Peripheral Biomarker for Vascular Disorders

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ABSTRACT: Atherosclerosis is the underlying cause of most myocardial infarction (MI) and ischaemic stroke episodes. An early sign of atherosclerosis is hypertrophy of the arterial wall. It is known that increased intima media thickness (IMT) is a non-invasive marker of arterial wall alteration, which can easily be assessed in the carotid arteries by high-resolution B-mode ultrasound. Similarly, the other key element of MI and ischaemic strokes is the N-methyl-D-aspartate (NMDA) receptor which is an ionotropic glutamate receptor that mediates the vast majority of excitatory neurotransmission in the brain. NMDA activation requires the binding of both glutamate and a coagonist like D-serine to its glycine site. A special enzyme, serine racemase (SR), is required for the conversion of L-serine into D-serine, and alterations in SR activities lead to a variety of physiological and pathological conditions ranging from synaptic plasticity to ischemia, MI, and stroke. The amount of D-serine available for the activation of glutamatergic signalling is largely determined by SR and we have developed ways to estimate its levels in human blood samples and correlate it with the IMT. This research based short communication describes our pilot study, which clearly suggests that there is a direct relationship between the SR, D-serine, and IMT. In this article, we will discuss whether the activity of SR can determine the future consequences resulting from vascular pathologies such as MI and stroke.

KEYWORDS: peripheral markers, vascular diseases, atherosclerosis, ischemia, NMDA receptors, serine racemase, D-serine, intima media thickness

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Background

Serine racemase levels and glutamatergic signalling

N-methyl-D-aspartate (NMDA), the ionotropic receptor channel of glutamate, requires its 2 binding sites (the glutamate site and the glycine site) to be occupied for its activation. In the literature, it is indicated that the D-serine, rather than glycine, is required to endogenously trigger the N-methyl-D-aspartate receptor (NMDAR) function.[1] D-serine is produced by the racemization of L-serine, by the action of a specific enzyme serine racemase (SR), which was identified more than a decade ago.[2] SR has therefore emerged as a new potential target for NMDA-receptor-based diseases.[3] Human serine racemase (hSR) has significant sequence homology (28% identity) with human serine dehydratase (hSDH); however, hSDH only catalyses the conversion of L-serine into pyruvate[4] and decreases the concentration of D-serine and hence the NMDA activities.

The release of glutamate, D-serine, and adenosine triphosphate (ATP) from astrocytes has diverse synaptic actions. Presynaptically, glutamate can access metabotropic glutamate and kainite receptors, whereas postsynaptically glutamate can act on the extrasynaptic NMDARs with D-serine as a coagonist to promote depolarization and excitation.

SR activities and products

SR plays a yin-yang role and act both as a generator and as a metabolizer of D-serine. SR converts the L-isomer into the D-isomer and can simultaneously degrade both L-serine and D-serine to NH_3 and pyruvate. The following is a brief explanation of SR biochemistry and all the factors such as pyridoxal 5′-phosphate (PLP), divalent cations, and ATP responsible for its inhibitory and excitatory actions. The highest levels of D-serine are found in astrocytes and neurons, where SR is localized, suggesting that D-serine is synthesized by SR.[5] SR belongs to a PLP-dependent enzyme.[6] In addition to PLP, SR binds to divalent cations (mainly Mg^{2+} but also Ca^{2+}) and has a nucleotide binding site which binds to the complex Mg-ATP with high affinity. Chelating Mg^{2+} greatly reduces the activity of the enzyme[6] and it is likely that the Mg binding site is important for proper folding of SR. The discovery of physiological cofactors of SR also disclosed the main chemical reaction catalysed by SR, that is, α, β elimination of water from L-serine to form D-serine, pyruvate, and NH_4.[6] For each molecule of D-serine, about 4 molecules of pyruvate are produced. SR also catalyses robust elimination of L-serine O-sulphate which is 500 times faster than the physiological racemization reaction, generating sulphate, ammonia, and pyruvate. This reaction provides the most simple and sensitive assay to detect the SR enzyme activity so far. L-serine O-sulphate is a better

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substrate for SR than L-serine and also an inhibitor of D-serine synthesis. Inhibition of SR provides a new strategy to selectively decrease the NMDAR coactivation and this may be useful in conditions in which overstimulation of NMDARs plays a pathological role in causing cerebrovascular ischemia.

Several plasma biomarkers such as high-sensitivity C-reactive proteins (hs-CRPs), low-density lipoprotein-cholesterol (LDL-C), lipoprotein-associated phospholipase A2, myeloperoxidase, oxidized LDL, lipoprotein (a), and isoprostanes are emerging non-specific biomarkers.[7] Thus, it is essential to have a very specific affordable biomarker for the early detection of stroke.

SR, D-serine, and blood vessels

SR is most active as a non-covalent dimer containing one or more free sulphydryls in the enzyme's active or modulatory site,[8] and this gave rise to the possibility of SR sensitivity to oxidative stress, which is the underlying basis of stroke[9] (Figure 1) acting through the astrocytic network to regulate the cerebrovascular system.[10]

Astrocytes express receptors for many neurotransmitters such as glutamate and ATP and their activation results in internal calcium oscillation and the accumulation of arachidonic acid (AA) causing both vasodilatory and vasoconstrictive actions through at least 2 of its metabolic pathways, that is, cyclooxygenase-2 (COX)-dependent accumulation of prostaglandin E2 (PGE2) leads to vasodilation, whereas the diffusion of AA to the smooth muscle, which contains high levels of cytochrome P450 4F2 (CPY4F2), is responsible for the conversion of AA into 20-hydroxyeicosatetraenoic acid (20-HETE) and causes vasoconstriction. Whereas these 2 opposing actions appear to be conflicting, because both have been observed to occur in vivo, the challenge is to identify the conditions that select for the respective actions (Figure 1).

Astrocytes are also considered to play an important role in brain pH homeostasis. Astrocyte processes surround synapses and possess transporters for the uptake of various neurotransmitters, including glutamate,[11] mainly through ionotropic NMDAR which needs D-serine for its activation. We hypothesized that altered D-serine concentration is associated with vascular pathology and as a coagonist of NMDAR; it plays an important role in the metabolism of excitatory amino acid glutamate through NMDAR. The pathophysiology of cerebrovascular disorders such as stroke has not been fully understood, but overstimulation of NMDAR appears to play a central role. This is of particular interest because D-serine concentration might be pharmacologically manipulated as the synthesizing and metabolizing enzymes of D-serine are known. We hypothesized that D-serine (product of SR) as a coagonist of NMDAR plays an important role in vascular pathologies such as stroke. We first tested our hypothesis in an animal model.

Materials and Methods

In vitro pilot study on rodents

Animal studies were approved by the Animal Ethics Research Committee, Faculty of Health Sciences, University of Brunei Darussalam. A total of 16 Wistar rats (200-250 g) were obtained for each sham and stroke model. Four rats per time point in both experimental and sham groups were anesthetized intraperitoneally with chloral hydrate (500 mg/kg). A skin incision was made between the eye orbit and ear to make the middle cerebral artery (MCA) visible. The MCA was coagulated by bipolar diathermy using a Gyrus Plasma Kinetic device to occlude MCA and the incision was sutured. For sham experiments, the MCA was exposed in the same manner as described above, but was not occluded. Animals from both the control (sham) and the occluded middle cerebral artery (OMCA) groups were decapitated at different time intervals of 1 hour and 4, 8, and 15 days. Both ischaemic (ipsilateral to surgery) and non-ischaemic (contralateral to surgery) cortices from the same rat were dissected.

High-performance liquid chromatography (HPLC) was used for the detection of L-serine and D-serine in the normal and OMCA brains. The sample preparation requires only homogenization in perchloric acid and centrifugation at 20 400*g* for 10 minutes before injection onto the column. The mixture was derivatized with a LiChrosorb RP-18 (10pm (polar mode)) column and a mobile phase consisting of a phosphate (NaH_2PO_4, 0.1 M)-methanol mixture with octylsulphonate (2.6 X M) at pH 8.35. For normal rats, L-serine was 576 ± 102 mmol/g of wet tissue and D-serine 69 ± 22 mmol/g of wet tissue; for stroke model rats on day 7 the concentration of L-serine in the ipsilateral (I) cortex obtained by HPLC was 745 ± 108 mmol/g of wet tissue and D-serine on day 7 and same side of occlusion was 235 ± 44 mmol/g of wet tissue.

Statistical analysis. Data are presented as the mean ± SEM. Comparisons between 2 groups were statistically made using the Student's *t*-test. Multiple comparisons were evaluated using an analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test, indicating a significant ($P < .05$) increase of D-serine concentration in OMCA rodents (Table 1). We observed higher values for both D-serine and L-serine, within an hour after the occlusion of medial cerebral artery, on both ipsilateral (I) and contralateral (C) sides. Then the concentration of L-serine dropped back to its original value within a week. In 15 days, the concentration of D-serine declines and that of L-serine increases indicating the exhaustion of SR activities. Table 1 suggests that the animal model of OMCA mimics stroke and the concentration of D-serine increases as a result of escalated SR activities. Hence, the cytotoxic effect of hyperactive NMDAR in the presence of its coagonist D-serine indicated an increase in SR activities before, during, and after the stroke.

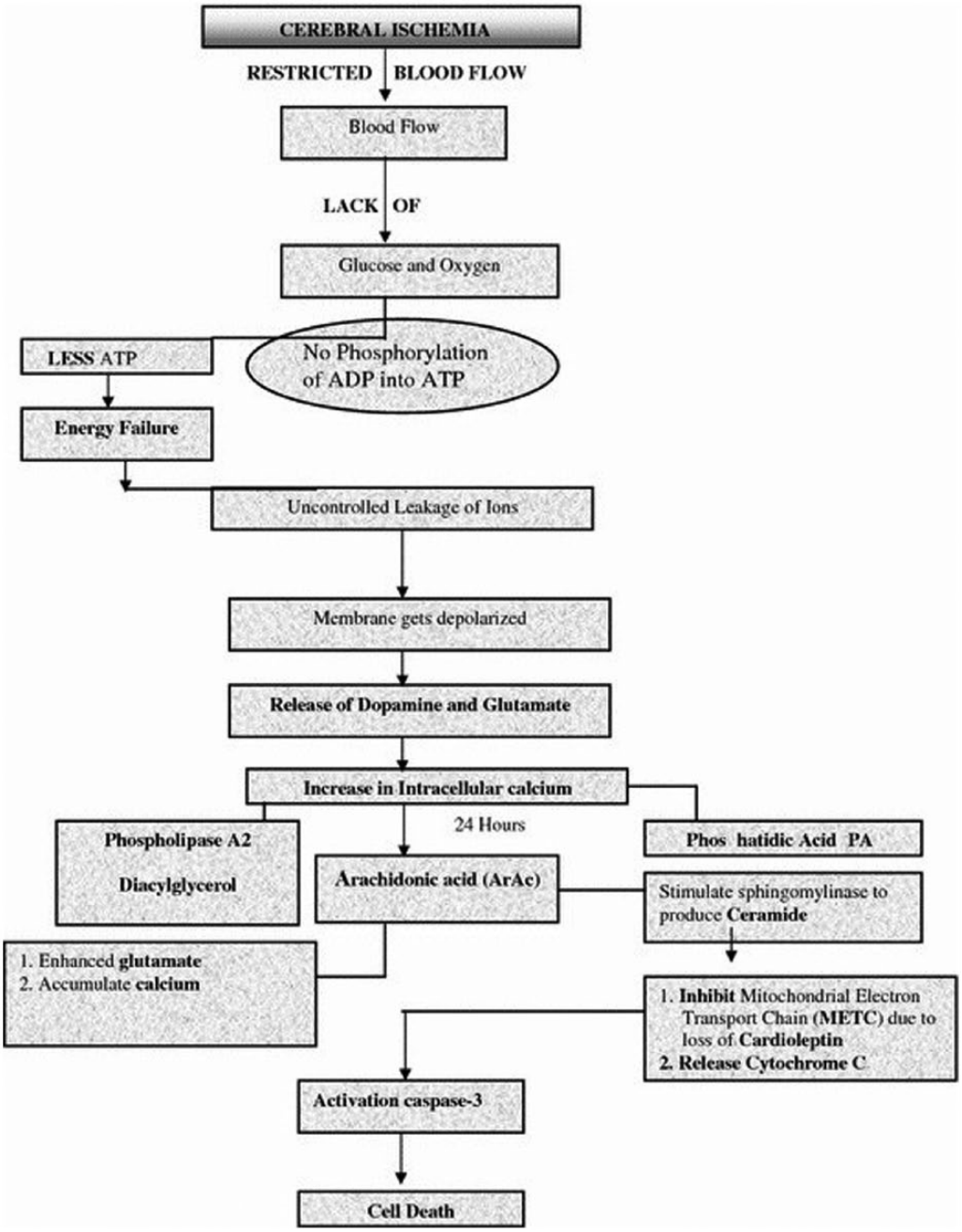

Figure 1. The inter-relationship of cerebral ischemia and resultant cell death.
Source: Adapted from Haydon and Carmignoto.[10]
ADP, adenosine diphosphate; ATP, adenosine triphosphate.

Animal studies were performed to see the effect of ischaemic brain (due to experimental occlusion of the medical cerebral artery) on the levels of L-serine and D-serine before measuring these chemicals in human subjects (suffering from transient ischaemic attacks [TIA]) and their normal counterparts.

Pilot study in a cohort of humans

The cardiovascular system is essential for supplying oxygen and nutrition and for the exchange of gasses, and any obstruction in the system gives rise to multiple pathologies. The walls of the blood vessels of both cardiovascular and cerebrovascular systems are susceptible to thickening, impeding the blood flow and causing ischemia and tissue death of the brain, heart, and other vital organs. Thus, it is essential to find out the early markers of atherosclerosis, as this condition remains silent till the cerebral, coronary, renal, and peripheral artery diseases occur.

This is a prospective case-control non-randomized study to find out the early markers of atherosclerosis. This study was performed after obtaining approval from the Human Ethic Committee, Raja Isteri Pengiran Anak Saleha (RIPAS) hospital, Ministry of Health Brunei Darussalam. A written informed consent was also obtained from each of the

Table 1. D-serine and L-serine contents in sham and OMCA rats (n = 4 for each time point).

TIME	D-SERINE (MMOL/G OF WET TISSUE) SHAM I AND C	L-SERINE (MMOL/G OF WET TISSUE) SHAM I AND C	L-SERINE AND D-SERINE RATIO SHAM I AND C
1 hour	69 ± 22 and 72 ± 19	745 ± 108 and 699 ± 88	10.799 and 9.708
4 days	79 ± 18 and 82 ± 17	694 ± 99 and 711 ± 78	8.785 and 8.671
7 days	88 ± 23 and 92 ± 31	805 ± 88 and 709 ± 60	9.148 and 7.707
15 days	91 ± 32* and 78 ± 16*	782 ± 78 and 725 ± 65	8.593 and 9.29
	OMCA I AND C	OMCA I AND C	OMCA I AND C
1 hour	110 ± 46* and 101 ± 38*	912 ± 89 and 839 ± 56*	8.291 and 8.307
4 days	121 ± 53 and 99 ± 24	881 ± 77 and 773 ± 54	7.281 and 7.808
7 days	235 ± 44* and 126 ± 51*	745 ± 108* and 801 ± 97*	3.170* and 6.357*
15 days	141 ± 42 and 110 ± 34	906 ± 81 and 887 ± 65	6.426 and 8.064

C, contralateral cortex; I, ipsilateral cortex; OMCA, occluded middle cerebral artery.
Values are expressed as mean ± SEM.
*$p < 0.05$.

Table 2. The demographic data along with average CIMT.

PARAMETER	TIA (N = 22)	CONTROL (N = 22)	*P* VALUE
Systolic blood pressure (mm Hg)	171 ± 26	122 ± 9.3	<.001
Diastolic blood pressure (mm Hg)	99.3 ± 15.5	79.3 ± 7.5	<.001
Blood sugar (mmol/L)	11.66 ± 1.32	5.8 ± 0.81	<.001
Kidney (ACR*) (mg/g)	283 ± 17	29 ± 49	<.001
Kidney (GFR*)	80 ± 11	92 ± 82	<.05
Cholesterol (mmol/L)	4.86 ± 0.82	3.91 ± 0.74	<.05
Triglyceride (mmol/L)	0.89 ± 0.47	0.72 ± 0.39	<.001
Low-density lipids (mmol/L)	2.95 ± 0.68	2.35 ± 0.41	<.001
High-density lipids (mmol/L)	0.89 ± 0.26	0.99 ± 0.38	<.05
CIMT left (mm)	1.1 ± 0.05	0.79 ± 0.02	<.001
CIMT right (mm)	0.98 ± 0.04	0.69 ± 0.02	<.001

ACR, albumin-to-creatinine ratio; CIMT, carotid intima media thickness; GFR, glomerular filtration rate; TIA, transient ischaemic attack.
Values are expressed as mean ± SE.
*$p < 0.05$

participants before running the biochemical tests and B-mode ultrasonography. Newly diagnosed TIA patients along with their age- and sex-matched healthy control subjects were recruited. There were 22 people in each group after screening them through the exclusion criteria of target organ damage after TIA such as stroke, heart failure, and kidney damage. The exclusion study was done using electroencephalography (EEG), electrocardiography (ECG), and renal function tests. Subjects with dyslipidaemia, diabetes, smokers, adrenal gland disease, and lung disease and pregnant and lactating subjects were also excluded. This information was obtained from their medical records.

To circumvent inter-observer variation, a single radiologist was assigned to perform all carotid examinations and was blinded to the status of all subjects. Measurements for carotid intima media thickness (CIMT) were repeated 3 times on each left and right carotid artery. To avoid any intra-observation differences, the average values for each set of 6 readings are represented as the mean ± SE for CIMT of each side (Table 2). CIMT increment is correlated with blood pressure, blood cholesterol, blood sugar, and kidney markers (Table 2).

We explored the possibility of finding a blood marker which imitates the ultrasound results of blood vessels, as ultrasound is expensive and cannot be done routinely. We observed for the first

Table 3. The concentrations of L-serine, D-serine, and total serine, measured by HPLC in both normal and TIA patients.

SUBJECTS	MG/DL	MMOL/L[a]	SERUM LEVELS
Control	1.960 ± 0.32	186.592	Total serine
TIA[b]	1.440 ± 0.21	137.088	Total serine
Control	0.025 ± 0.002	2.38	D-serine
TIA[b]	1.040 ± 0.002	99.08	D-serine
Control	1.935 ± 0.42	184.212	L-serine
TIA[b]	1.400 ± 0.12	133.28	L-serine

HPLC, high-performance liquid chromatography; TIA, transient ischaemic attack.
[a]Serum values in this column were obtained by multiplying column 1 with the conversion factor 95.2.
[b]CIMT > 1 mm.

time that the serum levels of D-serine (product of an enzyme SR) can tentatively predict the values of intima media thickness (IMT) of blood vessels obtained with B-mode ultrasonography. D-serine correlates with increased concentration and activity of SR in subjects from the TIA group (ie, individuals with more than 1 mm of CIMT), compared with their healthy counterparts.

We further examined the association between the concentrations of D-serine in the blood with the IMT of coronary vessels and found a direct relationship between them. HPLC was used to separate and quantify total serine, L-serine, and D-serine in both control and TIA patients. Thus, the measurement of D-serine will accurately reflect changes in SR levels and activity. Participants with normal levels (2.38 µmol/L) of D-serine have a CIMT of about 0.65 mm compared with

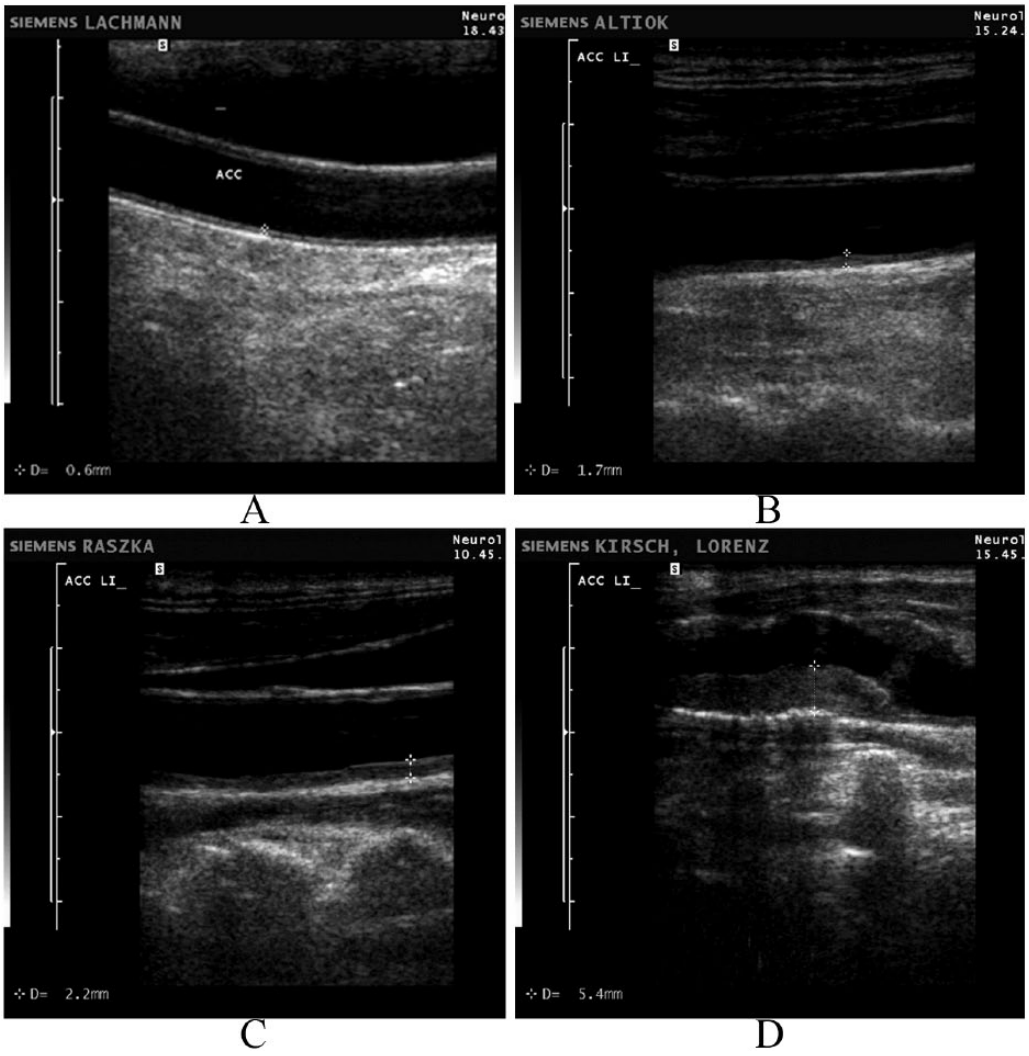

Figure 2. (A-D) Different degrees of thickness for the 2 innermost layers (intima and media) of blood vessels, which are measured using B-mode ultrasonography.
The * symbol towards the lumen represents the interface between the lumen and tunica intima of the blood vessel, whereas lower * exhibits the boundaries between the media and adventitia layers. These measurements are considered to be highly reliable markers for the status of atherosclerosis and will be very beneficial for correct diagnosis especially in asymptomatic conditions.

at-risk individuals with higher levels (3.808 µmol/L) of D-serine, where the CIMT was greater than 1 mm.

We found that both total serine and L-serine were low in TIA patients as compared with their normal counterparts, whereas D-serine concentration in patients with TIA was significantly higher ($P < .01$) than that in their healthy counterparts (Table 3), suggesting that the activity of SR may have increased in TIA patients.

Imaging data showed that the increase in the CIMT was highly correlated with the activity of SR and the concentration of its product D-serine.

The CIMT images (Figure 2) were obtained using *B-mode ultrasonography*. We found that the control subjects with a low concentration of D-serine had CIMT of less than 1 mm (Figure 2A), both of which increased with time (Figure 2B), whereas TIA patients with a CIMT value of 2.2 mm had a higher concentration of D-serine (Figure 2C). In some patients, with further increase in the concentration of D-serine, we observed higher values of IMT (5.4 mm). This increase in thickness could be due to the presence of calcified plaque in combination with intima hyperplasia (Figure 2D). Power Doppler recordings are shown in Figure 2.

Increasing CIMT over time correlates with increasing atherosclerosis, whereas a decrease in CIMT is indicative of atherosclerotic regression. In our experiments in humans, we found a direct relationship between increasing CIMT and elevating D-serine levels.

Significance and future directions

In total, 80 million adults (1 in 3 people) are affected by cardio/cerebrovascular diseases. Myocardial infarctions (MIs) and strokes occur because of atherosclerotic plaque formation, but unfortunately atherosclerosis remains asymptomatic, which leads to delay in treatment until it is too late. Our data indicate that there is a significant increase in the levels of D-serine which correlates with increased concentration and activity of SR in subjects from the 'at-risk group' (ie, individuals with more than 1 mm of CIMT), compared with their healthy counterparts. Thus, the measurement of D-serine may accurately reflect changes in SR levels and activity. Participants with normal levels (2.38 µmol/L) of D-serine had a CIMT of about 0.65 mm compared with at-risk individuals with higher levels (3.808 µmol/L) of D-serine, where the CIMT was greater than 1 mm.

Further studies are needed in a larger cohort to validate the exciting findings of our preliminary biochemical data supported by imaging, demonstrating that the activity of SR and the concentration of its product D-serine are correlated with the IMT.

Author Contributions

All authors contributed equally to the manuscript.

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