DOCTORAL (Ph.D.) THESIS

Biomarker studies in acute ischemic stroke

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ABBREVIATIONS

ACTIVE-W: Clopidogrel plus aspirin versus oral anticoagulation for atrial fibrillation in the Atrial fibrillation Clopidogrel Trial with Irbesartan for prevention of Vascular Events Trial

ACS: acute coronary syndromes

ADMA: asymmetric dimethylarginine

AF: atrial fibrillation

AHA: American Heart Association

AICS: acute ischemic coronary syndromes

AIS: acute ischemic stroke

ASA: acetilsalicyilic acid

ASA: American Stroke Association

ASCO: A for atherosclerosis, S for small vessel disease, C for cardiac source, O for other cause

ASCOD: ASCO + 'D' for dissection

ASPECTS: Alberta stroke program early CT scores

ASSERT: Asymptomatic Stroke and Atrial Fibrillation Evaluation in Pacemaker Patients

AUC: area under the curve

BBB: blood-brain barrier

CABG: coronary artery bypass graft

cAF: chronic atrial fibrillation

CCL-2: chemokine ligand 2

CCT: cranial computed tomography

CD-40L: Cluster of differentiation- 40L

CHA2DS2-VASc score:

CISS: chinese ischemic stroke subclassification

CKD: chronic kidney disease

CNS: central nervous system

COPD: chronic obstructive pulmonary disease

CRP: C-reactive protein

CT: computer tomography

CCS: Causative Classification System

CSF: cerebrospinal fluid

DALY: disability-adjusted life-years

DDAH: dimethylarginine dimethylaminohydrolase

DNA: deoxyribonucleic acid

DWI: diffusion-weighted imaging

EAE: experimental autoimmune encephalomyelitis

ECG: electrocardiogram

EDRF: endothelium derived relaxing factor

EEG: electroencephalography

EF: ejection fraction

ENGAGE AF-TIMI: The Effective Anticoagulation with Factor Xa Next Generation in Atrial Fibrillation–Thrombolysis in Myocardial Infarction Trial

eNOS: endothelial NOS

ESC: European Stroke Commitee

Gal3: galectin-3

GFAP: glial fibrillary acidic protein

HbA1c: Hemoglobin A1c

HDL: high-density lipoprotein

hsCRP: high-sensitivity C-reactive protein

hs-cTnT: high-sensitivity cardiac troponin T

IBM: International Business Machines

ICAM: intercellular adhesion molecule

IGT: impaired glucose tolerance

IL-6: interleukin-6

iNOS: inducible NOS

IQR: interquartile range

IV: intravenous

LDL: low-density lipoprotein

LPM: L-arginien pathway metabolites

LVEDV: left ventricular end diastolic volume

LVESD: left ventricular end systolic diameter

MBP: myelin basic protein

MCA: middle cerebral artery

MCP-1: monocyte chemoattractant protein 1

MIC-1: macrophage inhibitory cytokine 1

MMP: matrix metalloproteinase

MPV: mean platelet volume

MRI: magnetic resonance imaging

mRS: modified Rankin Scale

NADPH: nicotinamide adenine dinucleotide phosphate

NIHSS: National Institute of Health Stroke Scale

NLR: neutrophil-lymphocyte ratio

NLR: neutrophil-lymphocyte ratio

nNOS: neuronal NOS

NO: nitric oxide

NOS: NO synthase

NSE: neuron-specific enolase

NT-proBNP: N-terminal pro b-type natriuretic peptide

NY: New York

OAC: oral anticoagulant

OGTT: oral glucose tolerance test

PAF: paroxysmal atrial fibrillation

PNS: peripheral nervous system

PRMT: protein arginine methyltransferases

PTX3: pentraxin-related protein-3

RATE: Registry of Atrial Tachycardia and Atrial Fibrillation Episodes

ROC: receiver operating characteristic

rtPA: recombinant tissue plasminogen activator

SD: standard deviation

SDMA: symmetric dimethylarginine

SOS AF: Stroke prevention Strategies based on Atrial Fibrillation

SPAF-III: Prevention in Atrial Fibrillation Study III

SPSS: Statistical Package for the Social Sciences

SSS-TOAST:

TGFβ1: transforming growth factor beta 1

TOAST: Trial of ORG10172 in Acute Stroke Treatment

tPA: tissue plasminogen activator

UCH-L1: ubiquitin C-terminal hydrolase-L1

USA: United States of America

VCAM: vascular cell adhesion molecule

WBC: white blood cells

WHO: World Health Organization

I. Introduction

1.1 *Definition and epidemiology of acute ischemic stroke*

Stroke is defined by WHO as the clinical syndrome of rapid onset of focal (or global, as in subarachnoid haemorrhage) cerebral deficits lasting more than 24 hours or leading to death, with no apparent cause other than a vascular one [1]. Stroke was already diagnosed in the ancient times. For hundreds of years the treatment of this disease has changed dramatically. The first breakthrough in ischemic stroke therapy was the introduction of aspirin (ASA), followed by intravenous thrombolysis using recombinant tissue plasminogen activator (rtPA) and in recent years thrombectomy was introduced to therapy. Of all strokes, 87% are ischemic, 10% are intracerebral hemorrhage, and 3% are subarachnoid hemorrhage strokes [2]. It is now widely accepted that not all brain cells die immediately after stroke. Surrounding a core of severe and rapid tissue injury, brain cell death evolves more slowly in a heterogeneous area that has been called the penumbra [3-4]. Awakening with or experiencing the abrupt onset of focal neurologic deficits is the key feature of the diagnosis of ischemic stroke. In AIS, neuroimaging is essential, as it may identify the etiology of stroke, location of the lesion, potential stroke mimics, or contraindications to thrombolysis.

In 2013, stroke was the second most common cause of deaths (11.8% of all deaths) worldwide, after ischemic heart disease (14.8% of all deaths), and the third most common cause of disability (4.5% of disability-adjusted life-years [DALY] from all cause) after ischemic heart disease (6.1%). The highest stroke DALYs and mortality rates in 2013 were observed in Russia and Eastern European countries [5]. Costs to treat stroke are projected to more than double and the number of people having strokes may increase 20 percent by 2030, according to the American Heart Association/American Stroke Association. These cost projections for stroke are equivalent to those for other high-impact chronic diseases, like cancer and cardiovascular disease [6].

1.2 *Etiology of ischemic stroke*

The system that is mostly used by the clinicians to categorize ischemic stroke has been developed in the multicenter study known by the short name TOAST (Trial of ORG10172 in Acute Stroke Treatment). The TOAST classification system includes five categories: 1) large-artery atherosclerosis, 2) cardioembolism, 3) small-artery occlusion (lacune), 4) stroke of

other determined etiology, and 5) stroke of undetermined etiology. Diagnoses are based on clinical features and on data collected by tests such as brain imaging (CT/MRI), cardiac imaging (echocardiography, etc.), duplex imaging of extracranial arteries, arteriography, and laboratory assessments for a prothrombotic state [7]. Many etiologic stroke classifications have been proposed, varying between causative (classifies patients into a single etiologic subtype through a decision-making process) and phenotypic (uses clinical and diagnostic test findings and organizes this information into major etiologic groups) approaches. Main causes of ischaemic stroke can be seen on **Figure 1.1**. Nearly one third of ischemic strokes are of cardiogenic origin **Figure 1.2**.

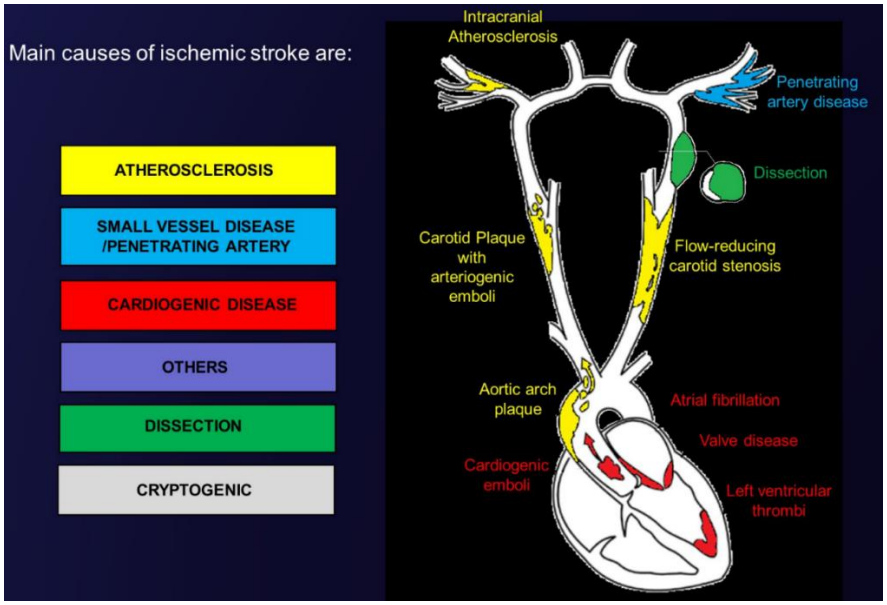

Fig. 1.1 Main caueses of ischemic stroke[8-9]

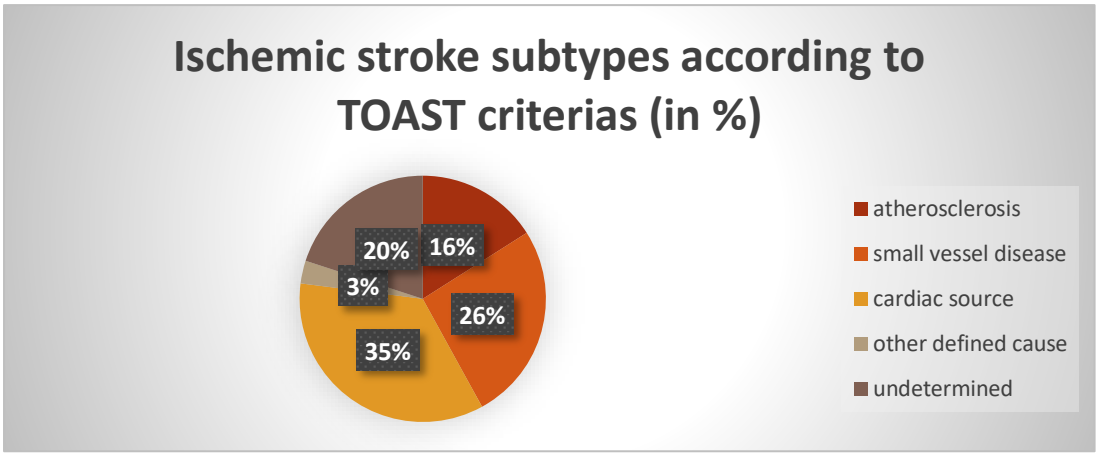

Fig. 1.2. Ischemic stroke subtypes according to TOAST criterias. [10]

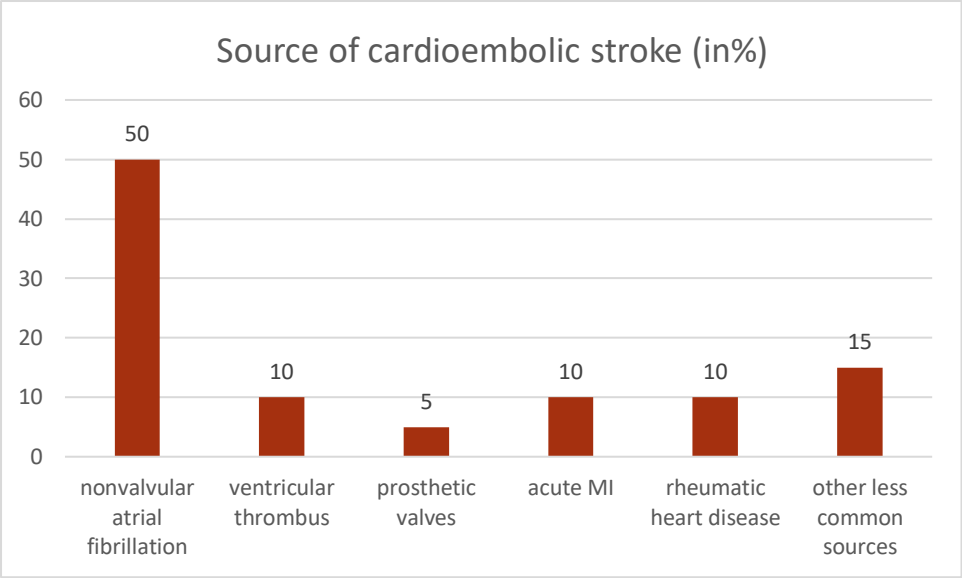

Fig. 1.3. Source of cardioembolic stroke [11]

1.2.1 *Atrial fibrillation in ischemic stroke*

Atrial fibrillation (AF) is the most common arrhythmia diagnosed in clinical practice [12].

According to a recent research project, the number of adults age 55 and over with AF will more than double in the European Union by 2060 [13]. Screening for silent atrial fibrillation emerges as an important opportunity to prevent atrial fibrillation associated complications [14]. AF is responsible for 50% of cardiogenic strokes **Figure 1.3**. Cardiac embolism causes more severe strokes than other ischemic stroke subtypes [15] and AF-related strokes result in more impairments (modified Rankin scale), more dependency (Barthel Index), and higher mortality [16-17]. Structural abnormalities reported in patients with AF are the following: extracellular matrix alterations (fibrosis, inflammatory changes, amyloid deposits), myocyte alterations (necrosis, apoptosis), microvascular changes and endocardial remodelling [18].

1.3. *Pathophysiology of ischemic stroke*

The pathophysiology of ischemic stroke is extremely complex and involves several processes **Figure 1.4**. This affected region of brain in acute ischemic stroke was found to be electrically silent but sufficiently active metabolically to sustain membrane potentials. Neurons within the penumbra are functionally impaired but still viable. Without reperfusion, the penumbra

collapses, brain cells die, and the lesion expands. Following ischemic stroke, neurons are deprived of oxygen and energy with detrimental effects on energy-dependent processes in neuronal cells [20]. Immediately after ischemic insult, neurons are unable to sustain their normal transmembrane ionic gradient and homoeostasis. This elicits many processes that lead to cell death: excitotoxicity, oxidative and nitrative stress, inflammation, and apoptosis. These pathophysiological processes are seriously injurious to neurons, glia, and endothelial cells [21-24].

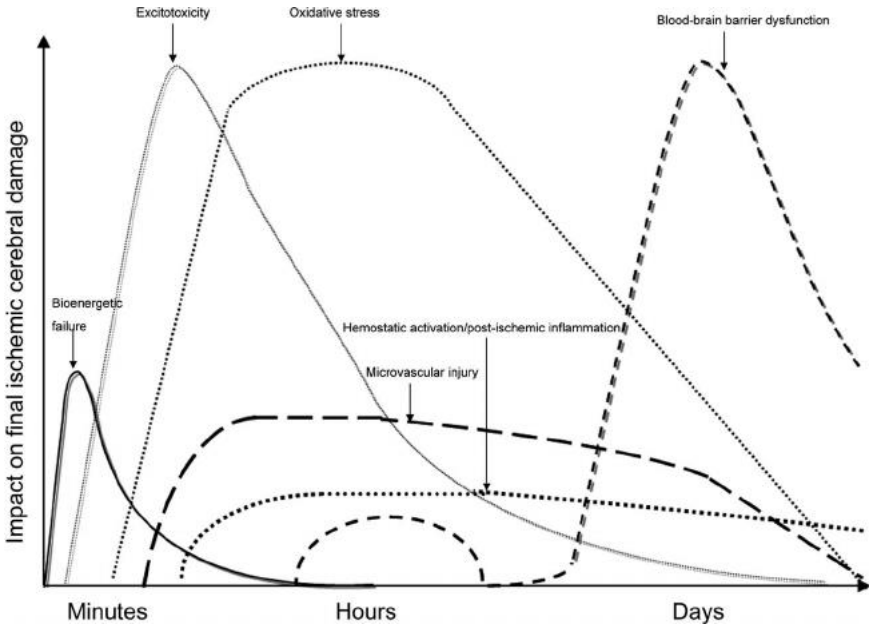

Figure 1.4. Timing of events in the ischemic cascade [19].

1.3.1 *Energy failure*

The depletion of cellular energy store due to failure of mitochondria causes further energy depletion and may trigger cell death due to apoptosis. Ischemia additionally causes loss of potassium and ATP, which are essential for energy exchange. It has been observed that energy failure does not precipitate immediate cell death, but 5–10 min of occlusion may lead to irreversible brain injury [25].

Other important mechanism is loss of membrane ion pump function and its harmful effects: Ischemia, leading to inadequate energy supply at the cellular level, leads to malfunction of ion gradient, resulting loss of potassium in exchange of sodium, chloride, and calcium ions.

This is accompanied by an inflow of water, resulting in rapid swelling of neurons and glia (cytotoxic edema) [26].

1.3.2 *Excitotoxicity*

Excitotoxicity, defined as cell death resulting from the toxic actions of excitatory amino acids and it leads to a number of harmful consequences, including impairment of cellular calcium homeostasis, generation of free radicals and oxidative stress, activation of the mitochondrial permeability transition, secondary excitotoxicity, and activation of several transcription factors and their genes expression [27]. Excitotoxic death requires the excessive influx of the extracellular Ca^{2+} via receptor-operated channels or voltage-sensitive Ca^{2+} channels [28]. The excessive intracellular Ca^{2+} initiates a set of molecular events that culminate in neuronal death. The increase in the extracellular glutamate concentration initiates a positive feedback loop, with further activation of glutamate receptors in adjacent neurons, and as a result, more Na^{+} inflow to neurons via monovalent ion channels that decrease ionic gradients and consume ATP, both of which promote further release of glutamate [29]. Excessive glutamate within the synapses ends up in glutamate receptors, at a pathophysiological level, triggering a series of events that can result in neuronal dysfunction and death [27].

1.3.3 *Oxidative stress*

Oxidative stress is generally defined as an imbalance that favors the production of free radicals over their inactivation by antioxidant defense systems [30].

Oxidative stress describes a condition in which cellular antioxidant defense are inadequate to keep the levels of free radicals below a toxic threshold. This may be either due to excessive output of “free radicals,” loss of antioxidant defenses, or both. A “free radical” is any chemical species capable of independent existence having one or more unpaired electrons. Free radicals are highly reactive and can directly oxidize and damage macromolecules such as proteins [31].

As we previously mentioned at 1.3.1, the depletion of cellular energy store results in insufficient ATP level. This leads to the inability of the neuron to maintain the ionic homeostasis; the increase in the formation of superoxide radical attached to a decrease in antioxidant activity can lead to an oxidative stress. The elevation of the free radicals leads the mitochondria to increase the production of free radicals and an oxidative stress in the

cell, resulting in the oxidation of proteins and lipids components of the structure of the cell membrane and DNA fragmentation. The result is necrotic cell death [32-33].

1.3.4 *Disruption of the blood–brain barrier (BBB)*

The blood–brain barrier (BBB) is a set of specialised structures located in the cerebrovasculature that tightly control the passage of molecules between the blood and brain parenchyma. After cerebral ischaemia the integrity of the BBB is compromised, allowing uncontrolled entry of molecules into the brain parenchyma that worsens damage caused by ischaemia [34]. BBB dysfunction after stroke appears to be biphasic, particularly after reperfusion [35]. Endothelial basal lamina dissolution starts as soon as 2 hours after the onset of ischemia [36] and is rapidly followed by an increase in BBB permeability [37]. An early reperfusion may temporarily alleviate BBB alterations, but if delayed, reperfusion will likely exacerbate the endothelial injury [38-39]. After the early BBB opening, there is a second phase of severe BBB injury taking place within 24–72 hours after infarction. This phase is characterized by leukocyte infiltration and marked elevation of MMP expression resulting in greater tissue damage [40-41].

1.3.5 *Inflammation*

Pathological features of ischemia such as necrotic cells, cell death debris, and increased reactive oxygen species (ROS) can induce neuroinflammation by activating resident microglia and astrocytes as well as attracting infiltrating leukocytes from circulating blood [42]. Stagnant blood flow and altered rheology induce shear stress on the vascular endothelium and platelets. This results in the deployment of P-selectin to the cell surface. P-selectin is stored in Weibel–Palade bodies in the endothelium and α-granules in platelets and can be released within minutes after activation. Selectins are crucial in slowing down circulating leukocytes by attracting them to the endothelial surface by interacting with P-selectin glycoprotein ligand-1 expressed on most leukocytes. Platelet P-selectin can bind also to leukocytes and act as a bridging molecule that promotes cluster formation of leukocytes causing intravascular clogging that further contributes to ischemic damage [43].

The complement system, the humoral branch of innate immunity, has been consistently implicated in the pathobiology of stroke and its activation is associated with unfavorable outcome [44]. In the setting of cerebral ischemic process, the complement system might be

activated intravascularly by proteases of the coagulation cascade, at the level of C3 and C5 cleavage, thus bypassing classical, alternative, and lectin pathways. However, activation of the alternative and lectin pathways might also contribute to ischemic brain injury. While intravascularly generated active complement proteins might gain access to the brain tissue through a damaged BBB, there is also evidence of increased complement synthesis by microglia [45].

The first-line responders to (CNS) injury are microglia and astrocytes. Microglia are the resident macrophages of the brain and a key modulator of immunologic responses after ischemic stroke [46]. Once activated by extracellular signals, they function to sweep debris and toxic substances by phagocytosis, thereby helping maintain normal cellular homeostasis in the brain [47]. Activated microglia also increase secretion of cytokines and leukocyte adhesion molecules within cerebral vasculature, all within 24 hours of the ischemic insult [48]. Astrocytes are the most prevalent cells in the brain. In uninjured brain tissues, astrocytes offer structural and nutritive support for neurons. After ischemic stroke, astrocytes play an important role in wound healing and repair by mediating reactive gliosis and glial scar formation [49] **Figure 1.5**.

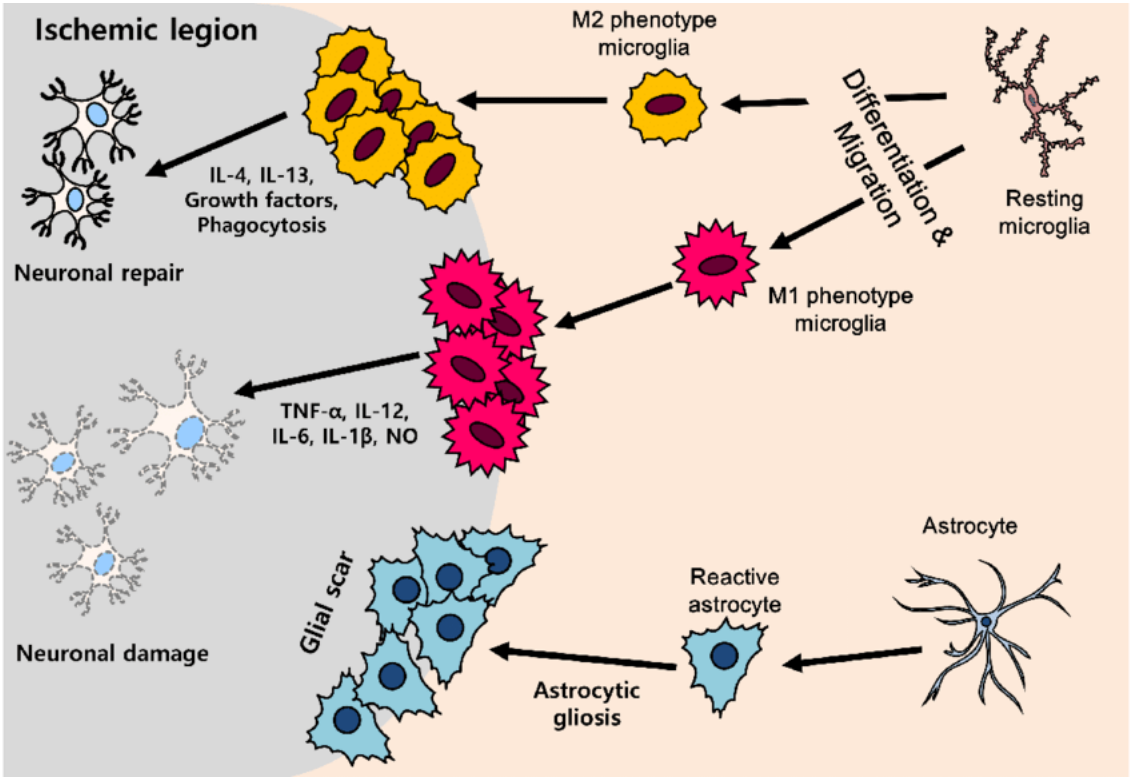

Figure 1.5. Immune signaling of microglia and astrocyte after ischemic injury. Resting microglia may be polarized to either the M1 or M2 phenotype. M1 microglia contribute to neuronal injury by pro-inflammatory mediators, whereas M2 microglia improve neuronal protection through antiinflammatory mediators and phagocytic functions. Astrocytes accumulate at the borders of the lesion, become reactive, and begin the formation of a glial scar [42].

1.3.6 *Hemostatic activation*

Hemostatic activation may itself exert a negative effect on prognosis, by stimulating thrombus propagation or recurrence, or trigger complicating venous thromboembolism. The magnitude of hemostatic changes in acute stroke may thus reflect the extent of cerebral damage, and also have implications for the course of disease, the effect of treatment, and the eventual outcome [50]. Activation of the coagulation cascades further generates inflammatory cues [51].

1.3.7 *Necrosis and apoptosis*

Many neurons in the ischemic area may undergo apoptosis after several hours or days, and thus they are potentially recoverable for some time after the onset of ischemic injury. In contrast to necrosis, apoptosis appears to be a relatively orderly process of energy-dependent programmed cell death to dispose of redundant cells [52]. There are two general

pathways for activation of apoptosis: the intrinsic and extrinsic pathways, **Figure 1.6**. Precipitating factors of apoptosis include oxygen free radicals, death receptor ligation, DNA damage, protease activation and ionic imbalance.

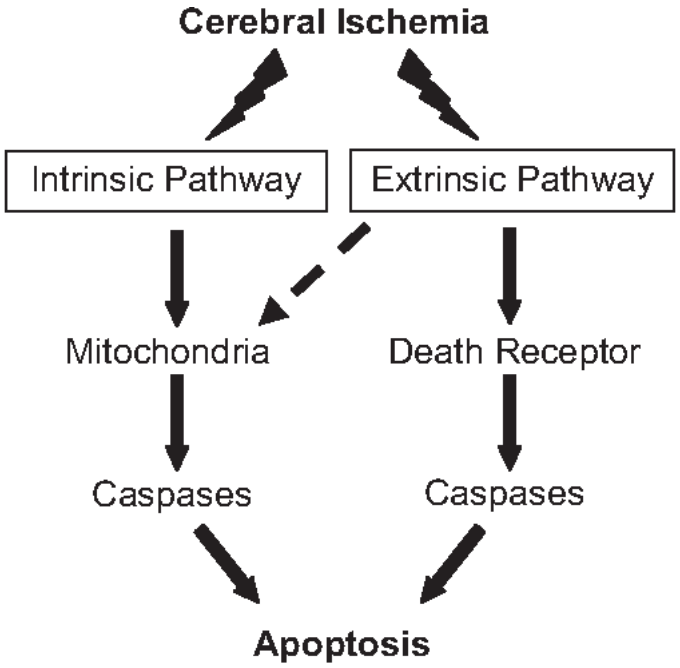

Fig. 1.6. Apoptotic signaling cascades after cerebral ischemia. Apoptosis can be initiated by internal events (ie, "Intrinsic Pathway") involving the disruption of mitochondria and the release of the cytochrome C, which leads to the downstream activation of caspases. Alternatively, cell surface receptors can be activated by specific ligands that bind to "death receptors" (ie, "Extrinsic Pathway") [53].

The alternative way to apoptotic cell death is necrosis, which is considered to be a toxic process where the cell is undergo an energy-independent mode of death. Although the mechanisms and morphologies of apoptosis and necrosis differ, there is some overlap between these two processes. Evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical process described as the "apoptosis-necrosis continuum"[54].

1.4. *Treatment and outcome of acute ischemic stroke*

The primary goal of treatment in acute ischemic stroke is to preserve tissue in the ischemic penumbra, where perfusion is decreased but sufficient to stave off infarction. Tissue in this area of oligemia can be preserved by restoring blood flow to the compromised area and optimizing collateral flow and regain functions of affected area of brain. Recanalization strategies, including thrombolysis and thrombectomy, attempt to establish revascularization so that cells in the area with decreased perfusion can be rescued before irreversible injury occurs. Restoring blood flow can mitigate the effects of ischemia only if performed quickly [55].

Thrombolysis with intravenous administration of recombinant tissue plasminogen activator (rt-PA) is only useful within a period of fewer than 3 or 4.5 hours. Moreover, the recanalization rate is less than 50%. Indications for endovascular therapy in AIS by intra-arterial thrombolysis or mechanical thrombectomy include patients with large-vessel occlusions and patients in the early postoperative phase when the systemic effects of IV rt-PA are not desirable [56].

Advances in acute stroke care have improved short-term (90-day) survival of patients with stroke and survivors have better short term functional outcomes. The adoption of stroke unit care with better management of stroke-related complications and effective reperfusion therapies have significantly contributed to improved survival. The largest degree of functional improvement after the stroke occurs in first 3 months and functional gains beyond this period are small [57].

II. Biomarkers in ischemic stroke and atrial fibrillation

2.1 *Definition*

The National Institute of Health Biomarker Definitions Working Group (1988) defines a biological marker (biomarker) as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention’ [58]. The definition is rather broad, as it includes a wide range of test areas (lab diagnostics, pathology, imaging procedures, ECG-EEG etc.) and classifies highly different technologies into one group. Sensitivity refers to the ability of a biomarker to detect the presence of a disease when the disease is present and specificity refers to its ability to exclude the disease when it is not present [59,60]. However, recently exlpored blood biomarkers are used with limitations in the care of patients with stroke.

2.2 *Development of a biomarker*

The recognition of a new marker is always preceded by a long (basic) research period. Pathological events of a given disease are centered on molecules whose quantitative/qualitative changes are correlated with certain clinical parameters of the disease. Here, a specific hypothesis is the basis for biomarker identification. This is the first phase, also called the discovery phase of biomarker development. Next, three phases can be distinguished. The second stage is the qualification phase, when the candidate biomarker level is determined in the body fluid used as a diagnostic sample and it is demonstrated that different concentrations can be measured in the samples of individual patients and their controls. In the third verification phase, population-level studies are performed on healthy controls and the goal is to verify the specificity of the test. Finally, the validation phase provides opportunity for development and testing of a clinical assay, in which samples are taken from patients and their controls. In addition to the clinical sensitivity/specificity data, potential clinical applications of the new marker are also investigated [61].

Strategies for the development of stroke biomarkers have taken two broad approaches. One approach is to identify molecules by targeting specific pathways known to be involved in cerebral infarction or hemorrhage, such as apoptosis, inflammation, hemostasis, cell death or oxidative damage. Another is the ‘-omic’ approach, which ranges from global profiling of

whole plasma or brain tissue to more focused analyses of vascular components including platelets, leukocytes and erythrocytes [62].

2.3 *Role of biomarkers in stroke*

The pathophysiology of cerebral ischemia has played a important role in guiding biomarker research in ischemic stroke. The expanding use of biomarkers in the field of stroke has made a substantial impact in our understanding of the pathophysiology of stroke and the treatment. Several categories of biomarkers are studied in stroke – physical markers, imaging markers, electrophysiological markers, histological markers, genetic markers, systemic (serum) markers and neuronal markers. The primary focus of molecular biomarkers of stroke has been their application to diagnosis. The model for a diagnostic marker is the cardiac isoenzyme test. Development of high-sensitivity techniques to detect the cardiac isoenzymes of troponin I and troponin T – products of myocardial degradation – led to the eventual revision of the clinical definition of myocardial infarction emphasizing the role of biomarkers in diagnosis [63]. The key property of the troponin test that led to its widespread incorporation into clinical care was a nearly absolute specificity for myocardial tissue (unlike creatine kinase-MB), as well as high sensitivity. Although a troponin-like diagnostic biomarker for stroke would be of interest, several other biomarker applications are possible and more likely to be of clinical importance [64] **Table 2.1.**

- Predict the risk of stroke
- Clarify pathological mechanisms
- Provide early and accurate diagnosis of stroke in the acute setting (differentiate stroke from mimics, ischemia from hemorrhage)
- Identify penumbra/core ratio and estimate risk of progression/worsening
- Explore stroke evolution (early deterioration)
- Predict outcome and estimate long-term prognosis
- Predict complications (haemorrhagic transformation)
- Help in decision-making (guidance of therapy)
- Clinical research and drug development

Table 2.1. Emerging role of stroke related biomarkers

2.4 *Type of biomarkers in stroke*

Stroke is the end point of a long pathophysiological process. The main factor is atherosclerosis, which gradually becomes a manifest clinical disease. Substantial data indicate that atherosclerosis is a life course disease that begins insidiously with the evolution of risk factors, giving rise to a subtle subclinical disease that culminates in overt cerebrovascular events [65]. Ischemic stroke affects the large intra- and extracranial arteries and small vessels, as well as a result of an embolic phenomenon of blood thrombus from the heart or aorta, resulting in an interruption and severe reduction of blood flow within the cerebral circulation [66]. Depending on the degree of hypoperfusion, an area with complete absence of flow can result in the infarct core, where neuronal death occurs within a few minutes, and a surrounding area called the penumbra, which suffers from a moderate reduction of blood flow and contains functionally impaired but viable brain tissue [67]. In the ischemic core region, brain cells undergo necrotic cell death producing an area that is electrically, metabolically and functionally inactive. By contrast, neurons in the ischemic penumbra are thought to be metabolically active, but electrically and functionally compromised. The penumbra has a variable outcome. If blood flow is not restored within a relatively short time, the penumbra undergoes the same destiny as the core region [66]. The ischemic cascade is characterized by the following biochemical events - energy failure, ion imbalance, acidosis, excitotoxicity, oxidative stress and inflammation, before culminating in cell death via necrosis or apoptosis [68]. In practice, the benefits of arterial recanalization and reperfusion are weighed against the risk of intracranial hemorrhage that is associated with early neurologic deterioration, malignant infarction and a high mortality [69]. The use of biological signatures of cerebral ischemia that takes into account the complex biology of stroke is appealing to clinicians as this facilitates an objective assessment of benefits and risks under different clinical scenarios [70].

All of the aforementioned pathophysiological mechanisms are targeted in biomarker research. We will try to give an overview of the mechanical basis of the non-exhaustive list of biomarkers, which looks at different aspects of stroke pathogenesis, with particular regard to diagnosis, stroke severity, outcome and cardiovascular etiology in **Table 2.2.**

Type	Biomarker	Description	Localization	Application in ischemic stroke
Brain injury marker	S100B	Calcium binding protein, Involved in cell cycle progression & differentiation	Astrocytes, Schwann cells; Melanocytes; Adipocytes	Could be used in stroke prognosis and prediction of infarct volume. Correlates with severity, and functional outcome
	NSE	Neuronal glycolytic enzyme	Neurons, Neuroendo-crine neoplasms	Prediction of infarct volume
	GFAP	Intermediate filament protein, role in cell structure,BBB, communication	Astrocytes, Testis, Liver	Differentiation between IS and HS
	H-FABP	involved in intra-cellular fatty acid transportation	Cytosolic protein	Early diagnosis of stroke. Potentially useful in stroke prognosis if used in combi- nation with other markers
	MBP	Myelination of CNS>PNS	Structural protein of the myelin membrane proteolipid produced by oligodendroglia cells	Can be useful for early diag- nosis of stroke, infarct volume, stroke severity
	UCH-L1	Play an important role in maintaining the structure and function of neuromuscular junction	Cytoplasmic enzyme from ubiquitin carboxy-terminal hydrolases family, expressed mainly in neurons and to a lesser degree in the gonads.	significantly increased serum UCH-L1 levels was detected in patients with ischemic stroke compared with the healthy control group [71]
Inflammatory markers	CRP	Acute phase penta-mericprotein. CRP binds and aggra-vates a variety of soluble ligands. Activates the clas-sica complement pathway.	Found in blood, and produced in the liver in response to IL-6	Increased levels have been correlated with increased risk of stroke, recurrent stroke, infarct volume, stroke severity, and long-term outcome. Can serve as a prognostic factor for functional outcome in the early phase of stroke.
	Il-6	Acute phase response, fever	Cytokine from T-cells+ macrophages	Infarct volume, stroke severity, poor functional outcome after stroke [72]
	Adhesion molecules (VCAM, ICAM-1)	Transmembrane immunoglobulin proteins involved in leukocyte endo-thelial cell signal transduction	soluble forms exist as a result of proteolytic cleavage from the cell surface and can be measured in biological fluids such as plasma serum or CSF	Can be useful makers of stroke severity, recurrent stroke and poor outcome.
	Matrix Metalloproteinases (MMP-9, MMP-2)	It is a Zinc-binding proteolytic (matrix degrading) enzyme with critical role in CNS via BBB break-down, demyelination, axonal injury and activation of inflammation.	Released from astrocytes and microglia involved in an inflammatory response.	Serum concentration of MMP-9 correlates to NIHSS scores and initial and final DWI infarct volumes
Markers of oxidative stress	uric acid	Purine metabolism product; has both antioxidant and pro-oxidant properties	Cytosolic protein	Inversely associated with the extent of neurological deficits on admission
Apoptotic pathway markers	caspase-3	Constitute the main molecular cascade in the apoptosis pathway	Located in different intracellular compartments	Plasma levels have been found to be elevated in acute stroke and they correlate with infarct growth and short and long-term neurological outcome
Neuroendocrin	copeptin	copeptin's function is still not fully under-stood. It is thought to have an effect on the cardio-vascular system and on the kidney	Hypothalamic stress hormone. It is a stable by-product of arginine-vasopressin synthesis	Risk factor for stroke. May be useful in prediction of outcome recurrent stroke.

Table 2.2. Potential blood biomarkers involved in the pathophysiology of stroke [73,74].

2.5 *Biomarkers related to atrial fibrillation*

While biomarkers have already implemented in the diagnosis and management of myocardial infarction and heart failure, the use of biomarkers has became less popular in atrial fibrillation management. Guideline recommendations on the use of biomarkers in atrial fibrillation were virtually non-existent until the 2016 European Society of Cardiology guidelines, which offered a class IIb recommendation to consider using biomarkers such as high-sensitivity troponin and natriuretic peptide to further refine stroke and bleeding risk in patients with atrial fibrillation [75]. Atrial fibrillation is one of the most important cause of ischemic stroke. Prediction of stroke risk in patients with AF based on clinical factors, electrocardiographic markers, serum biomarkers (inflammation and fibrosis) and on structural and functional markers [76].

2.5.1. *Electrocardiographic markers*

Patients with paroxysmal AF treated with aspirin plus clopidogrel or OAC have a similar risk for thromboembolic events than patients with sustained AF according to the ACTIVE-W trial conducted in 2007 [77]. In a large cohort of Japanese patients with AF, PAF (n=1588) was independently associated with lower incidence of stroke/systemic embolism than sustained AF (n=1716) [78]. In ENGAGE AF-TIMI 48 trial, patients with paroxysmal AF suffered fewer thromboembolic events and deaths compared with those with persistent and permanent AF [79]. A meta-analysis including 99,996 patients in 12 studies found that the rate of thromboembolism was higher in patients with non-paroxysmal AF as compared to those with paroxysmal AF [80].

Although previous data show that patients with paroxysmal AF (PAF) have a stroke risk similar to those with persistent or permanent AF, recent studies suggest that PAF is associated with a lower rate of stroke. Overall, there is a growing consensus that the risk of thromboembolism increases as patients progress from paroxysmal to sustained to permanent AF [76].

A similar correlation was found between the duration of atrial fibrillation and stroke risk. In ASSERT study, subclinical atrial tachyarrhythmias, without clinical atrial fibrillation, occurred frequently in patients with pacemakers and were associated with a significantly increased

risk of ischemic stroke or systemic embolism [81]. The SOS AF study, which included 10,106 patients with implanted cardiac devices who underwent at least 3 months of follow-up, found an association between AF episodes of duration 5 minutes or longer and stroke [82]. In the RATE study it was found that short-duration (20 sec>) atrial fibrillation did not increase stroke risk, but the duration longer than 20 sec associated with increased stroke risk [83].

2.5.2. *Inflammatory biomarkers in atrial fibrillation*

There is an increasing need for blood biomarkers that are capable of identifying patients at a significant risk of PAF. Currently, the optimal marker is still unknown. An ideal biological marker has the following characteristics: high sensitivity, high specificity, high predictivity, and rapid, simple, accurate, inexpensive, and reproducible detection in all relevant patients [84]. Based on their structure and function, four groups of biomarkers can be categorized: markers of inflammation, markers of fibrosis, markers with hormonal activity, and other markers. Active inflammation can generate AF, which in return causes an inflammatory response that further escalate atrial remodeling, resulting in arrhythmia, the so-called “AF begets AF” phenomenon. The progress is similar to a spiral: inflammation begets AF, and AF *per se* induces inflammation [85]. Pentraxin-3 (PTX3), a member of the long pentraxin family, seemed to be a promising inflammatory marker, however it was found a weak predictor of the recurrence of AF in a prospective trial with 382 patients [86].

The neutrophil-to-lymphocyte ratio (NLR) is a pivotal marker of subclinical inflammation, and it has been widely investigated in the prediction of cerebro- vascular diseases [84]. In a cohort study with 32,912 adult patients with AF revealed that the incidence rate of stroke increased across NLR quartiles and came to the conclusion that NLR is directly associated with the risk of stroke in patients with atrial fibrillation [87].

Anderson JL et al. found that high levels of CRP independently predicted an increased risk of AF supporting the theory that pathogenesis of AF may have an inflammatory basis [88] In a sub-study of SPAF-III (recruited 880 subjects), it was found that all-cause mortality, and vascular events, but not stroke, were more common in patients with high CRP levels [89].

2.5.3 *Markers of fibrosis*

Galectin-3 (Gal3) is a member of the galectin family, which consists of animal lectins that bind β -galactosides [90]. Plasma Gal-3 concentrations were measured in 3,306 patients and

found that higher circulating Gal-3 concentrations were associated with increased risk of developing AF over the subsequent 10 years in age- and sex-adjusted analyses but not after accounting for other traditional clinical AF risk factors [91]. Serum galectin-3 levels were also significantly higher in patients with persistent AF than those with paroxysmal AF according to Gurses et al., however their results come from a much lower number (76) of patients [92].

Numerous other atrial fibrosis marker are known in the literature, the most important are the following. Transforming growth factor β1 (TGFβ1) was one of the most important factors for accelerating atrial fibrosis. Lin et al. found that TGF-b1 increased in chronic AF (cAF) and paroxysmal AF (PAF) group compared with sinus rhythm group, but no difference was found in TGF-b1 levels between the cAF group and the PAF group [93]. Endogenous enzymes involved in extracellular matrix remodelling include the matrix metalloproteinases (MMPs), whose substrates are different types of collagen; consequently, the serum level of MMP-9 is considered to be a marker of extracellular collagen degradation [94]. Li et al. showed in a prospective study that MMP-9 levels increase gradually from paroxysmal AF through persistent AF vs. permanent AF [95].

Growth/differentiation factor 15 (GDF15) was first identified as Macrophage inhibitory cytokine-1 or MIC-1 [96]. It has several physiological functions including the regulation of proliferation and apoptosis in normal, injured, and transformed cells, but it also has pathological functions such as growth inhibition and overexpression in cancer cells [97]. A study included 67 patients with nonvalvular AF and 67 healthy persons found that patients with paroxysmal AF had a significantly higher serumlevel of GDF- 15 compared to the control group. According to multivariable analyses, GDF-15 was independently associated with paroxysmal AF [98].

2.5.4 *NT-proBNP and troponin*

Natriuretic peptides (NPs) are produced in the heart and released into the circulation in response to pressure and volume overload. In recent years, brain natriuretic peptide (BNP), its N-terminal prohormone (NT-proBNP), emerged as possible biological markers of atrial fibrillation. BNP levels were measured in 72 outpatients with chronic atrial fibrillation (cAF) and in 49 control patients without AF. BNP levels were significantly higher in patients with

AF (median value 131 pg/ml) than without AF (median value 49 pg/ml; p <0.001), and remained significantly higher after controlling for demographic and clinical variables [99].

In a cohort, consisted of 99 consecutive patients with acute cerebral infarction (23 patients with permanent and 13 with paroxysmal atrial fibrillation and 40 had noncardioembolic stroke) cardioembolic subtype was strongly predicted with > 95% accuracy assessed by plasma BNP and left atrial appendage flow [100]. The relation between NT-proBNP and AF was studied in 5445 Cardiovascular Health Study participants and found that its levels were strongly associated with prevalent AF. After a median follow-up of 10 years, NT-proBNP remained the strongest predictor of incident AF after adjustment for an extensive number of covariates, including age, sex etc. [101].

Troponin is a protein that plays a role in myocyte contractile function and determination of its exclusively cardiac-specific form that has provided a significant progress for the early diagnosis of coronary events [102]. Troponin consists of three subunits (troponin I, T, and C), which, together with tropomyosin, regulate the interaction between myosin and actin filaments of muscle contraction [102-103]. Troponin C does not have cardiac specificity and thus no assays have been developed to measure it [104]. It means that every time a method detects elevation of Troponin T or I, we may assure that the cardiac isoform is elevated. Troponin assays have improved over the past 10 years, and cTn high-sensitivity (hs) assays have been introduced in clinical practice since 2012. High-sensitivity cTn assays have two main advantages over conventional assays. First, an improved diagnostic accuracy of TnT when measured with the hsTnT assay, specially in patients presenting early after the onset of symptoms. Second, an incremental prognostic value of the high-sensitive assay, allowing improved risk stratification also in patients with negative troponin T levels (measured with conventional assays) on admission [105]. Nevertheless, it is demonstarted that elevated cardiac troponin levels may occur in conditions other than acute coronary syndromes, as it happens with supraventricular tachyarrhythmias [106]. In ARISTOTLE trial, hs-TnI was analyzed in 14 821 atrial fibrillation patients. The hs-TnI assay detected troponin (≥1.3 ng/L) in 98.5% patients, and 9.2% had levels ≥23 ng/L (considered elevated). During a median of 1.9 years follow-up, the hs-TnI level is independently associated with a raised risk of stroke, cardiac death, and major bleeding [107].

III. Aims

The aim of the present thesis is to test if biomarkers released by pathyphysiological processes during acute ischemic stroke have a potential to predict outcome or disease progression.

Specific aims:

1 Our first aim was to prove evidence that L-arginine pathway metabolites (LPM) have a potential to discriminate between types of atrial fibrillation in ischemic stroke. Therefore, we aimed to explore the absolute concentration of L-arginine, ADMA, SDMA as well as their ratios in patients with different types of AF or sinus rythm and analyze the relationship among the LPM and clinical variables in the subacute phase of acute ischemic stroke.

2. Secondly, we also intended to investigate the relationship between early L-arginine and dimethylarginine plasma levels and outcome of ischemic stroke at discharge and 6 months later in a prospective study. Moreover, we explored whether L-arginine pathway metabolites predict worsening of 6-months clinical outcome compared to baseline.

3. Thirdly, we investigated the association of serum concentration of cardiac troponin (high-sensitivity cardiac troponin T [hs-cTnT]) with thrombo-inflammatory markers, in patients with acute ischemic stroke (AIS) without cardiovascular complications.

IV. L-arginine pathway in acute ischemic stroke

4.1. *L-arginine pathway in general*

The vascular endothelium offers numerous functions, all of which serve to maintain vascular tone as well as blood fluidity and provide homeostasis in the event of vascular injury. Specific endothelial functions include control of vascular tone, modulation of vascular structure by regulation of angiogenesis and proliferation, maintenance of a selective permeability barrier, regulation of lipid oxidation and mediation of immune responses [108]. Additionally, the endothelium plays a major role in interactions between circulating blood and the vessel wall by responding to hemodynamic influences as well as neurohumoral and inflammatory factors [109]. Perturbations, such as those that may occur at sites of inflammation or high hydrodynamic shear stress, disrupt these activities and induce endothelial cells's to create a prothrombotic and antifibrinolytic microenvironment [110]. Local endothelial dysfunction can stimulate leukocyte infiltration, smooth muscle cell migration from the media to the intima, smooth muscle cell proliferation and the formation of foam cells. These mechanisms form the basis of atherogenesis [111].

Nitric oxide (NO) is commonly considered as a toxic gas, but it was found to transmit biological information as a signal molecule 40 years ago. At first, it was recognized that the endothelium released a factor which relaxed vascular smooth muscle cells and subsequently caused vasodilatation in the late 1970s [112-113]. The molecular structure of this factor was unknown, it was named endothelium-derived relaxing factor (EDRF) until Furchgott and his colleagues confirmed that EDRF was NO, a colorless, odorless gas 10 years later [114] (receiving the Nobel Prize in Physiology or Medicine for this discovery—shared in 1998 with Louis Ignarro and Ferid Murad) [115]. The main physiological functions of NO include the maintenance of vascular tone, the reduction of inflammation response, the balance of thrombotic-thrombolytic homeostasis and the regulation cell growth [116]. Endogenous NO is derived largely from enzymatic pathways, but a non-enzymatic pathway also exists. Enzymatic NO formation is catalyzed by NO synthase (NOS) through a series of redox reactions, with degradation of L-arginine to L-citrulline and NO, and in the presence of oxygen and NADPH[117-118]. Three isoforms of NOS are recognized: endothelial NOS (eNOS

or NOS3), neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS or NOS2). iNOS-derived NO and nNOS-derived NO play neurotoxicity, but eNOS-derived NO plays a neuroprotective role in acute ischemic stroke. The toxic effects of NO produced by iNOS and nNOS are mainly due to the production of nitrates and the release of free radicals, which directly damage mitochondrial enzymes and genetic materials [119-122]. On the contrary, neuroprotective effects of NO produced by eNOS are achieved primarily by regulating vascular bed and peripheral nerve tissue [123].

NO synthesis can be inhibited by an endogenous compound, NG,NG-dimethylarginine (asymmetrical dimethylarginine, ADMA) [124]. Symmetrical dimethylarginine (SDMA) is the structural isomer of the NOS inhibitor asymmetric dimethylarginine. SDMA does not directly inhibit NOS but is a competitor of arginine transport [125]. ADMA and symmetric dimethylarginine (SDMA) are protein breakdown products of L-arginine [126]. The biochemical pathways related to the synthesis and metabolism of SDMA and ADMA are illustrated in **Figure 4.1.**

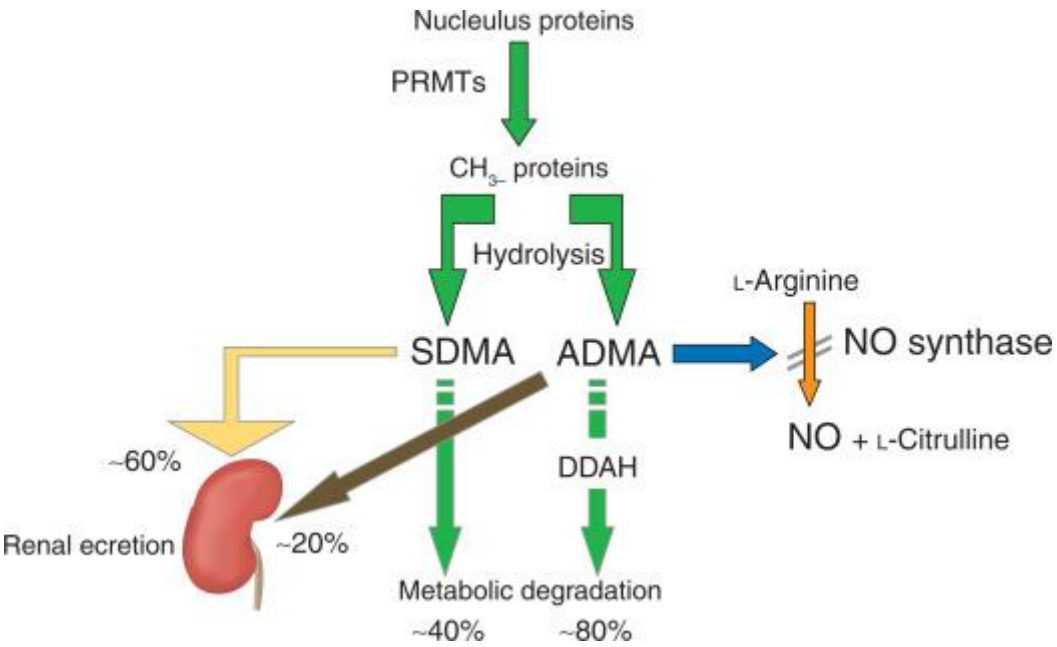

Fig. 4.1. Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are continuously generated during protein turnover. The primary source of these methylarginines are nucleolar proteins. Protein arginine methyltransferases (PRMTs) methylate these proteins, and ADMA and SDMA are then generated by hydrolysis. The bulk of ADMA (80%) is degraded by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), and the remaining 20% is eliminated by the renal route. SDMA is mainly excreted in the urine, and it is not degraded by DDAH.

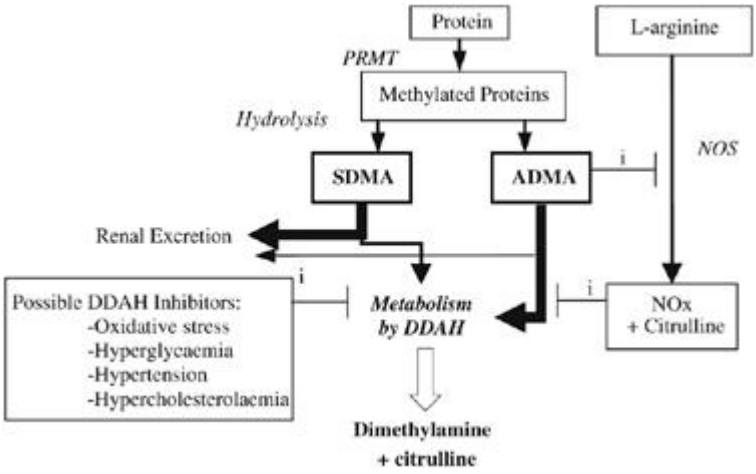

Fig. 4.2. Diagram representing production and degradation of ADMA and SDMA and the synthesis of nitric oxide. NOS, nitric oxide synthase; PRMT, protein arginine methyltransferase; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; NOx, nitric oxide; DDAH, dimethylarginine dimethylaminohydrolase; i, potential inhibition.

Free ADMA and SDMA are released following proteolysis **Figure 4.2**. A healthy adult produces 60 mg (~300 µmol) ADMA per day, of which approximately 20% is excreted in urine via the kidneys [127]. In contrast to ADMA, SDMA is present at only ~50% of the levels of ADMA and the elimination of SDMA is largely dependent on urinary excretion [128]. In adults, plasma ADMA levels increase with age and the mean plasma concentration of ADMA for a healthy adult is between 0.4 and 0.6 µmol [129]. At physiological extracellular L-arginine and ADMA concentrations, intracellular NOS is well saturated with the substrate L-arginine and physiological levels of NO are produced. In the presence of pathological concentrations of ADMA, NOS activity decreases, resulting in a reduction of NO. Cellular ADMA levels can be 5- to 20-fold higher than those in the plasma and can fall in the range known to inhibit NOS [130].

4.2. *Role of L-arginine pathway in atherosclerosis*

Numerous clinical studies have demonstrated elevated ADMA and SDMA levels in a wide spectrum of human diseases especially in vascular conditions. Increased plasma ADMA levels are associated with clinical conditions mainly associated with endothelial dysfunction such as hypertension [131], peripheral arterial occlusive disease [132], hypercholesterolemia [133], preeclampsia [134], diabetes mellitus [135-136], stroke [137], obesity [138], coronary artery disease [139].

4.3. *L-arginine pathway and atrial fibrillation*

4.3.1. *Atrial fibrillation and endothel dysfunction*

Several studies have demonstrated that a beat-to-beat variation in the blood flow, which occurs in patients with AF, adversely affects the endothelial function by modulating the production of vasoactive substances produced by endothelial cells [140]. Myocardial blood flow alterations caused by decreased generation of nitric oxide (NO) causes endothelial dysfunction [141]. Elevated level of plasma asymmetric dimetylarginine (ADMA) contribute to the development of vascular injury and increased cardiac oxidative stress [142]. A study demonstrated that systemic ADMA levels were increased in patients with AF compared to sinus rhythm (SR) and after successfull cardioversion, ADMA declined to normal levels suggesting that the higher ADMA levels are a consequence of AF [143]. Evidences suggested that the persistent form of AF may independently contribute to endothelial dysfunction [144]. Moreover, endothelial dysfunction is reversible in patients with AF after sinus rhythm restoration with cardioversion or catheter ablation [144-146].

Nitric oxide (NO) plays a role in maintaining vascular integrity [147]. NO is synthetized by the oxidation of L-arginine, which can be inhibited by ADMA, symmetric dimethylarginine (SDMA) competes with arginine uptake and antagonizes the effects of L-arginine [148].

ADMA and SDMA, were first isolated from human urine in 1970 [149].

Emerging clinical and experimental evidences indicate that ADMA and SDMA are involved in the pathophysiology of endothelial dysfunction, atherosclerosis, oxidative stress, inflammation and apoptosis [150]. All of these pathological processes play pivotal role in the development of AF. Therefore, we aimed to explore the absolute concentration of L-arginine, ADMA, SDMA as well as their ratios in patients with different types of AF or sinus rythm and analyze the relationship among the L-arginine pathway metabolites and clinical variables in the subacute phase of acute ischemic stroke.

4.3.2. *Patients and methodes*

The study was approved by the Local Ethics Committee of the University of Pecs. An informed consent was obtained from each patient.

4.3.2.1. *Subjects and measured parameters*

A total of 46 patients with acute ischemic stroke and 10 healthy subjects were enrolled into this prospective study after admission to hospital, between January 2015 and April 2016, at the Department of Neurology, University of Pecs, Hungary. Exclusion criteria were primary intracranial haemorrhage, refusing to participate in the study, any known immunological disturbances and chronic renal failure (estimated glomerular filtration rate, eGFR <50 and/or creatinine >120 μmol/l at two distinct measurement). Ischemic stroke was defined as an acute focal neurological deficit with cranial computed tomography (CCT) evidence of infarction. To define the size of infarct, we dichotomized Alberta stroke program early CT scores (ASPECTS) [145]: larger area involved: 0–7 or smaller area involved: 8–10 scores.

The severity of stroke was measured by the National Institute of Health Stroke Scale (NIHSS) on admission. Baselinne NIHSS 0-1 was considered as mild stroke, 2-8 as moderate stroke and ≥9 as severe stroke. Clinical data recorded from all patients included demographic characteristics, smoking status, stroke risk factors such as hypertension, diabetes, serum lipid parameters and creatinine on admission. Neuroimaging was performed by Siemens SOMATOM Definition 64 Slice CT Scanner on admission. The neutrophil-lymphocyte ratio (NLR) as a novel systemic inflammatory marker and a prognostic indicator of cardiovascular disease was calculated using data obtained from the complete blood count differential analysis. NIHSS was also recorded 24 hours later. NIHSS on discharge and modified Rankin Scale (mRS) at 6 months after stroke were used as outcome measures.

4.3.2.2. *Atrial fibrillation classification*

The patients were divided into three subgroups based on diagnosis of either sinus rhythm, paroxysmal AF or permanent AF, which were made according to the relevant ESC guideline [151]. All patients in the control group have sinus rhythm.

To clearly define the type of rhythm in each patient, we followed an inpatient and outpatient screening method based on the recommendation of the AHA/ASA joint statement of stroke prevention [152]. Patients with documented long-standing persistent (lasting longer than 1 year) AF were defined as permanent AF group. Paroxysmal AF group consisted of two category: 1. Every patient who presents with AF for the first time but during hospitalization proved to be self-terminated (within 48 h) were considered paroxysmal AF. 2. Previously

documented self-terminating AF with admission sinus rhythm also considered paroxysmal AF. Patients with sinus rhythm on admission and with no evidence of AF on inpatient and outpatient screening methods (continuous ECG monitoring or Holter ECG, Nihon Kohden Cardiofax GEM ECG-9022K) considered as sinus rhythm group. At follow-up visit after 6 months, a 12-lead ECG was recorded to document the rhythm and rate, and to investigate disease progression. All patients underwent transthoracal echocardiography (General Electric Vivid E9) during hospital stay, performed by skilled and trained cardiologist to record atrial parameters, seeking evidence of heart failure or any relevant structural abnormality of the heart.

4.3.2.3. *Blood collection*

Plasma samples were drawn from the patients 24 hours after admission. Venous blood samples from 10 healthy controls and 46 patients were collected. The samples were immediately centrifuged at 3000/min for 15 minutes. The supernatant was stored at −80°C until analysis. Concentration of L-arginine, ADMA, and SDMA were measured in the plasma by high-performance liquid chromatography (HPLC) as described previously [153].

4.3.2.4. *Statistical analysis*

Statistical analyses were performed using SPSS Statistics version 20.0 (IBM, 35 Armonk, NY, USA). Categorical data were summarized by means of absolute and relative frequencies (counts and percentages). Quantitative data were presented as mean and 95% confidence interval, as well as mean ± SD. The distribution of values was assessed by using the Shapiro-Wilk test. Non-normally distributed data were presented as median and interquartile range (25th-75th percentiles). Due to normally distributed data, parametric methods (chi-square test for categorical data, Student- t test for continuous data) were used for demographic and clinical data. Comparison of the variables between the subgroups was performed by using the Kruskal-Wallis test, because these parameters did not follow the normal distribution. Correlation analysis was performed calculating Spearman's correlation coefficient (r). A p-value < 0.05 was considered statistically significant.

4.3.3. *Results*

Forty-six patients (mean age 66.8 ± 8.4, male 57%, female 43%) were prospectively enrolled within 24 hours after onset of ischemic stroke. In the total study population, 46% of the patients were smokers, 89% had hypertension, 30% had a previous ischaemic heart disease or MI and 22% had diabetes.

Demographics, risk factors, biomarkers are summarized by rhythm status in **Table 4.1**. Individuals with either paroxysmal or permanent AF were about 10 years older and had a higher CHA2DS2-VASc score than those with sinus rhythm. Patients with any type of AF tended to have higher level of creatinine on admission, as well as a larger left atrial diameter ($p<0.001$). NLR also showed a significant higher value in individuals with both paroxysmal and permanent AF ($p=0.029$). In the total study population, serum glucose concentration (0.436; $p=0.002$), age (0.341; $p=0.02$) (**Figure 4.1**) and antiplatelet therapy (-0.357; $p=0.015$) showed significant associations with NLR on cross-sectional analysis. There were no clinically relevant differences in the left ventricular ejection fraction between groups.

Table 4.1. Demographic and clinical characteristics of stroke patients according to rhythm groups

	sinus rhythm (n=18)	paroxysmal AF (n=17)	permanent AF (n=11)	*p*-value
Age (y)	60±7	72±5	70±6	0.000
Female (%)	5 (11)	10 (59)	3 (27)	0.807
BMI (kg/m^2)	27±3	29±3	29±5	0.078
Current smoker (%)	10 (56)	4 (24)	7 (64)	0.935
Hypertension (%)	16 (89)	15 (88)	10 (91)	0.897
Diabetes (%)	4 (22)	3 (18)	3 (27)	0.845
Severe stroke (%)	5 (28)	2 (12)	4 (36)	0.892
Moderate stroke (%)	10 (56)	13 (76)	5 (45)	0.523
mRS score at follow-up	2±1.9	1.8±2	2.7±2.1	0.440
History of IHD (%)	2 (11)	4 (27)	6 (55)	0.042
Anticoagulant therapy (%)	1 (6)	11 (65)	10 (91)	0.000
CHA_2DS_2-VASc score	2.5 (2-3)	5 (5-6)	6 (5-7)	0.000
CRP (mg/L)	3.75 (3-8)	4.7 (2-17)	6 (2-25)	0.174
creatinine (mmol/L)	71±12	80±20	85±34	0.165
WBC (G/L)	9.1±2	8.4±4	10±3	0.942
LDL (mmol/L)	2.62±0.95	2.82±1.5	2.32±0.7	0.498
HDL (mmol/L)	1.48±0.4	1.4±0.4	1.2±0.4	0.066
cholesterin (mmol/L)	5±1.3	4.9±1.5	4±1.1	0.073
NLR	2.7±1.5	3.8±3	4.8±3.1	0.029
MPV	8±1.1	8.1±0.9	9±2	0.218
LVESD (mm)	31±2	30±3	36±10	0.432
LVEDV (mm)	49±3	48±4	50±8	0.716
EF (%)	59±6	60±5	52±9	0.051
left atrial area (cm^2)	19±3.1	22.5±3.6	24.4±4.7	0.000

Note: BMI: body mass index; Severe stroke: NIHSS 2-9 on admission, moderate stroke: NIHSS >9 on admission; mRS: modified Rankin Scale; CHA_2DS_2-VASc score: congestive heart failure, hypertension, age 75 years (doubled), diabetes, stroke (doubled), vascular disease, age 65 to 74 years, sex category (female); CRP: C-reactive protein; IHD: ischemic heart disease; WBC: white blood cells; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; NLR: neutrophil-lymphocyte ratio; MPV: mean platelet volume; LVESD: left ventricular end systolic diameter; LVEDV: left ventricular end diastolic volume; EF: ejection fraction. The discret variables are expressed as absolute numbers and percentage and continous variables as mean ± SD or median and interquartile range (25th - 75th percentiles).

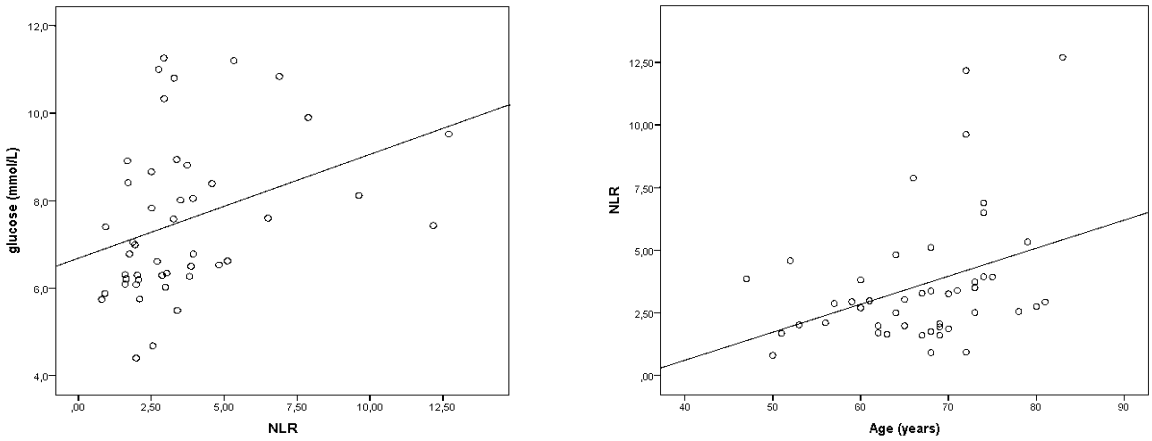

Fig. 4.1. Correlation of neutrophil-lymphocyta ratio (NLR) and serum glucose level 24 hours after stroke onset (A). Correlation of NLR and age (B). Data are presented as mean and 95% confidence interval.

4.3.3.1. *L-arginine metabolites in atrial fibrillation subgroups*

Concentration of ADMA was significantly higher in the plasma of patients with permanent AF compared with paroxysmal AF (median: 0.894, IQR: 0.86-1.37 vs. 0.568, 0.47-0.7, p=0.001) (**Figure 2**). Plasma concentration of SDMA was significantly higher in patients with permanent AF compared to both either with sinus rhythm or paroxysmal AF (permanent AF: 0.9, 0.71-1.22 vs. sinus rhythm: 0.544, 0.48-0.61 or paroxysmal AF: 0.517, 0.48-0.72, p=0.001 and p= 0.002, respectively) (**Figure 4.2. A and B)**. Accordingly, L-arginine/SDMA ratio was significantly lower in patients with permanent AF compared to those with sinus rhythm (permanent AF: 78, 54-88 vs sinus rhythm: 129, 96-158, p=0.031) (**Table 4.2)**.

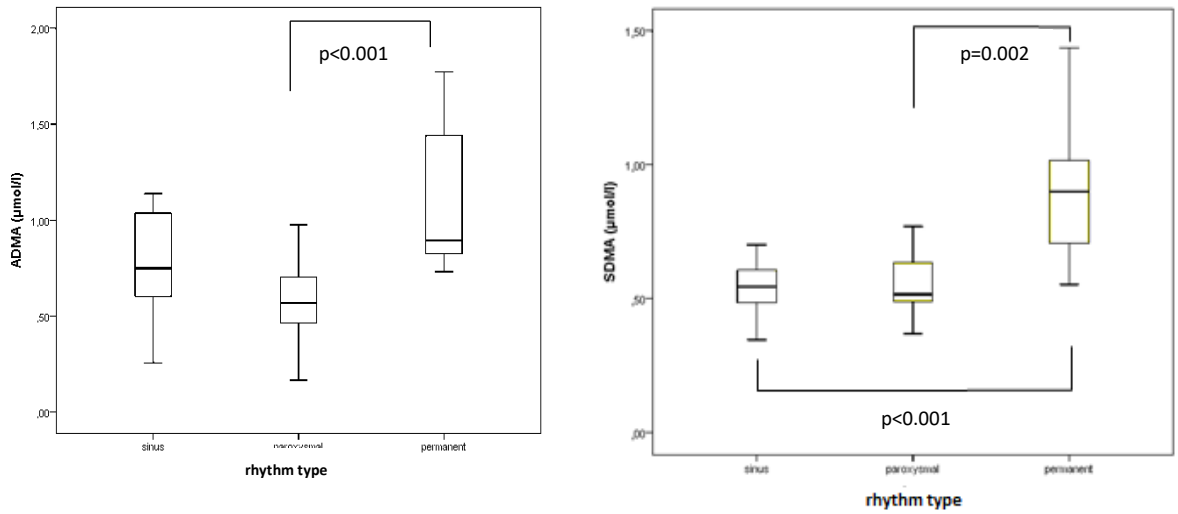

Fig.4.2. A. Plasma concentration of ADMA (µmol/l) is shown in ischemic stroke patients 24 hours after admission with sinus rhythm, paroxysmal atrial fibrillation (AF) or permanent AF. Data are presented as mean and 95% confidence interval. **B.** Plasma concentration of SDMA (µmol/l) is shown in ischemic stroke patients 24 hours after admission with sinus rhythm, paroxysmal atrial fibrillation (AF) or permanent AF. Data are presented as mean and 95% confidence interval

Table 4.2. Plasma concentration of L-arginine pathway metabolites and ratios in different type of atrial fibrillation and sinus rhythm

	permanent (n=11)	sinus (n=18)	*p*-value
ADMA (µmol/l)	0.89 (0.9-1.4)	0.75 (0.5-1.5)	NS
SDMA (µmol/l)	0.9 (0.7-1.2)	0.54 (0.5-0.6)	<0.001
L-arginine (µmol/l)	62 (50-75)	61.9 (50-88)	NS
L-arginine/ADMA	60 (47-72)	80 (66-112)	NS
L-arginine/SDMA	78 (54-88)	129 (96-158)	<0.031
	permanent (n=11)	paroxysmal (n=17)	
ADMA (µmol/l)	0.89 (0.9-1.4)	0.57 (0.5-0.7)	<0.001
SDMA (µmol/l)	0.9 (0.7-1.2)	0.52 (0.5-0.7)	0.002
L-arginine (µmol/l)	62 (50-75)	43 (37-59)	NS
L-arginine/ADMA	60 (47-72)	74 (53-148)	NS
L-arginine/SDMA	78 (54-88)	84 (64-111)	NS
	paroxysmal (n=17)	sinus (n=18)	
ADMA (µmol/l)	0.57 (0.5-0.7)	0.75 (0.5-1.5)	NS
SDMA (µmol/l)	0.52 (0.5-0.7)	0.54 (0.5-0.6)	NS
L-arginine (µmol/l)	43 (37-59)	61.9 (50-88)	NS
L-arginine/ADMA	74 (53-148)	80 (66-112)	NS
L-arginine/SDMA	84 (64-111)	129 (96-158)	NS

Note. ADMA: asymmetric dimethylarginine, SDMA: symmetric dimethylarginine, NS: non significant. Data are shown as median (25th–75th percentiles).

Based on a ROC analysis, the cut-off value of the plasma concentration of SDMA ≥ 0.639 µmol/L measured 24 hours after AIS discriminated permanent AF from paroxysmal AF or sinus rythm with a sensitivity of 90.9% and a specificity of 77.1% (Area: 0.894, 95%CI: 0.796-0.991, p< 0.001) (**Figure 4.3).**

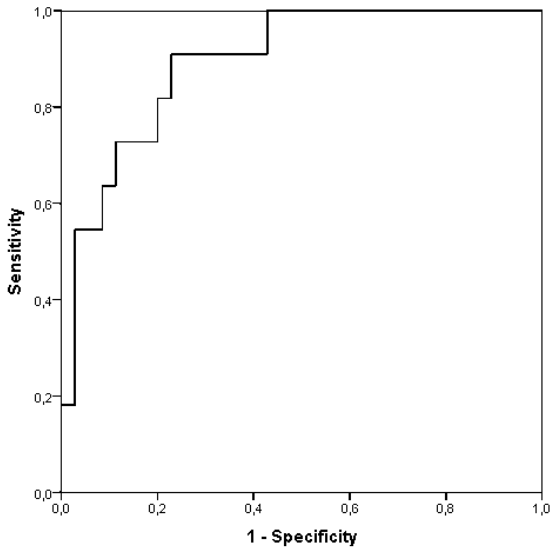

Fig. 4.3. Optimized cut-off value determined for SDMA using receiver operating characteristic curve (ROC) analysis.

Concentration of SDMA> 0.639 µmol/L (AUC:0.894, 95% confidence interval 0.796-0.991; p<0.001) discriminates permanent AF from both, paroxysmal AF or sinus rhythm with a 90.9% sensitivity and 77.1% specificity.

4.3.3.2. *Dimethylarginines and clinical variables*

All L-arginine pathway metabolites, except ADMA were significantly correlated with various factors in univariate analysis (**Table 4.3.**) L-arginine/ADMA ratio associated with clinical variables such as BMI, creatinine, NIHSS. Moreover, L-arginine/SDMA ratio showed association with NIHSS on admission, serum LDL and AF status, whereas serum concentration of SDMA only correlated with AF status.

Table 4.3. Correlation among clinical datas, biomarker variables and L-arginin pathway metabolites in cross-sectional analysis

Variable	ADMA	SDMA	L-arginine	L-arginine/ADMA	L-arginine/SDMA
Age	- 0.202	0.006	- 0.198	- 0.168	- 0.242
BMI	0.226	0.187	- 0.134	- 0.295*	- 0.135
creatinine	- 0.029	0.169	0.303*	0.299*	0.149
NIHSS	0.130	0.077	- 0.266	- 0.321*	- 0.320*
ASPECT score	- 0.095	- 0.082	0.058	0.055	0.130
glucose	- 0.009	0.141	- 0.094	- 0.073	- 0.199
WBC	0.125	0.073	0.215	0.199	0.221
hsCRP	- 0.034	- 0.094	- 0.136	- 0.101	- 0.080
NLR	- 0.206	0.029	- 0.155	0.015	- 0.185
MPV	- 0.046	- 0.115	- 0.047	- 0.024	- 0.059
cholesterine	- 0.248	- 0.169	0.021	0.189	0.116
LDL	- 0.102	- 0.165	0.252	0.278	0.340*
HDL	- 0.086	- 0.156	- 0.223	- 0.187	- 0.121

Note: BMI: body mass index, NIHSS: National Institute of Health Stroke Scale, ASPECT: Alberta stroke program early CT score, WBC: white blood cell, hsCRP: high sensitivity C-reactive protein, NLR: neutrophil-lymphocyte ratio, MPV: mean platelet volume, LDL: light density lipoprotein, HDL: high-density lipoprotein,
Values are Spearman correlation coefficients. *p<0.05, **p<0.01

4.3.3.3. *Discussion*

In this study we evaluated the association between L-arginine pathway metabolites and type of rhythm (sinus vs. AF) at 24 hours after acute ischemic stroke. Moreover, we aimed to explore the relationship between AF status (paroxysmal vs permanent), subclinical inflammation and characteristics of acute ischemic stroke patients. The most important finding in our study was the significantly higher levels of SDMA in permanent AF group compared to either patients with paroxysmal AF or sinus rhythm. These results are to some degree in line with results from the study by Stamboul K et al who found gradually increased level of SDMA across groups of patients characterized by type of AF (no AF, silent AF, symptomatic AF) [154]. In a large cohort higher concentrations were observed in participants with AF for SDMA compared to those with sinus rhythm and SDMA was also positively correlated with left atrial area and p wave duration [155]. In contrast, only AF status showed significant association with SDMA in our cohort, but inflammatory markers such as CRP, WBC, infection and outcome measures such as NIHSS, mRS did not. Data from literature emphasized that ADMA and SDMA levels are expected to increase in the state of chronic inflammation and to decrease in the state of acute inflammation [156-157]. Marcus et al demonstrated that serum IL-6 levels were similar in both patients with and without previous AF episodes, but were significantly increased in blood samples taken during AF attack [158].

Serum TNF-α blood levels were greater in patients with AF compared with those in SR, and in persistent and permanent AF compared with paroxysmal AF [159]. These data support the concept that chronic AF represents a continuous subclinical inflammatory state. In accordance, the significantly higher plasma level of SDMA in our cohort as well as in other studies [160-161] also support the role of chronic inflammation in AF.

Increased creatinine level showed significant positive association with L-arginine and L-arginine/ADMA ratio measured after 24 hours on cross-sectional analysis. A gradually increased creatinine level was observed in patients with paroxysmal and permanent AF, however it was not significant, only a trend could be seen. Several studies have highlighted the increased incidence of AF among those with worsening renal function [162]. ADMA and baseline creatinine are significantly associated with progression of non-diabetic kidney diseases [163].

There might be a bidirectional relationship between AF and kidney injury. Importantly, SDMA level was considered to be closely related to renal function because SDMA is exclusively excreted by the kidney [164], nevertheless we did not find strong correlation between baseline creatinine and SDMA here. Despite, less than 15% of total ADMA is excreted by the kidneys in a non-metabolized form [165] patients with mild to moderate CKD had elevated plasma levels of ADMA compared to individuals without CKD [166].

We found elevated plasma ADMA level in patients with permanent type of AF compared to those with paroxysmal AF. Interestingly, plasma level of ADMA in patient with sinus rhythm was higher (although non-significantly) compared to patients with paroxysmal AF. This unexpected result might be due to other risk factors influencing the circulating ADMA level in our cohort. The number of current smokers are more than double in the sinus rhythm group compared to the paroxysmal AF group and almost the same in the permanent AF group. Staab et al. reported an increased serum concentration of ADMA following exposure to cigarette smoke. They found an increased ADMA concentration in the bronchial epithelial cells in mouse [167]. Recently, Schnabel et al. [168] found a higher ADMA concentration in smokers compared to non-smokers in a population with preexisting CHD. ADMA plasma concentrations are regulated by multiple factors and its concentrations tend to rise under conditions of oxidative stress [169] so current smoking status may contribute significantly to elevated ADMA level in this case.

In our study we found a positive association between AF status and NLR value. Various studies evaluated the role of NLR in relation to AF. Nam HJ et al. found that NLR were significantly higher in the AF group of patients than in those with no atrial fibrillation, but NLR in individuals with persistent AF were also higher compared to paroxysmal AF group [170]. An other study found that the prevalence of AF has a significant and positive association with NLR in both sexes [171]. The preablation NLR value was found to be one of the strongest and independent predictor of AF recurrence after cryoablation [172] however NLR was insufficient to predict recurrence rate after electrical cardioversion in persistent AF patients40. A meta-analysis from 2014, showed that baseline NLR and post-NLR was a remarkably consistent and strong predictor of AF [174]. Our findings are in accordance with these publications and suggest that both paroxysmal and permanent AF may be related to increased NLR and support the theory that neutrophil accumulation might participate in atrial remodeling by the release of activated substances and increase the prevalence of AF in stroke population too.

Blood glucose level showed a positive correlation with NLR value in our study. NLR was found to be higher in people with impaired glucose tolerance (IGT), those newly diagnosed with diabetes by OGTT and those previously diagnosed with diabetes compared to individuals with normal glucose tolerance [175]. According to Sefil et al. positive correlation was found between HbA1c levels and NLR [176]. Lymphocyte levels were found to be reduced due to hyperglycaemia and was accompanied by impaired tissue oxygenation during the hyperglycaemic episode [177]. Patients with diabetes mellitus have been suggested to have insufficient proliferation of lymphocytes [178]. Reduction in lymphocyte count and thus elevated NLR, may be related to stress, since cortisol production is increased under stress conditions resulting in a decreased lymphocyte count in the peripheral blood46. Sen N et al. provided evidence that the NLR was associated with impaired myocardial perfusion and long term adverse outcome in patients with ST-elevated myocardial infarction undergoing primary coronary intervention [180]. In patients after CABG, AF was strongly associated with peak post-operative blood glucose concentration, independently from diabetes mellitus [181]. According to a recent meta-analysis, elevated serum HbA1c levels may be associated with an increased risk of AF [182]. Interestingly, in prediabetic patients with impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) atrial conduction

times and P wave dispersion, the noninvasive predictors of atrial fibrillation, were longer before the development of overt diabetes [183].

In summary, we assumed that the persistent subclinical inflammatory state among patients with atrial fibrillation is well established, but if AF a cause or a consequence of inflammation is still remained unclear.

From the viewpoint of clinical practice, the presence of a subclinical inflammatory environment reflected by either ADMA, SDMA and its ratios and NLR are likely to become a novel tool for AF progression and monitoring, but their practical value in guiding AF management is not well established.

Our study should be evaluated with some limitations in mind. First, the results are from a single-center experience and should not be generalized to all populations. Low patient number decreased the power of study and precluded detailed multivariate analysis. Because of single measurement of L-arginine pathway metabolites at 24 post-stroke hours, we were not able to explore association between change in concentrations of markers and further AF status here.

4.4. *L-arginine pathway and ischemic stroke*

Stroke is the third most common cause of disability worldwide [184]. Prognostic tools that monitor the neurological condition and predict the final outcome in clinical practice are scarce. Although several blood biomarkers have been proposed as promising biomarkers, e.g. C-reactive protein (CRP), tumor necrosis factor (TNF), interleukin-6 (IL-6), and CD40L, no biomarker is used in clinical practice [185].

4.4.1. *Nitric oxide (NO) in stroke*

Nitric oxide (NO) plays a role in maintaining vascular integrity [147]. NO is synthetized by the oxidation of L-arginine, which can be inhibited by asymmetric dimethylarginine (ADMA). Symmetric dimethylarginine (SDMA) competes with arginine uptake and antagonizes the effects of L-arginine [148]. The plasma concentration of L-arginine and its dimethylated derivatives ADMA and SDMA were associated with long-term mortality in patients followed up to 7 years after an acute stroke [186]. SDMA level in the plasma was strongly associated with adverse clinical outcome during the first 30 days after ischemic stroke [187]. Increased plasma levels of both ADMA and SDMA within the first 72 hours after onset of acute ischemic stroke predict poor functional outcome 90 days after stroke onset [188].

4.4.2. *Materials and methodes*

4.4.2.1 *Study population*

The study was approved by regional ethics committee, and patients or their legal representatives gave informed consent.

Between January 2015 and April 2016, 46 patients with acute ischemic stroke were prospectively enrolled. Patients underwent systemic thrombolysis according to international guideline [189], or received conservative treatment according to recent ESC Guideline of acute ischemic stroke [190].

The etiology of stroke was classified according to Trial of ORG 10172 in acute stroke treatment (TOAST) criteria [191]. Neuroimaging was performed by Siemens SOMATOM Definition 64 Slice CT Scanner at admission.

4.4.2.2. *Comorbidities*

A history of hypertension was defined as the use of antihypertensive drugs or blood pressure more than 140/90 mmHg on at least two separate occasions. Diabetes mellitus was defined as the use of anti-diabetic drugs or a fasting plasma glucose value ≥7.0 mmol/L. An evidence-based guideline was followed to detect infectious complications: in short, physical and laboratory measures including white blood count (WBC), high sensitivity C-reactive protein (hsCRP), procalcitonin, fever, abnormal urine, chest X-ray, or positive cultures [192]. Body mass index (BMI) equal to or below and above 26.3 kg/m2 were classified as “low BMI” and “high BMI” based on a large cohort of similar high risk population [193].

4.4.2.3. *Outcome measures*

To define the size of infarct, we dichotomized Alberta stroke program early CT scores (ASPECTS) [194]: larger area involved: 0–7 or smaller area involved: 8–10 scores.
The severity of stroke was measured by the National Institute of Health Stroke Scale (NIHSS) on admission. NIHSS 0-1 was considered as mild stroke, 2-8 as moderate stroke and ≥9 as a severe stroke [195].
We also recorded the modified Rankin scale (mRS) at discharge and 6 month after the onset of stroke: score 0-1 was considered as reflecting good recovery, 2-4 as moderate recovery, and 5-6 as poor recovery. Patients were categorized into 3 distinct groups based on the difference between the mRS at discharge and 6 month after stroke: (i) improved, if mRS after 6 months was lower than mRS at discharge; (ii) unchanged, if mRS after 6 months persisted; (iii) and worsened, if mRS after 6 months was higher than at discharge. To assess the mRS after 6 months, we either requested hospital visit or conducted a phone interview with the patient, the spouse, or the general practitioner.
Exclusion criteria were historical mRS higher than 0 on admission, (functional deficits prior to index event), history of malignant tumor, chronic renal failure (estimated glomerular

filtration rate, eGFR <50 and/or creatinine> 120 µmol/l at two distinct measurement), any known immunological disturbances, severe hypoxia, systemic infection or sepsis.

4.4.2.4. *Blood collection*

Plasma samples were collected from venous blood 24 hours after admission. The samples were immediately centrifuged (Clinspin Horizon 853VES Centrifuge) at 3000 rpm/min for 15 minutes. The supernatant was stored at −80°C until analysis. L-arginine, ADMA, and SDMA were measured in the plasma by high-performance liquid chromatography as described previously [153].

4.4.2.5. *Statistical analysis*

Statistical analyses were performed using SPSS Statistics version 20.0 (IBM, 35 Armonk, NY, USA). Categorical data were summarized by means of absolute and relative frequencies (counts and percentages). Quantitative data were presented as mean and 95% confidence interval, as well as mean ± SD. The distribution of values was assessed by using the Shapiro-Wilk test. Non-normally distributed data are presented as median and interquartile range. Due to normally distributed data, parametric methods (chi-square test for categorical data, Student- t test for continuous data) were used for demographic and clinical data. Comparison of the variables between the subgroups was performed by using the Kruskal-Wallis test, because these parameters do not follow the normal distribution. Correlation analysis was performed calculating Spearman's correlation coefficient (r). A p-value <0.05 was considered statistically significant.

4.4.3. *Results*

4.4.3.1. *Demographics*

Based on the change of mRS between hospital discharge and 6 months after stroke onset, patients were categorized as improved (33%, n=15), unchanged (41%, n=19) or worsened (26%, n=12). There were no significant differences in gender and age among these three outcome groups. Demographic and clinical characteristics of total study population and the three outcome groups based on mRS are shown in **Table 4.4.**

The median NIHSS score on admission was 6 (mean:6.3±4.8). According to NIHSS classification, 15% of the ischemic stroke was mild (NIHSS 0-1, n=6), 61% moderate (NIHSS 2-8, n=29) and 24% severe (NIHSS ≥9, n=11). Two-third of the patients (n=28, 61%) had atrial fibrillation prior to stroke, 14 (30%) had a previous ischemic heart disease or myocardial infarction, and 89% of the patients had hypertonia. Twenty-one subjects (46%) were active smoker. According to TOAST criteria, 54% of the stroke was of cardioembolic origin, 17% was caused by large artery atherosclerosis, 20% was lacunar, and 9% was undetermined. Sixteen patients (35%) underwent thrombolysis and 30 (65%) had conservative treatment.

Table 4.4.
Demographic and clinical characteristics of stroke patients on admission according to outcome after 6 months

	Patients *n*=46	Improved *n*=15	Unchanged *n*=19	Worsened *n*=12	*p* value
Age, years	66.8 ± 8.4	65.5 ± 8.7	67.6 ± 8.5	66.8 ± 8.5	0.470
Female, n (%)	20 (43)	8 (53)	8 (42)	5 (42)	0.506
Hypertension, n (%)	41 (89)	13 (87)	17 (89)	11 (92)	0.868
Diabetes, n (%)	10 (21.7)	4 (27)	3 (16)	3 (25)	0.856
Smoking, n (%)	21 (45.6)	5 (33)	12 (63)	4 (33)	0.844
BMI	28.3 ± 3.9	27.3 ± 4.9	28.8 ± 3.6	28.5 ± 3.6	0.726
Creatinine, umol/L	78 ± 22	87 ± 35	78 ± 17	70 ± 17	0.082
WBC, G/L	9.1 ± 3	9.2 ± 3	9.7 ± 4	8.1 ± 2	0.371
CRP, mg/l	9.1 ± 13	5.1 ± 5	11.8 ± 18	8.8 ± 8	0.338
Stroke severity, n (%)					0.269
Mild	15 (6)	7 (1)	16 (3)	16 (2)	
Moderate	61 (29)	67 (10)	58 (11)	67 (8)	
Severe	24 (11)	26 (4)	26 (5)	17 (2)	
Discharge mRS score	2.11 ± 1.73	2.73 ± 1.4	1.84 ± 1.8	1.75 ± 1.9	0.078
6-month mRS score	2.11 ± 2	1.2 ± 1.2	1.84 ± 1.8	3.67 ± 2.3	0.004
ASPECT score	8.7±1.6	8.8±1.4	8.7±1.7	8.4±1.8	0.640
Stroke etiology, n (%)					0.990
Cardioembolic	25 (54)	8 (53)	10 (53)	7 (58)	
Atherothrombotic	8 (17)	4 (27)	3 (16)	1 (9)	
Lacunar	9 (20)	1 (7)	5 (26)	3 (25)	
Undetermined	4 (9)	2 (13)	1 (5)	1 (8)	
Pulmonary, n (%)	9 (19)	4 (27)	2 (11)	4 (33)	0.608
Thrombolysis, n (%)	16 (35)	8 (53)	3 (16)	4 (33)	0.402

Note. BMI = body mass index; WBC = white blood cells; CRP = C-reactive protein; ASPECT = Alberta stroke program early CT score. Data are mean ± SD and number of cases (percentage). Outcome was measured by change of modified Rankin score (mRS) between hospital discharge and 6 months after the stroke. Pulmonary include history of COPD or severe asthma

4.4.3.2. *L-arginine metabolite levels at 24 post-stroke hours*

The median plasma concentration of L-arginine was 56.1 µmol/l [IQR: 41.7-71.7], the median ADMA level 0.74 µmol/l [0.56-0.99], the median SDMA level 0.58 µmol/l [0.5-0.75], the median L-arginine/ADMA ratio 74.1 [53.5-90.0], and the median L-arginine/SDMA ratio 88.1 [57.6-130.9].

4.4.3.3. *L-arginine metabolites levels and outcome*

In the three stroke severity groups based on NIHSS at admission, the median L-arginine/ADMA ratio was significantly different (mild group: median: 88.7, IQR: 78-152; moderate group: 67.8, 48-87; and severe group: 60.1, 41-80, $p<0.05$). L-arginine, ADMA, SDMA and L-arginine/SDMA showed no significant difference between the groups.

None of the examined L-arginine metabolites or ratios showed any significant correlation with either short-term outcomes (discharge mRS) or long-term outcomes (6-month mRS).

Patients with worsened mRS by 6 months had significantly higher L-arginine plasma concentrations at 24 post-stroke hours compared to patients with improved mRS ($p<0.001$) and unchanged mRS ($p<0.005$). The L-arginine/ADMA and the L-arginin/SDMA ratios at 24 hours were significantly higher among patients with worsened compared to improved mRS ($p<0.004$ and $p<0.002$, respectively). The plasma concentration of L-arginine and L-arginine/SDMA ratio was also significantly higher in the unchanged compared to the improved mRS group ($p<0.005$ and $p<0.006$, respectively) (**Table 4.5.)**.

Table 4.5.
Plasma concentration of L-arginine pathway metabolites and ratios in different outcome groups at 6 months after stroke

	improved (n=15)	worsened (n=12)	*p*-value
ADMA (µmol/l)	0.58 (0.46-0.8)	0.7 (0.59-1.04)	NS
SDMA (µmol/l)	0.56 (0.49-0.73)	0.61 (0.51-0.83)	NS
L-arginine (µmol/l)	38.9 (25.2-47.9)	79 (64.6-93.5)	<0.000
L-arginine/ADMA	55.3 (40.9-76.4)	102.8 (68.8-151.9)	<0.004
L-arginine/SDMA	53.2 (39.8-77.3)	131.4 (89.5-166.9)	<0.002
	improved (n=15)	unchanged (n=19)	
ADMA (µmol/l)	0.58 (0.46-0.8)	0.87 (0.72-1.02)	NS
SDMA (µmol/l)	0.56 (0.49-0.73)	0.59 (0.51-0.77)	NS
L-arginine (µmol/l)	38.9 (25.2-47.9)	57.7 (50.6-76)	< 0.005
L-arginine/ADMA	55.3 (40.9-76.4)	74.6 (59.6-84.9)	NS
L-arginine/SDMA	53.2 (39.8-77.3)	95.2 (77.6-132.1)	< 0.006
	worsened (n=12)	unchanged (n=19)	
ADMA (µmol/l)	0.7 (0.59-1.04)	0.87 (0.72-1.02)	NS
SDMA (µmol/l)	0.61 (0.51-0.83)	0.59 (0.51-0.77)	NS
L-arginine (µmol/l)	79 (64.6-39.5)	57.7 (50.6-76)	NS
L-arginine/ADMA	102.8 (68.8-151.9)	74.6 (59.6-84.9)	NS
L-arginine/SDMA	131.4 (89.5-166.9)	95.2 (77.6-132.1)	NS

Note. ADMA: asymmetric dimethylarginine, SDMA: symmetric dimethylarginine, NS: non significant. Data are shown as median (25th–75th percentiles). Outcome was measured by change of mRS between hospital discharge and 6 months after the stroke.

4.4.3.4. *Correlation of L-arginine pathway metabolites with clinical and risk factors*

ADMA, L-arginine and L-arginine/ADMA and L-arginine/SDMA ratios in the acute phase correlated with various factors (clinical and plasma molecular markers) in cross-sectional analysis (**Table 4.6.**). Plasma concentration of ADMA was significantly higher among smokers compared to non-smokers (median: 0.91, IQR: 0.7-1.1 vs. 0.64, 0.5-0.82, $p<0.004$). Higher L-arginine concentration was associated with higher creatinine concentration. Higher L-arginine/ADMA and L-arginine/SDMA ratios were associated with lower NIHSS at admission (-0.321, p=0.029, -0.320, p=0.03, respectively). Higher L-arginine/ADMA ratio was also associated with lower BMI, and higher creatinine concentration. Higher L-arginine/SDMA ratio was significantly associated with higher LDL levels on admission, and lower discharge mRS. No correlation was found between L-arginine metabolites and the size of infarct defined by the ASPECT scores, hsCRP, WBC, neutrophil/lymphocyte ratio, mean platelet volume, glucose, HDL, and cholesterol **Table 4.6.**

We also correlated L-arginine concentration, L-arginine/SDMA and L-arginine/ADMA ratio measured 24 hours after the onset of stroke with changes in mRS after 6 months. All of these values negatively correlated with change of mRS between hospital discharge and at 6 months (ΔmRS) (**Table 4.6** and **Figure 4.4**).

Table 4.6.
Correlation among clinical data, biomarker variables and L-arginin pathway metabolites in cross-sectional analysis

Variable	ADMA	SDMA	L-arginine	L-arginine/ADMA	L- arginine/SDMA
Age	- 0.202	0.006	- 0.198	- 0.168	- 0.242
BMI	0.226	0.187	- 0.134	- 0.295*	- 0.135
creatinine	- 0.029	0.169	0.303*	0.299*	0.149
smoking	0.419**	0.284	0.104	- 0.192	- 0.071
NIHSS	0.130	0.077	- 0.266	- 0.321*	- 0.320*
ASPECT score	- 0.095	- 0.082	0.058	0.055	0.130
glucose	- 0.009	0.141	- 0.094	- 0.073	- 0.199
WBC	0.125	0.073	0.215	0.199	0.221
hsCRP	- 0.034	- 0.094	- 0.136	- 0.101	- 0.080
NLR	- 0.206	0.029	- 0.155	0.015	- 0.185
MPV	- 0.046	- 0.115	- 0.047	- 0.024	- 0.059
cholesterine	- 0.248	- 0.169	0.021	0.189	0.116
LDL	- 0.102	- 0.165	0.252	0.278	0.340*
HDL	- 0.086	- 0.156	- 0.223	- 0.187	- 0.121
mRS discharge	0.052	0.144	- 0.211	- 0.216	- 0.326*
mRS 6 month	0.246	0.254	0.312*	0.080	0.070
ΔmRS	- 0.205	- 0.071	- 0.672**	- 0.447**	- 0.517**

Note: BMI: body mass index, NIHSS: National Institute of Health Stroke Scale, ASPECT: Alberta stroke program early CT score, TOAST: Trial of Org 10172 in Acute Stroke Treatment Criteria, WBC: white blood cell, hsCRP: high sensitivity C-reactive protein, NLR: neutrophil-lymphocyte ratio, MPV: mean platelet volume, LDL: light density lipoprotein, HDL: high-density lipoprotein, mRS: modified Rankin score; mRS: mRS discharge-mRS 6 months All markers were measured at 24 hours after stroke. Values are Spearman correlation coefficients. *p<0.05, **p<0.01

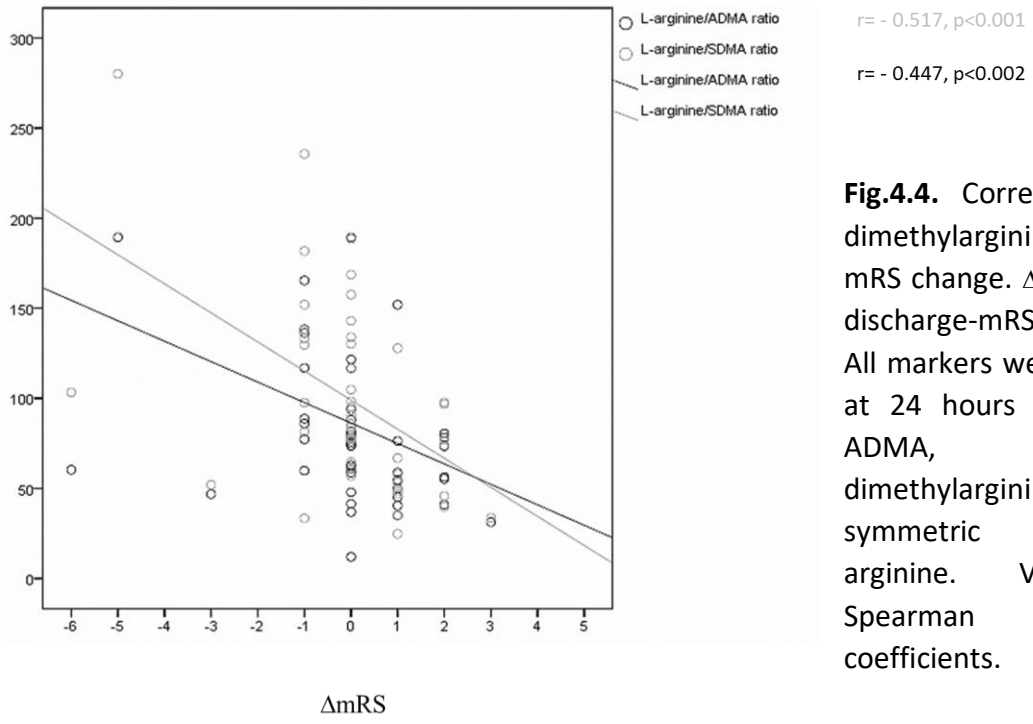

Fig.4.4. Correlation among dimethylarginine ratios and mRS change. ΔmRS: mRS at discharge-mRS at 6 months. All markers were measured at 24 hours after stroke. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethyl-arginine. Values are Spearman correlation coefficients.

4.4.3.5. *L-arginine pathway metabolites measured at 24 hours and treatment*

L-arginine/ADMA (median: 77, IQR: 61-100 vs. 52, 40-78, p=0.022) as well as L-arginine/SDMA ratio (median: 98, IQR: 71-133 vs 64, 36-94, p=0.019) was significantly higher in the conservative treatment vs thrombolysis group. Although patients treated with thrombolysis had a higher baseline NIHSS than those without thrombolysis (median of NIHSS: 7.7, IQR: 5.5-10 vs 4, 3.7-7.5, p=0.103), the mRS score at 6-month follow-up showed no significant difference between the two groups (median of mRS: 2, IQR: 1.1-3.4 vs 1, 1.3-2.7, p=0.737). No patients had undergone mechanical thrombectomy.

4.4.4. *Discussion*

In this study, first we explored association of the L-arginine pathway metabolites with clinical and risk factors, as well as with stroke severity, short-term outcome (mRS at discharge) and molecular markers in the acute phase of ischemic stroke. In a prospective part of the study, we investigated the plasma concentration of L-arginine pathway metabolites in 46 patients with improved, unchanged or worsened mRS by 6 months after acute ischemic stroke.

High BMI showed significant negative association with L-arginine/ADMA ratio measured after 24 hours. Obesity is associated with elevated level of NO and markers of nitrosative and oxidative stress [193]. This may be reflected by the lower L-arginine/ADMA ratio. The higher hsCRP in patients with high BMI in our cohort presumably reflects the chronic low grade inflammation. L-arginine/SDMA ratio and SDMA at 72 hours were independently associated with post-stroke infections [196]. Both L-arginine/ADMA and L-arginine/SDMA ratios were significantly higher in patients with conservative treatment compared to those underwent thrombolysis. Importantly, patients with higher baseline NIHSS in the thrombolysis group reached the same mRS score at 6-month follow-up compared to the conservative treatment group indicating the efficiency of recanalization therapy. Significant increase of L-arginine serum level was noticed at immediately after rtPA administration in comparison to patients treated with conservative therapy [197]. Stroke patients who were treated with rtPA showed significantly lower ADMA levels than patients who received placebo [198]. These findings are in line with our results.

Interestingly, ADMA per se was unrelated to the mRS endpoints at discharge, after 6 months and change of mRS by 6 months. It is in accordance with others findings. Schulze et al. suggested that ADMA has a less important role in stroke outcome than in primary cardiovascular disease [186]. Accordingly, ADMA was poorly associated with laboratory parameters and clinical factors in this cohort. In contrast, SDMA and L-arginine/SDMA ratio showed strong association with atrial fibrillation in our study. Similarly, SDMA, but not ADMA was closely associated with the presence of cardio-embolic stroke [199]. Higher concentration of SDMA was observed in participants with atrial fibrillation compared to patient with sinus rhythm refered by Ramuschkat M et al. [155].

ADMA directly inhibits NOS activity, whereas SDMA indirectly influences NO formation interacting with the transport of L-arginine, which may lead to an intracellular depletion of L-arginine, causing endothelial dysfunction [200].

Interestingly, a lower median L-arginine plasma concentration was observed at 24 poststroke hours than in our previous study measured in the serum at the same time point. However, more patients with mild severity (mean of NIHSS on admission: 6.3±4.8) were recruited in this cohort compared to our previous study population (mean of NIHSS on admission: 11.5±5.4) [196].

Regarding the long-term outcome, here we found that higher plasma L-arginine level, L-arginine/ADMA, and L-arginine/SDMA ratios at 24 post-stroke hours showed inverse correlations with worsening mRS during the first 6 months. Although plasma ADMA and SDMA concentration at 24 post-stroke hours were higher in patients with worsening mRS, but the differences were not significant. Our data indicate that lower L-arginine level, lower L-arginine/ADMA and L-arginine/SDMA ratio at 24 hours is associated with higher mRS on discharge i.e. worse outcome on the short-term; but these patients improve on the long-term resulting in association of lower L-arginine/SDMA with improving mRS. Similarly, patients with lower L-arginine/ADMA ratio improves on the long-term. In contrast, patients with higher L-arginine, L-arginine/ADMA and L-arginine/SDMA ratios and lower discharge mRS improve fast, but will not improve or may even worsen by 6 months indicating that its biological effect is time-dependent. Early elevation of L-arginine in the acute phase of ischemic stroke may have a beneficial effect on cerebral perfusion by increasing NO, thus may be protective in the acute phase of ischemic stroke [196]. Among healthy individuals

with a low risk of cardiovascular disease, those with plasma ADMA levels above 0.71 µmol/L had a higher risk of future cardiovascular and cerebrovascular events, as compared to those with plasma ADMA levels below this treshold [201]. Pikula et al. observed that silent brain infarcts in MRI occur more frequently in subjects in the upper three age-specific quartiles of plasma ADMA concentrations compared to the lowest quartile [202]. Plasma ADMA levels in the acute stage of stroke independently predicted adverse outcome at 90 days. Similarly, an increased plasma ADMA concentration was found here in patients who are not improved by 6 post-stroke months. ADMA levels ≥0.566 µmol/L at day 3 and ≥0.530 µmol/L at day 7 after symptom onset were independent predictors of an unfavorable outcome [203]. Infusion of ADMA increased vascular stiffness and decreased cerebral perfusion in healthy subjects [204]. We observed the elevation of ADMA and L-arginine at 24 post-stroke hours in patients with lower mRS on the short-term. We hypothesize that the detrimental effect of ADMA may be antagonized by an increased L-arginine level as a regulatory mechanism resulting in increased NO production and vasodilatation as we stated in our previous work [196]. Clinical studies found evidence that increased ADMA levels are associated with a higher risk of cerebrovascular events [205]. Based on our findings, an increased systemic concentration of ADMA less likely to be the result of acute stroke, rather a preceding factor contributing to cerebrovascular events. In accordance with this, coincidence in both, lower level of ADMA and L-arginine, suggests a lower vascular risk and a better outcome in the long term.

In our cohort, we found almost the same prevalence of smokers among patients with improving and worsening mRS. In contrast, more smokers were found in the unchanged outcome group showing a trend that may reflect ischemic preconditioning, but the difference was not significant. In previous studies, higher ADMA level was observed in smokers compared to non-smokers [206]. This connection is confirmed in our study; nevertheless it was not associated with worse outcome. Theoretically, smoking status combined with other cardiovascular risk factors increases circulating ADMA level, and may affect negatively the restoration of ischemic damage. ADMA was positively associated with thrombo-inflammatory markers in the acute phase of ischemic stroke [207].

In conclusion, our cross-sectional data indicate that patients with high L-arginine plasma concentration 24 hours after stroke improve fast, but will not improve further by 6 months suggesting that these patients might be in various stage of atherosclerosis. In addition, the

beneficial effect of L-arginine seems time-dependent [8]. In contrast, lower L-arginine/SDMA ratio is associated with worse outcome on the short-term, but these patients may improve on the long-term.

There are some limitations of our study: (i) low number of enrolled patients decreased the power of this study and precluded detailed multivariate analysis; (ii) lack of follow up imaging study in all patients tackled us to explore relationship between penumbral or hypoperfused area and markers; and (iii) using single measurement of L-arginine pathway metabolites at 24 post-stroke hours, we were not able to explore association between change in concentrations of markers and functional outcome here.

V. Troponin and thrombo-inflammatory molecules in ischemic stroke

5.1 *Troponin elevation in condition other than acute ischemic coronary syndromes (AICS)*

Cardiac troponins are sensitive and specific biomarkers used in the diagnosis of myocardial infarction that are released into the bloodstream when cardiac myocytes are damaged by acute ischemia. However, troponin elevation indicates the presence, not the mechanism, of myocardial injury [208]. There are many clinical conditions other than myocardial infarction that cause troponin elevation.

Up to 71% of patients with chronic kidney disease (CKD) had elevated cTnT in the absence of clinical evidence for acute ischemia [209]. Several theories have been proposed for the mechanism of elevated troponin levels in CKD. Irrespective of our uncertainty regarding mechanism, studies have shown that there is a strong prognostic implication of elevated troponin levels; and that it is predictive of increased risk of mortality and cardiovascular events [210].

Troponin levels may rise without apparent ischemia in heart failure [211]. The exact mechanisms leading to cTn elevation in AHF are unknown, but multiple process including both ischaemic and non-ischaemic (inflammatory cytokines, neurohormones etc.) mechanisms may contribute [212]. Recent studies have reported the rate of cardiac injury markers as being 20%–40% in the presence of subarachnoid haemorrhage [213]. The degree of neurological injury as measured by the Hunt-Hess grade is a strong, independent predictor of myocardial necrosis after SAH supporting the hypothesis that cardiac injury after SAH is a neurally mediated process [214].

Role of troponin in AF was discussed in 2.5.4.

5.2 *Role of troponin is stroke*

Elevated levels of cardiac troponin (cTn) in the serum reflect myocardial damage and also predict poor prognosis in acute ischemic stroke (AIS) [215]. Ischemic stroke is a frequent complication in cardiac diseases, and cardiac complications commonly cause early clinical worsening and death after stroke [216]. In addition, acute myocardial infarction is a common

complication of AIS. Increased cTn concentrations in the serum in patients with acute coronary syndrome (ACS) indicate ongoing myocardial necrosis. Beyond ACS, it implies an increased risk for adverse outcome in patients with various cardiovascular conditions such as heart failure, stroke, myocarditis, Takotsubo cardiomyopathy, aortic dissection, arrhythmias, valvular diseases [217]. The prognostic value of high-sensitivity cardiac troponin T (hs-cTnT) in a low-risk outpatient population also provided excellent risk stratification on all-cause mortality, acute myocardial infarction, and stroke [218]. However, the reason of cTn elevation in stroke patients without apparent cardiovascular damage is not yet explored. Recently, the association between poststroke mortality and abnormal results of cardiac troponin/ echocardiogram was evaluated in a large number of patients with AIS, who presented to an emergency department: elevated troponin concentration was associated with increased mortality at 1 and 3 years even in the absence of concomitant myocardial infarction [219].

5.3 *Thrombo-inflammatory molecules in ischemic stroke*

5.3.1 *P-selectin*

P-selectin is a protein that in humans is encoded by the SELP gene [220].

P-selectin is constitutively expressed in megakaryocytes (the precursor of platelets) and endothelial cells [221]. P-selectin expression is induced by two distinct mechanisms. First, P-selectin is synthesized by megakaryocytes and endothelial cells, where it is sorted into the membranes of secretory granules [222]. When megakaryocytes and endothelial cells are activated by agonists such as thrombin, P-selectin is rapidly translocated to the plasma membrane from granules [223]. Secondly, increased levels of P-selectin mRNA and protein are induced by inflammatory mediators such as tumor necrosis factor-a (TNF-a), LPS, and interleukin-4 (IL-4) [224]. The elevated synthesis of P-selectin may play an important role in the delivery of protein to the cell surface.

On the cell surface of the endothelial cells P-selectin initiates the earliest phase of leukocyte recruitment into inflammatory sites. Expression of P- selectin on activated platelets is important in the recruitment of leukocytes to thrombi and in the induction of fibrin production during hemostasis [225-227]. In ischemic stroke patients, plasma P-selectin concentration was reported to be highly correlated to plasminogen activator inhibitor-1

activity and tissue plasminogen activator activity [228]. Homozygous deletion of P-selectin in mice has been associated with a significant reduction in infarct volume following MCA:O [229]. Change of P-selectin from 6 to 72 hours by 1 unit increased the incidence of poststroke infections with an odds ratio of 22.7, therefore p-selectin emerged to predict outcomes besides hsCRP [230].

5.3.2 *MCP-1*

The monocyte chemoattractant protein 1 (MCP1) is also referred to as chemokine (C-C motif) ligand 2 (CCL2) and small inducible cytokine A2. CCL2 is a small cytokine that belongs to the CC chemokine family. CCL2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection [231-232]. CCL2 is implicated in pathogeneses of several diseases characterized by monocytic infiltrates, such as psoriasis, rheumatoid arthritis and atherosclerosis [233].

CCL2 is involved in the neuroinflammatory processes that takes place in the various diseases of the central nervous system (CNS), which are characterized by neuronal degeneration [234]. CCL2 expression in glial cells is increased in epilepsy,[235-236] brain ischemia [237] Alzheimer's disease [238] experimental autoimmune encephalomyelitis (EAE),[239] and traumatic brain injury [240]. Shear stress-induced overexpression of MCP-1 contributes significantly to the development of protective collaterals in the heart, but little is known about its role in the cerebral vasculature [241].

5.3.3 *sCD-40L*

CD40L is a 48-kDa trimeric transmembrane protein belonging to the tumor necrosis factor superfamily originally identified on cells of the immune system [242]. Both CD40L and its receptor CD40 are found in platelets [243]. Whereas CD40 is constitutively expressed on platelets, CD40L rapidly appears on the platelet surface following activation, on which it is subsequently cleaved, generating a soluble fragment of 18-kDa, termed sCD40L, accounting for 95% of its plasmatic concentration [244]. Circulating levels of sCD40L in patients have now emerged as strong indicators of cardiovascular risk, as there appears to be a significant correlation between levels of sCD40L and vascular complications such as atherosclerosis and acute coronary syndromes (ACS) [245-247].

5.3.4 *Tissue plasminogen activator*

Tissue plasminogen activator (tPA) is a protein involved in the breakdown of blood clots. Conradi (1902) identified tPA, at this time named fibrokinase, later characterized to mediate fibrinolysis [248]. tPA is a protein of 527 amino-acids including three glycosylation sites and 17 disulfide bridges [249]. Collen and Lijnen then provided evidence that tPA could facilitate the dissolution of blood clots by inducing the degradation of fibrin in a plasminogen-dependent manner [250]. Depending on the study, endogenous tPA was reported as deleterious or beneficial for neurons. Although it is difficult to reconcile these findings, some propose that tPA is neuroprotective at low levels, but neurotoxic at higher levels [251].

5.3.5 *High-sensitive C-reactive protein*

C-reactive protein is a homopentameric acute-phase inflammatory protein, a highly conserved plasma protein that was initially discovered in 1930 by Tillet and Francis while investigating the sera of patients suffering from the acute stage of Pneumococcus infection and was named for itsreaction with the capsular (C)-polysaccharide of Pneumococcus [252]. C-reactive protein exhibits elevated expression during inflammatory conditions such as rheumatoid arthritis, some cardiovascular diseases, and infection [253]. The average levels of CRP in serum in a healthy Caucasian is around 0.8 mg/L, but this baseline can vary greatly in individuals due to other factors, including polymorph-isms in the CRP gene [254]. The highest concentrations of CRP are found in serum, with some bacterial infections increasing levels up to 1,000-fold [255]. CRP plasma levels increase from around 1 μg/mL to over 500 μg/mL within 24–72 h of severe tissue damage such as trauma and progressive cancer [256]. Increased high-sensitivity C-reactive protein (hsCRP) in the subacute stage is an independent

predictor of death [257]. Several prospective studies have reported the association between higher CRP levels and increased disability risk of ischemic stroke [258].

5.3.6 *S100B*

S100 B is an astroglial protein, which is present in high concentrations in glial cells and Schwann cells of the central and peripheral nervous systems as well as in Langerhans cells and cells of the anterior pituitary [259].

S100B in the peripheral blood is a sensitive marker of both blood–brain barrier dysfunction and ischemic brain damage and predictor of stroke outcome [260,196].

5.4 *Materials and methodes*

The study protocol was approved by the Local Ethics Committee at University of Pecs, Faculty of Medicine, and informed consent was obtained from each patient.

5.4.1 *Subjects*

Thirty-five patients were enrolled within 6 hours after onset of first-ever AIS at the Department of Neurology, University of Pecs. Initial assessment of each patient, including cardiac anamnesis, 12-lead electrocardiogram recording, and physical examination, was performed to enroll only patients without acute cardiovascular event into this prospective study. Severity of stroke was measured by the National Institutes of Health Stroke Scale (NIHSS) on admission and on a daily basis until discharge or death.

5.4.2 *Inclusion and exclusion criterias*

Inclusion criteria were (1) first-ever ischemic stroke; (2) onset of AIS within 6 hours; (3) duration of neurologic symptoms over 24 hours; (3) computed tomography or magnetic resonance result showed infarction in the brain area corresponding to the clinical symptoms and signs. The enrolled patients were conservatively treated; therefore, we were exclusively assaying the circulating levels of endogenous tPA after stroke. Exclusion criteria were (1) hemorrhagic stroke; (2) patients with ACS, myocardial infarction, stable angina, or the presence of clinical symptoms indicating acute infections, and other chronic infectious diseases; (3) patients with immune disorders, liver or kidney dysfunction, myopathy, and tissue injury outside of the brain; (4) recent use of both prescribed and over-the counter anti-inflammatory drugs.

5.4.3 *Sampling and analysis of markers*

Venous blood samples were taken for the measurement of hs-cTnT within 6 hours after onset of first neurologic symptoms and 24 hours later. P-selectin, MCP- 1, sCD40L, and tPA, hsCRP, and S100B were measured within 6 hours after onset and at 72 poststroke hours. Blood samples were centrifuged at 3000 × g for 10 minutes. Supernatants were frozen and stored at −80°C until analysis. hs-cTnT concentrations were measured by a fully automated solid phase electrochemiluminescence immunoassay (Roche), (Roche Diagnostics, GmbH, Mannheim, Germany) using a Cobas e 411 analyzer (Roche). Serum levels of S100B were examined by automated electrochemiluminescent immunoassay (Liaison Sangtec 100 system, DiaSorin, Bromma, Sweden). Serum levels of hsCRP were examined by automated fluorescence immunoassay (BRAHMS Kryptor, Berlin, Germany). Concentration of P-selectin, MCP-1, sCD40L, and tPA were examined by immunoassay (BMS711F, Bender Gmbh, Campus Vienna Biocenter 2, Vienna, Austria).

5.4.4 *Statistical analysis*

Data were evaluated using SPSS software package (Version 19.0, IBM Corp., Armonk, NY). Categorical data were summarized by means of absolute and relative frequencies (counts and percentages). Quantitative data were presented as mean and 95% confidence interval, as well as mean ± SD. The Kolmogorov- Smirnov test was applied to check for normality. Chi-square test for categorical data and Student t test for continuous data were used for analysis of demographic and clinical factors. Nonparametric Mann– Whitney U test was used for non-normally distributed markers. Binary logistic regression analysis was applied to confirm independent predictors. Correlation analysis was performed calculating Spearman correlation coefficient (r). A P value <.05 was considered statistically significant.

5.5 *Results*

The mean age for patients was 66.8 ± 11.7 years (ranging from 40 to 90). Demographic and clinical data are summarized in **Table 5.1.** There were significantly more patients with diabetes (male: 6 of 16 versus female: 2 of 19, p= 0.04) and smokers (male: 8 of 16 versus female: 0 of 19, p = 0.003) among male subjects.

Table 5.1. Demography and clinical characteristics of patients with improved and worsened NIHSS by poststroke 24 hours

	all patients	NIHSS improved	NIHSS worsened	p
N	35	13	22	
Age (year)	67±11	61±10	70±11	0.02
Male	16	7	9	NS
BMI	27.3±4.4	26.8±4.6	27.7±3.0	NS
smoking	8	3	5	NS
Hypertension	29	10	19	NS
Diabetes mellitus	8	4	4	NS
Dyslipidemia	10	4	6	NS
Cardioembolic	12	2	10	NS
Atherothrombotic	21	9	12	NS
Lacunar	2	1	1	NS
NIHSS on admission	12±6	9±6	14±6	0.04
NIHSS day 2	12±6	7±5	15±6	0.05
NIHSS on discharge	9.5±6	3.5±1	11±6	0.04
Death	7	0	7	0.02

Abbreviations: BMI, body mass index; N, number of cases; NIHSS, National Institutes of Health Stroke Scale; NS, not significant. Sampling time is time in minutes between stroke onset and the first sample draw. Data are presented as mean ± SD or number (percentage).

5.5.1 *Progression of Neurologic Deficit*

The concentration of MCP-1 within 6 hours after stroke was increased in the serum of patients with worsened NIHSS by 24 hours compared with patients with stable or improving NIHSS (p= 0.009). The concentration of hscTnT was elevated at both within 6 hours and 24 hours after stroke onset in the serum of patients with worsened NIHSS at discharge from hospital (p= 0.026 and p= 0.001, respectively). A cutoff value 9.4 ng/L or greater for hs-cTnT measured 24 hours after AIS predicted worsened NIHSS at hospital discharge with a sensitivity of 81% and a specificity of 74% using a receiver operating characteristic curve analysis (area: 0.808, p = 0.002) **Table 5.2.**

Table 5.2. Median serum concentration of biomarkers in patients with improved and worsened NIHSS by poststroke 24 hours and hospital discharge

	$NIHSS_{24}$ improved n=13	$NIHSS_{24}$ worsened n=22	*p*	$NIHSS_{discharge}$ improved n=19	$NIHSS_{discharge}$ worsened n=16	*p*
within 6 hours after onset of stroke						
hs-cTnT	5.9	16.6	0.001	7.4	16.6	0.023
S100B	0.09	0.20	0.001	0.10	0.30	0.005
hsCRP	3.9	5.3	NS	4.1	5.9	NS
MCP-1	9146	18927	<0.001	10220	13755	NS
tPA	22918	41315	0.005	26711	31827	NS
sCD40L	41008	353008	0.03	109986	250015	NS
P-selectin	1129	911	NS	911	984	NS
at post-stroke 72 hours						
hs-cTnT*	6.4	17.7	0.001	6.5	18.2	0.002
S100B	0.07	1.01	0.001	0.10	0.31	NS
hsCRP	3.1	19.2	0.003	3.4	38.3	0.001
MCP-1	13083	15640	NS	13940	15984	NS
tPA	23441	42915	NS	34954	28836	NS
sCD40L	63873	336485	0.05	114396	45196	NS
P-selectin	1062	944	NS	887	1091	NS

Abbreviations: hsCRP (mg/L), high-sensitivity C-reactive protein; hs-cTnT (ng/mL), high-sensitivity cardiac troponin T; MCP-1 (pg/mL), monocyte chemoattractant protein-1; N, number of cases; NIHSS, National Institutes of Health Stroke Scale; sCD40L (pg/mL), soluble CD40 ligand; tPA (pg/mL), tissue plasminogen activator. Data are presented as median. P-selectin (ng/mL); S100B (ng/mL). *hs-cTnT was measured at 24 h.

5.5.2 *Biomarkers and Outcome*

Higher concentration of hs-cTnT within 6 hours and 24 hours after AIS was found in the serum of nonsurvivors (n = 7) compared with survivors (6 hours: median: 17.4, 25th-75th percentiles: 15.6-25.5 versus 7.8, 5.1-17.2; after 24 hours: 19.2, 16.3-26.2 versus 7.1, 6.4-20.5, P = .03, respectively). However, nonsurvivors were significantly older (80 ± 8 versus 67 ± 11 years, p < 0.001), and we found a significantly positive correlation between age and hs-cTnT measured at both 6 and 24 hours after AIS (r = .492, P = .003 and r = .538, P = .001, respectively). S100B concentration within 6 hours after AIS was also significantly higher in patients (n = 7) who died compared to survivors (median: 1.17, 25th-75th percentiles: .17-2.24 versus .13, .09-0.29, p= 0.009). Serum concentration of hsCRP (mg/L) 72 hours after AIS was also higher among nonsurvivors (median: 114.1, 25th-75th percentiles: 40.8-204 versus 4.0, 2.7 14.2, p= 0.001).

5.5.3 *Association between Cardiac Troponin and Thrombo-Inflammation*

Higher serum concentration of hs-cTnT within 6 hours and 24 hours after AIS was associated with higher concentration of hsCRP measured 72 hours after AIS (r = .592 and .596, both P = .001, respectively); higher concentration of tPA within 6 hours of AIS (r = .550 and .534, P = .001 and P = .002, respectively); and higher concentration of MCP-1 within 6 hours of AIS (r = .465 and .442, P = .01 and P = .015, respectively). We found no correlation between hs-cTnT and S100B levels, but serum concentration of MCP-1 measured within 6 hours after onset of stroke positively correlated with S100B measured both within 6 hours and 72 hours after AIS (r = 0.379 and 0.456, p= 0.04 and 0.019, respectively) **Table 5.3.** Based on binary logistic regression analysis including age, gender, and biomarkers, only hs-cTnT measured 24 hours after AIS was an independent predictor of NIHSS worsening at hospital discharge (odds ratio: 1.58, 95% confidence interval: 1.063-2.370, p= 0.024), but not of death.

Table 5.3. Correlations between cardiac troponin T and S100B with thrombo-inflammatory markers

	hs-cTnT	*p*	S100B	*p*
	on admission			
hs-cTnT	NA	NA	0.307	NS
S100B	0.307	NS	NA	NA
hsCRP	0.345	NS	0.111	NS
MCP-1	0.464	0.009	0.379	0.042
tPA	0.550	0.001	0.298	NS
sCD40L	0.280	NS	0.121	NS
P-selectin	-0.314	NS	0.074	NS
	at 72 hours			
hs-cTnT*	NA	NA	0.069	NS
S100B	0.069	NS	NA	NA
hsCRP	0.596	0.001	0.547	0.007
MCP-1	0.303	NS	0.133	NS
tPA	0.439	0.013	0.354	NS
sCD40L	0.183	NS	0.205	NS
P-selectin	-0.194	NS	0.057	NS

Abbreviations: hsCRP, high-sensitivity C-reactive protein; hscTnT, high-sensitivity cardiac troponin T; MCP-1, monocytechemoattractant protein-1; NA, not applicable; NS, not significant. sCD40L, soluble CD40 ligand; tPA, tissue plasminogen activator.

Spearman correlation; data are presented as correlation coefficient (r) and *P* value. *hs-cTnT was measured at 24 hours.

5.5.4 *Discussion of results*

The ultimate goal of this study was to explore associations among serum levels of cardiac troponin T, the brain damage marker S100B, the acute phase protein hsCRP, and thrombo-inflammatory markers such as tPA, sCD40L, P-selectin, and MCP-1 in patients with first-ever AIS without acute cardiac events.

We found that higher concentration of hs-cTnT in the hyperacute (within 6 hours) and subacute stage (after 24 hours) of ischemic stroke was associated with elevated levels of thrombo-inflammatory molecules such as tPA, MCP-1, and hsCRP in patients with first-ever AIS. Although hs-cTnT and MCP-1 concentration in the hyperacute stage also predicted NIHSS worsening by 24 hours, only the elevated concentration of hs-cTnT at poststroke 24 hours remained an independent predictor of progressing neurologic deficit, indicating poor functional outcome on hospital discharge. High-sensitivity cardiac troponin I was elevated in about 20% of patients with AIS in a very recent study, but ACS that required treatment was diagnosed only in a minority group. Thus, high-sensitivity cardiac troponin I elevation without dynamic changes can occur in stroke patients without apparent ACS due to different reasons that stress the heart [261]. It is not clear whether increased troponin T reflects critical coronary artery disease or its elevation is due to other causes such as supraventricular tachycardia or myocardial fibrosis [262]. We also found a positive correlation between age and hs-cTnT independent from comorbidities, similar to others [263-264].

Furthermore, hs-cTnT was recently described as an atherosclerosis marker [265]. We found a positive correlation between hs-cTnT levels and concentration of MCP-1 and tPA in the hyperacute stage, and hsCRP measured at poststroke 72 hours. The higher serum concentration of MCP-1 in the hyperacute stage of ischemic stroke may indicate the severity of atherosclerosis preceding the acute event [266]. High baseline MCP-1 appeared to be a useful biomarker for selecting patients with AIS, who can benefit from early treatment with acetylsalicylic acid combined with extended release dipyridamole [266]. MCP-1 elevation presumably predicts early progression of neurologic deficit but not the late outcome, as no association was found between MCP-1 levels and mortality in this cohort. Indeed, MCP-1 is a key regulator in “opening” the blood–brain barrier in animal models and in in vitro studies [267-268].

Beside hs-cTnT and MCP-1, serum concentration of endogenous tPA in the hyperacute stage was also increased in patients with AIS with worsened NIHSS by 24 hours, suggesting that hemostatic variables also play an important role in developing acute ischemic episodes. It has been shown that increased coagulation activity and disturbed fibrinolysis are predictors of future vascular events, including stroke, particularly in older population [269]. The acute phase reactant hsCRP measured at 72 poststroke hours, but not in the hyperacute stage, was significantly increased in patients with declining NIHSS both at 24 hours and hospital discharge. Previously, an increase in concentration of CRP between 12 and 24 hours after symptom onset was found to be a predictor of an unfavorable stroke outcome [270]. Importantly, hsCRP showed a strong positive correlation with both hs-cTnT and S100B in the subacute stage of AIS in our cohort, suggesting a link between the cerebral ischemia induced inflammatory response and infarct size propagation [271]. It has been previously shown that both hsCRP and lesion area were independently associated with S100B, indicating that the degree of systemic inflammation is associated with astroglia-derived S100B concentration in AIS [272]. In general, there is a great body of evidence that inflammation following ischemic brain injury correlates with adverse outcome. Our findings provide an additional dimension indicating that elevated acute and subacute concentration of serum cardiac troponin T indicates an additional increased risk for adverse outcome in patients with cerebrovascular conditions without manifest cardiovascular events. Recently, biomarkers such as hscTnT were associated with cerebral microinfarcts on 3T magnetic resonance imaging in an older patient population attending a memory clinic [273]. Others also found association between high levels of hs-cTnT and steeper cognitive decline in the elderly independent of cardiovascular diseases [274]. We also found a positive correlation between serum concentration of MCP-1 and S100B in the hyperacute stage of AIS that persisted up to at least 72 poststroke hours. This association may suggest that more severe atherosclerosis reflected by increased MCP-1 is accompanied by higher infarct size [275-276]. In contrast to cardiovascular studies, our findings did not confirm the hypothesis that an increased serum concentration of baseline MCP-1 reflecting better collateralization might be protective in cerebrovascular diseases as well [277].

5.6. *Conclusion*

In summary, our findings suggest that hs-cTnT, similar to other atherosclerosis markers, such as hsCRP or MCP-1, might be an indicator of preceding atherosclerotic burden in patients with first-ever ischemic stroke. Nevertheless, serial measurement of hs-cTnT and expert cardiological workup is mandatory for best medical treatment, in order to decide whether elevated hs-cTnT supports a coexisting ACS in patients with AIS or rather predicts a progression of neurologic deficit reflected by declining NIHSS [261].

VI. Novel findings and conclusions

Ad Aim 1.

Our studies have shown that permanent atrial fibrillation exhibits marked endothelial dysfunction, compared with paroxysmal atrial fibrillation and sinus rhythm indicated by high ADMA and SDMA values observed in permanent atrial fibrillation. Our findings suggest a link between sustained inadequate atrial contractions and elevated dimethylarginine derivatives, but the exact mechanism is not elucidated so far. One hand, we presume an association between atrial wall stress and endothelial damage generated by increased ADMA production, on the other SDMA appeared to be a predominant marker in patients with permanent atrial fibrillation here as a novelty.

Ad Aim 2.

Although plasma ADMA *per se* showed no correlation with short and long term outcomes, patients with more severe neurological symptoms on admission had lower L-arginine / ADMA ratio in our cohort.

In the acute phase, in patients with milder neurological symptoms on admission, higher L-arginine / ADMA ratio was observed suggesting the protective role of an increased L-arginine level, probably improving the cerebral perfusion by the beneficial effect of the NO level.

In the subacute phase, lower L-arginine level, lower L-arginine/ADMA and L-arginine/SDMA ratio were associated with higher mRS on discharge respectively, indicating a worse short-term outcome. Interestingly, patients with lower L-arginine/ADMA and L-arginine/SDMA ratios improved on the long-term.

In contrast, patients with higher L-arginine, L-arginine/ADMA and L-arginine/SDMA and lower discharge mRS improved fast, but not later or even worsened by 6 months indicating that its biological effect is time-dependent.

Based on our findings, an increased systemic concentration of ADMA less likely to be the result of acute stroke, rather a preceding factor contributing to cerebrovascular events. In accordance with this, coincidence of the lower level of ADMA and L-arginine may suggest a lower vascular risk and a better long-term outcome.

Ad Aim 3.

A higher concentration of hs-cTnT in the hyperacute (within 6 hours) and subacute stage (after 24 hours) of ischemic stroke was associated with elevated levels of thrombo-inflammatory molecules such as tPA, MCP-1, and hsCRP in patients with first-ever AIS. Although hs-cTnT and MCP-1 concentration in the hyperacute stage also predicted NIHSS worsening by 24 hours, only the elevated concentration of hs-cTnT at poststroke 24 hours remained an independent predictor of progressing neurologic deficit, indicating poor functional outcome on hospital discharge.

MCP-1 elevation presumably predicts early progression of neurologic deficit but not the late outcome.

Serum concentration of endogenous tPA in the hyperacute stage was also increased in patients with AIS with worsened NIHSS by 24 hours, suggesting that hemostatic variables also play an important role in developing acute ischemic episodes.

Serum concentration of hsCRP showed a strong positive correlation with both hs-cTnT and S100B in the subacute stage of AIS in our cohort, suggesting a link between the cerebral ischemia induced inflammatory response and infarct size propagation

Our findings provided an additional dimension indicating that elevated acute and subacute concentration of serum cardiac troponin T indicates an additional increased risk for adverse outcome in patients with cerebrovascular conditions without manifest cardiovascular events.

VII. List of publications

7.1. Publications related to the thesis

1. **Csecsei P**, Pusch G, Ezer E, Berki T, Szapary L, Illes Z, Molnar T. Relationship between Cardiac Troponin and Thrombo-Inflammatory Molecules in Prediction of Outcome after Acute Ischemic Stroke. J STROKE CEREBROVASC DIS. 2018;27(4):951-956. **IF: 1.598**

2. **Csecsei P**, Nagy L, Keki S, Szapary L, Illes Z, Farkas N, Molnar T. L-Arginine Pathway Metabolites Predict 6 Months Outcome after Acute Ischemic Stroke. INT J NEUROREHABILITATION. 2018;5: 315. DOI: 10.4172/2376-0281.1000315 **IF: 1.01**

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