Age-related cerebrovascular changes: formation of cerebral microbleeds and development of neurovascular uncoupling

DOCTORAL THESIS

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List of abbreviations and terms

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ABP	arterial blood pressure
ATP	adenosine triphosphate
AR	autoregulation
BP	blood pressure
BBB	blood - brain barrier
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium
CO_2	carbon-dioxide
CNS	central nervous system
CBF	cerebral blood flow
CBFV	cerebral blood flow velocity
CMB	cerebral microbleed
CPP	cerebral perfusion pressure
CVCi	cerebrovascular conductance index
COX	cyclooxygenase
eNOS	endothelial nitric oxide synthase
EET	epoxyeicosatrieonic acids
GABA	gamma-aminobutyric acid
HDL	high density lipoprotein
20-HETE	20-Hydroxyeicosatetraenoic acid
iNOS	inducible nitrogen oxide synthase
IP_3	inositol trisphosphate
IGF-1	insulin-like growth factor 1
IGF-1 R	insulin-like growth factor-1 receptor
ICAM	intercellular adhesion molecule
ICP	intracranial pressure
K_{IR}	inward rectifying K channel
LOC	loss of consciousness
LDL	low density lipoprotein
MMP	matrix – metalloproteinase
MRI	magnetic resonance imaging

MAP	mean arterial pressure
MCA	middle cerebral artery
mTBI	mild traumatic brain injury
mmHg	millimetres of mercury
NIRS	near infrared spectroscopy
nNOS	neuronal nitrogen oxide synthase
NPY	neuropeptide Y
NVC	neurovascular coupling
NVU	neurovascular unit
NADPH	nicotinamide adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
NO	nitric oxide
NMDA	N-methyl-D-aspartate
Nrf2	nuclear factor erythroid 2–related factor 2
NFKB	nuclear factor kappa b
O_2	oxygen
PCO_2	partial pressure of carbon dioxide
PO_2	partial pressure of oxygen
PLA_2	phospholipase-A_2
PCA	posterior cerebral artery
PTA	posttraumatic amnesia
K	potassium
ROS	reactive oxygen species
s	second
SMC	smooth muscle cell
TrK/B	tropomyosin receptor kinase B
TMT	trail making test
TCD	transcranial doppler ultrasound
TBI	traumatic brain injury
TMB	traumatic microbleed
VIP	vasoactive intestinal peptide

INTRODUCTION

Age-related cerebrovascular changes

Globally there are more than 1 billion people who is over 60 years old and 10% of the word population is over 65 years today. [1-4] The aging population (65 years and older) is expected to rise from 10% to 16% by 2050 furthermore, and in the next 30 years the number of people who is aged 80 and over is predicted to triple worldwide. [2, 3, 5, 6] Prevalence of cerebrovascular diseases is increasing by age. [5, 7] Even during healthy aging several cellular-, micro-, and macro-cerebrovascular changes appear, which have been linked to age – related cognitive decline, dementia or gait abnormalities, placing an enormous burden on the healthcare systems worldwide. Aging-related cerebrovascular dysregulation includes both structural and functional changes. [5, 8-11] Among others increased vascular stiffness, atherosclerosis, formation of cerebral microbleeds have been linked to age-related cognitive decline. [7, 12-14] Also, age-related neurovascular uncoupling, i.e. attenuated increase in blood flow to neuronal activation also contributes to age-related dysfunction of the central nervous system. [7, 8, 15, 16]

The weight of the brain is approximately 2% of the total body weight, while around 15% of the cardiac output is allocated to the brain to meet the metabolic need of cerebral tissue. [7, 17] Even in rest, 25% of the total oxygen and glucose consumption is associated to brain activity, which does not have local cellular energy stores. [18, 19] Consequently, the stable and constant cerebral blood supply is crucial for healthy brain function. [5, 7, 11, 17] Besides, the brain is in the cranium, where volume expansion is limited, because increase in one of the intracranial compartments (blood, cerebrospinal fluid, brain tissue) will lead to increases in intracranial pressure (Monroe-Kelly doctrine). Therefore, regulation of intracranial blood volume is of utmost importance. According to the unique metabolic demand together with the specific location in the closed space, cerebral blood flow (CBF) and perfusion is strictly regulated by multilevel, interrelated mechanisms. Mechanism responsible for stable blood perfusion of cerebral tissue independent from systemic blood pressure is autoregulation (AR) of

cerebral blood flow, and mechanisms adjusting local CBF to neuronal activation is called neurovascular coupling (NVC). [5, 7, 10, 20] The anatomical unit responsible for neurovascular coupling is called neurovascular unit (NVU). [18, 19]

NVU is an anatomical structure which includes neurons, glial cells, mural cells (as pericytes and vascular smooth muscle cells) supplying vascular endothelium cells and the surrounding cerebral extracellular matrix. [19, 21] Proximal from the neurovascular unit the cerebrovasculature exhibits a well-defined segmental structure. Accordingly, large cerebral arteries (ie. middle cerebral artery) form the circle of Willis at the brain base, then they are running in sulci towards the cortical surfaces, having an extrinsic innervation by peripheric ganglia. [21, 22] The pial arteries and arterioles are on the surface of the brain within the subarachnoid space and have an excessive smooth muscle cell layer. [19, 22] As pial arteries penetrate the cerebral cortex, they form penetrating arterioles with finer smooth muscle layer in the Virchow-Robin space. [18] When the penetrating arterioles progress deeper to the parenchyma, the smooth muscle cell (SMC) layer thinners and subsequently turns into single or intermittent muscle cells with direct astrocyte end feet connections. [19] The parenchymal vessels will branch extensively in the cerebral parenchyma generating a dense capillary system. [18, 19] Capillaries do not have a smooth muscle cell layer but embedded with contractable pericytes which are integrated with the basement membrane and also connected to astrocyte end-feet. [18, 19, 21, 22] Postcapillary vessels will form veins in which the muscle cells have elongated shape and less contractility. [22] Besides controlling local CBF for active and functioning cerebral areas, NVU has an important role in maintaining the blood-brain-barrier (BBB), as well. [7, 23] BBB is made of endothelial cells, pericytes and astrocyte end-feet and serves as a physical, transport and metabolic barrier between the systemic circulation and cerebral tissue. [1, 22-24] The above mentioned structures are prone to be impaired even during healthy aging, leading to age-related cerebrovascular changes, in which oxidative stress, proinflammatory, prothrombotic environment and decreased metabolism play a significant role. [9, 10, 15, 16, 21, 23, 25, 26]

Cerebrovascular endothelium has a very important role in mediating local CBF changes, mainly based on biochemical signalling pathways (eg. endothelial nitric oxide synthase) which will act on vascular smooth muscle cells and induce vascular tone modification. [9, 24] Physiological aging is associated with increased reactive oxygen

species (ROS) production and defective antioxidant mechanisms, leading to oxidative environment and stress. [14] The increased ROS production is multifaceted, partly explained by mitochondrial ROS production and increased NAPDH oxidase expression and activation. [10, 14, 27, 28] These changes appear in neurons, astrocytes and vessels, as well. [27] Beyond increased ROS production, dysregulation of nuclear factor erythroid 2–related factor 2 (Nrf2) pathways and the downregulation of antioxidant mechanisms (superoxide dismutase or catalase) leads to increased net ROS production. [13, 14, 29] ROS directly damage mitochondrial and nuclear DNA, therefore lead to abnormal protein formation, genomic instability and altered neurosynaptic plasticity initiating neurodegeneration. [10, 14, 16] ROS directly react with NO, forming peroxynitrite and decrease bioavailability of NO which strongly affects endothelial function, which will further decrease the amount of attainable NO. [5, 18, 19, 26, 30, 31] Decreased expression of endothelial nitric oxide synthase (eNOS) is also a key factor to age related CBF changes and neurovascular uncoupling. [10, 14, 16, 26, 27, 32]

By aging, the prevalence of atherosclerosis and arteriolosclerosis increases, causing direct ischaemic damage and altered vascular adaptation to changes in perfusion pressure, resulting in hypoperfusion and consequent dysfunction. [5, 7, 33] Atherosclerosis is the accumulation of low density lipoprotein (LDL) and cholesterol in the vascular intima, causing structural changes and inflammation in the vessels, leading to abnormal function. [26] Aging itself increases the concentration of LDL in the vascular intima, by defective removal and diminished transformation of LDL to high density lipoprotein (HDL). [26, 33] Endothelial damage due to hypertension and ROS facilitates intimal accumulation of LDL, thus leads to further inflammation and modified LDL deposition. [5, 16, 33] These lesions will eventually turn to atherosclerotic plaques and attract inflammatory cells, cytokines and cell growth factors, causing myocyte transformation and collagen production (I and III), resulting in further damage and cerebrovascular impairment. [26]

Further vascular changes are seen in normal aging due to increased calcification, which ab ovo increases arterial stiffness and causes changes of smooth muscle cell phenotype: replacement by collagenous connective tissue along with abnormal protein accumulation, leading to intimal thickening. [26, 33] Senescence smooth muscle cells have an increased inducible nitrogen oxide synthase (iNOS) activity, ICAM-1

expression and angiotensin system activation. [10, 26, 33] These age-related changes reverse the elastin/collagen ratio, leading to altered vascular wall structure with increased stiffness, permeability and triggers atherosclerotic lesion formation. [26, 33] In senescence the oxidative environment and decreased bioavailability of NO alter the normal matrix – metalloproteinase activation (MMP) and promotes MMP2 and MMP9 activation, leading to vascular wall disintegration, weakening and fragility. [13, 27] These structural changes, which will lead to increased arterial stiffness and inflammation, lead to narrowing of the vessels and altered haemodynamic regulation due to increased vascular resistance. [8, 10, 27]

Aging leads to functional and structural derangement of the BBB. [23, 26] Senescence causes basement membrane thickening, thinning of the endothelium, resulting in capillary looping and twisting with aberrant transport protein and receptor expression, resulting in defective transmission. [8, 23, 33] Additionally, age-related complex derangement involves other components of the NVU, such as decreased number and altered function of pericytes, astrocyte retraction and swelling of astrocytic end-feet, leading to detachment from the basement membrane of endothelial cells. [23, 26] Preclinical and clinical studies showed, that injured BBB will induce further activation of the inflammatory cascade and cellular infiltration along with vascular and parenchymal damage such as microinfarcts or cerebral microbleeds (CMB). [9, 23, 34]

CMB formation in aging

Autoregulation and structural changes in aging: basis for formation of cerebral microbleeds

Autoregulation (AR) stands for a mechanism which aims to secure constant CBF when cerebral perfusion pressure (CPP) changes. [24, 35, 36] If AR is intact, cerebral vessels will respond to mean arterial pressure (MAP) changes by dilation or constriction to adjust cerebrovascular resistance. [24, 35] By the so-called myogenic response, activated by changes of intraluminal pressure, cerebral vessels will constrict to increases and dilate to decreases in CPP, maintaining (more or less) constant CBF. [9, 24, 35] Accordingly, a relatively unchanged CBF can be obtained regardless of blood pressure (BP) changes between 50-150 mmHg MAP. [17, 22, 24, 36] This is the

so called static AR, describing CBF-CPP relation at a steady state pressure value and in response to steady state pressure steps. [24, 35] Dynamic AR was also introduced, which demonstrates how fast autoregulation is able to compensate sudden changes in MAP and to prevent its propagation to changes in CBF. [24, 25, 35]

In aging vascular stiffness, calcification, atherosclerosis, altered protein structure and function and smooth muscle cell depletion result in altered AR. [1, 5, 9, 10, 13, 22] Defective myogenic response will lead to a narrowed AR range increasing the lower limit of autoregulation by 20 mmHg leading to maladaptation to rapid pressure changes and hypoperfusion (during for example orthostatic hypotension). At higher pressure values of the autoregulatory curve, due to insufficient myogenic constriction, resistance vessels are unable to protect the distal microvessels from hydrostatic overload, leading to BBB disruption, extravasation of blood-borne substances into the brain parenchyma, resulting in inflammation and CMB formation. [10, 24, 26] Both the prevalence and number of CMBs increase with aging due to increased production and activation of MMPs, increased NF-KB activation causing proinflammatory environment and vascular wall fragility, atherosclerosis and modification of collagen and elastin content of the vascular wall. [10, 14, 15, 24, 26, 27, 31] In addition to the direct effect of aging, the prevalence of other known risk factors of CMBs is increasing by age, such as diabetes mellitus, hypertension, metabolic diseases, or traumatic brain injury (TBI). [10, 14, 16, 26, 32]

CMB formation in TBI and Aging

Globally, there is 500-800 TBI cases per 100.000 people annually. [37] Traumatic brain injury is the most frequent cause of hospitalization and death in the elderly population (above 75 years) causing a global healthcare demand, and with an aging society the burden is still expanding. [6, 37, 38] Mild traumatic brain injury (mTBI) is the most frequent form of brain trauma with the estimated incidence of 300/100.000 people per year. [38, 39] However, the definite number of patients with mTBI is most likely significantly higher, since most of mTBI patients do not get hospitalized. [6, 37, 39] MTBI is defined as Glasgow Coma Scale (GCS) 14-15, and it specifically affects elder individuals. Elderly is prone to fall and suffer TBI due to altered vascular adaptation to orthostatic position, frequent vision abnormalities, dehydration, impaired balance and

decreased defence mechanisms or compensations against falls due to aged neuro-musculoskeletal system. [6, 40]

Besides direct brain injury, TBI initiates secondary injury of cerebral tissue. [12, 39] This is especially important in the elderly because even mild TBI has been linked to secondary cerebral changes leading to gait disorders, psychiatric or cognitive decline. [6, 38, 39] TBI-associated secondary changes are initiated by oxidative stress, mitochondrial dysfunction, inflammation and redox-dependent activation of MMPs leading to BBB destruction, neurovascular impairment and CMB formation. [12, 13, 39, 41, 42]

CMBs or cerebral microhaemorrhages are small, 5-10 mm diameter hemosiderin accumulations around the cerebral microvessels due to injury-related bleeding of small arteries, cerebral arterioles or capillaries. [12, 13, 42, 43] CMBs have been linked to cognitive decline: altered attention, processing speed or executive dysfunction in addition to psychiatric disorders and depression. [12, 13, 40, 41] Furthermore, CMBs are associated with balance, coordination, and gait dysfunction, further increasing the prevalence of TBI. [6, 12, 41]

Aging is an independent risk factor for CMB formation, as well, partly because of the senescence-related oxidative stress, atherosclerosis, MMP activation, vascular collagen modification and increased fragility together with BBB dysfunction. [8, 13, 34]

The pathobiological pathways which have been demonstrated in age-related CMB formation and TBI-induced development of CMBs are partly identical. Furthermore, aging increases the prevalence of hypertension and decreases the presence and effect of vasoprotective factors (such as insulin like growth factor) exaggerating the vascular damage and complications of TBI leading to cerebrovascular lesions (Figure 1).

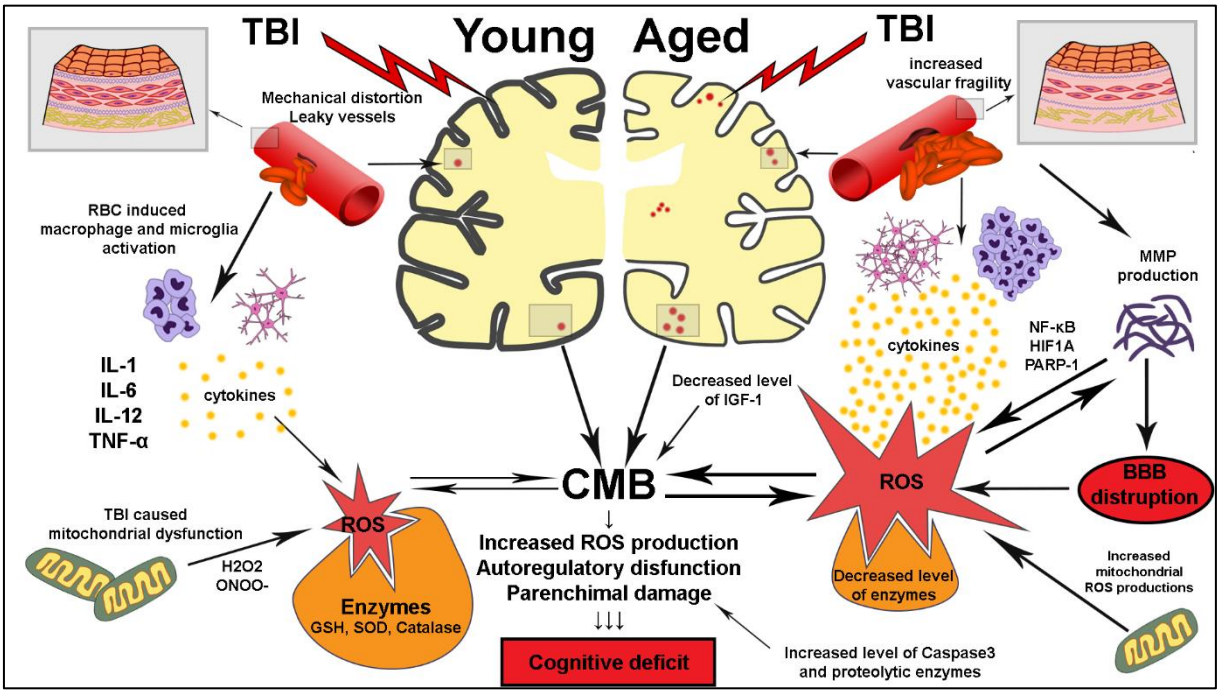

Figure 1 Hypothetical mechanisms through which TBI and aging interact to promote the development of CMBs. Right: the aged cerebral vascular wall is more fragile and vulnerable due to altered collagen content and calcified elastin. In aging the CMBs are partly responsible for ROS production by activating greater extent of macrophages and microglia cells, causing prolonged and marked inflammatory response. Furthermore, senescence mitochondria are prone to produce more ROS, still the defensive enzymes, as glutathione peroxidase and superoxide dismutase are less effective. These cellular and histological mechanisms together result in more pronounced parenchymal damage clinically manifesting in cognitive and gait disturbances in the elderly following TBI.

Hypothesis I

Based on the aforementioned, we hypothesized that TBI and aging synergize to induce the formation of cerebral microbleeds, therefore TBI will lead to an increased number of CMBs in the elderly.

Neurovascular coupling and uncoupling

Metabolism of an active brain area is highly increased with very limited local energy stores; therefore cerebral perfusion has to be strictly adjusted to the actual need of cerebral tissue. [18, 19] Cerebral perfusion is increased during neuronal activation via NVC which is a second – to - second adjustment of cerebral blood flow to activated neuronal metabolism and maintained by the NVU. [18, 19] NVC or functional hyperaemia aims to provide oxygen and glucose for the activated neurons and remove metabolic end- and by-products (eg. CO_2, lactate) in a precisely controlled manner by complex signalling mechanism throughout the NVU. [18, 19]

NVC is initiated upon neuronal activation via glutamate which will act both on the synapsing neurons, interneurons (by postsynaptic glutamate receptors: NMDA, AMPA) and endothelial cells triggering an increase in intracellular Ca^{2+} concentration, resulting in neuronal cyclooxygenase (COX-2) and neuronal nitrogen oxide synthase (nNOS) activation, and production of vasoactive substances, such as NO and prostanoids, leading to vasodilation. [1, 18, 19, 36] Simultaneously, glutamate acts on astrocytes, as well, which are important to transmit the neuronal activation to cerebral vessels. [18, 19, 21] Excitation induced depolarization increases intracellular Ca^{2+} in astrocytes via metabotropic glutamate receptors (IP_3) inducing phospholipase-A_2 (PLA_2) and cyclooxygenase – 2 to produce vasodilatory substances (such as epoxyeicosatrienoic acids (EETs), prostaglandins). [19, 22] Additionally, K+ channel activation on astrocyte end feet causes SMC relaxation and capillary vasodilation. [18, 19] Preclinical studies showed the importance of other vasodilatory mediators and cells in the NVC process, such as ATP derived adenosine, activation of GABA interneurons, neuropeptide Y (NPY) mediated vasoconstriction and VIP dependent vasodilation, as well. [19, 22]

Recent trials investigated the difference between the mechanism of NVC on capillary vs. arteriolar level. [19] At the arterial level, the glutamate-activated NMDA-related NO production is the main mediator of vasodilation. [18, 19] NVC stands for local hyperaemia of active neurons, however retrograde propagation of vasodilation can be observed to more proximal segments of the cerebrovascular tree by endothel cells. [19, 21, 24, 36] The main mediator of retrograde propagation is the K ion. [18] The K current transmitted via gap junctions or myoendothelial junctions and acts on inward rectifying ion channels (K_{IR}), consequently causing spreading hyperpolarization from

capillaries to arterioles, resulting in conducted vasodilation via SMC relaxation. [19, 24] Apart from ion transfer, the metabolic activity-related decreased oxygen availability will increase blood viscosity at the capillary level and therefore increase blood flow consequently. This results in increased shear stress on arteriolar endothelial cells, production of endothelial NO and ultimately vasodilation due to SMC relaxation. [18, 19] Furthermore, the dilatory effect of local metabolites such as ATP and adenosine is added to ion- and flow-dependent dilation of arterioles and small arteries. [19, 24]

Intact neurovascular coupling is essential for higher cortical functions and requires healthy cerebral vasculature and homeostasis. Altered NVC has been demonstrated in various age-related disorders, and even in normal aging. [1, 15, 22] Accordingly, in aging, generally decreased metabolic rate is characteristic, therefore the neuronal activation induced energy demanding vasodilation is limited due to reduced available ATP level. [1, 10, 15] Aging increases NADPH activation, resulting in ROS production, which is highly reactive with NO and forms inactive peroxynitrite, decreasing the bioavailability and vasodilatory effect of NO. [10, 15] Oxidative environment will cause significant endothelial damage and dysfunction, resulting in decreased vascular response to local CO_2, bradykinin and acetylcholine, further exacerbating decreased dilator capacity of the cerebrovasculature. [1, 18, 21, 44]

Apart from endothelial dysfunction, oxidative stress and aging will cause alteration in the NVU, as well, facilitating neurovascular uncoupling. [10, 15, 21] The decrease in pericytes will affect BBB structure, leading to increased accumulation of potentially toxic substances (eg. Aß) in the cerebral tissue, which will concentrate in pericytes causing fatal modification and further pericyte loss, consequently defective vasodilation, and neurovascular uncoupling. [1, 19, 34]

Advanced age affects the number, function, and structure of the astrocytes. Aging will generate flattened and swollen astrocyte structure with growth arrest and increased lysosomal mass, leading to proinflammatory secretion profile, which will further aggravate the microstructural damage and abandon normal functions. [21, 45] Besides, the production of vimentin and glial fibrillary protein is increased in elderly, leading to astrocyte activation and transformation to reactive neuroinflammatory phenotype. [19, 45] Due to the oxidative environment, the astrocytes' glutamate signalling is markedly affected having a fundamental role in neurovascular uncoupling.

[15, 19, 21, 26, 45] Senescence shifts the mediator synthesis in astrocytes, and results in the production of the vasoconstrictor prostaglandins 20-HETE, further decreasing neurovascular hyperaemia. [15, 21, 45]

Aging induced oxidative stress and structural changes with consequent neuroinflammation, metabolic and functional alterations play a significant role in the development of cognitive decline, especially in aging-related memory and learning difficulties. [19, 26, 28, 29, 45, 46]

Together with the abovementioned cell autonomous changes (as mitochondrial dysfunction, oxidative stress, inflammatory environment, energy depletion) recently non-cell autonomous changes were identified as important contributors to vascular aging and concomitant neurovascular dysfunction. [47] The non-cell autonomous interactions mainly include secreted substances or molecules which induce changes in other cells' function, such as anti-geronic factors (which are important to prevent or modify cellular aging). Insulin-like growth factor (IGF-1) is a liver produced anabolic hormone which has a systematic role in maintaining healthy body mass, however recent preclinical studies proved critical role on cerebral development and suggested important effects on the central nervous system (CNS) in plasticity, neuronal structure and angiogenesis. [48-50] The level of IGF-1 is significantly decreasing with aging in humans and in laboratory animals due to an age-related decline in GH production/release contributing to age related cerebromicrovascular changes and dysfunction. [49-54] IGF-1 deficiency-induced neuronal impairment is likely multifaceted. Alteration in NMDA signalling, altered plasticity and reduced elimination of potentially toxic proteins (eg. Aß) and cerebrovascular changes contribute to decline in cognitive dysfunction. [49, 50, 55] Importantly, preclinical studies demonstrate that circulating IGF-1 deficiency in transgenic mouse models impairs both endothelium-mediated and astrocyte-dependent NVC responses, mimicking the aging phenotype. [30, 56] Additionally, disruption of IGF1R signalling specifically in endothelial cells (*VE-Cadherin-Cre^{ERT2}/Igf1r$^{f/f}$*) or astrocytes (*GFAP-Cre^{ERT2}/Igf1r$^{f/f}$*) significantly impairs NVC responses in mice, similar to the effects of circulating IGF-1 deficiency and aging. [57, 58] These findings provide strong evidence that circulating IGF-1 modulates NVC responses in the murine brain. Despite these advances, the role of age-related IGF-1 deficiency in neurovascular dysfunction in older adults has not been determined.

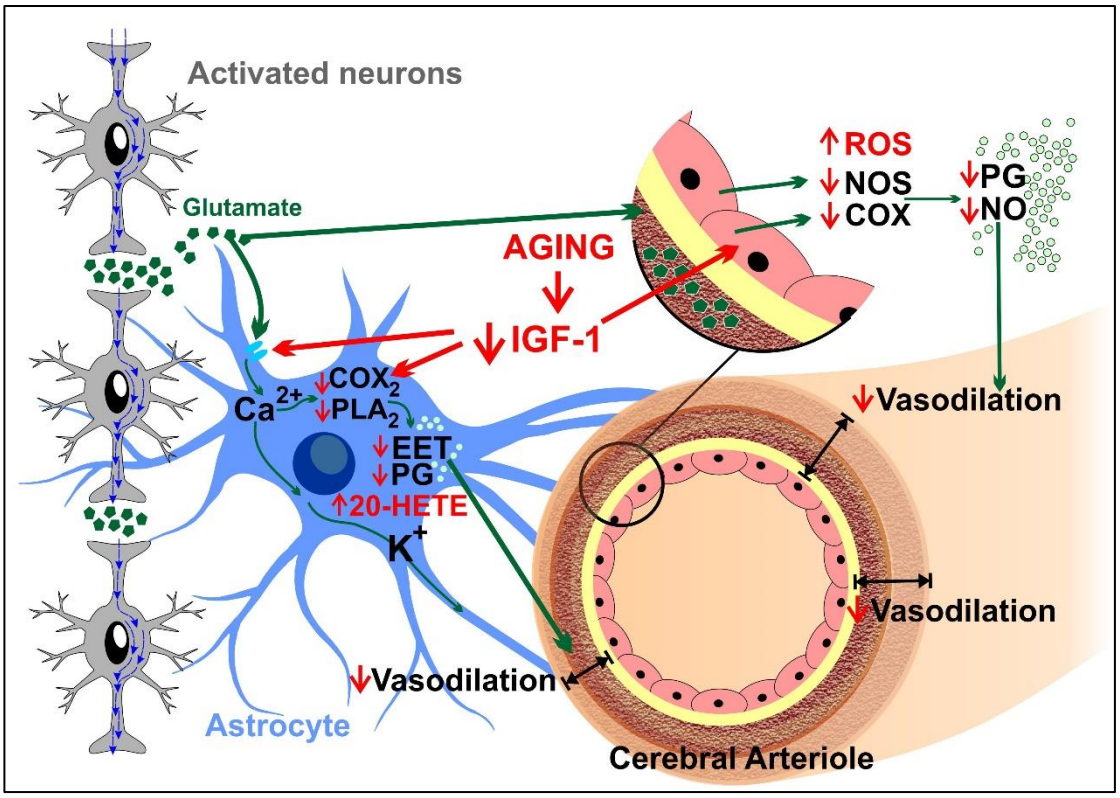

Figure 2 shows the mechanisms of neurovascular coupling and the effect of age-related IGF-1 deficiency. Glutamate released from active neurons acts on the cerebral arteriole's endothel cells and on the astrocytes as well. Glutamate acts on astrocytic metabotropic receptors, leading to increased intracellular Ca^{2+} level and promoting phospholipase-A_2 (PLA_2) and cyclooxygenase – 2 (COX2) activation and production of vasodilatory substances (epoxyeicosatrienoic acids (EETs), prostaglandins (PG)). Additionally, K+ channel activation on astrocyte end feet causes SMC relaxation and capillary vasodilation. IGF-1 deficiency decreases endothelial NO production due to increased ROS production, and it attenuates COX activation, resulting in decreased vasodilatory PG levels and increased production of vasoconstrictor 20- HETE. IGF-1 deficiency also alters astrocytes' sensitivity to glutamate by altering receptor expression on astrocytes.
Mechanisms related to decreased IGF-1 level are marked with red arrow.

Hypothesis II

Based on the aforementioned, we hypothesized that age-related IGF-1 deficiency is associated with neurovascular uncoupling in healthy elderly.

METHODS

All studies were carried out at University of Pécs, Neurosurgery Department and approved by National Hungarian and Regional Ethical Committees, registered under 6552–4/2020/EUIG, 7270-PTE 2018.

CMB detection in elderly volunteers and patients after mild traumatic brain

Enrolled patients

To evaluate the effect of mild traumatic brain injury (mTBI) on CMB formation in young and elderly we carried out a retrospective study and analysed the medical history and MRI images of patients who attended our Clinic between April of 2014 and September of 2019. For the TBI group we enrolled 34 patients (14 males and 20 females) who suffered mTBI with admission Glasgow Coma Scale (GCS) 14–15. As control group 43 aged-matched patients' (17 males and 26 females) MRI without TBI in the medical history were analysed, as well. For the TBI groups, the inclusion criteria were: young: age is between 18 and 40 years; aged: above 60 years old at the time of the injury; an mTBI in the history within 6 months of the MRI; an mTBI according to Mayo criteria: GCS 14–15, absence or a maximum of 30 min of loss of consciousness, and the absence of post-traumatic amnesia. Exclusion criteria: any conditions associated with CMB formation in the medical history, such as epilepsy, any previous TBI, stroke, transient ischemic attack, cavernous malformations, cerebral amyloid angiopathy, chronic hypertensive encephalopathy, acute haemorrhagic leukoencephalitis, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), Alzheimer's disease, cerebral vasculitis, cerebral metastases, haemorrhagic micrometastases, intracranial embolism, intravascular lymphoma, posterior reversible encephalopathy syndrome (PRES), progressive facial haemiatrophy, thrombotic microangiopathies, intracranial infection, and COL4A1 brain small-vessel disease. For the control group, an additional exclusion criterion was a TBI

in the medical records. Both in the TBI and control groups, two age groups were defined in a 2 × 2 study design: young (Y): n = 20, 10 females, 10 males, age: 25 ± 6 years; young + mTBI (Y + mTBI): n = 17, 11 females, 6 males, age: 25 ± 10 years; aged (A): n = 23, 16 females, 7 males, age: 68 ± 5 years; aged + mTBI (A + mTBI): n = 17, 9 females, 8 males, age: 72 ± 7 years (Table 1).

Detection of CMBs, imaging protocol

CMBs are small, rounded or ovoid-shaped hemosiderin deposits 5-10 mm in diameter around cerebral vessels. Microbleeds are invisible on computed tomography (CT) or on conventional spin-echo MRI sequences, but on T2 -weighed gradient-recalled echo or susceptibility-weighted imaging (SWI) MRI they appear as hypointense foci. SWI MRI is particularly sensitive to blood, since the strong magnetic field, high resolution and full velocity corrected gradient echo imaging, sensitive to substances which distort the magnetic field, such as iron. Based on that, susceptibility- and T1-weighted magnetization-prepared rapid acquisition with gradient echo (MPRAGE) and fluid-attenuated inversion recovery (FLAIR) MR images were obtained using 3T (Magnetom Trio/Prismafit) Siemens MR scanners (Siemens, Munich, Germany). MPRAGE is suitable for structural brain imaging with high contrast and tissue contrast while FLAIR imaging is useful for subtle changes in hemispheric regions. The T1-weighted high-resolution images were then obtained using a three-dimensional (3D) MP-RAGE sequence [TI = 900–1,100 ms; TR = 1,900–2,530 ms; TE = 2.5–2.4 ms; slice thickness = 0.9–1 mm; field of view (FOV) = 256 mm × 256 mm; matrix size = 256 × 256], while 3D SWI images were acquired as follows: TR = 27–49 ms; TE = 20–40 ms; slice thickness = 1.2–3 mm; FOV = 137–201 mm × 230–240 mm; matrix size = 125–182 × 256–320, with no inter-slice gap. For image evaluation, the 3D Slicer 4.8.11 software was used.

Microbleed Analysis

Three independent neuroradiologists evaluated the images individually, blinded to medical history. In order to precisely identify CMBs, the exclusion of SWI lesions that

mimic CMBs (intersection of veins, bottom of sulci, calcium deposits, artifacts caused by air-tissue interfaces, or macroscopic bleeding caused by, e.g., an intraventricular drain) was carried out. The number and location of CMBs were obtained according to the clinically validated Microbleed Anatomic Rating Scale (MARS). This scale distinguishes the number of definite and possible lesions and precisely localizes the CMBs according to anatomic regions as follows: (1) infratentorial: brainstem or cerebellum; (2) deep: basal ganglia, thalamus, internal or external capsule, corpus callosum, or either the periventricular or deep white matter; (3) lobar: cortex or subcortical white matter. In this study, we present only the definite lesions (Figure 3).

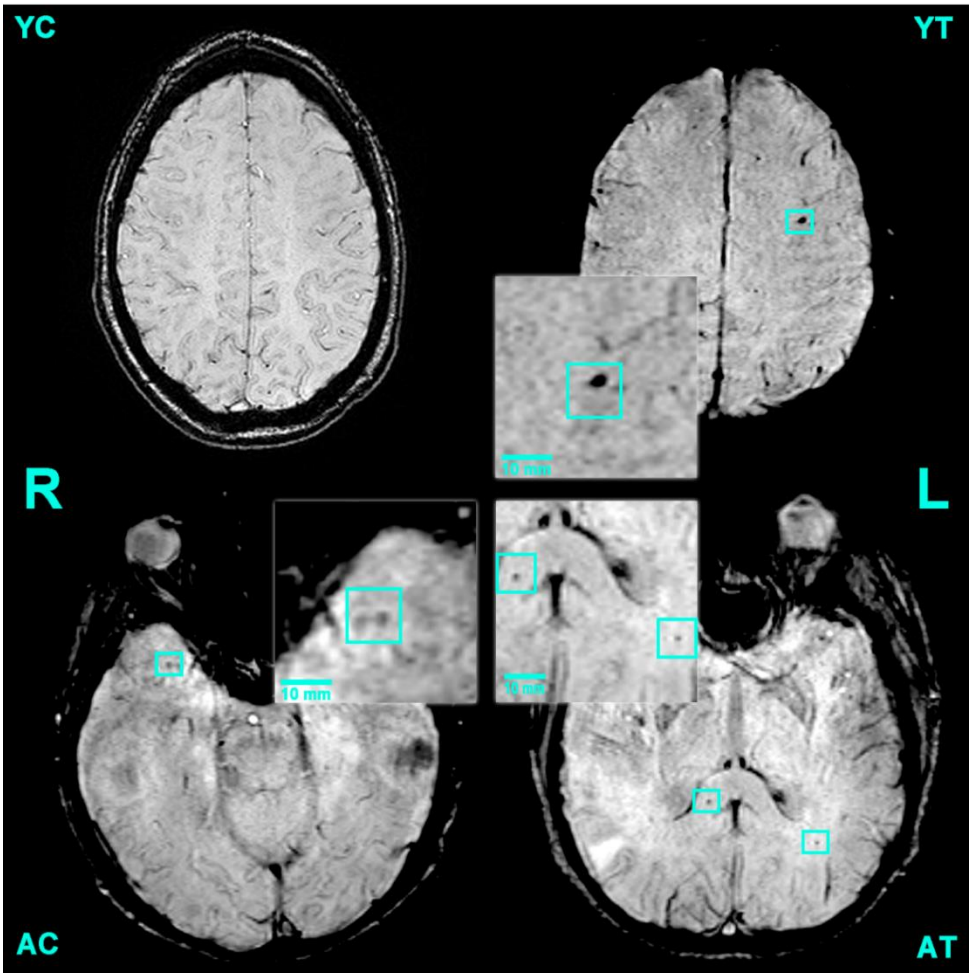

Figure 3: Axial susceptibility weighted MRI (3 Tesla) shows cerebral microbleeds as hypointense elliptical lesions highlighted by squares, in a young control patient (AC), in a patient following mild traumatic brain injury (YT), in an aged control patient (AC), and in an aged patient following mild TBI (AT). R: right side, L: left side

Methods to assess neurovascular coupling (NVC) in elderly volunteers

Enrolled patients

The participants were prospectively enrolled on a voluntary basis. The inclusion criteria were 18–40 and over 60 years of age and a written informed consent from each study participant. The exclusion criteria were previous or ongoing neurological diseases; any condition that could affect IGF-1 levels, including neoplasia, nephrectomy, renal disease, endocrine disorder, pregnancy, uncontrolled diabetes mellitus, starvation, and corticosteroid therapy; and non-penetrable acoustic window in the temporal area. Total of 63 participants were enrolled in this study, with 31 young (mean age: 28.4 ± 4.2 years, 11 females, 20 male) and 32 older adults (mean age: 67.9 ± 4.1 years, 18 females, 14 male) (Table 2).

To assess NVC traditionally, blood oxygen level-dependent signal is measured with functional magnetic resonance imaging, but it does not present information about cerebral haemodynamic changes. [1] Therefore, recently transcranial doppler ultrasound (TCD) or near infrared spectroscopy (NIRS) is used to measure NVC. [1, 18, 19] TCD is the most frequently used, relatively unexpensive device, which enables to non-invasively and easily measure cerebral blood flow velocity (CBFV) during cognitive, verbal or motor tasks (functional TCD). [18, 24] During functional TCD, posterior or middle cerebral artery (PCA or MCA) could be examined and an average 5-15% increase in CBFV is expected and could be measured. [17, 18, 36] For the measurement it is important to consider blood pressure and local gas tensions which also could affect vascular reactivity. [18, 19, 22, 24] To assess NVC responses a Doppler ultrasound system (DWL Multi-Dop® T digital, Singen, Germany) was used with 2 MHz transducers. The probes were secured bilaterally to the temporal acoustic window to measure the CBFV in the MCAs. MCAs were located manually, and velocities were recorded on the depth of 45-60 mm, signal towards the probe (Figure 4). Since systemic blood pressure and CO_2 could affect the cerebral vascular reactivity, parallel to the CBFV measurement arterial blood pressure was taken by a finger-cuff non-invasive continuous blood pressure monitor system (CNAP Monitor 500 HD,

CNSystems, Graz, Austria) and a capnograph (Promed, Kwun Tong, Kowloon, Hong Kong) was used to measure end tidal CO_2.

The minimum, mean and maximum flow velocities were measured with TCD and integrated to further analyse by ICM+® software (Cambridge, England) (Figure 4). During measurements ABP, MAP and for NVC response mean cerebral blood flow velocity on both sides were recorded continuously in the middle of MCAs in cm/s. During postprocessing cerebrovascular conductance index (CVCi) was calculated on both sides of MCA as the quotient of CBFv and MAP in cm/s/mmHg to eliminate the effect of blood pressure change on the cerebral blood flow variation.

To analyse the NVC response in MCAs trail making test was used. [59, 60] Trail making test (TMT) is a widely used and popular neuropsychological assessment, which gives information about visual search, scanning, processing and executive functions based on an easily achieved task. [60] The tests consists of 25 encircled numbers from 1-25 randomly placed on a sheet of paper and that has to be connected in sequential order by drawing lines by pen or pencil. For the evaluation, both the time needed to perform the test and the mistakes are evaluated. [59, 60]

After the installation of the measurement system (TCD, blood pressure monitor system, capnograph) the volunteers were asked to relax for few minutes. Thereafter the baseline CBFV was measured in resting state for 60 seconds (s) followed by the TMT test and further 60 seconds rest respectively. To objectively evaluate the NVC response positive and negative control tests were performed as well. As a positive control breath hold test was implemented, when the participants were asked to hold their breath for at least 45 s or as long as they can with no inhalation to avoid Valsalva maneuver. After another resting state and normalized CBFV and MAP, a negative control test was carried out with hyperventilation for 60 s respectively.

Measurement of serum IGF-1 level

Blood samples were collected before assessment of NVC responses, centrifuged at 3500 revolutions per minute for 15 min and processed for serum collection. IGF-1 levels were determined by enzyme-amplified chemiluminescence immunoassay (CLIA) using an IGF-1 assay kit (Siemens Healthcare Diagnostics, Los Angeles, USA,

catalogue nr: L2KGF2) on a Siemens IMMULITE 2000 platform (Siemens Healthcare GmbH, Erlangen, Germany).

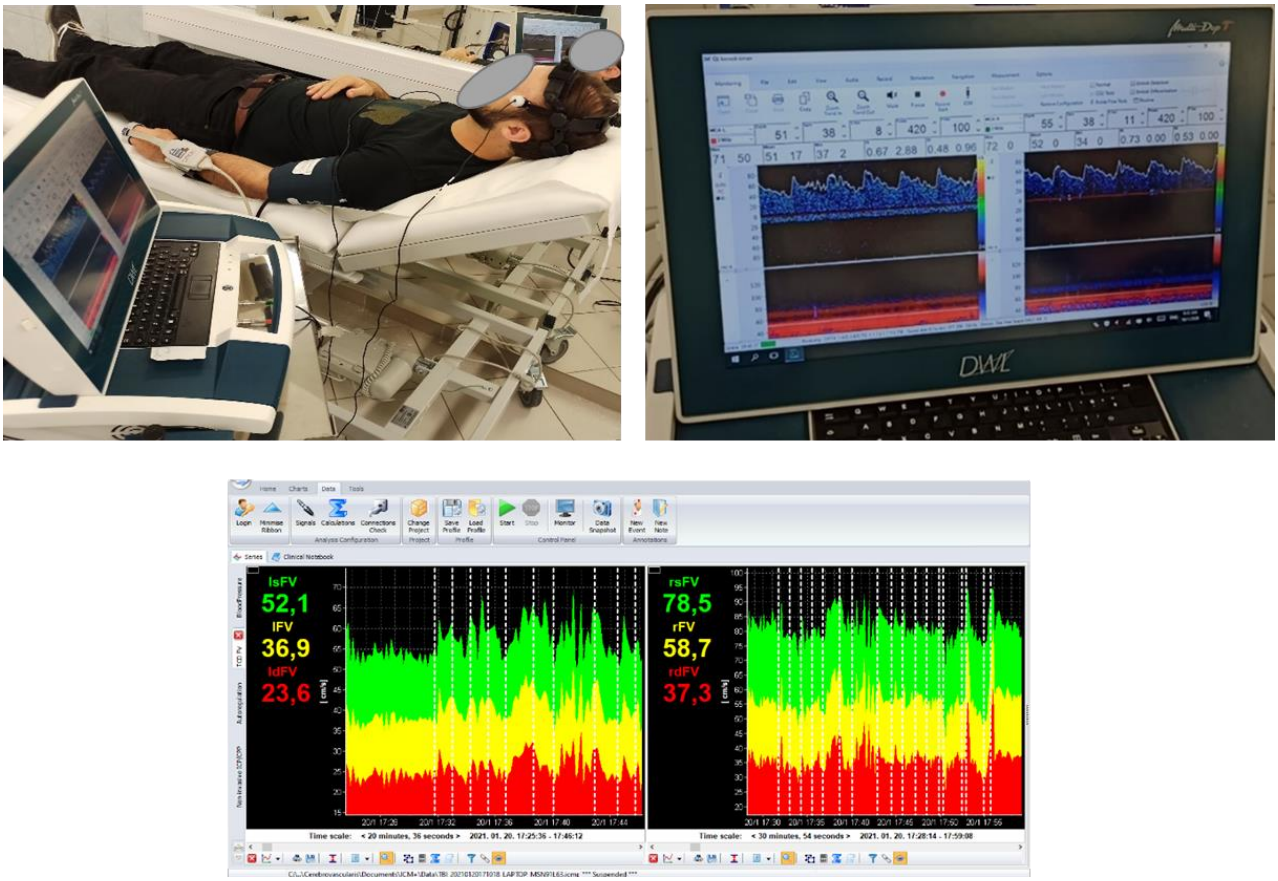

Figure 4: The setup of the NVC measurement: TCD and ICM+® software for data analysis

Magnetic resonance imaging (MRI): flow analysis

MRI measurements were performed on a 3T MRI scanner (MAGNETOM PrismaFit, Siemens Healthcare, Erlangen, Germany) with a 20-channel head/neck coil. The flow was measured in the M1 segments of both left and right MCAs using a parasagittal two-dimensional single-slice phase contrast sequence with peripheral pulse gating and the following parameters: TR/TE = 89.22/9.03 ms, flip angle = 15 degrees, slice thickness = 4 mm, FOV = 140 × 140 mm2, matrix size = 256 × 256 interpolated to 512 × 512, receiver bandwidth = 130 Hz/pixel, averages = 3, number of phases = 25,

and velocity encoding = 100 cm/s in through-plane direction. The imaging plane was arranged perpendicular to the longitudinal axis of the vessels (Figure 5) using a native 3D time-of-flight MR angiography with the following parameters: TR/TE = 22/3.86 ms, flip angle = 18 degrees, slice thickness = 0.7 mm, FOV = 167 × 222 mm2, matrix size = 202 × 384, receiver bandwidth = 178 Hz/pixel, 4 overlapping (27.08%) slabs, and a total of 153 axial slices (48 slices/slab). Flow analysis of phase contrast MRI data were performed using Argus software (Leonardo workstation; Siemens Healthcare, Erlangen, Germany). After loading the magnitude, phase, and rephased images into Argus, vessel contour of MCA was manually outlined on the first cardiac phase image and then automatically propagated to all other cardiac phases. The propagated contours (i.e., automatically outlined contours) were carefully checked for each cardiac phase and manually adjusted to ensure accurate vessel boundary delineation (Figure 5). To avoid phase aliasing near vessel borders, velocity range was adjusted from ± 100 cm/s to − 50/ + 150 cm/s. After applying background correction, average flow (ml/s) and average MCA area (cm2) were automatically calculated by the software.

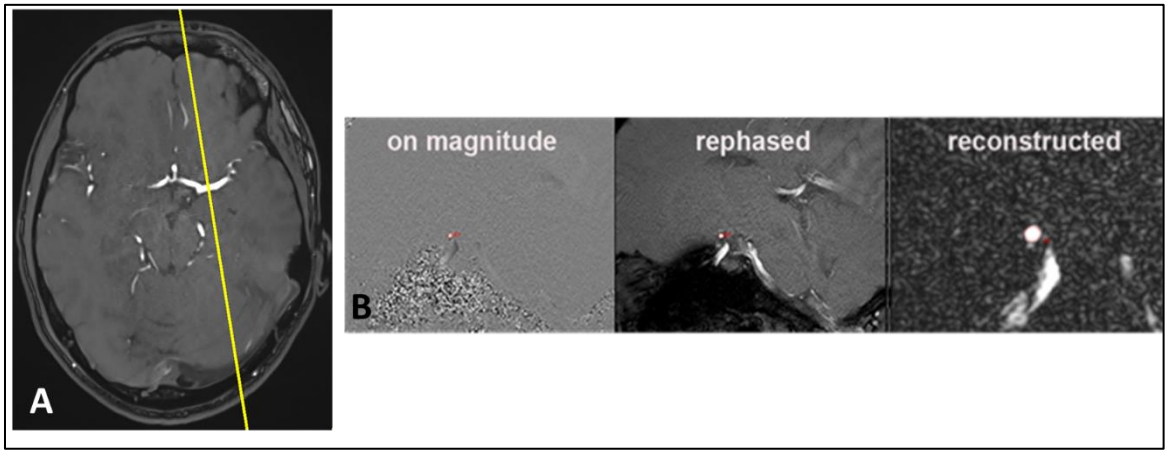

Figure 5 shows the phase contrast MRI for mean cerebral blood flow measurement in the middle cerebral arteries (MCAs) bilaterally. ***A:*** *Representative MRI image of the brain of a 65-year-old study participant. The yellow line shows parasagittal cross section of the left MCA, where the baseline flow velocity was measured on time-of-flight sequence.* ***B:*** *Representative figure showing magnitude, rephased, and reconstructed sections of the left MCA in parasagittal view. The red circle with hyperintense signal shows the cross section of MCA, and the small red circle shows the reference point in the cerebral parenchyma.*

Gait analysis

For the gait analysis a non-invasive marker based Diers® Pedogait system was used. The volunteer was instructed to walk on a comfortable speed for the gait analysis. The participants were trained before the measurement to get used to the treadmill platform. For the dynamic gait analysis the participant was asked to walk for 30 seconds on a comfortable, previously set treadmill speed. During that, both leg's step lengths (cm), stride lengths (cm), step width in cm, speed of walking (km/h) and percent of the stance phase as well as percent of swing phase was assessed. (Figure 6)

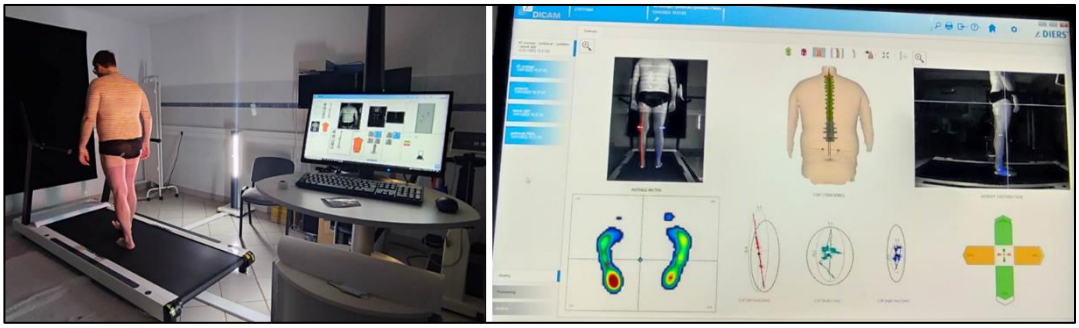

Figure 6 shows the Diers Pedogait gait analysis system we used for the research, including the treadmill with pressure sensors, non-invasive cameras and the software.

RESULTS

Development of cerebral microbleeds in young and aged patients following mild traumatic brain injury

Study population

77 patients were enrolled to this study and the results were analysed in 4 subgroups, namely: young trauma (Y+mTBI), young control (Y), aged trauma (A+mTBI) and aged control (A). Medical history and cerebrovascular risk factors of the enrolled individuals were evaluated. There was no difference in the assessed parameters between the study groups.

Group	Age (Mean ± SD)	Sex		Hypertension		Smoking		Urea		Creatinine		Total cholesterol		Low density lipoprotein	
		Female	Male	Yes	No	Yes	No	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Young control (Y)	25.09 ± 5.63	50%	50%	10.0%	90.0%	5.0%	95.0%	85.0%	15.0%	85.0%	15.0%	90.0%	10.0%	95.0%	5.0%
Young trauma (Y + mTBI)	24.65 ± 10.22	61.1%	35.3%	5.88%	94.12%	0%	100%	88.24%	11.76%	76.47%	26.53%	94.12%	5.88%	100.0%	0%
Aged control (A)	68.36 ± 4.88	69.6%	30.4%	60.87%	39.13%	4.35%	95.65%	91.3%	8.7%	91.3%	8.7%	56.52%	48.43%	78.26%	21.74%
Aged trauma (A + mTBI)	71.86 ± 7.31	52.9%	47.1%	88.24%	11.76%	17.65%	82.35%	82.35%	17.65%	52.94%	47.06%	82.35%	17.65%	100.0%	0%

Table 1: Characteristics highlighting the comorbidities of the study groups.

Aging increased CMB development

In line with previous preclinical and clinical research showing aging is an independent risk factor for CMB formation in our study we found significantly higher number of CMBs in aged participants (Figure 7). In young patients (*n* = 20, age: 25.09 ± 5.63 years) no microbleed was found, in young patients after an mTBI (*n* = 17, age: 24.65 ± 10.22 years) 0.75 ± 0.41 CMBs were detected, whereas aged control patients (*n* = 23, age: 68.36 ± 4.88 years) had 1.88 ± 1.1 lesions, and in aged patients with mTBIs (*n* = 17, age: 71.86 ± 7.31 years) 2.64 ± 1.51 CMBs were detected (Figure 8). We also found that, aging significantly increased the prevalence of microbleeds ($p<0.05$), regardless of the number of lesions in the aged group. Interestingly, mild TBI

did not increased significantly the number or the prevalence of CMBs found in ageing (Figure 9).

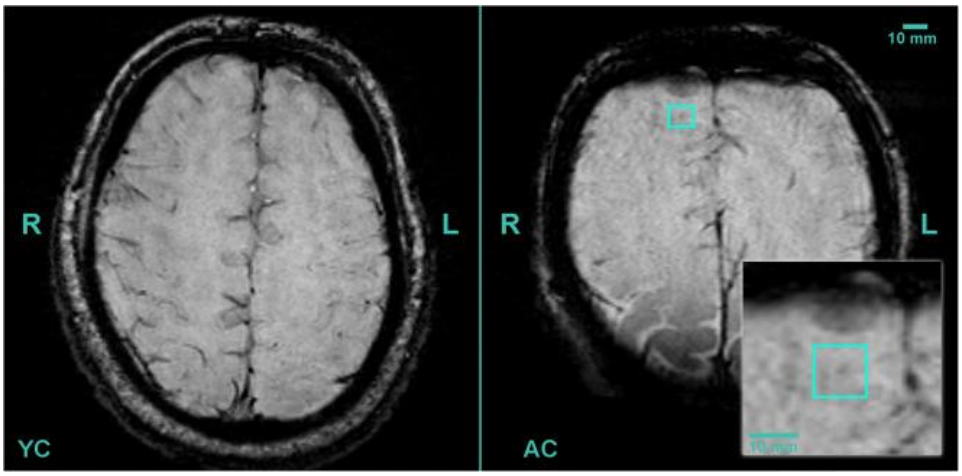

Figure 7: Axial (3 T) SWI MR images of an aged (AC) and a young (YC) control patient (without trauma). The young control (YC) patient had no cerebral lesion on MRI. Aged control (AC) patient had a single cerebral microbleed in right corona radiate, highlighted with blue square. R: right, L: left

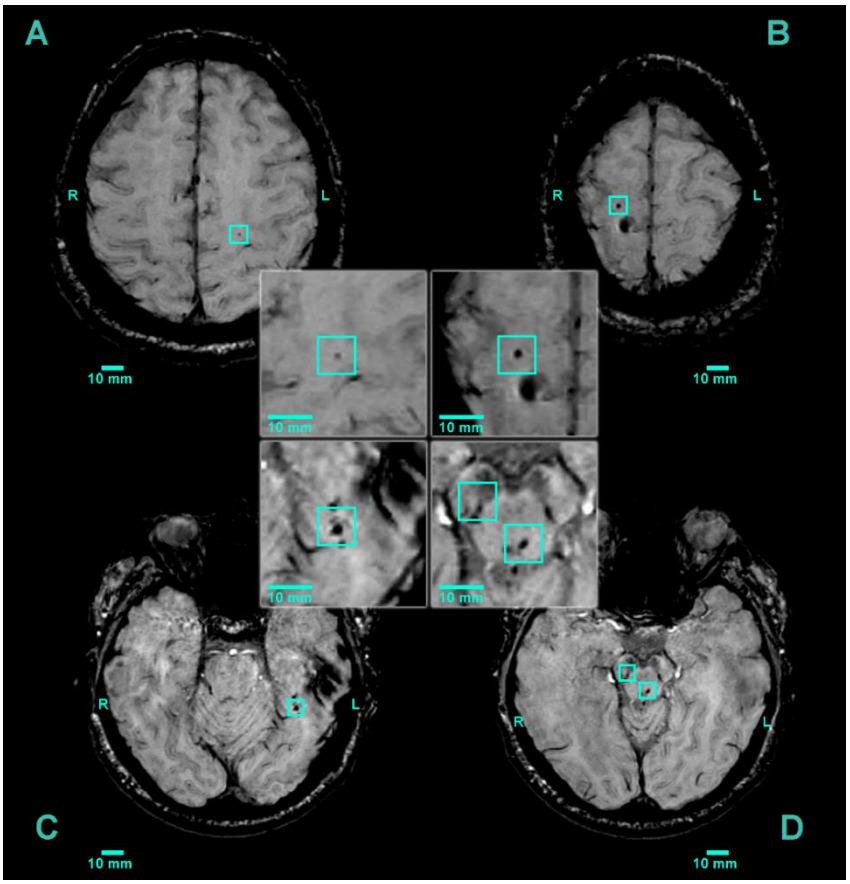

Figure 8 presents the cerebral microbleeds found on the axial SWI MRI (3 Tesla) in an aged patient, who suffered mild trauma (AT).

CMBs are highlighted by the blue boxes: ***A****.: left corona radiate* ***B****.: right corona radiate* ***C****.: left parahippocampal gyrus* ***D****.: crus cerebri, medial longitudinal fasciculus*

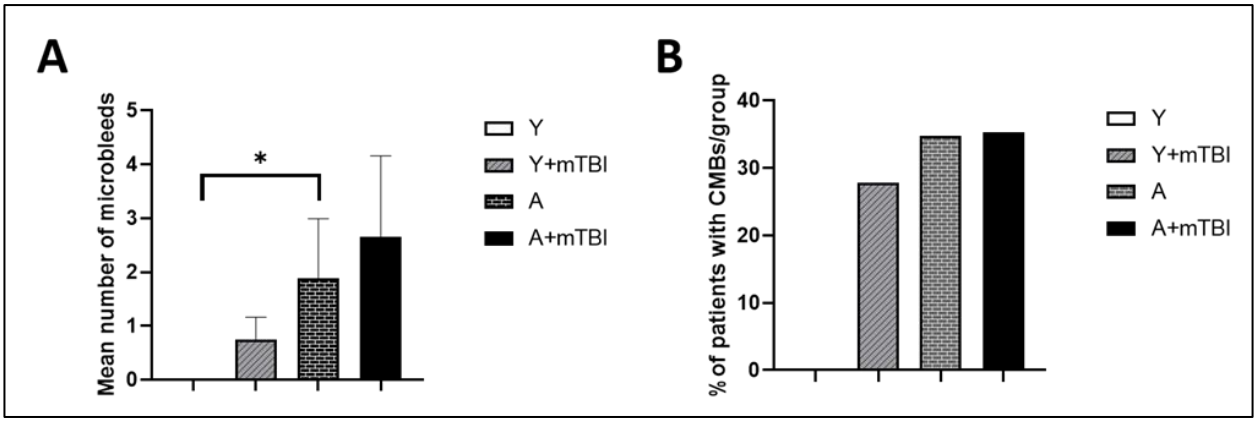

*Figure 9 shows the mean number ± SEM of CMBs found in the evaluated age groups (**A**). The difference between the mean number of CMBs was significant in young vs. aged group. *p<0.05*

*Panel **B** shows the number of patients with CMB expressed as % of the given group (Y: 0%, Y+mTBI: 27.78%, A: 34.78%, A+mTBI: 35.29%) and A+mTBI was significantly higher than young patients. p<0.05*

(Y: young control, Y+mTBI: young with mild TBI, A: aged control, A+mTBI: aged participants with mild TBI)

Localisation of CMBs in aged and young patients after mTBI

Further analysis revealed that, most of the CMBs were located in the supratentorial compartment (lobar and basal ganglion) (Figure 8). Minority of the CMBs appear in the infratentorial location (brainstem or cerebellum) in aged patients after an mTBI, however the difference did not reach significance level (p>0.05) (Figure 10). As the majority of the lesions were located in the supratentorial, lobal region, the localisation in the cerebral lobes (frontal, temporal, parietal, and occipital) were further analysed. Aging enhanced the number of parietal and occipital CMBs after an mTBI vs. young mTBI patents ($p < 0.05$), furthermore an mTBI provoked parietal lobe CMBs in aging compared to young participants ($p < 0.05$) (Figure 10).

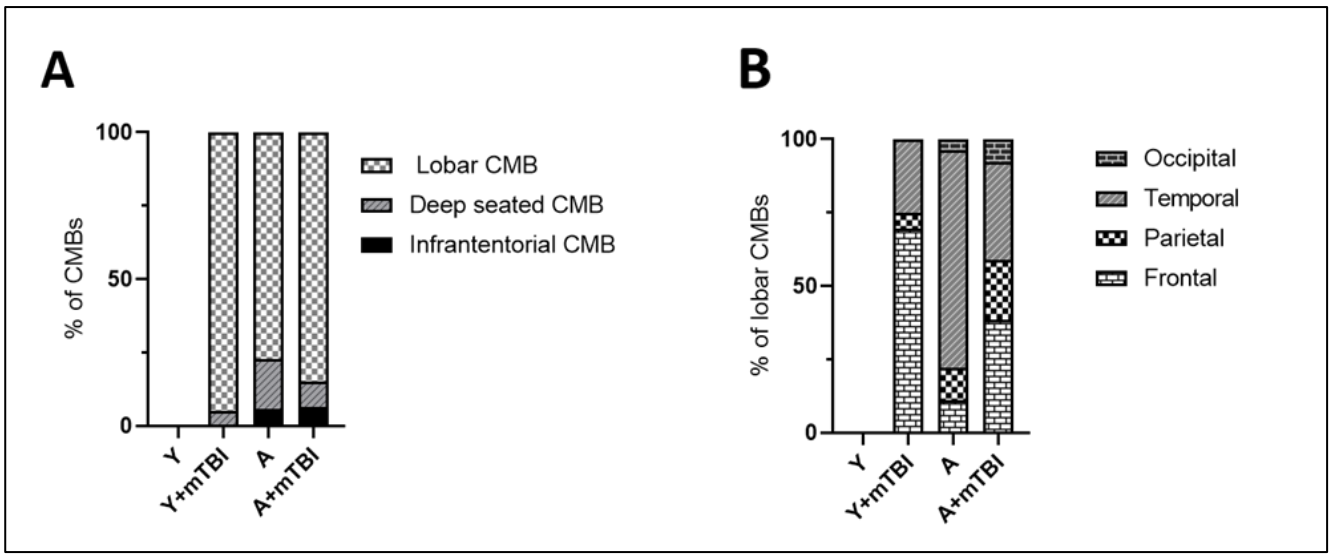

Figure 10 presents the localisation expressed in % of all CMBs in the different study groups based on MARS classification. ***A****: Lobar CMBs were the most prevalent in all of the study groups regardless of age or TBI. In the aged mTBI group a modest number of CMBs are found in infratentorial localisation compared to aged without trauma, but the difference did not reach significance level.* ***B:*** *The most frequently seen lobar localisation was further analysed and found that aging increased the number of parietal and occipital CMBs after mild traumatic brain injury (mTBI) (P<0.05 vs. Y+mTBI), and that mTBI leads to the formation of more CMBs in the frontal, parietal and occipital lobes in aging (P<0.05 vs. A).*

(Y: young control, Y+mTBI: young with mild TBI, A: aged control, A+mTBI: aged participants with mild TBI)

Age-related neurovascular coupling measurements

Mean arterial pressure and end-tidal CO_2 levels were in the physiologic range during the examinations and there was no significant difference between the two age groups. Basic cardio-cerebrovascular risk factors were reviewed, and significantly higher mean arterial blood pressure was found in the elderly compared to young individuals, despite the lack of hypertension. Smoking was more frequent among young volunteers. Blood glucose metabolism and cholesterol levels were not different between the study groups. Participants with diabetes mellitus were on oral medication, and their blood sugar levels and haemoglobin a1c levels were in the normal range.

		Young group	Aged group
n		31	32
Age (in years)		28.37 + / – 4.2	67.9 + / – 4.1
Sex	Female	11	18
	Male	20	14
Baseline MAP (mean + / – SD in Hgmm)		90.66 + / – 14.8	107.05 + / – 16.24*
Baseline heart rate (beat/min)		69.02 + / – 8.9	67.25 + / – 10.56
Smoking	Yes	25%#	6%
	No	75%	94%
Diabetes mellitus	Yes	3%	15%
	No	97%	85%
Total cholesterol level (mean + / – SD in mmol/l)		4.73 + / – 0.83	5.69 + / – 3.3
Low density lipoprotein level (mean + / – SD in mmol/l)		2.61 + / – 0.72	3.49 + / – 2.88

Table 2: Summary of the study participants. Main cardiovascular risk factors are summarized. MAP: mean arterial pressure. Significant differences were found in MAP ($p < 0.0001$ vs. young) and smoking (#$p = 0.0337$ vs. aged).*

Aging decreased basal cerebral blood flow

A subgroup of patients (young n=9, aged n=12) were examined with phase contrast MRI to assess the basal cerebral blood flow and MCA area. The mean cross-sectional areas of the left MCAs (lMCA) (young: 0.08 ± 0.012, aged: 0.065 ± 0.022) and right MCAs (rMCA) (young: 0.08 cm^2 ± 0.02, aged: 0.067 ± 0.02 values expressed as mean

± SEM) were not significantly different between the two groups evaluated by two sample t-test (Figure 11).

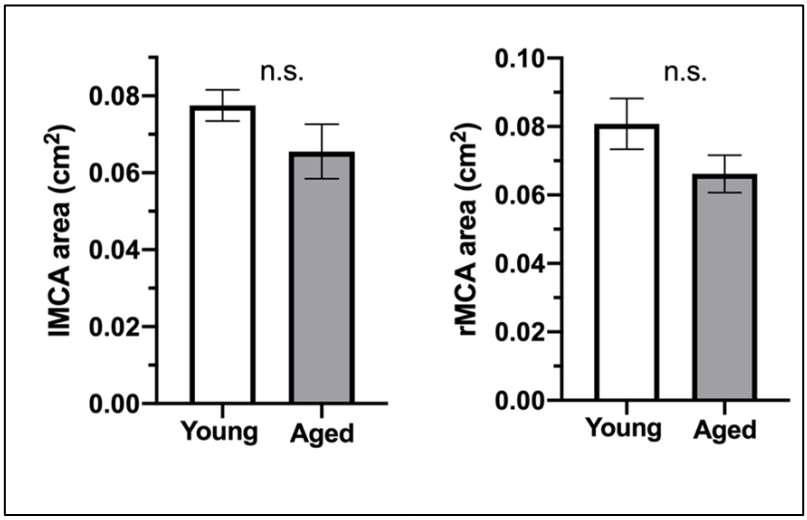

Figure 11: The area of the left and right MCA measured by phase contrast MRI in young and aged volunteers expressed in cm². Data are mean ± S.E.M.

Based on the measurements the mean flow velocities in both MCAs were significantly decreased in older subjects as compared to younger subjects (young left MCA FV: 2.39 ± 0.56 ml/s, right MCA FV: 2.57 ± 0.65 ml/s vs. aged left MCA FV: 1.71 ± 0.65 ml/s, aged right MCA FV: 1.79 ± 0.43 ml/s) (Figure 12).

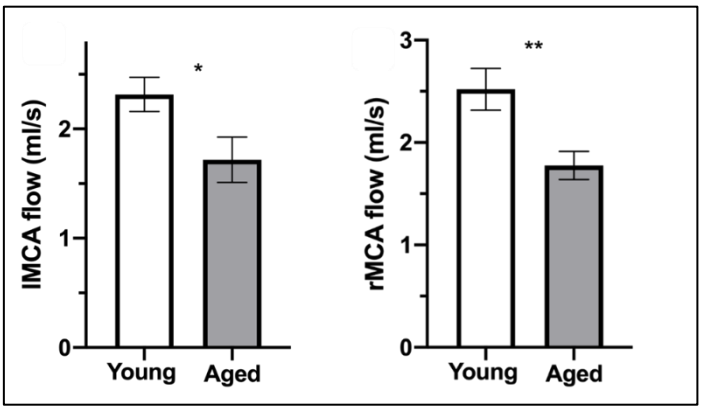

*Figure 12: The mean flow velocity (ml/s) of the left and right MCAs of young (n = 9) and aged (n = 12) volunteers. Data are mean ± S.E.M.*p < 0.05, **p < 0.003*

Aging altered NVC response

NVC response was expressed by measuring CVCi during the trail making test. TMT significantly increased CVCi in left and right MCA in both young and aged groups compared to baseline CVCi expressed in % change during TMT performance. The CVCi % change was significantly higher in young individuals (young group n=31). Mean left MCA CVCi change in % and SEM: 16 +/- 0.02, mean right MCA CVCi change % and SEM: 16 ± 0.02, aged group n=32, right MCA CVCi change: 2 ± 0.01, left MCA CVCi change: 3 ± 0.01. (Figure 13)

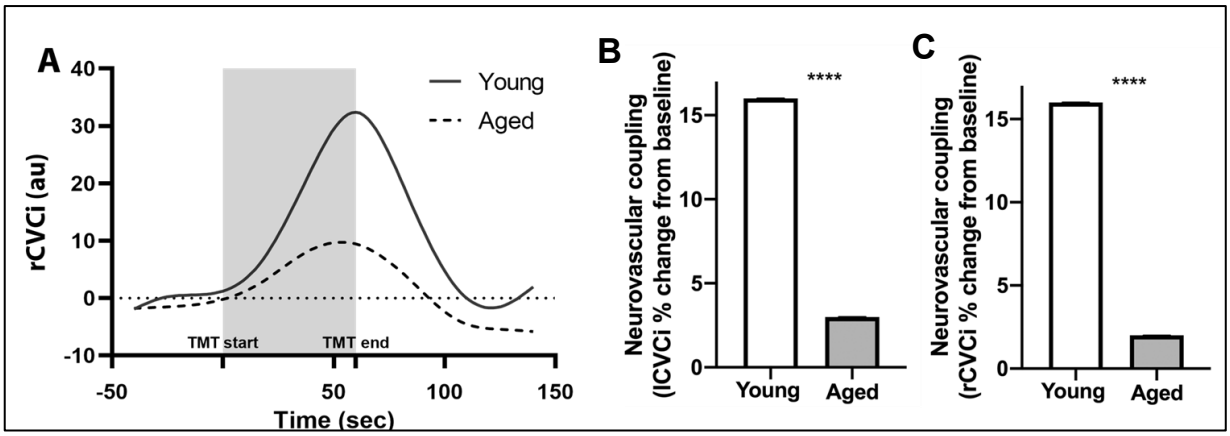

Figure 13 shows the impaired neurovascular coupling responses in elder individuals. ***A****: Representative figure of the CVCi changes in the right MCA during TMT in a 25-year-old (young) and a 65-year-old (aged) study participant. The gray area shows the period while the TMT test was performed. The task-evoked maximal NVC response in the young and old study participant was 32.4% and 9.7%, respectively, as compared to baseline.* ***B – C****: bar graph shows the summation of left (B) and right (C) CVCi change in % ± SEM compared to baseline during TMT in young and aged.*
***** p<0.0001*
(CVCi: cerebrovascular conductance index, r:right, l:left)

Breath hold test was performed as the positive control test and induced significant increase in CVCi in both age groups (young mean left MCA CVCi change (expressed in % change compared to baseline and SEM): 21 ± 0.02, right CVCi change: 20 ± 0.02, aged group left MCA CVCi change: 20 ± 0.02, right MCA CVCi change: 18 ± 0.03) but

the difference between the two groups did not reach statistical significance, indicating similar cerebrovascular reactivity in the studied groups (Figure 14).

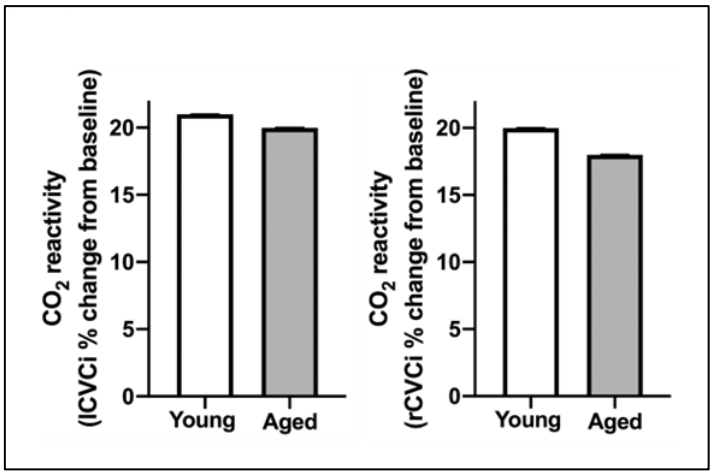

Figure 14 shows the changes of cerebrovascular conductance index (CVCi) of the left and right MCAs in young and aged volunteers during breath hold test

IGF-1 level is decreased in the elderly

Serum IGF-1 level (ng/ml) was significantly decreased in aged participants compared to young volunteers (young n=31, mean IGF-1 concentration ± S.E.M.: 176.22 ± 8.84 ng/ml vs. aged n=32, mean IGF-1 concentration: 115.70 ± 7.59 ng/ml). Decrease of serum IGF-1 level significantly correlated to age (R=0.566) (Figure 15). Sex related differences of serum IGF-1 were not observed in the study.

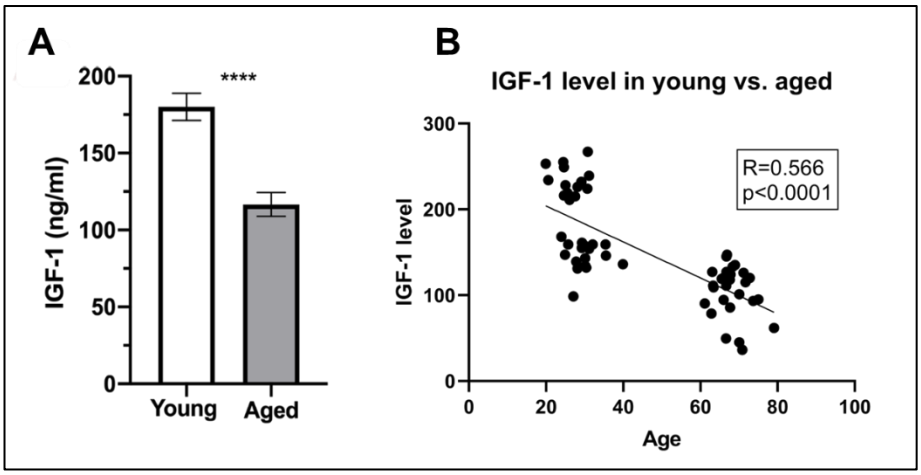

Figure 15 shows serum IGF-1 level in young and aged volunteers. ***A****: IGF-1 is significantly higher in young individuals **** p<0.0001* ***B****: Correlation between serum IGF-1 levels (ng/ml) and age (years) in young (n = 31) and aged (n = 32) volunteers*

Decreased IGF-1 level is associated with decreased basal cerebral blood flow

Decreased serum IGF-1 level was seen in aged study participants. More importantly, serum IGF-1 level significantly correlated to basal cerebral blood flow measured by phase contrast MRI in both of the study groups (R=0.53, p=0.02). (Figure 16)

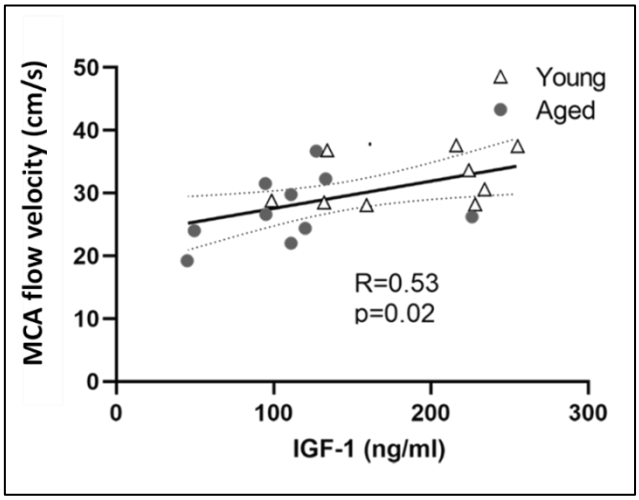

Figure 16 shows the correlation between serum IGF-1 levels (ng/ml) and MCA flow velocity in young (n = 10) and aged (n = 11) volunteers

IGF-1 level correlates with neurovascular uncoupling

Importantly, serum IGF-1 levels significantly correlated with changes in CVCi in both MCAs during performing the trail making test compared to baseline CVCi in the studied participants (left MCA CVCi R=0.32, p=0.02, right MCA CVCi: R=0.32, p=0.03), indicating that serum IGF-1 level correlates with neurovascular coupling and its dysfunction in aging (Figure 17).

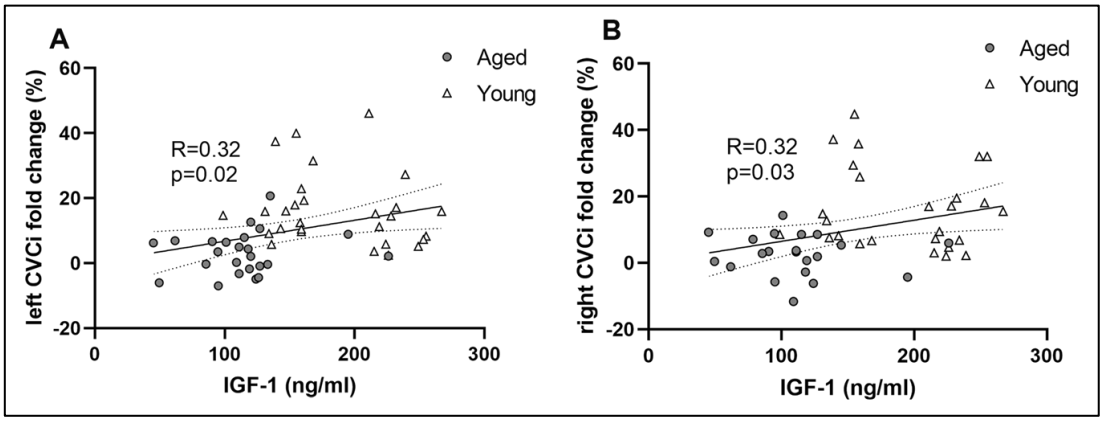

*Figure 17 shows the correlation of cerebrovascular conductance index (CVCi) (expressed as fold change from baseline) and IGF-1 level (ng/ml) in aged and young on the left (**A**) and right MCA (**B**)*

Gait function is impaired in aged individuals

Measurement of gait function showed that the stance phase (represents the phase of walking, when both legs are in contact with the ground and expressed in % of the gait cycle) of the elderly was significantly longer compared to young participants: young right mean: 63.03 ± 0.48 %, young left mean: 37.69 ± 0.46 % vs. aged right mean: 68.79 ± 1.17%, aged left mean: 68.56 ± 1.44%. Consequently, the swing phase was significantly shorter in aged compared to young participants (swing phase starts when one of the foot is raised off from the ground and ends when the same foot reaches the ground and expressed as % of the gait cycle): young n=31, right mean: 63.03 ± 0.48% vs. aged n=32, right mean: 68.79 ± 1.17%, young left mean: 37.69 ± 0.46% vs. aged left mean: 31.44 ± 1.44 % (Figure 18). Mean step length in conjunction with mean stride length were significantly higher in young individuals measured on both legs compared

to aged participants (young mean step length right: 54.79 ± 1.65 cm, step length left: 55.23 ± 1.57 cm, mean stride length 87.94 ± 2.59 cm, vs. aged step length right: 29.74 ± 2.50 cm, step length left: 29.88 ± 2.25cm, stride length: 47.52 ± 3.41 cm) p<0.0001) (Figure 18). We observed significantly higher step width in the aged group compared to the young individuals (young mean: 9.58 ± 0.69 cm, whereas aged mean: 11.85 ± 0.50 cm, p<0.05) (Figure 18). All these parameters reflect altered and unstable gait cycle in aged volunteers compared to young ones.

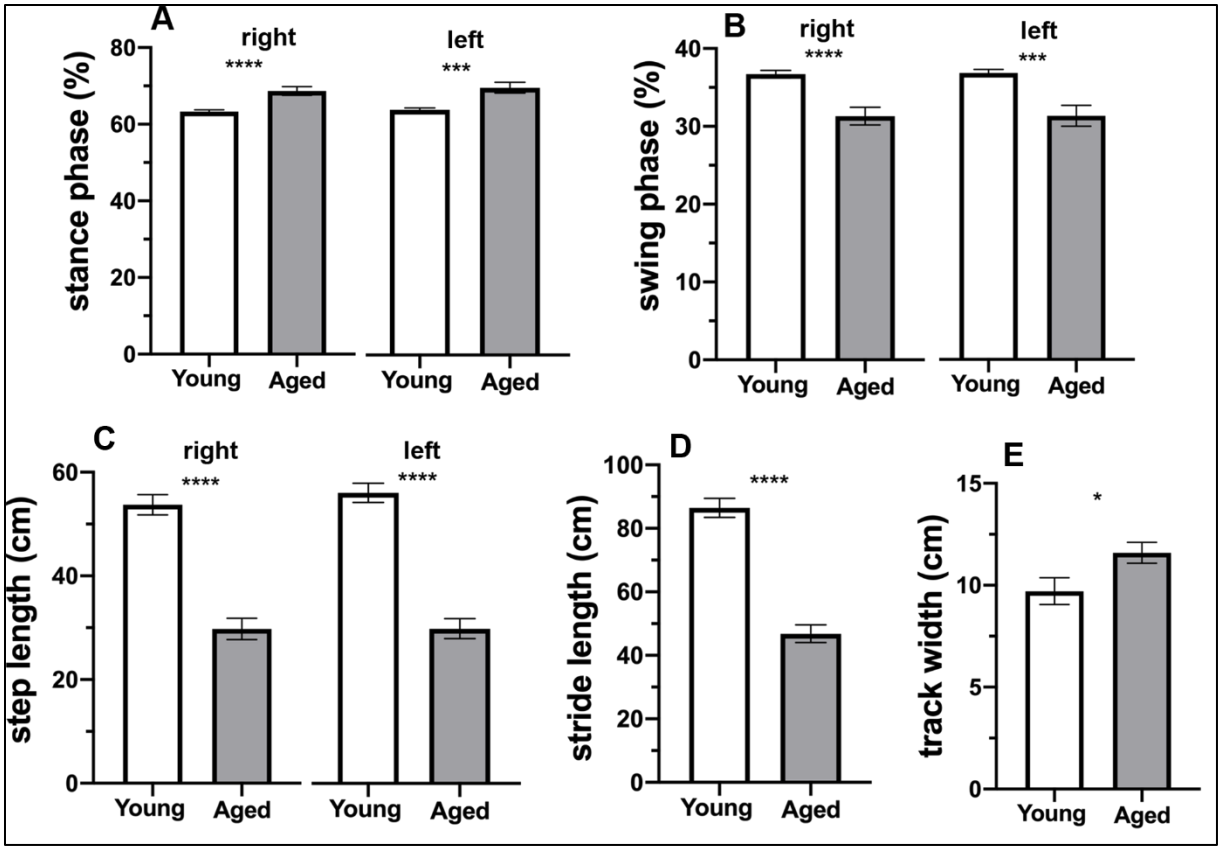

*Figure 18 shows altered gait function in aged participants. **A** and **B** panels show stance phase and the swing phase during gait cycles for both legs (left, right) in the examined age groups (young group n=31, aged group n=32, **** P<0.,0001, ***P<0.003). **C**: The graph shows the difference in the mean step length (in cm) for right and left legs in the studied young and aged groups. The mean step length (cm) was significantly higher in young individuals for both legs compared to elder persons (**** P<0.003). The mean stride length (cm) (**D**) and was significantly (**** P<0.0001) longer and track width (cm) (**E**) was significantly (* P<0.05) wider in the young group compared to the aged group of volunteers.*

Gait dysfunction correlates with neurovascular uncoupling and decreased IGF-1 levels in aged individuals

Our data showed strong correlation between mean step length (in cm) and both % change of cerebrovascular conductance index (CVCi) in MCAs during trail making test (R=0.52, p<0.00001) and serum IGF-1 levels (R=0.49, p<0.0001) in the studied participants, indicating that gait dysfunction correlates with age-related neurovascular uncoupling and age-related decline in serum IGF-1 in humans (Figure 19). We also found that track width (in cm) significantly correlated with neurovascular dysfunction (R=-0.29, p=0.0419) (Figure 19. C) and tended to correlate with serum IGF-1 levels (R=0.10, p=0.45) in an age-dependent manner in young (n=31) and aged (n=32) participants (Figure 19.D).

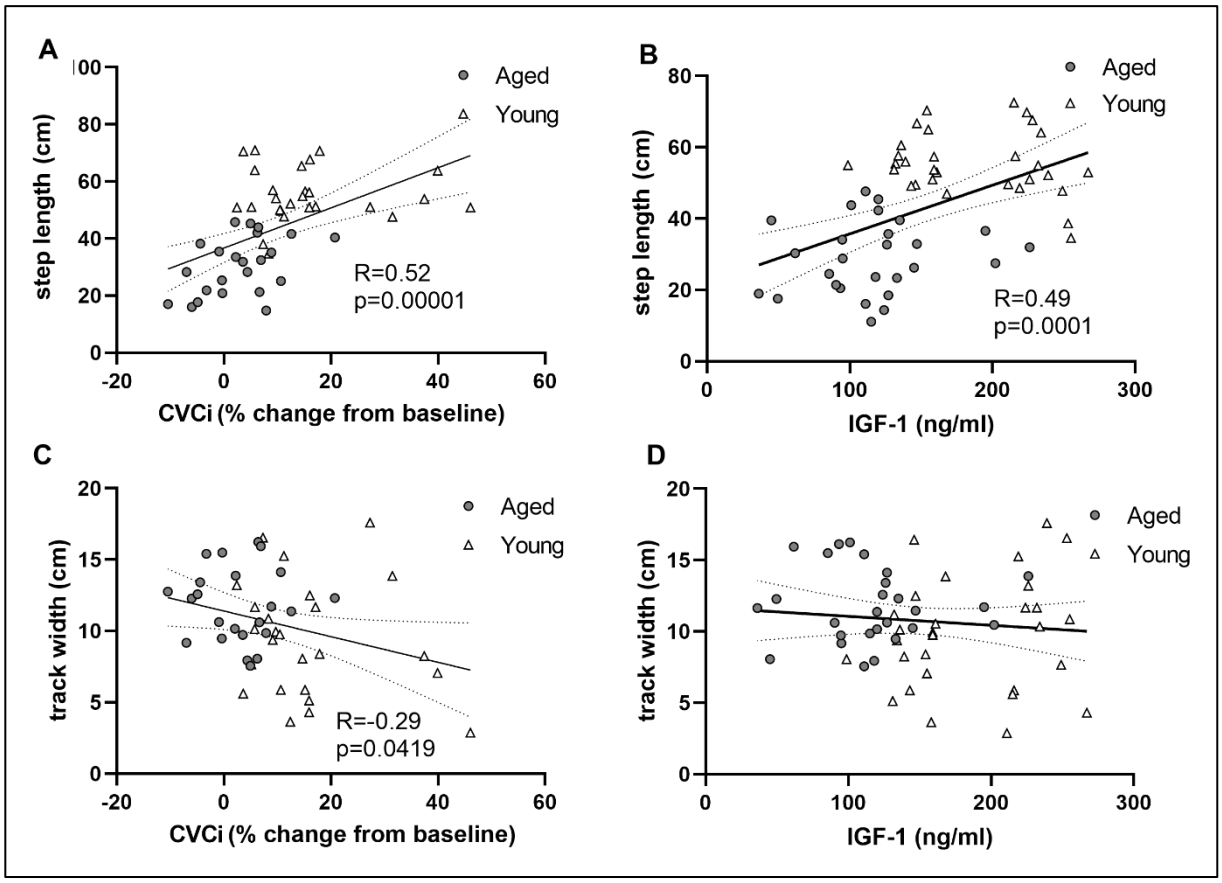

Figure 19 Age-related gait dysfunction correlates with neurovascular uncoupling and IGF-1 deficiency in humans. ***A****: Correlation between mean step length (cm) and changes of cerebrovascular conductance index (CVCi) during cognitive test*

(expressed as % change from baseline) in right MCA of young and aged volunteers (R=0.52, P=0.00001). ***B****: Correlation between mean step length (cm) and IGF-1 serum concentration of young and aged volunteers (R=0.49, P= 0.0001).* ***C-D****: The figures show the correlation between the track width in cm and % change of CVCi during cognitive test normalized to baseline (C) and serum IGF-1 concentration (D) in young and aged volunteers (R=-0.29, p=0.0419 and R=0.10 p=0.45, respectively).*

DISCUSSION

Formation of cerebral microbleeds in aging and traumatic brain injury

We hypothesized that aging and mTBI interact to promote the development of cerebral microbleeds, but contrary to our hypothesis we did not detect a significant difference in the number of cerebral microhaemorrhages between aged (A) and aged patients with mild traumatic brain injury (A+mTBI) (Figure 9). Although we showed that significantly more microbleeds can be found in the aging human brain than in young healthy individuals, confirming the results of previous studies, importantly our results suggests that aging and mTBI do not synergize in the induction of the development of CMBs. [13, 42, 43, 61]

Earlier studies investigated the effects of CMBs on brain function and found that these lesions are not clinically silent, but have been associated with cognitive decline, mental disorders and gait abnormalities. This is presumably due to the cumulative effect of CMBs, or the anatomical location-related domain specific decline in neurological function, or both. [12, 13, 42, 62-64]

For example, damage of the fronto - subcortical circuits linking prefrontal areas to basal ganglia is associated with impairment in executive function, and disarrangement of pathways from the mentioned areas projecting to thalamus results in memory disturbances. [42, 62, 63, 65] Based on our results, the distribution of CMBs following mTBI are altered in aged patients. (Figure 10) Specifically, we found that the number of occipital and parietal microbleeds were significantly higher in aged following mTBI compared to young participants.

This finding might explain important functional consequences of these CMBs. Accordingly, parietal and occipital lobes have crucial roles in integrating visuospatial and sensory information, cognitive processing, posture and motor control, visual transmission and incorporation, also the visuospatial and cognitive fine tuning of coordination along with rapid movement corrections. [41, 66-71] Based on that, as a future direction special visuospatial and cognitive domain tests e.g. trail making test, Beck’s depression test, Montreal Cognitive Assessment, Balance Evaluation System

Test or Lower Extremity Motor Coordination Test should be included in the mTBI research to access and comprehensively interpret the effect of CMBs in a region-specific approach in both aged and young individuals. This potential associations should be tested and established in the future.

Possible clinical importance: gait and cognitive dysfunction

Coordinated and harmonious gait and posture is a higher cortical function which requires interdependent activity and participation of cortical (primary motor cortex, premotor cortex and supplementary motor area) and subcortical areas (basal ganglia, thalamus, cerebellum, the limbic system, midbrain, pons, medulla and spinal locomotor network). [69, 71, 72] Based on previous research, the development and presence of CMBs result in gait dysfunction presumptively by damaging these primary and supplementary motor centres and disrupting the communicating pathways between them. [42, 73-75] In aged people, altered gait parameters, characterized by increased double support time, step width and step variance along with decreased stride length, walking speed, cadence and timed up and go test were seen if CMBs were present in lobar (mainly temporal and frontal) regions, basal ganglia and projection areas as corona radiata. [73, 74, 76]

Interestingly, only one case series investigated the effect of traumatic microbleeds on vestibular and gait dysfunction by dynamic posturography and the dynamic gait index. [77] Based on the results, the SWI positive posttraumatic patients suffered vestibular or balance abnormalities, particularly abnormally slow gait speed and varying vestibular deficits. [77]

It is logical to postulate that TBI exacerbates gait dysfunction in the elderly, and gait disturbance of the elderly most likely is a central factor in the increased incidence of TBI amongst them due to increased propensity to fall. Future clinical studies are evidently needed to clarify the possible interactions between ageing and TBI on gait dysfunction and the possible role of cerebral microbleeds. Furthermore, the functional connection between cognition and gait coordination especially following TBI should be investigated in the future, as well.

However the relation of location of CMBs to different etiological factors and cognitive decline have been widely analysed, the direct link between the number and location of the lesions and the mechanisms for cognitive deterioration is still not completely understood. [12, 13, 42, 43, 61-63, 78]

Similarly, to gait dysfunction it is suggested that the cumulative effect and the focal damage of special anatomical regions or connections caused by microbleeds are responsible for the development of CMB-associated cognitive decline. [13, 61, 63] The cumulative effects of CMBs could be explained partly by the increased number of lesions, leading to more severe cognitive disorder in several domains measured e.g. by Montreal Cognitive Assessment (MoCA). [63, 79] Contrary to that, other studies claimed that the location of the CMBs are more important than number of lesions on cognitive outcome and found in healthy non-demented elderly that strictly lobar lesions were associated with decreased speed of information processing, worsened executive function and gait abnormalities; whereas the deep lesions were related to decline in global cognitive function. [43, 61, 65] Damage of fronto-subcortical circuits linking prefrontal areas to basal ganglia was demonstrated to be associated with impairment in executive function of healthy individuals in all age groups of patients with vascular disease, and disarrangement of pathways projecting from the mentioned areas to the thalamus results in memory disturbances. [61-63, 65] Similar mechanisms can be responsible for the cognitive decline caused by TBI-induced microbleeds. Accordingly, single case study suggested direct neuropathological connection between cognitive dysfunction and TMBs following mTBI in a previously healthy 57-year-old male patient. [80] This was further substantiated by studies showing that in mild TBI patients' number of traumatic CMBs correlated with general cognitive decline, impaired short-term memory, concentration difficulties and depression. [12, 40, 64, 75, 81] Interestingly, the number of lesions in the acute stage predicted the progress of post-concussion syndrome and decline in psychomotor and processing speed a year after the injury. [75] Although it seems logical to posit that (even mild) brain trauma results in enhanced cognitive disturbances in elderly individuals, to our best knowledge, no studies have tested this hypothesis. Thus, future clinical research should investigate the synergistic effect of ageing and TBI-related formation of cerebral microbleeds on cognitive decline.

To our best knowledge, there is no specific treatment to prevent the development of CMBs. [13, 42] CMB formation is frequently associated with cardiovascular risk factors

or previous cardiovascular events, therefore treating these factors and the use of anticoagulation therapy may slower the progression of CMBs. However, some studies reported increased incidence of CMBs related to antiplatelet therapy in patients. [82]

Limitations and perspectives

The major limitations of our studies are the retrospective design and the relatively small sample size. Future prospective studies should verify our findings on a large number of control healthy volunteers. We used the Mayo criteria to define mTBI. Since other guidelines suggest slightly different scoring systems, it would be important to compare CMB formation in TBI groups defined by various scoring systems. Aging and mTBI may interact in altering regulatory mechanisms of cerebral blood flow (CBF) in a functional manner. Accordingly, changes in neurovascular coupling, autoregulation of CBF and cerebrovascular reactivity should be assessed and correlated with cognitive and gait function in different age groups after mTBI. Finally, the possible mechanisms through which aging and TBI may interact to alter cerebrovascular function and formation of CMBs should be studied, with special focus on mitochondrial oxidative stress, activation of redox-sensitive matrix metalloproteinases, modification of the cerebrovascular wall, production of proinflammatory cytokines and disruption of the blood-brain barrier.

Age-related neurovascular uncoupling

In the aging societies worldwide, age-related cerebrovascular dysfunction plays a central role in the development of neurological disorders, such as cognitive decline and gait dysfunction, affecting the elderly population. [15, 83-85] Neurovascular coupling leads to reactive hyperaemia of the active brain areas in a well-regulated, feed forward manner, providing the needed metabolic agents for active neural tissue, and washing out by products. [15, 83, 85-88] It is logical to consider that normal neurovascular coupling is essential for maintaining healthy brain function. Indeed, recent preclinical studies from our group and others showed that pharmacological uncoupling of neurovascular signalling leads to impaired hyperaemic response in active brain areas, which is associated with impaired cognitive and gait function in mice. [86, 89, 90] Aging was shown to be associated with attenuated neurovascular coupling responses and brain dysfunction in laboratory animals. [15, 87, 90] Here we show that in healthy elderly humans reactive hyperaemic response during cognitive tasks is attenuated compared to young volunteers, confirming the mentioned observations made in laboratory animals. Specific age-related disruption of neurovascular mechanisms are further suggested by our results that CO_2-reactivity was found to be intact in the elder group of patients. It has to be noted, that in a few persons studied even negative hyperaemic responses were recorded during the cognitive tasks, suggesting a severely impaired neurovascular coupling in these individuals. Further studies should establish the factors that determine the magnitude of impairment of neurovascular coupling in aging. Importantly, we confirm previous studies that basal cerebral blood flow is also decreased by aging, which further decreases the capability of the impaired neurovascular responses in these patients to fulfil metabolic needs of active brain areas by shifting baseline perfusion to lower values. [91, 92] Further studies should establish the separate effects of neurovascular uncoupling and decreased basal CBF on cognitive and gait function in the elderly.

The mechanisms by which aging impairs neurovascular coupling in humans have not been established, yet. Age related IGF-1 deficiency, which is the most common neuroendocrine change with aging, has been linked to decline of neural function in aging. [49, 50, 55, 93] We recently provided evidence in mice that disrupting IGF-1 signalling in the brain either by adenovirus-associated knock-down of IGF-1

production in the liver or blocking IGF-1 receptors on endothelial cells of cerebral vessels lead to a significant decrease in neurovascular responses of these animals during neuronal activation, which is associated with both gait and cognitive dysfunction. [30, 94, 95] Based on this here we tested the hypothesis that IGF deficiency is associated with neurovascular uncoupling in elder humans. We show a significant age-related decrease in serum IGF-1 level in the elder group of participants (Figure 15) and demonstrate that IGF-1 levels significantly correlate with neurovascular dysfunction (Figure 17). Despite the limitation of correlative data in demonstration of casualty, these observations together with our previous preclinical results strongly suggest that IGF-1 deficiency disrupts neurovascular signalling leading to neurovascular uncoupling in aging humans.

The mechanisms by which IGF-1 deficiency disrupts neurovascular coupling in aging is multifaceted. First, IGF-1 deficiency results in decreased expression of metabotropic glutamate receptors, NMDA receptors and glutamate transporters in astrocytes isolated from mice after viral knockdown of IGF-1 production, leading to decreased sensitivity of astrocytes to the glutamate signal released from activated neurons. [30, 50] Second, IGF-1 deficiency induces a disbalance of astrocyte-derived factors mediating arteriolar dilation during neurovascular coupling, leading to an increased level of constrictor factors produced in astrocytes. [15, 88, 96] Accordingly, the production of dilator prostaglandins (PGE_2) and eicosatrieonic acids (EETs) is decreased and the constrictor 20-hydroxytrienoic acid (20-HETE) is increased in response to glutamate-stimulation on brain slices isolated from IGF-1 deficient mice, due to an IGF-1 dependent dysregulation of the producing enzymes. [30, 88, 96] IGF-1 deficiency also induces increased production of reactive oxygen species in cerebral endothelial cells, leading to decreased endothelium-dependent dilation of pial and cortical arterioles in the brain. [95-97] Future studies should establish these pathways in humans and test pharmacological and biotechnological interventions to restore IGF-1 levels, such as the use of nanofibrous substitutes to restore neurovascular coupling and neural function. [50, 98, 99] In addition to ageing, diabetes mellitus and GH deficiency has been linked to decreased IGF-1 level, future studies should establish neurovascular function and the role of IGF-1 deficiency in these patient population. It is important to note that IGF-1 deficiency has significant effects on capillary density and synaptic function, as well. [30] Evidence shows that mice lacking IGF-1 exhibit a decreased density of brain capillaries in the hippocampus and impaired synaptic

function. [30, 56, 94, 96] Further studies should examine the effect of IGF-1 on synaptic function and capillary density in humans.

As mentioned above, a line of evidence suggest that neurovascular hyperaemia is essential to maintain normal neural function in laboratory animals. [85, 89, 90, 100] Here we show for the first time that gait dysfunction observed in older individuals significantly correlate with neurovascular uncoupling (Figure 19 A,C). The causative role of neurovascular dysfunction in the development of neural impairment is strongly suggested by preclinical studies showing that 1) decreased neurovascular coupling is associated with impaired cognitive and gait function in animal models of cerebrovascular risk factors, such as hypertension, diabetes, as well as aging, and 2) direct pharmacological inhibition of neurovascular coupling disrupts normal cognition and gait in mice. [86, 89, 90, 101] This is further supported by our findings that IGF-1 deficiency significantly correlates with gait dysfunction in the studied elder group of participants (Figure 19 B,D). Future studies should test whether increasing IGF-1 levels would restore neurovascular coupling and gait and cognitive function in elderly humans.

Conclusion, theses and perspectives

Age-related cerebrovascular changes have been recognized to have great impact on age-related cognitive decline and gait dysfunction, placing an enormous burden on the aging societies worldwide. In the present theses we studied two aspects of age-related cerebrovascular alterations, the formation of cerebral microbleeds in normal aging and after mild traumatic brain injury, and neurovascular dysfunction associated with aging, both of which have been shown to contribute to age-related deterioration of cerebral function.

Thesis 1:

Our results showed that aging leads to enhanced number of cerebral microbleeds compared to young volunteers, and ***mild traumatic brain injury and aging do not synergize to increase the number of CMBs***. *W*e found that aging altered the CMB distribution and caused more occipital and temporal lobe lesions in elderly compared to young traumatic patients, which might have an impact on long term consequences and complications of traumatic microbleeds. The major limitations of our studies are the retrospective design and the relatively small sample size. Future prospective studies should verify our findings on a larger number of patients. Also, the possible mechanisms through which aging and TBI may interact to alter cerebrovascular function and formation of CMBs should be studied, with special focus on mitochondrial oxidative stress, activation of redox-sensitive matrix metalloproteinases, modification of the cerebrovascular wall, production of proinflammatory cytokines and disruption of the blood-brain barrier.

Thesis 2:

We showed that ***age-related IGF-1 deficiency correlates with aging-induced neurovascular un-coupling and decreased basal cerebral blood flow in humans, and both of them correlates with age-related gait dysfunction***. Future studies should establish the possible clinical interventions to restore age-related neurovascular dysfunction to prevent age-related cognitive decline and gait impairment.

Publication list (Impact factor: 59.629)

Publications, the present thesis is directly based on

- Toth L, Czigler A, Horvath P, Kornyei B, Szarka N, Schwarcz A, Ungvari Z, Buki A, Toth P. Traumatic brain injury-induced cerebral microbleeds in the elderly. Geroscience. 2021 Feb;43(1):125-136. doi: 10.1007/s11357-020-00280-3. Epub 2020 Oct 3. PMID: 33011936; PMCID: PMC8050119. (Q1, Impact factor: 7.581)

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