BIOMARKER STUDIES IN ACUTE ISCHEMIC STROKE

PhD thesis

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Pécs, 2019

I. INTRODUCTION

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. Of all strokes, 87% are ischemic, 10% are intracerebral hemorrhage, and 3% are subarachnoid hemorrhage strokes. The TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification is based on clinical symptoms as well as results of further investigations. Accordingly, a stroke is classified as either thrombosis or embolism due to atherosclerosis of a large artery, an embolism originating from the heart (cardiogenic stroke), a complete blockage of a small blood vessel (lacunar) or other determined/undetermined cause. Cardioembolic stroke accounts for 14-30% of ischemic strokes and atrial fibrillation (AF) is responsible for about 50% of cardiogenic strokes. Cardiac embolism causes more severe strokes than any other ischemic stroke subtypes resulting in more impairments (modified Rankin scale), more dependency (Barthel Index), and higher mortality. Several blood biomarkers have been emerged in the last decade in association of the evolution, diagnosis and prognosis of ischemic stroke. Some of them are organ specific (e.g. astrocyte damage marker - S100B), others reflect the preceding atherosclerosis such as hsCRP or indicators of endothelial dysfunction or thrombo-inflammation.

S100B in the peripheral blood is a sensitive marker of both blood–brain barrier dysfunction and ischemic brain damage and predictor of stroke outcome. Increased high-sensitivity C-reactive protein (hsCRP) in the subacute stage is an independent predictor of death. Thrombo-inflammatory molecules (tissue plasminogen activator [tPA], soluble CD40 ligand [sCD40L], P-selectin, interleukin-6, interleukin-8, monocyte chemoattractant protein-1 [MCP-1]) connect the prothrombotic state, endothelial dysfunction, and systemic/ local inflammation in acute vascular events such as myocardial infarction or ischemic stroke. Besides other factors, MCP-1 plays a crucial role in atherogenesis. On the other hand, 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. Cardiac troponins (cTn) are sensitive and specific biomarkers used in the diagnosis of myocardial infarction (MI). Primarily, cTn is released into the bloodstream when cardiac myocytes are damaged by acute ischemia. Beside MI, there are several clinical conditions, such as stroke accompanied by troponin elevation. However, the exact mechanism of such increased concentration of cTn in stroke patients without apparent myocardial damage is not fully explored.

Nitric oxide (NO) plays a role in maintaining vascular integrity. 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. 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 ischemic stroke. In addition, SDMA level in the plasma was strongly associated with adverse clinical outcome during the first 30 days following ischemic stroke. Furthermore, a subacute elevation of both ADMA and SDMA proved to be predictors of poor functional outcome at 90 days.

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. Myocardial blood flow alterations caused by decreased production of nitric oxide (NO) lead to endothelial dysfunction. Emerging clinical and experimental evidences indicate that ADMA and SDMA are involved in the pathophysiology of endothelial dysfunction, atherosclerosis, oxidative stress, inflammation and apoptosis. All of these pathological processes play pivotal role in the development of AF.

II. AIMS

In general, the aim of the present thesis was to explore clinical utility of potential biomarkers in association with acute ischemic stroke particularly focusing on prediction of the outcome and/or disease progression.

Specific aims:

1. Our first aim was to prove that L-arginine pathway metabolites (LPM) have a potential to discriminate between types of atrial fibrillation in patients with 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. We also aimed to 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 follow-up study. Moreover, we aimed to explore 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 high-sensitivity cardiac troponin T (hs-cTnT) with thrombo-inflammatory markers, in patients with acute ischemic stroke (AIS) without cardiovascular complications.

III. L-ARGININE PATHWAY IN ACUTE ISCHEMIC STROKE

3.1. L-arginine pathway and atrial fibrillation

3.1.1 Subjects and methodes

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.

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. 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.

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. All patients in the control group have sinus rhythm.

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.

3.1.2. 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 3.1**. 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 3.1**) 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 3.1. 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).

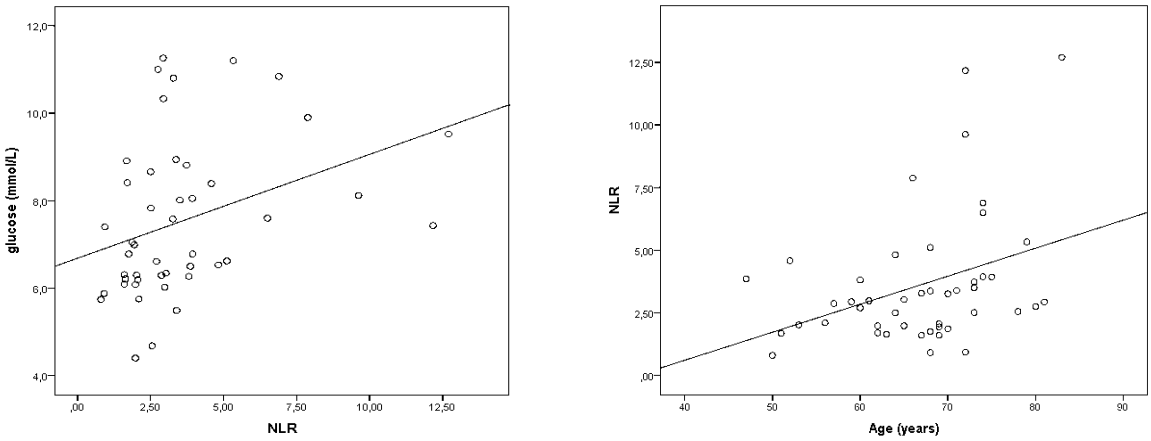

Fig. 3.1. Correlation of neutrophil-lymphocyta ratio (NLR) and serum glucose level 24 hours after stroke onset (A). Correlation of NLR and age (B). Data are presented as mean and 95% confidence interval.

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). 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 3.2. 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 3.2)**.

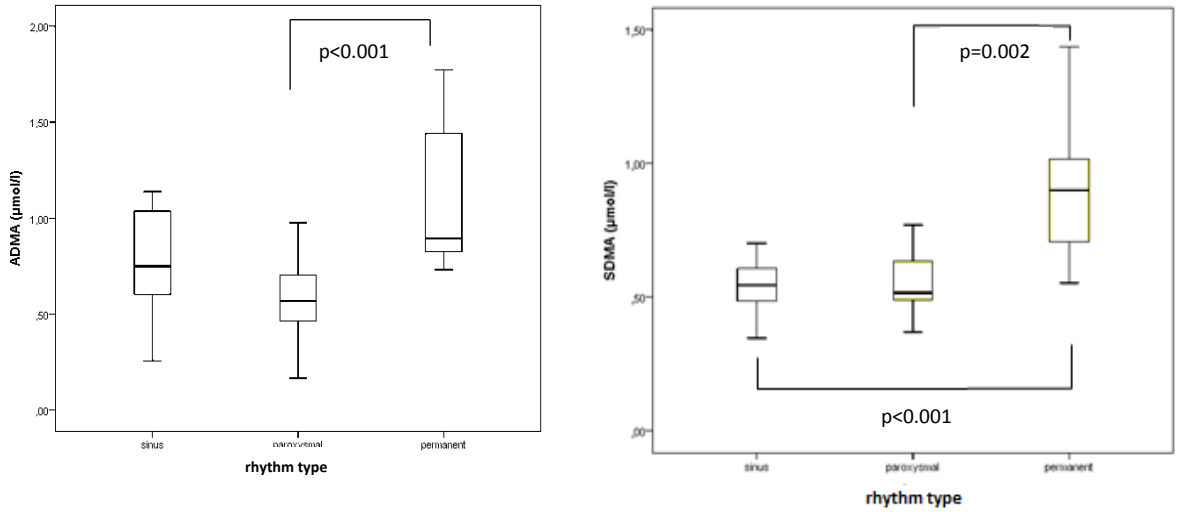

Fig.3.2. A. Plasma concentration of ADMA (µmol/l) is shown in ischemic stroke patients 24 hours after admission with sinus rhythm, paroxysmal atrial fibrillation (AF) or permanent AF. Data are presented as mean and 95% confidence interval. **B.** Plasma concentration of SDMA (µmol/l) is shown in ischemic 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 3.2. 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 3.3).**

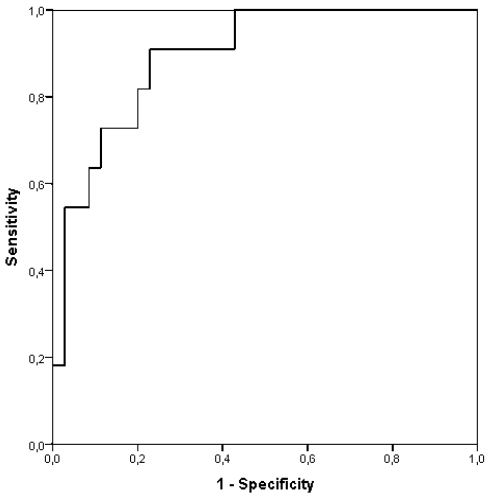

Fig. 3.3. Optimized cut-off value determined for SDMA using receiver operating characteristic curve (ROC) analysis.

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.

Dimethylarginines and clinical variables

All L-arginine pathway metabolites, except ADMA were significantly correlated with various factors in univariate analysis (**Table 3.3.**) 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 3.3. 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

3.2. L-arginine pathway and ischemic stroke

3.2.1 Subjects and methodes

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. All relevant demographic and clinical parameters of the patients were recorded.

Outcome measures

We have 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.

3.2.2. Results

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 3.4.**

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 3.4.
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

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].

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 3.5.)**.

Table 3.5.
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.

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 3.6.**). 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 3.6.**

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 (∆mRS) (**Table 3.6** and **Figure 3.4**).

Table 3.6.

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
∆mRS	- 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

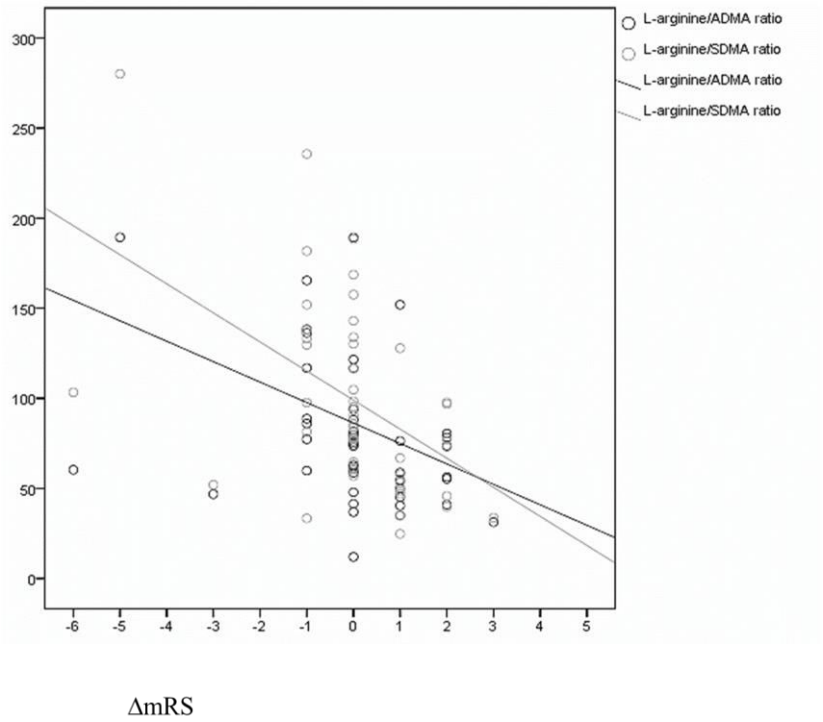

Fig.3.4. Correlation among dimethylarginine ratios and mRS change. ΔmRS: mRS at discharge-mRS at 6 months. All markers were measured at 24 hours after stroke. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethyl-arginine. Values are Spearman correlation coefficients.

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.

IV. TROPONIN AND THRMBO-INFLAMMATORY MOLECULES IN ISCHEMIC STROKE

4.1. 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.

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.

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.

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).

4.2. 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 4.1.** 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 4.1. 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).

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 4.2.**

Table 4.2. 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.

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 ($r = 0.492$, $P = 0.003$ and $r = 0.538$, $P = 0.001$, 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 = 0.592 and 0.596, both P = 0.001, respectively); higher concentration of tPA within 6 hours of AIS (r = 0.550 and 0.534, P = 0.001 and P = 0.002, respectively); and higher concentration of MCP-1 within 6 hours of AIS (r = 0.465 and 0.442, P = 0.01 and P = 0.015, 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 4.3.** 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 4.3. 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.

V. NOVEL FINDINGS AND CONCLUSION

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 risk 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.

VI. LIST OF PUBLICATIONS

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**

3. **Csecsei P**, Varnai R, Nagy L, Keki S, Molnar T, Illes Z, Farkas N, Szapary L. L-arginine pathway metabolites can discriminate paroxysmal from permanent atrial fibrillation in acute ischemic stroke. IDEGGYÓGYÁSZATI SZEMLE. In Press, accepted: 09/04/2018 **IF: 0.252**

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Cumulative impact factor related to the thesis: **5.443**

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Total impact factor not related to the thesis: 11.743

Number of independent citations (MTMT): 34

ACKNOWLEDGEMENTS

First of all I would like to express my thank to my supervisors *Laszlo Szapary* and *Tihamer Molnar* who have guided and supported me in the last years and also for providing me a „multidimensional" scientific thinking.

I would like to thank *Prof. Sandor Keki and Lajos Nagy* for providing the laboratory background of my studies at the Department of Applied Chemistry, at the University of Debrecen.

I would like to emphasize my gratitude to *Professor Zsolt Illes* for not regreting the time and effort to correct and improve the quality of my manuscripts.

I greatly appreciate the valuable suggestions from *Nelli Farkas*, who guaranteed the precise statistical analysis of my data.

I am especially grateful for my Professors, *Samuel Komoly and Jozsef Janszky* for guiding and managing my scientific progress by constant inspiration, critical notes and providing me a continous encouragement in my work.

At last but not least, I would like to thank to *my family* for their love, patience and continous support, creating me a calm background, which was necessary to my research work.