunar vascular infarcts and chronic hypoperfusion syndrome [73, 74,75,76]. Chronic reduction of the cerebral blood flow can affect the control of the cholinergic networks, but it happens that a proper cholinergic function is compulsory to well-regulation of the regional brain blood flow [77, 78]. In animal SVD models, there is a concomitant reduction of vasopressin and histamine, interpreted as a result of the tracts' interruption, which comes from the supra-optic and tuberomammillary nuclei and ends in the basal forebrain [79]. It has been conveyed that the cholinergic impairment is not mediated by a direct loss of the cholinergic neurons of Nucleus Basalis of Meynert [80, 81], but is a consequence of the secondary cholinergic deficits due to the indirect, cholinergic endothelial effect, aforementioned. Nevertheless, the number of muscarinic cholinergic receptors is markedly reduced in mixed dementia patients [81] and SVD dementia. Cholinergic poorness promotes a less efficacious endothelium relaxation, even due to an altered nitric oxide synthase and loss of efficacy of the GABA interneurons [82, 83].

The pathological cascade of events, which occurs as a consequence of all the pathological alterations described, determines a decrease of the vascular tone, with a release of the blood-brain barrier permeability, with a loss of the internal vascular remodeling, and with major vascular rarefactions. As a result, hypoperfusion at rest occurs in the brain and is associated with an impairment in the moment-to-moment control of CBF. There is also a decrease in adaptive vascular responses and the diminishment of the neurovascular coupling and auto-regulation system [76,84].
Even if the endothelium is one of the main targets of the redox altered process and inflammation (and both these processes are highly activated in SVD), the brain endothelium, even in severe SVD (presenting an almost complete loss of myocytes and other mural cells) remains intact [85, 86, 87, 88]. This paradoxical survival of the brain endothelium is also evident in patients with CADASIL [85, 86]. On the contrary, systemic endothelium activation is quite different in SVD.

Thus, indirectly, brain endothelium suffers in SVD conditions. Mitochondrial senescence of the endothelium walls has a catastrophic effect on cerebral endothelial cells [89]; this alteration, which is over-expressed in SVD [90], is generally related to an impaired response to the three major endothelium-derived nitric oxide–vasodilators [91], prostacyclin [92] and endothelium-derived hyperpolarizing factors (EDHF) [93]. The reduction of NO production is derived from an impairment of the mitochondrial functions, caused by a hyperproduction of the anti-oxidative defense system, and an increased O2 anions reaction with NO, producing peroxynitrite [94]. The activity of endothelial NO synthase (eNOS), which catalyzes the production of NO, declines with aging [95] but is even more impaired in SVD, where an important downstream target of Rho is the Rho-associated protein kinase (ROCK) [96]. These ubiquitously expressed serine/threonine protein kinases are involved in diverse cellular activities, including apoptosis, smooth muscle contraction, cell adhesion, and remodeling of the extracellular matrix [97]. In the regulation of endothelial cell, migration ROCK interacts with ezrin, radixin, and moesin, also known as the ERM proteins that function as cross-linkers between the plasma membrane and actin filaments [96], and are indispensable for the leukocyte adhesion molecules coordination, essential for barrier function [98]. Moreover, the ROCK/RhoA complex regulates, as previously exposed, the endothelial nitrous oxide synthase (eNOS) [96]. Nitric oxide (NO)-induced vasodilation occurs via myosin light chain phosphatase (MLCP) activation in a cGMP dependent manner. RhoA/ROCK counteracts this through MLCP inactivation and calcium desensitization [96, 99]. ROCK/Rho decreases eNOS expression and affects the availability of nitric oxide (NO) [100]; even if these effects have been largely studied in major vessel disease (coronary), it has also been proven in brain small vessels [8]. Three potentially functional eNOS polymorphisms (T-786C, intron 4ab, G894T) located toward the 5’ flanking end of the gene are known to be considered as being present in SVD and also in isolated lacunar infarction and ischemic leukoaraiosis [101]. RhoA inhibition overwhelms vascular endothelium growth factor (VEGF)-enhanced endothelial cell migration in response to vascular injury, without, or better said, with minimal effect, on basal endothelial cell migration [102, 103]. The endothelial barrier’s maintenance is a prior role of the endothelium cells, mainly through the operative system of RhoA [104], also mediated through the regulation of VE-cadherins [105]. In diabetes (one of the main risk factors associated to SVD), advanced glycation end products (AGEs) accumulate in the vasculature, triggering a series of purposeful and morphologic changes of endothelial cells, such as the increase of the activation of the RhoA/ROCK pathway; the significant consequence is an increased endothelial permeability [106]. It can also act as a VEGF inducer, which indirectly causes microvascular endothelial hyper-permeability [107].

Therefore, it should be argued that, even if morphologically and structurally undamaged, the endothelium seems to be functionally impaired in SVD [108].

The endothelial NO downregulation in SVD is a marker of decreased endothelial regulatory capacity, in response to external stimuli, such as hypercapnia [109, 110]. Living studies have demonstrated a significant baseline CBF reduction in SVD–affected subjects, together with an impaired CBF autoregulation [111,112, 113]. Endothelial activation refers to the change in many different surface markers [114, 115, 116, 117]. These circulating markers of endothelial activation include intercellular adhesion molecule-1 (ICAM-1), which has been considered as a generic expression of white matter progression [118], soluble thrombomodulin (sTM), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor, and others [86, 119,120,121]. Moreover, upregulation of hypoxia-endothelial-related markers has been proven, such as HIF 1 alpha, VEGFR2, and neuroglobin, when white matter lesions appear to be confluent [122]. The matter is even more impressive when it appears evident that endothelium in overall activated, as above described, but, according to some authors, not specifically in the human gray matter [88, 120, 123, 124]. Though, the brain endothelium NO dysregulation implies not only a direct inhibition of the vessel tone but indirectly, more critical, a decrease of the dynamic neurovascular control mechanism [125, 126].
Moreover, it should be taken into account the permanent status of oxidative stress-induced, which causes a superimposed macroscopic alteration of the cerebral endothelium.

The immediate consequence of the endothelial dysfunction has two significant consequences, the reduction of the resting flow in the marginally perfused white matter and macroscopic alterations of the BBB permeability [126]; these two aspects lead to additional oxidative stress by inducing tissue hypoxia and extravasation of the plasma proteins [126], and both of them potentiate inflammation pathway, through NFkB dependent transcription. The modern view gives the endothelium the control role of propagating vasomotor signals [127] even if the question is still unresolved. In systemic vessels, the endothelium is well known to participate in the retrograde propagation of vascular signals [7, 128], but in the brain, the mechanisms by which endothelium interacts with the spread of the vascular signal is still debated. It has been proven that a highly localized lesion of the endothelium failed to propagate beyond the lesion site, and altered the amplitude and the temporal dynamics of the go-ahead vascular sign, with weaker temporal coordination [129]. It has been demonstrated that brain endothelium is enriched with KIR channels, and not by KCa channels. These channels are sensitive to high K flow, derived from neural activity, and transmitted by the synapses or astrocytic end-feet [128,130]. K+ is recognized in the endothelium, and the upstream penetrating arteriole is the effector of the vasodilatation [130]. Its rapid propagation is probably conducted by ionic currents traveling through the endothelium via gap junctions and then through the myoendothelial junctions [128]. Therefore, a KIR suppression avoids the increase of CBF produced by cortical activation [130]. The most intriguing aspect of the endothelial conductance is that the conducted vasomotor responses, either being a dilatation or vasoconstriction, can be generated by different neuromodulators, ie. Acetylcholine, ATP, prostaglandin F2alpha, NO, but their effects on neurovascular coupling has never been determined [131]. The evidence is increasing on pial arterioles: signals generated by the neuronal activity, deep in the brain, should be conveyed to upstream arterioles, remote from the area of activation, to increase flow efficiently [128]. Vascular mapping and fMRI demonstrated that vascular responses are first seen in the deep cortical lamina during somatosensory activation, and then, more superficially, suggesting a retrograde propagation of the vascular response [132]. A possible scenario for the transmission and coordination of the vascular response is described [128] as follows: the activation-induced increase in extracellular potassium triggers hyperpolarization of capillary endothelial cells and pericytes [133]. The hyperpolarization propagates upstream and reaches smooth muscle cells in penetrating arterioles producing relaxation [128, 130]. Simultaneously, metabolic modifications (reduced viscosity, increased deformability of blood cells) on the endothelium of feeding arterioles increment the smooth muscle cell relaxation (the so-called flow-mediated vasodilation). In upstream pial arterioles, remote from the activation site, there is vasodilation, by propagation from arteriole downstream and acting as a local flow-mediated and myogenic response. For all the conditions mentioned above, SVD is defective in neurovascular coupling, even for endothelial and pericytes failure.

**Homocysteine and brain**

Hcy is a sulfur-containing non-protein toxic intermediary amino acid related to methionine metabolism [134], either degraded via the remethylation pathway or converted, via the transsulfuration pathway, into cysteine. The methionine synthesis is anticipated by the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-methylTHF) catalyzed by the flavin-containing methylenetetrahydrofolate reductase [135].

Homocysteine (Hcy) is related to the production of 5,10-methylenetetrahydrofolate, a fundamental step for the synthesis of thymidylate, purines, and methionine, employing vitamin B12 and folate as cofactors [136, 137,138, 139]. Methionine adenosyltransferase (MAT) catalyzes S-adenosylmethionine (AdoMet) in a reaction involving methionine and ATP [140, 141]. Every reaction made by methyltransferases produces S-adenosylhomocysteine (AdoHcy), which is a potent inhibitor of most of them [142, 143]. An AdoHcy hydrolase (SAHH) acts on AdoHcy, producing adenosine and homocysteine, and they need to be metabolized or transported out of the cell to prevent their accumulation [144]. This hydrolysis is a reversible reaction that favors S-Adenosyl-L-homocysteine (SAH) synthesis. The S-Adenosyl-Methionine = AdoMet (SAM) to SAH ratio defines the cell's methylation potential [145]. If homocysteine is allowed to accumulate...
in normal conditions, it will be rapidly metabolized to SAH, which competes with SAM for the active site on the methyltransferase system [146, 147, 148, 149]. Homocysteine is also methylated in the entire body, but not in the brain, by betaine [150, 151]. Homocysteine remethylation is catalyzed by the methionine synthase (MTR) enzyme, which requires vitamin B12 (Cbl) as a coenzyme [152, 153]. During the transsulfuration pathway, homocysteine is irreversibly degraded to cysteine. Cysteine is a precursor of glutathione, the most vital endogenous anti-oxidant [154]. In most tissues, homocysteine is either remethylated or exported out of the cell. Moreover, the methylation reactions are strongly necessary for the brain for the fact that SAM is the sole donor in numerous methylation reactions involving proteins, phospholipids, and biogenic amines [155], and for the packaging of many phospholipids [156].

Normal levels of Homocysteine (Hcy) range between 5-15 micromol/L, and in physiological conditions, plasma total (t) Hcy levels are <15 micromol/L, as reported by the majority of investigations. Less frequently, a threshold of 13 micromol/L has been reported, depending on the method of detection employed. Hyperhomocysteinemia (HHcy) is typically defined as levels >15 mol/L in reported studies; levels between 15–30 are considered moderate HHcy; levels at 30–100 micromol/L are considered severe HHCy; levels above 100 micromol/L are considered fatal HHcy [156]. The physiologic levels of Hcy in a healthy population are determined primarily by the dietary intakes of methionine [157], folate [158], and vitamin B12 [159]. Recent studies are generally confident with the fact that lifestyle conditions, such as smoking, alcohol consumption, and physical inactivity, may help the elevation of Hcy [160,161]. Aging is considered a strong determinant for homocysteine increase [162]. HHcy-induced oxidative stress, endothelium dysfunction, inflammation, smooth muscle cell proliferation, and endoplasmic reticulum stress have been considered to play an essential role in the pathogenesis of several diseases including atherosclerosis. Different epidemiological researches propose that increased homocysteine level is an independent risk factor for vascular diseases, including stroke and neurodegenerative disorders. The clinical role of Hcy, and especially of its accumulation, is frequently controversial in standard clinical practice [163]. It seems quite evident that Hcy is not relevant, per se, at the moment, rather than its accumulation.

The best and undoubted cases are the ones determined by a genetic deficiency of Cystathionine - Synthase (CBS) and to other genetic alterations of remethylation and trans-sulfuration pathways, which induced severe HHCY (total Hcy > 50 microM) or homocystinuria. Severe HHCY (>100 microM) in children with a CBS defect correlates with a 10-fold increase in concentrations of Hcy in cerebrospinal fluid (CSF) [164]. This genetic defect limits purines and thymidine synthesis due to a severe alteration/ inhibition of the transmethylation pathway. The final result is a severe delay or almost an abolition of the neural tube closure [165]. It has been demonstrated in animal models that Hcy could be intrinsically toxic, compromising the blood-brain barrier [166]; the same mechanism has been postulated for human beings [167, 168]. The most crucial aspect of HHcy is the constant tendency to decrease the cell's methylation potential, interfering with the SAM/SAH ratio [169]. Therefore, HHcy promotes a generalized DNA hypomethylation, together with the transcription of cyclin A in endothelial cells; opposite results induced by HHcy are the up-regulation of the p66shc expression, promoting oxidative stress [145, 169]. Homocysteine, therefore, could contribute SVD pathology with four mechanisms: promoting neurodegeneration, favoring neuro-inflammation, damaging endothelium, and finally activating oxidative stress. Nevertheless, there are not many studies directly concerning Hcy or HHcy and SVD [162, 170, 171, 172, 173, 174].

Our review is an attempt to review the four principal mechanisms of HHcy induced damage with a focus on SVD.

**Homocysteine and Neurodegeneration**

Many clinical works try to focus on the possible direct consequences of Hcy inside neurodegenerative disorders: it is well-accepted that Hcy increases in CSF with aging, for not precise reasons. It has been demonstrated that there is a favorable condition, mediated by HHcy, of Abeta1-40 deposition in the brain of AD patients [175], mediated by an endoplasmic protein-HCY related (HERP), which potentiates the c-secretase enzyme activity, and a direct potentiation of the intra- and extracellular accumulation of Abeta42...
Moreover, it is accepted that DNA hypomethylation mediated by HHcy up-regulate the presenilin genes, in particular, presenilin 1 (PS1), which promotes the amyloid precursor (APP) synthesis [180, 181]. Besides, covalent protein modification by the metabolite of Hcy, Hcy thiolactone (HTL) (see afterward), has now been shown to be another cause of cellular Hcy toxicity. This mechanism, termed as "protein N-homocysteinylatation," is known to result in protein denaturation, enzyme inactivation, and even amyloid formation, and favor its oligomer depositions [182]. The role of protein N-homocysteinylation and the resulting consequences are an imbalanced mechanism, which lies in between inflammation and neurodegeneration. It is probably a mixer-result between potentiating misfolded protein accumulation and consequent neuronal death by starvation or hyper-activating inflammation. Hcy is indirectly bound to the tau protein, too. Tau protein has many functions, mainly being the principal actor of the microtubules' assembly, and therefore, being the principal responsibility of the axonal nutrients transport. Tau protein needs a dephosphorylating system, regulated by protein phosphatase methyltransferase 1 and 2A (PPM1, PP2A), whose methylation is SAM-dependent [183, 184, 185, 186, 187]. Hyperphosphorylated tau (induced by the reduced methylation capacity by HHcy, or to an increased SAH) is an invalid protein, which inhibits the microtubules' congregation, whose precipitation determines the deposition of the neurofibrillar tangles, and the neuronal death by starvation [188, 189, 190]. The hyperphosphorylation of tau does not occur only in neurodegenerative conditions; the induced depletion of folic acid in neuroblastoma cultured cells, causing HHCY condition, produced an increase of P-TAU by 66% [191]. Moreover, HHcy has direct toxic effects when it is artificially induced inside brain models either by pressure ejection or iophorohes [192]. Hcy acts as an agonist of the endogenous glutamate NMDA receptors [193, 194, 196, 196]. The Hcy-NMDA binding effect largely depends on glycine medium concentration; when glycine is in average concentration, Hcy acts as a partial antagonist of the NMDA receptors [155, 193]. When glycine medium concentration upraises, i.e., in brain ischemia, or functional arterial functional spastic condition, such as protracted migraine crisis, even low concentration of Hcy could become dangerous, acting as an agonist on NMDA [197, 198], enhancing overwhelming calcium influx currents [198]. It has also been hypothesized that Hcy can compete with GABA and directly activates group I metabotropic glutamate receptors, favoring substantial calcium influx [197]. HHcy can determine an extracellular signal-regulated kinase in the hippocampus, effect blocked by three types of glutamate receptor antagonists (NMDA, non-NMDA, and metabotropic receptors) [155, 199]. By activating ionotropic and metabotropic receptors, HCY indirectly increases intracellular calcium level and activates several kinases [155, 199]. It has also been evidenced that HHcy leads to a severe reduction of dopamine turnover in the striatum, and that probably occurs due to a high affinity for Hcy in the third loop of the D2 receptor, and therefore Hcy exerts an allosteric antagonist activity of D2 receptors [200, 201].

Concerning SVD, HHcy has two significant implications: it has been found that HHcy increases Abeta (1-40) toxicity on the smooth muscle cells of small arteries in the brain, and that appears more evident in the cerebral amyloid angiopathy (CAA), which is frequently involved in SVD [202]. Nevertheless, it is also clear that Hcy induces the m-TNA production of the C-reactive protein (CRP), augmenting, therefore, the NR1 subunit of NMDA receptor expression. This way, HHcy, by CRP hyper-production, enhanced continuously by a combined NMDA-ROS-erk1/2/p38-nKBeta signal pathway [203] mediates a pro-inflammatory cascade inside the smooth cells of small arteries. All along with, it seems relatively easy to think that HHcy can promote atherosclerotic process, but also a substantial impedance of smooth muscle activity. Therefore, small arteries fail their precarious autoregulatory response, extend their diminishment of perfusion capabilities, and promote white matter alterations, accelerating the entire cascade of SVD events [204].

Homocysteine and neuroinflammation

It is challenging to talk about the relationship between neuroinflammation and HHcy and leave the crucial point of concomitant reduced anti-oxidative properties. It is well known that HHcy induces and accelerate the disruption of the redox system in vascular and neuronal cells [205], and these are the two processes that induce the lipid peroxidation sequel of events [206, 207]. Even though, HHcy has been directly claimed on the general principle of inducing inflammation, per se. It has been demonstrated that in severe inflammatory status, i.e., sepsis, comatose states, multiple traumatic lesions, there is a constant tendency of HHcy, not
related to vitamin B12 and folate poorness [208]; by the way, it has been implemented all these cases with generous doses of vitamin B12 and with folate. The implementation does not reduce HHcy and does not affect inflammatory markers, such as IL-6, TNFα, CRP, etc.; on the contrary, folate integration only reduces neopterin, suggesting a possible modulating role of folic acid in the inflammatory cascade [208]. In different clinical scenarios, the systemic inflammatory response is constantly related to a poor clinical outcome [209], which is probably mediated by sustained HHcy activation of the macrophage-system cascade, with a consequent increment of ROS production, and therefore a potentiation of the oxidative stress. The virtual loop is an overwhelming, auto-potentiated system. Animal models definitely show that HHcy induced a constant increase of TNF-α and IL-1 beta, together with a decrease of H2S and cystathionine-gamma-lyase in macrophages. HHcy upregulates methyltransferase expression and induces hypermethylation in many different macrophage promotor regions [210, 211, 212]. Cultured macrophages exposed to HHcy show a so-called memory response, probably derived by an epigenetic mutation, which strongly influences the expression of inflammatory-inducers genes and endothelium altered-response inflammation [210]. There is also an in-vitro demonstration of altered transcriptional fibroblast growth factor 2 induced by HHcy exposure [213]. HHcy possesses a direct effect on the endothelium, and, even if without a note mechanism, it releases inflammatory cytokines from vascular endothelial cells, such as IL-6, IL-8, and TNF-alpha [214, 215, 216]. There is an interesting response in endothelium to TNF-alpha, high production of cathepsins [217]. The serum level of cathepsins is associated with cardio- and cerebrovascular diseases [218], especially cathepsin V [219, 220]. Cathepsins are lysosomal cysteine proteases and belong to the papain family of proteases that comprises 11 members, while endothelial cells mainly express cathepsin K, B, S, L, and V [221, 222]. Cathepsins are involved in vascular remodeling and inflammation [223]. One of the unresolved problems is that cathepsin V is mainly expressed outside of the nucleus. Therefore, to determine its effects on remodeling and the regeneration of affected endothelium, it should exist a nuclear translocation mechanism capable of transferring the cathepsin V signal into the nucleus. A very recent experimental data sequence shows that a high methionine diet-induced HHcy mouse model was used to assess cathepsin V expression and vascular inflammation [224]. The authors firstly demonstrate that Cathepsin L (human cathepsin V homologous) was increased in the thoracic aorta endothelial cells of hyperhomocysteinemic mice [224]. Moreover, high concentrations of N-[[1,1-dimethyl ethoxy)carbonyl]-L-tryptophan-2-[[2-(2-ethyphenyl)amino]-2-oxoethyl]thio]carbonyl]hydrazide (SID) suppressed the activity of cathepsin V. It reversed the up-regulation of inflammatory cytokines (IL-6, IL-8, and TNF-α), adhesion and chemotaxis of leukocytes and vascular inflammation induced by L-homocysteine in vivo and in vitro [224]. Cathepsin S enhances the VEGF/ERK1/2 signaling pathway [225]. Moreover, cathepsin V upregulated the expression of IL-6 and TNF-α, but not that of IL-8, via the ERK1/2/STAT1 pathway [224]. ERK1 and ERK2 are related to protein-serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade. This cascade participates in regulating a large variety of processes, including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription. In physiological conditions, ERK1/2 is unphosphorylated in the cytosol but is translocated to the nucleus upon phosphorylation. ERK1/2 phosphorylation is maintained by a balance between MAP kinase and ERK kinase (MEK1/2) activation and is negatively controlled by compounds such as the Dual-specificity phosphatases (DUSPs). DUSPs inactivate ERK1/2 by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues [224]. The authors demonstrate that increased expression of cathepsin V promotes the phosphorylation and subsequent nuclear translocation of ERK1/2, possibly through degradation of DUSP6 and DUSP7. Phospho-ERK1/2 can phosphorylate and activate its downstream signaling molecule (such as transcription factor STAT1) in the nucleus, and phospho-STAT1 will promote the transcription of target genes (such as IL-6 and TNF-α) [224, 25]. Genome-wide analysis revealed that the hypomethylation (predispersed by HHcy status) of chromosomal DNA predominates in atherosclerotic plaques [226], where inflammation status predominates. Moreover, it has been described a B-activation cascade of events, induced by HHcy, probably induced by an up-regulation of pyruvate kinase muscle isozyme 2 (PKM-2) in B cells, which mainly promotes the inflammatory basis of atherosclerosis cascade, demonstrated in vivo and in vitro and which is primarily inhibited by shikonin [227]. There is a possible link between the excitotoxic effect of Hcy and the pro-inflammatory role of
Hcy: the NMDA receptors are found not only in neurons (see above in the text), but also on neutrophils and macrophages. The activation of these receptors, as well as in the cerebral context, arises the cytoplasmatic calcium influx, and activates a pro-inflammatory cascade, with an accumulation of ROS species [228]. In fact, in the Rheumatoid Arthritis, as an example, [229] HHcy is two times more frequent than in the general population and HHcy contributes to the oxidative stress, and, indirectly, by the excess of ROS released, it induces an up-regulation of the Nuclear Factor Kappa B, considered as one of “the master regulator of the expression of inflammatory genes” [230]. In this context, a recent series of studies put in evidence that HHcy might relate to an overproduction of other products, such as asymmetric dimethylarginine (ADMA), that is an endogenous inhibitor of endogenous nitric oxide synthase (eNOS), the enzyme catalyzing the formation of nitric oxide (NO) from arginine. This endogenous inhibitor of endothelial nitric oxide synthase (eNOS) competes with the natural substrate, L-arginine, limiting the formation of NO, similarly to Hcy.

Plasma levels of ADMA have been reported to be positively correlated with plasma homocysteine levels [231, 232, 233, 234]. Protein arginine N-methyltransferases (PRMTs) 1 and 2 are involved in the methylation of protein arginine residues. PRMTs utilize SAM as a methyl donor and generate SAH (and ultimately Hcy) as a byproduct [222]. Hcy enhances the activity of PRMT 1 and also increases the proteolysis of proteins with methylated arginine residues. The major pathway for ADMA's metabolism is the formation of citrulline and methylamine mediated by dimethylarginine dimethylaminohydrolase (DDAH) [234, 235]. Similarly, Hcy can influence the activity of DDAH, which would prevent the metabolism of ADMA and thus bring down the levels of NO. Also, Hcy may elevate ADMA levels by inducing ER stress and cell death, leading to increased proteolysis of proteins that contain methylarginine residues [101, 102]. The likelihood of HHcy enhancing PRMT activity is controversial since it results in high levels of SAH [101]. It remains possible, however, that the inhibitory effect of high levels of SAH in HHCy might compensate for the increased expression of PRMT 1 [234, 235, 236, 237, 238].

HHcy upregulates the matrix metalloproteinases-9 (MMP-9) expression, a pro-inflammatory proteinase that is over-expressed when there is either in clinical or in the experimental condition rupture of the atherosclerotic plaque [234, 239]. Circulating apoptotic endothelial cells have been observed in severe HHcy patients’ bloodstream, suggesting that endothelial dysfunction. A pathologically relevant level of homocysteine can induce apoptosis in cultured endothelial cells mediated by endoplasmic reticulum (ER) stress and unfolded protein response (UPR) [240]. It is widely known that any accumulation of unfolded and aggregated proteins results in ER stress, causing a cascade of events, the UPR [241]. It is well-accepted that UPR upregulates the ER, mediating an increased production of chaperons, and downregulating the ER protein load, via a control on transcription and translation process [241, 242]. Whenever there is a persistent hyper-activation of ER protein folding capacity, UPR cannot resolves it, and damaged cells should be removed. HHHCy is an elicitor of ER stress condition [243]; therefore, an HHcy condition leads to hyper-activation of UPR responses, and when the system is under stress, in a dose-dependent relationship, promoting apoptotic responses. The first is the activation of CHOP/ GADD153 (growth arrest- and DNA-damage inducible gene 153). The second is the activation of TDAG51 (T-cell death-associated gene 51) and the initiation of detachment-mediated apoptosis [243, 244, 245]. Over-expression of the latter in endothelial cells has been shown to cause cell rounding up, an extension of cell pseudopods, dramatic loss of cell adhesion, and caspase-dependent cell death [243, 245]. Moreover, other facts occur in HHHCy condition. When the SAH/SAM is high, there is a low methylation condition. That is the main determinant of the fact that the enzyme that links methionine to its conjugate t-RNA (the methionyl-tRNA synthase (MetRS)) attacks homocysteine instead of methionine, causing the thiol group and the carboxyl group of homocysteine to join with each other by thioester linkage [243, 245]. That results in the generation of cyclic derivative homocysteine thiolactone [243, 246, 247]. The thioester group of thiolactone actively reacts with any lysine residues in proteins to form amide bonds in a process known as N-homocysteinylation [243, 245, 248]. Naturally, the rate of N-homocysteinylation proportionally increases with the number of lysine residues [243, 247, 249]. N-homocysteinylated proteins tend to aggregate: the most interesting aspect is that multiple lysine-rich proteins include fibrinogen [247, 248], high-density lipoprotein [248], lysine oxidase [246], and cytochrome c [249], potential and virtual target of the N-homocysteinylation [243]. Homocysteine thiolactone induces apoptosis directly in endothelial cell cultures in vitro and in vivo models [243]. HHcy due to N-
homocysteinylolation also induces a prothrombotic condition for its effect on fibrinogen [247, 248, 250, 251], and enhanced platelet activation, enhanced coagulation [252], and attenuated fibrinolysis [253, 254]. Moreover, enhanced expression of the receptors for advanced glycation end products, vascular cell adhesion molecule-1, tissue factor, and MMP-9 was found in a mouse model with methionine-induced hyperhomocysteinemia [243, 255]. However, contradicting results have indicated that the inflammatory response induced by homocysteine occurred in vascular smooth muscle cells, and not at the endothelium, which could be a target of HHcy by redox impairment, by stimulating CRP production, which is mediated through the NMDAr-ROS-ERK1/2/ p38-NF-kB signal pathway [243, 256].

HHcy-induced toxicity is thought to be also mediated by an indirect effect on the global “Cellular protein quality control” (PQC). PQC is essential for maintaining proteome integrity and cell viability, and its failure contributes to the development of multiple diseases [257]. Chaperones, UPR, ubiquitin-proteasome system (UPS), and autophagy are analogous strategies of PQC that maintain cellular proteome integrity. Recently, multiple studies reported that HHcy responsible for the perturbation of PQC by reducing chaperone levels, activating UPR, and impairing autophagy [257, 258, 259]. Besides, HHcy also induces cytotoxicity, inflammation, protein aggregation, and apoptosis. It has been shown that some of the factors, including altered sirtuin1/heat shock factor 1/heat shock protein axis (SIRT1-HSF1 axis) and irreversible homocysteinylolation of proteins, are responsible for folate and/or B12 deficiency or HHcy-induced impairment of PQC [260].

It has been demonstrated that HHcy treatment enhanced brain injury, induced activation of microglia, and triggered the expression of pro-inflammatory cytokines in the om mice models [261]. This is the first study [261] to provide evidence that changes in the signal transducer and activator of transcription 3 (STAT3), a member of the STAT protein family of transcription factors activities located in microglia. The STAT3 has been extensively described as a central signaling molecule that controls cellular adaption in response to environmental stimuli or stress. Several groups have shown that the STAT3 is activated in vitro and in vivo experimental models of stroke. Subsequently activated - STAT3 promotes numerous genes responsible for neural injury and repair [262, 263]. Data on recovery progressions STAT mediated are quite controversial [264], and most of the newest data converge towards their implication in a poorer stroke outcome [265]. Nevertheless, what is almost evident is the role of STAT3 as a regulator of inflammatory gene expression in microglial pro-inflammatory reactivity to various stimuli [266]. In the model reported [264], HHCy treatment activated microglial cells, with consequent enlargement of the infarction volume, with a subsequent increment of IL-1Beta, TNF-alpha, and IL-6, aggravating the brain damage [267, 268].

Homocysteine and oxidative stress

Mechanisms of action of Hcy are an ongoing development, but the most substantial evidence derives from its antioxidant effect, the primary biochemical mechanism responsible for hHcy-related pathogenesis [166, 248, 269, 270]. Oxidative stress is defined as a severe imbalance between the production of reactive species and antioxidant defenses and can result from diminished levels of antioxidant and/or increased production of reactive species [206, 271]. Redox reactions are fundamental in endothelium regulation, platelet aggregation, and atherosclerosis induction. Oxidative stress is generated during oxidation of the free thiol group of Hcy when Hcy binds via a disulfide bridge with plasma proteins - mainly albumin - or with other low-molecular plasma thiols, or with a second Hcy molecule. Accumulation of oxidized biomolecules, such as superoxide anion (O2-) and hydrogen peroxide (H2O2), mainly during its auto-oxidation, alters the biological functions of many cellular pathways [206]. Increasing evidence suggests that elevated plasma Hcy affects the body’s oxidant-antioxidant balance following endothelial injury [272]. Five mechanisms have been proposed for Hcy-induced oxidative stress [206]. They include

1. inhibition of cellular antioxidant enzymes,
2. Hcy auto-oxidation,
3. nitric oxide synthase (NOS)-dependent generation of superoxide anion via uncoupling of endothelial NOS (eNOS),
4. disruption of extracellular superoxide dismutase from endothelial surfaces, and
5. activation of NADPH oxidases [206].
ROS and oxidative stress promote nitrotyrosine, an indicator of NO and superoxide radical reaction, resulting in the formation of strong oxidant peroxynitrite [206]. Peroxynitrite leads to tyrosine nitration, which induces significant cellular dysfunctions [273].

HHcy also increases NAD(P)H oxidase activity, which in turn triggers microglia activation and stimulates the secretion of pro-inflammatory molecules such as arachidonic acid and cytokines [274, 275]. Homocysteine also directly inhibits antioxidants’ activity, thereby disrupting SOD, activating NADPH oxidases (NOXs), and subsequently producing superoxide anion, causing an accumulation of ROS. The generated ROS further activates the transcriptional activity of NF-κB, which results in the expression of pro-inflammatory genes and vascular inflammation [3, 276]. HHcy plays an essential role in the pathogenicity of cardiovascular diseases. In atherosclerosis (AS) (a progressive disease of multifactorial origin, which occurs in response to endothelial injury) has been shown that HHcy is the primary cause of endothelial dysfunction due to oxidative stress and DNA methylation. Several mechanisms have been indagated to understand the role of HHcy. Recent work has demonstrated that HHcy injured the endothelial through the effect of methylation and trans-sulfuration metabolism of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) through toll-like receptor 4(TLR4)/nuclear factor (NF)-κB/DNA methyltransferase (DNMT)1. Following an attack to the endothelial cells, lipids accumulate in the subendothelial layer to promote atherosclerosis formation [277]. The endothelial damage is mediated by one of the precursors, hydrogen sulfide (H2S), formed during the transsulfuration process [162, 278]. The disruption of the redox system in vascular and neuronal cells induces and accelerates the lipid peroxidation sequel of events [205]. The vascular endothelium is a single layer of dynamic cells that produces vasoactive substances to maintain the vascular tone and regulate blood flow to the tissues through a variety of stimuli. Among these, this effect was attributed to a substance(s), subsequently identified as nitric oxide (NO) [279].

In animal models, HHcy, increasing oxidative stress, induces the upregulation expression of proteins that promote blood coagulation, exacerbates traumatic brain injury-associated blood-brain barrier dysfunction, and promotes the infiltration of inflammatory cells into the cortex [206]. An increase of brain injury-induced lesion size and aggravated anxiety-like behavior has been observed, suggesting that moderate HHcy exacerbates traumatic brain injury outcomes and that Hcy catabolic dysregulation may be a significant biological variable that could contribute to traumatic brain injury pathophysiology heterogeneity [280].

Endothelial dysfunction also results from a disruption in the cellular integrity, leading to impaired endothelium-dependent relaxation mainly due to a reduction in the nitric oxide (NO) bioavailability. NO is produced from its precursor L-arginine by endothelial nitric oxide synthase (eNOS). Under physiological conditions, following production, NO diffuses across the endothelial cell membrane into the vascular smooth muscle cells to activate guanylate cyclase, leading to subsequent cyclic guanosine-30,5-monophosphate (cGMP)-mediated vasodilation. Several molecules, such as acetylcholine, bradykinin, serotonin, and substance P, can induce eNOS. HHcy-induced ROS production decreases NO production and bioavailability, triggering increased redox signaling. Impaired NO production during HHcy can also occur due to inhibition of Dimethylarginine dimethylaminohydrolase (DDAH), causing Asymmetric dimethylarginine (ADMA) accumulation [281, 282, 283,284].

Another critical stimulus is the shear stress exerted by the flowing blood, which can cause ion channel activation for a rapid response or through a phosphorylation process to induce the sustained release of NO to maintain vasodilation [285]. HHcy has been linked to an increment of ROS and deactivation of nitric oxide and the well-known inflammation cascade [206, 207]. Oxidative stress as a result of ROS accumulation is the primary mechanism that mediates homocysteine-induced vascular injury [286]; finally, a possible link between Hcy and lowered melatonin production has been reported [287], and it has been demonstrated that melatonin scavenges free radicals [288] and counteracts Hcy by a direct antioxidant effect and by apoptosis modulation [289, 290].

Recently, a well-conducted study [291] conducted on neuroblastoma cells incubated with Hcy determined some different and time- and concentration-dependent results. The highest concentration of Hcy determines cellular death after five days of incubation. Forty microM Hcy conducted to a 35% of cell death after five days of incubation. Quite impressive, cell exposure to Hcy for three days does not induce any change in Reactive Oxygen Species (ROS); exposure to Hcy for five days elevated to a 4.4 fold increase ROS production. A five days incubation with Hcy induced a 2-fold increase of Bax mRNA and 14-fold of Bcl-2 mRNA. A
three-days incubation with Hcy induces an increase of 2-fold for cyclin D1 mRNA, 6-fold for cyclin E1 mRNA, and 5-fold for cyclin A1 mRNA [3]. Unexpectedly, all the levels turn back to a normal range after five days of incubation. This study points out that there is a general upregulation of p21 and p-16 after five days of Hcy incubation, inducing a reduction of 35% of pRB, checkpoint regulators of G1 cell-cycle phase. This work suggests potential genotoxic stress, time-exposure, and Hcy concentration-related. In response to the higher Hcy level, endothelial cells produce NO to induce the formation of S-nitroso-Hcy, which acts as a protector of endothelium; however, with chronic exposure to Hcy, NO levels diminish [182.] and this fact, associated to the high levels of Hcy, promotes endothelial damage. The first by stimulation of muscle cells, vasoconstriction, and promoting inflammatory response, testified by an increase of c-reactive protein and cysteiny1 leukotrienes, was associated with an incremental increase in HMG-CoA reductase activity [292]. 

The activities of methionine synthase that mediate the clearance of Hcy are linked to the redox potential of the cells [293, 294], with an observed efficacy in the oxidative stress process; in this situation, more Hcy is converted into cysteine and glutathione. A disruption of the CBS causes altered redox homeostasis, and through a reduction of the cysteine and glutathione, it causes an alteration of the oxidative repairing process [295].

On the other side, different studies demonstrate that the antioxidants, such as N-acetyl cysteine, vitamin E, or C might reduce the potential pro-inflammatory response of Hcy in animal models [296,297]. Different in vivo reports recognized that the Th1-activity induced the Hcy inflammation response [298], and it appears that HHcy can be detected in chronic inflammatory conditions, even if vitamin B12 and folate are in range. A recent study promoted oxidative injury and apoptotic cell death in human umbilical vein endothelial cells by Hcy [299]. High level of Hcy promotes ROS accumulation in human umbilical vein endothelial cells through Hcy auto-oxidation that causes oxidative stress [286]. Hyperhomocysteinemia damages endothelial cell function, increases oxidative stress and enhances apoptosis via the intrinsic apoptotic pathway [300]. Indeed, increased levels of ROS and lipid peroxidation are the markers of oxidative damage. ROS and malondialdehyde generation are concurrent with Hcy-induced apoptosis [301]. ROS generation is significantly decreased by treating the cells with melatonin, vitamin E, or both.

Then, homocysteinylation is another mechanism of damage, strongly related to the three pathogenic mechanisms (neurodegenerative, neuroinflammatory, and oxidative) by which Hcy does its damages. Two homocysteinylation systems have been detected: S-homocysteinylation and N-homocysteinylation, both considered posttranslational protein modifications [206]. S-homocysteinylation occurs when Hcy reacts, by its free thiol group, with another free thiol derived from a cysteine residue in a protein molecule. These changes can alter the thiol-dependent redox status of functional proteins [247]. N-homocysteinylation occurs after acylation of the free amino (e.g., lysine) groups of different proteins to form adducts under physiological conditions, and its degree depends on the Hcy levels [247]. It appears that the conversion of Hcy to Hcy-thiolactone followed by protein N-homocysteinylation largely contributes to manifestations of Hcy toxicity [246]. Naturally, the rate of N-homocysteinylation proportionally increases with the number of lysine residues [243, 247, 249]. N-homocysteinylated proteins tend to aggregate: the most exciting aspect is that multiple lysine-rich proteins include, as above reported, fibrinogen [247, 248], high-density lipoprotein [248], lysine oxidase [246] and cytochrome c [249], potential and virtual target of the N-homocysteinylation [243]. Homocysteine thiolactone induces apoptosis directly in endothelial cell cultures in vitro and in vivo models [243]. Moreover, enhanced expression of the receptor for advanced glycation end products, vascular cell adhesion molecule-1, tissue factor, and MMP-9 was found in a mouse model with methionine-induced hyperhomocysteinemia [243, 255].

**CONCLUSIONS**

To write on HHcy and SVD is a novelty, and even though some clinical studies have been done [162, 170, 171, 172, 173, 174], mechanisms, processes, and reactions have developed continuously and established as potential damage mechanisms. It is an extraordinary-ongoing cultural development and conquest. Hard to say, it is only an academic status at the moment. Firstly, because we, as many others, continue to describe HHcy role dividing it, for academic meticulosity into different ways of happening; the truest possibility is that inflammation, oxidative damages, misfolding, and neurodegeneration happen all together, in a very dynamic (and not at all static) photogram sequence.
Secondly, because biochemical and histological pieces of knowledge are very distant and their application far from a clinical application.

Thirdly, there is no adequate number of studies that interface, at the moment, lab to a hospital ward, and try to detect a specific point of sharing the vision in between lab developments and clinical outcomes (we are still debating on how SVD happens to become sVAD).

These obscure points will or should be the future targets of prospective studies to simplify the basic science knowledge and finally be keen to apply in clinical practice.

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