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Correlation between serum tight junction proteins with blood brain barrier integrity in ischemic stroke patients

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ABSTRACT

Background/aim: Blood brain barrier (BBB) breakdown is a proposed mechanism for ischemic stroke (IS). Platelet-derived growth factor B (PDGFB) and tight junction (TJ) associated proteins; zonula occluden-1 (ZO-1) and claudin-5 (CLDN5), play fundamental roles in keeping BBB integrity. The aim of the present work is to investigate the impact of some modifiable stroke risk factors on serum levels of CLDN5, ZO-1and PDGFB, as non-invasive markers for BBB integrity

Materials and methods: The present study is performed on 186 IS patients. Patients were classified into two groups; Group I: small vessel disease (SVD) patients; n= 114, and Group II: large vessel disease (LVD) patients; n= 72. Serum levels of ZO-1, CLDN5 and PDGFB were measured using human ELISA kits.

Results: Ischemic heart disease (IHD) was significantly ($p<$**0.05)** associated with decreased serum levels of ZO-1 and PDGFB in LVD patients, along with decreased levels of ZO-1 and CLDN5 in SVD patients. Smoking could not be associated with any reliable differences in any of the studied markers. Diabetes mellitus (DM) was significantly ($p<$**0.05)** associated with decreased levels of ZO-1 in both LVD and SVD patients, while hypertension (HTN) was significantly ($p<$**0.05)** associated with decreased levels of ZO-1 only in SVD patients.

Conclusion: IHD, DM and HTN are significantly associated with decreased levels of serum markers of BBB integrity, however, their impacts differ according to stroke subtype. Defining extent of BBB injury associated with each risk factor could both help predict patients' prognosis and serve as a useful tool in making individualized treatment plans.

1. Introduction

Stroke is an outstanding global health issue, aligned as the second leading cause of deaths and the third leading cause of disabilities worldwide [1]. The increased prevalence of traditional vascular risk factors such as hypertension, diabetes, smoking, and coronary heart disease lies behind the increased stroke incidence [2]. Large vessel disease (LVD) and small vessel disease (SVD) are two ischemic stroke (IS) subtypes that are overlapping, sharing similar risk factors, though they do have different pathophysiological mechanisms [3]. One of the proposed mechanisms for IS, is blood brain barrier (BBB) breakdown [4]. The BBB integrity deeply relies on a composite structure of tight junction (TJ) proteins, of which, claudin-5 (CLDN5) and zonula occluden-1 (ZO-1) are basic functional and structural elements that are commonly used as BBB integrity markers. When BBB is injured, those TJ proteins escape from the barrier into blood, where they become detectable [5].

In fact, BBB integrity also relies on the presence of pericytes (PCs), whose contraction and relaxation is necessary for the regulation of cerebral blood flow (CBF) [6]. After ischemia, oxygen deficiency induces PC relaxation, which in turn induces dilation of the blocked vessels, ultimately leading to restoration of the CBF. Several proteins have been discovered as regulators of PC contractility after stroke, of which, Platelet-derived growth factor B (PDGF-B) is an important one [7].

The aim of the present study is to investigate the impact of ischemic heart disease (IHD), diabetes mellitus (DM), hypertension (HTN) and smoking on the serum levels CLDN5, ZO-1and PDGFB in IS patients with LVD and SVD.

2. Materials and methods

2.1. Study design and Informed consent

The present study is approved by the ethical committee of Neuropsychiatry department, faculty of medicine, Ain Shams University, with an approval number FWA 000017585. Patients gave their permission for reviewing their medical records and for withdrawal of blood samples. Consent by first degree relative or guardian was obtained for patients who were severely ill or unconscious. The study was performed on 186 IS patients.

The diagnosis was based on clinical, biochemical, magnetic resonance imaging (MRI), and carotid duplex investigations. Patients were classified into two groups: **Group I**: SVD patients; n=114, and **Group II**: LVD patients; n=72. Each group was further subdivided according to the presence or absence of the studied risk factors as following: Diabetics vs non-diabetics patients were diagnosed as being diabetic as previously reported [8]: having FPG of 126 mg/dL (7.0 mmol/L), or 2-h PG of 200 mg/dL (11.1 mmol/L) or A1C 6.5% (48 mmol/mol)}, hypertensive vs normotensive {patients were diagnosed as being hypertensive as previously reported [9]: Grade 1 hypertension: having systolic blood pressure of 140-159 mmHg and/or diastolic blood pressure of 90-99 mmHg, Grade 2 hypertension: having systolic blood pressure of ≥ 160 mmHg and/or diastolic blood pressure of ≥ 100 mmHg, patients with IHD vs patients with non-IHD, and smokers vs non-smokers.

2.1.1. Inclusion Criteria

According to TOAST classification (Trial of Org 10172 in Acute Stroke Treatment), which divides strokes into five subtypes as follows: 1) large-artery atherosclerosis, 2) cardioembolism, 3) small-vessel occlusion, 4) stroke of other determined etiology, and 5) stroke of undetermined etiology, Egyptian patients presenting with ischemic stroke fulfilling the criteria of SVD and LVD were included.

2.1.2. Exclusion Criteria

Patients with evidence of cardio-embolic stroke, acute stroke of other determined etiology, stroke of undetermined etiology, intracranial hemorrhage, other immune-mediated disorders. patients who refused to participate in the study, age younger than 45 years old.

2.2. Methods

2.2.1. Routine Clinical Investigations

These included full history taking, detailed neurological examination, magnetic resonance imaging, carotid duplex, electrocardiogram and echocardiography

2.2.2. Biochemical analyses

These included the estimation of Human ZO-1, CLDN5 and PDGFB in Serum of the studied patients. This was achieved using human ELISA kits for in vitro quantitative detection of human ZO-1, CLDN5 and PDGFB in serum, respectively. All chemicals and reagents used in this study were of high analytical grade and were purchased from QIAGEN (Germany), Thermofisher Scientific (USA), and Bioassay Technology Laboratory (China).

3. Results

AS shown in table 1, the mean age in the SVD group was (69.47 ± 0.798 years) which was significantly higher (p< 0.001) than that in the LVD group (60.19 ± 1.87 years). While 68.4% of the SVD patients were hypertensive, only 50% of the LVD patients were hypertensive, with a significant difference between them (p= 0.04).

The distribution of smokers in the SVD group was significantly different (p= 0.001) from that in the LVD group, where the frequency of non-smokers, smokers and heavy smokers in the SVD group was 84.2%, 10.5%, and 5.3%, respectively, compared to 50%, 16.7% and 33.3%, respectively, in the LVD group. No significant differences could be observed comparing the frequency of ischemic heart disease between the two groups, meanwhile, the frequency of diabetics was significantly higher (p= 0.043) in the SVD group (63.2%), compared to only 41.7% in the LVD group.

AS revealed in table 2, comparing serum levels of ZO-1, CLDN5 and PDGFB between LVD patients with IHD vs those with non-IHD revealed significant differences only for ZO-1 and PDGFB which showed significantly (p<0.05) higher levels (12.60 ± 1.169) nd (178.17 ± 20.49), respectively, in the patients with non-IHD compared to those with IHD (6.46 ± 0.14 and 53.40 ± 5.69, respectively).

The same comparison between SVD patients with IHD vs those with non-IHD revealed significantly (p=0.021) higher levels of ZO-1 in the SVD patients with non-IHD (13.87 ± 3.38) compared to those with IHD (8.027 ± 0.85), and also significantly (p<0.001) higher levels of CLDN5 in the SVD patients with non-IHD (8.48 ± 0.66), compared to those with IHD (4.19 ± 0.24).

As shown in table 3, comparing serum levels of ZO-1, CLDN5 and PDGFB between diabetic and non- diabetic LVD patients revealed significantly (p= 0.018) higher levels of ZO-1in the non-diabetic patients (13.74 ± 1.59) compared to the diabetics (8.87 ± 0.92). The same comparison between diabetic and non-diabetic SVD patients revealed significantly (p= 0.017) higher levels of ZO-1in the non-diabetic patients (11.59 ± 1.69) compared to the diabetics (6.08 ± 0.27), with no significant differences in the levels of either CLDN5 or PDGFB.

As shown in table 4, comparing serum levels of ZO-1, CLDN5 and PDGFB between normotensive and hypertensive LVD patients did not reveal any significant differences. The same comparison between normotensive and hypertensive SVD patients revealed significantly (p= 0.037) higher levels of ZO-1 in the normotensive patients (13.007 ± 3.09) compared to hypertensive patients (7.97 ± 0.73).

AS shown in table 5, comparing serum levels of ZO-1, CLDN5 and PDGFB between smoker and non-smoker LVD patients did not reveal any significant differences. The same was true in the SVD patients.

Table 1. Demographic characteristics of LVD and SVD patients.

Groups Variable	LVD N= 72	SVD N= 114	*p*-value (Pearson Chi-square)	95% CI
Gender, No. (%)				
Male	60 (83.3%)	54 (47.4%)	**0.001****	**2.005~ 15.393**
Female	12 (16.7%)	60 (52.6%)		
Age (Years)				
Mean age ± SD	60.19 ± 1.87	69.47 ± 0.798	**0.000****	**-12.84 ~ -5.71**
Hypertension, No. (%)				
Normotensive	36 (50%)	36 (31.6%)	**0.04**	**0.917 ~ 5.118**
Hypertensive	36 (50%)	78 (68.4%)		
Smoking, No. (%)				
Non-smoker	36 (50%)	96 (84.2%)	**0.001****	
Smoker	12 (16.7%)	12 (10.5%)		
Heavy smoker	24 (33.3%)	6 (5.3%)		
Ischemic heart disease No. (%)				
Ischemic	12 (16.7%)	30 (26.3%)	**0.278**	**0.621 ~ 5.135**
Non-ischemic	60 (83.3%)	84 (73.7%)		
Diabetes, No. (%)				
Diabetic	30 (41.7%)	72 (63.2%)		
Non- diabetic	42 (58.3%)	42 (36.8%)	**0.043***	**1.022 ~ 5.635**

LVD: large vessel disease, SVD: small vessel disease, *p*-value < 0.05 was considered significant (*), while *P*-value ≤ 0.001 was considered highly significant (**).

Table 2. Comparing serum levels of ZO-1, CLDN5 and PDGF-B between LVD patients with IHD to those with non- IHD and SVD patients with IHD to those with non- IHD

Variable Mean ± SD	LVD patients		*p*- value	95% CI
	With non- IHD N= 60	With IHD N= 12		
ZO-1 (ng / ml)	12.60 ± 1.169	6.46 ± 0.14	**0.026***	0.76 ~ 11.51
CLDN5 (ng / ml)	8.78 ± 1.14	6.70 ± 1.32	0.439	-3.32 ~ 7.48
PDGF-B (ng /L)	178.17 ± 20.49	53.40 ± 5.69	**0.011***	30.40 ~ 219.14
	SVD patients			
	With non- IHD N= 84	**With IHD N= 30**		
ZO-1 (ng / ml)	13.87 ± 3.38	8.027 ± 0.85	**0.021***	-10.77 ~ -0.916
CLDN5 (ng / ml)	8.48 ± 0.66	4.19 ± 0.24	**0.000****	2.03 ~ 6.53
PDGF-B (ng / L)	122.96 ± 14.63	90.43 ± 11.38	0.208	-18.63 ~ 83.68

LVD: large vessel disease, SVD: small vessel disease *P*-value < 0.05 was considered significant (*), while *P*-value < 0.001 was considered highly significant (**).

Table 3. Comparing serum levels of ZO-1, CLDN5 and PDGF-B between diabetic vs non- diabetic LVD patients and diabetic vs non- diabetic SVD patients.

Variable Mean ± SD	**LVD patients**		***p*- value**	**95% CI**
	Non-diabetic N= 42	**Diabetic N= 30**		
ZO-1 (ng / ml)	13.74 ± 1.59	8.87 ± 0.92	**0.018***	0.873 ~ 8.86
CLDN5 (ng / ml)	9.23 ± 1.64	7.44 ± 0.84	0.37	-2.24 ~ 5.83
PDGF-B (ng / L)	187.94 ± 24.46	119.17 ± 26.99	0.068	-5.37 ~ 142.92
	SVD patients			
	Non-diabetic N= 42	**Diabetic N= 72**		
ZO-1 (ng / ml)	11.59 ± 1.69	6.08 ± 0.27	**0.017***	-9.99 ~ -1.03
CLDN5 (ng / ml)	6.79 ± 0.47	7.68 ± 0.82	0.438	-3.18 ~ 1.40
PDGF-B (ng / L)	108.30 ± 11.15	117.95 ± 16.77	0.684	-56.96 ~ 37.66

LVD: large vessel disease, SVD: small vessel disease *P*-value < 0.05 was considered significant (*), while *P*-value < 0.001 was considered highly significant (**).

Table 4. Comparing serum levels of ZO-1, CLDN5 and PDGF-B between normotensive vs hypertensive LVD patients and normotensive vs hypertensive SVD patients.

Variable Mean ± SD	**LVD**		***p*- value**	**95% CI**
	Normotensive N= 36	**Hypertensive N= 36**		
ZO-1 (ng / ml)	13.20 ± 1.68	9.95 ± 1.17	0.122	-0.918 ~ 7.4
CLDN5 (ng / ml)	10.02 ± 1.74	6.86 ± 0.808	0.11	-0.75 ~ 7.06
PDGF-B (ng / L)	172.15 ± 29.62	142.61 ± 23.42	0.44	-47.21 ~ 106.3
	SVD			
	Normotensive N= 36	**Hypertensive N=78**		
ZO-1 (ng / ml)	13.007 ± 3.09	7.97 ± 0.73	**0.037***	0.322 ~ 9.74
CLDN5 (ng / ml)	8.46 ± 1.23	6.82 ± 0.56	0.161	-0.68 ~ 4.01
PDGF-B (ng / L)	131.90 ± 22.62	106.32 ± 12.81	0.297	-23.10 ~ 74.27

LVD: large vessel disease, SVD: small vessel disease *P*-value < 0.05 was considered significant (*), while *P*-value < 0.001 was considered highly significant (**).

Table 5. Comparing serum levels of ZO-1, CLDN5 and PDGF-B between smoker vs non-smoker LVD patients and smoker vs non-smoker SVD patients.

Variable Mean ± SD	**LVD patients**		***p*- value**	**95% CI**
	Smokers N= 36	**Non-smokers N= 36**		
ZO-1 (ng / ml)	10.71 ± 1.23	12.44 ± 1.7	0.416	-2.54 ~ 6.00
CLDN5 (ng / ml)	7.87 ± 1.88	9.01 ± 0.64	0.569	-5.18 ~ 2.9
PDGF-B (ng / L)	167.18 ± 30.92	147.58 ± 22.01	0.609	-57.54 ~ 96.74
	SVD patients			
	Smokers N= 18	**Non-smokers N= 96**		
ZO-1 (ng / ml)	8.74 ± 0.86	13.92 ± 5.49	0.09	-0.909 ~ 11.27
CLDN5 (ng / ml)	7.51 ± 0.91	7.32 ± 0.63	0.904	-2.86 ~ 3.23
PDGF-B (ng / L)	68.00 ± 1.5	123.10 ± 13.05	0.07	-115.99 ~ 5.79

LVD: large vessel disease, SVD: small vessel disease *P*-value < 0.05 was considered significant (*), while *P*-value < 0.001 was considered highly significant (**).

4. Discussion

Ischemic heart disease: Studying the impact of IHD on serum levels of tight junction proteins in LVD and SVD IS patients revealed significantly decreased levels of ZO-1 and PDGFB in LVD patients with IHD compared to those with non-IHD, in addition to decreased levels of ZO-1 and CLDN5 in SVD patients with IHD compared to those with non-IHD. Although IS and IHD are the major symptoms for circulatory diseases, sharing common risk factors, and relying on the same underlying mechanism; ischemic injury and TJ alterations [10], but unlikely, studies concerning the impact of IHD on BBB integrity in IS patients are lacking. However, a few studies have addressed the impact of IHD on the expression levels of TJ proteins. Starting with ZO-1, the current results are in accordance with a previous study [11] which observed diminished ZO-1 expression levels in patients with IHD compared to healthy subjects, suggesting that ZO-1 is a key player in gap junction formation and stability. The current results are also in agreement with another study which revealed the importance of ZO-1 for appropriate heart function and that reduced ZO-1 levels are associated with IHD. The authors have also observed that variants within the ZO-1 encoding gene are associated with development of cardiomyopathy, which explains the reduced ZO-1 levels in patients with IHD [12].

Regarding PDGFB, the current results about decreased PDGFB levels in LVD patients with IHD, might be understood in light of a previous study [13] which revealed that inactivation of PDGFB signaling leads to cardiac abnormalities such as ventricular dilation and lack of coronary vascular smooth muscle cells (VSMCs). In addition, the deletion of PDGFRB (platelet-derived growth factor receptor B) from adult mice cardiomyocytes has been found to cause heart failure [14]. Our results are also in accordance with a previous study which recorded upregulation of PDGFRB and PDGFB seven days after ischemic reperfusion (IR) in mice, with blockage of the PDGFRB causing leaky blood vessels [15]. The current results can also be understood in light of previous studies which revealed that PDGFB plays essential roles in the development, homeostasis, and healing of cardiac tissues, and that it constitutes a promising drug target for the treatment of cardiovascular diseases. The authors stated that delivery of recombinant PDGF ligands reduces mortality and improves cardiac function in both rodents and porcine models during or after myocardial infarction [16].

On the other hand, the current results about decreased CLDN5 serum levels in SVD patients with IHD are in accordance with previous studies, which revealed that CLDN5 levels are reduced in at least 60% of patient with IHD compared to those with non-IHD [17]. The current results are also in agreement with a previous study which revealed that CLDN5 is reduced during myocardial IR, then increased again to ameliorate IR-induced cardiac dysfunction. The current results can be explained in light of the study which stated that CLDN5 is not only an integral part of the BBB, but is also essential for improvement of vascular structural integrity and barrier function of the myocardial vascular ECs [18].

Diabetes: In the current study, significantly decreased ZO-1 levels were observed in both diabetic LVD and SVD patients, with no significant changes in either CLDN5 or PDGFB levels. Although the current results are in contrast to some previous studies which indicated that BBB disruption is not observed in diabetics [19] and that there are no significant changes in ZO-1 levels in the brains of diabetic animals, however, several studies support the current results as they revealed that DM is an essential risk factor for a variety of central nervous system (CNS) disorders, including stroke [20] , in addition, several studies have revealed that DM induces BBB damage, and that BBB damage is the most proposed mechanism that adversely affects both the homeostasis and functions of CNS[21]. A progressive increase in the BBB permeability in mice with streptozotocin- induced DM has been reported; the midbrain, in specific, was susceptible to microvascular damage, induced by DM. In addition, even after control of hyperglycaemia, DM continued to induce microvascular damage [22].

Several mechanisms have been proposed to explain how DM leads to pericyte loss and BBB breakdown. It is assumed that hyperglycemia causes mitochondrial dysfunction and production of reactive oxygen species (ROS), ultimately leading to oxidative stress and inflammation [23]. In addition, it has been revealed that both hyperglycemia and the formation of advanced glycation end-products (AGEs) downregulate the TJ proteins claudin-5, ZO-1and occluding in pericytes and endothelial cells. Furthermore, the levels of occludin and claudin-5 markedly increase on the membrane-bound extracellular vesicles, which permits the influx and escape of blood constituents into the perivascular space [24].

The current results are also supported by previous studies which revealed an increased BBB permeability in both type 1 DM and type 2 DM [25], and that diabetes causes a progressive increase in BBB permeability by reducing the levels of TJ proteins [22, 23]. The current results are also concomitant with other studies which revealed that hyperglycemia down regulates ZO-1 in PCs and endothelial cells (ECs) of the BBB [24].

Hypertension: The current results about decreased serum levels of ZO-1 in hypertensive SVD patients are in accordance with the studies which revealed that TJ levels, including ZO-1, were gradually destroyed eight weeks after induction of HTN in stroke-prone hypertensive rats [26], suggesting that prolonged HTN can ultimately lead to disruption of BBB and induce white matter hyperintensities (WMHs), which are hallmarks for the pathogenesis of SVD. The current results are also in agreement with previous studies which revealed that chronic HTN causes both increased BBB permeability and compromised structure and function [27].

The current results are also concomitant with a previous study which reported BBB damage in brains of spontaneously hypertensive rats at the age of 6 months or older [28, 29]. However, not only prolonged or chronic HTN is associated with BBB leakage, but also short-term HTN. A previous study has revealed that short-term acute HTN is associated with changes in gene expression and remodeling of TJ proteins in ECs of cerebral microvasculature, and proposed that these are the major mechanisms of BBB breakdown during short-term acute HTN [30].

Smoking: In the current study, results did not reveal any significant differences, in any of the measured proteins, between smokers and non-smokers. In fact, studies investigating the impact of smoking on BBB integrity are numerous, but contradictory. Some studies suggested that nicotine contained in tobacco smoke negatively affects endothelial TJs and decreases ZO-1 expression [31], and that the deleterious impacts of nicotine are related to its content of reactive oxygen and nitrogen species (ROS and RNS) [32], which induces oxidative stress and inflammation, in addition to affecting ions [33] and glucose transporters [34] in cerebral microvessels.

On the other hand, other studies suggest that nicotine consumption does not affect the expression of TJ-associated proteins ZO-1 and CLDN5, and that smoking-induced BBB permeability is attributed to changes in the organization of TJ proteins rather than changes in their expression [35]. This hypothesis is supported by a previous study which provided an evidence that chronic infusion of nicotine induces a significant increase of BBB permeability; an effect that is mediated through altered distribution of ZO-1 by endothelial nicotinic acetylcholine receptors without any observation of decreased ZO-1 expression [31]. This assumption is also supported by a previous study which investigated the harmful effects of smoking on the expression and distribution of TJ proteins in the BBB, and did not reveal any significant changes in ZO-1 expression levels [36].

It is worth noting that the current results can be explained in light of the sample size; the number of smokers in the LVD group was 36 patients, opposed to an equal number of non-smokers, furthermore, the number of smokers in the SVD group was only 18 patients, opposed to 96 non-smoker patients, so that, studies on a larger sample size might reveal different results.

5. Conclusion

IHD, DM and HTN are significantly associated with decreased levels of serum markers of BBB integrity, and hence, they are associated with BBB injury; however, their impacts differ according to stroke subtype. Studies on larger sample size are needed to clarify whether smoking is also associated with BBB injury or not. Defining the extent of BBB injury associated with each risk factor could both help predict patients' prognosis and serve as a useful tool in making individualized treatment plans.

6. Abbreviations

IS: ischemic stroke; BBB: blood brain barrier, PDGFB: platelet-derived growth factor B; TJ: tight junction; ZO-1: zonula occluden-1; CLDN5:claudin-5; IHDL: ischemic heart disease, DM: diabetes mellitus; HTN: hypertension; LVD: large vessel disease; SVD: small vessel disease; PC: pericyte; CBF: cerebral blood flow; MRI: magnetic resonance imaging; PDGFRB: platelet-derived growth factor receptor B; VSMCs: vascular smooth muscle cells; IR: ischemia reperfusion; WMHs: white matter hyperintensities; ECs: endothelial cells. TOAST: Trial of Org 10172 in Acute Stroke Treatment.

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