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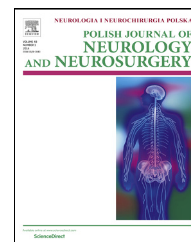

The rtPA increases MMP-9 activity in serum during ischaemic stroke

Piotr Gołąb [a], Anna Boguszewska-Czubara [b], Michał Kiełbus [c], Jacek Kurzepa [b,*]

[a] Department of Internal Medicine, The Holy Family Specialist Hospital in Rudna Mala, Rzeszów, Poland
[b] Department of Medical Chemistry, Medical University of Lublin, Lublin, Poland
[c] Department of Biochemistry and Molecular Biology, Medical University of Lublin, Lublin, Poland

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Background and purpose: To find the relationship between rtPA treatment vs. MMP-9 activity, MMP-3, and TIMP-1 serum levels related to patients' neurological status during acute ischaemic stroke (IS).

Material and methods: 35 IS patients were enrolled. 14 of them underwent thrombolytic therapy with Actylise (rtPA group). The serum samples were obtained at 3 time-points for rtPA group (time-point 0: 1st–4th hour of stroke; time-point 1 – immediately after rtPA administration; time-point 2 – on day 5–7 from stroke onset). Remaining patients had venous blood collection at two time-points: time-point 1 – 5th–10th hour of stroke and time-point 2 – on day 5–7 of stroke. MMP-9 was analyzed with gelatin zymography, MMP-3 and TIMP-1 serum levels were analyzed with ELISA method. NIHSS improvement ratio (IR) was calculated as a difference between NIHSS score at the admission and discharge of patient.

Results: The active form of MMP-9 (86 kDa) was not observed in any analyzed samples. Total MMP-9 activity was significantly elevated at time-point 1 in rtPA group in comparison with non-rtPA group. MMP-3 serum level significantly decreased during rtPA administration in comparison with non-rtPA group and it was restored at time-point 2. MMP-3 negatively correlated with IR values ($p = 0.06$).

Conclusions: Thrombolysis applied for IS treatment increases MMP-9 activity in serum, however, rtPA does not facilitate the conversion of pro-MMP-9 into the active form. Our results also suggest the involvement of MMP-3 to the biochemical processes occurring during acute phase of IS.

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1. Introduction

The ischaemic stroke (IS) is one of the leading causes of death and disability worldwide. In spite of well-developed diagnostic tools for rapid detection of cerebral ischaemia the recommended treatment is limited to recombinant tissue plasminogen activator (rtPA) [1]. However, due to restrict qualifying criteria for thrombolytic therapy it can be applied for a few percentage among of all ischaemic stroke patients [2]. In

* *Corresponding author at*: Department of Medical Chemistry, Medical University of Lublin, Chodzki 4a, 20-093 Lublin, Poland.
Tel.: +48 81 535 73 90; fax: +48 81 535 73 61.
E-mail address: kurzepa@onet.pl (J. Kurzepa).

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addition, the rtPA should be administrated at experienced centres in order to limit the appearance of side effects [3]. One of the serious side effects during thrombolytic therapy is an intracranial bleeding. This event is developed due to overactivity of anticlotting mechanisms trigged by rtPA [4]. Previous studies indicated that intracranial bleeding within the ischaemic focus potentially can occur by high baseline activity of matrix metalloproteinase(MMP)-9 (gelatinase B) [5,6]. MMP-9 enzyme belongs to MMPs family, capable to digest proteins located extracellularly. MMPs are involved in numerous physiological and pathological processes also during the IS [7]. The involvement of MMP-9 into the development of intracranial bleeding is caused by its ability to cleave type IV collagen – the constituent of basal membrane of endothelium. This process leads to the blood–brain barrier (BBB) destruction. In addition, MMP-9 can cleave occludin and claudin, the tight junctions proteins (TJPs) within the BBB [8].

Animal and clinical studies showed the increase of MMP-9 activity over the course of first 24 h of stroke [9]. MMP-9 expression is trigged by cerebral ischaemia and inflammatory process occurring within the brain tissue thereafter. Proinflammatory cytokines as tumour necrosis factor(TNF)-α and interleukin(IL)-6 activate the expression of MMP-9. MMP-9 is next involved into further activation of IL-1β and chemokine CXCL-8 [10].

Extracellularly, the activity of MMPs is regulated by proteolytic activation of pro-form and the presence of natural inhibitors of MMPs – tissue inhibitors of MMPs (TIMPs). Among four known TIMPs, TIMP-1 has the highest affinity to MMP-9. Different studies applied MMP-9/TIMP-1 ratio as an indicator of MMP-9 activity *in vivo* [11,12].

The proteolytic activation of MMP-9 is conducted by active forms of other metaloproteinases as MMP-2 (gelatinase A) or MMP-3. Under *in vivo* conditions MMP-2 is activated on the cells' surface by complex composed of membrane-type metalloproteinase (MMP-14) with tissue inhibitor of MMP–TIMP-2 [13]. However, MMP-3 can be activated by other proteases including plasmin [14]. Therefore, MMP-3 can be considered as a link between plasmin (activated by rtPA) and MMP-9. Finally the activation of MMP-9 facilitates the BBB destruction and secondary bleeding within the ischaemic focus [6].

Our objective was to find the relationship between rtPA treatment of acute ischaemic stroke and MMP-9 activity, MMP-3, TIMP-1 serum level related to patients' neurological status.

2. Materials and methods

2.1. Patients

Thirty-five patients with acute ischaemic stroke confirmed with computed tomography (CT) scan imaging performed at the admission were enrolled. The main inclusion criterion was the admission into the hospital before the 10th hour from the onset of appearance of focal neurological symptoms (the stroke onset). Fourteen patients met the criteria for thrombolytic treatment with rtPA (Actilyse, Boehringer Ingelheim). The rtPA was administrated according to standard protocol (0.9 mg/kg of body weight, 10% of drug was administrated in bolus, remaining part was administrated through 1 h in iv. drip). After enrollment of thrombolytic patients, twenty-one IS patients who did not met the criteria for rtPA administration were included into the study as a control group. For non-rtPA group thrombolytic therapy was not applied due to (1) extended time between stroke onset and admission to emergency department or (2) the presence of focal neurological symptoms during awakening in the morning (the onset of IS at night during sleeping, the exact start-hour unknown). Written informed consent was obtained from each patient (or from family members when necessary). The local Ethics Committee (Medical University of Lublin) accepted the protocol of the study (agreement No. KE-0254/260/2013).

Exclusion criteria were: (1) regression of neurological symptoms within 24 h from the onset (Transient Ischaemic Attack, TIA), (2) history or symptoms suggesting other reason

Table 1 – The characteristic of study group. The values of age, NIHSS and improvement ratio were expressed in median and 1st–3rd quartiles [square brackets].

		rtPA, $n = 14$	Non-rtPA, $n = 21$	χ^2
Age in years (median)		71.0 [67.5–72.5]	80.5 [70.0–80.5]	
Sex (female/male)		6/8	12/9	
Hypertension (yes/no)		12/2	18/3	
Type II diabetes (yes/no)		2/12	5/16	$p > 0.1$
CHD (yes/no)		8/6	12/9	
Atrial fibrillation (yes/no)		2/12	9/12	
Smoking (yes/no)		8/6	10/11	
OCSP classification	TACI	1	3	
	PACI	9	12	
	LACI	3	4	
	POCI	2	2	
Secondary bleeding (*n*)		1	1	
NIHSS at admission (median)		13 [5.5–16]	12.5 [7–20]	
NIHSS at discharge (median)		1 [1–12.5]	6 [4–16.25]	
Improvement ratio (median)		4 [2.5–10]	3 [0.5–6]	

CHD, coronary heart disease; OCSP, Oxford Community Stroke Project classification; TACI, total anterior circulation infarct; PACI, partial anterior circulation infarct; LACI, lacunar infarct; POCI, posterior circulation infarct.

of focal neurological symptoms. Characteristic of study group was given in Table 1.

2.2. NIHSS

The neurological status was evaluated at the admission and discharge with use of National Institutes of Health Stroke Scale (NIHSS). The neurological status Improvement Ratio (IR) was calculated as a difference between NIHSS score at the admission and discharge of patient.

2.3. Sample collection

For thrombolytic patients the venous blood was obtained at three time-points; time-point 0 – at the admission (1st–4th hour of stroke); time-point 1 – immediately after rtPA administration (4th–5th hour of stroke); time-point 2 – on day 5–7 from stroke onset. Remaining stroke patients had venous blood collection at two time-points: time-point 1 – at the admission to hospital (5th–10th hour of stroke) and time-point 2 – on day 5–7 of stroke. Time-point 0 (1st–4th hour of stroke) was established only for rtPA patients cause the non-thrombolytic patients were enrolled after the time designed to time-point 0.

2.4. Zymography

MMP-2 and MMP-9 activities were determined by gelatin zymography [15]. Briefly, the serum samples after incubation were diluted 1/50 with redistilled water. 9 μl of diluted serum was mixed with +3 μL sample buffer with 10% sodium dodecyl sulfate (SDS). Next, the proteins were separated by electrophoresis on a 10% polyacrylamide gel with 0.05% gelatin type A from porcine skin (G2500) (Sigma–Aldrich, St. Louis, MO, USA). Gels were washed for 1 h with 50 mM Tris–HCl buffer, pH 7.2, containing 10 mM $CaCl_2$, 0.02% NaN_3 and 2.5% Triton X-100. Incubation was performed for 20 h at 37 °C in the same buffer, instead using 1% Triton X-100. Gels were stained with 0.1% Coomassie Blue R-250 in 20% methanol and 10% acetic acid, and destained in 20% methanol and 10% acetic acid. MMP-2 and MMP-9 activities were detected as clear bands on a blue background. Gelatin zymography usually shows the activity of all gelatinase forms. Inactive pro-forms undergo non-proteolytic activation during incubation with sample buffer containing SDS. Enzymes were identified by comparing their localization with molecular mass standards (SM0441) (Fermentas Life Sciences, St. Leon-Rot, Germany), as well as with standards of both gelatinases (911-MP, 902-MP) (R&D Systems, Minnesota, MN, USA). Zymograms were captured by gel visualization system (Syngene, Cambridge, UK) and quantified with genetools software (Syngene, Cambridge, UK). The activities of gelatinases were expressed as the optical density (OD) of the substrate lysis zone.

2.5. ELISA

Commercially available ELISA kits were applied for analysis of MMP-3 and TIMP-1 serum levels (R&D Systems Inc., USA) according to manufacturer instructions. Briefly, for MMP-3 evaluation serum samples were diluted 10-times, for TIMP-1 - 100 times with diluent included to the kits. The plates were read by Microplate reader (Epoch, Biotek Instruments, USA) at wave length 450 nm corrected at 540 nm.

MMP-9/TIMP-1 ratio, estimated on the basis of zymography and ELISA results, was expressed in OD/ng/ml.

2.6. Statistical methods

The presence of Gaussian distribution was estimated with Lillefors test. In case of Gaussian distribution the repeated measures analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons post hoc test or *t*-test were used for statistical analysis. Otherwise Friedman test with Dunn post hoc test or Mann–Whitney test were applied. For paired or repeated measurements the paired *t*-test or Wilcoxon test as well as repeated ANOVA or Friedman test was used. Correlation between NIHSS improvement ratio and biochemical parameters were evaluated with Spearman correlation. χ^2 test was applied for estimation of difference of selected parameters expressed in Table 1. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Zymography

Gelatinolytic activity was detected at 72 kDa molecular weight (corresponding to pro-MMP-2), 92 kDa (corresponding to pro-MMP-9), 130 kDa (corresponding to MMP-9/NGAL, heterodimer with neutrophil gelatinase-B associated lipocalin) and ~200 kDa (corresponding to MMP-9/MMP-9 homodimer) in all analyzed samples. The activities derived from all forms of MMP-9 (92 kDa, 130 kDa and ~200 kDa forms) were summed and the result was treated as total MMP-9 activity during further calculations. The active forms of gelatinases were not detected in analyzed samples. Representative zymogram was shown in Fig. 1.

3.2. MMP-9, TIMP-1, MMP-9/TIMP-1 ratio

The activity of MMP-9 was significantly increased during the rtPA administration in comparison to time-point 0 ($p < 0.05$,

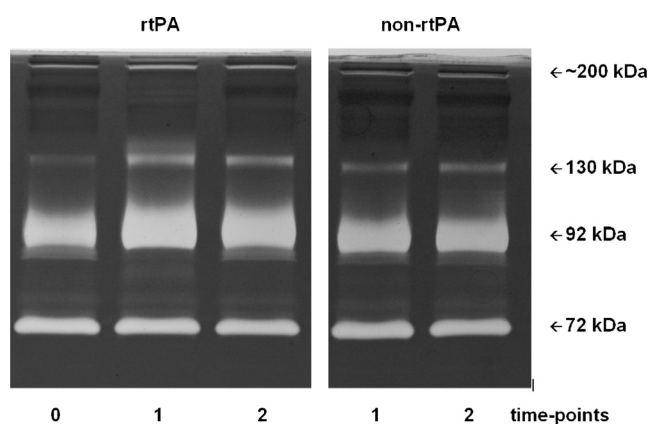

Fig. 1 – Representative zymograms of sera obtained from thrombolytic and non-thrombolytic patients. 72 kDa band corresponds to pro-MMP-2, 92 kDa to pro-MMP-9, 130 kDa to MMP-9/NGAL heterodimer (description in text), and 200 kDa to MMP-9/MMP-9 homodimer.

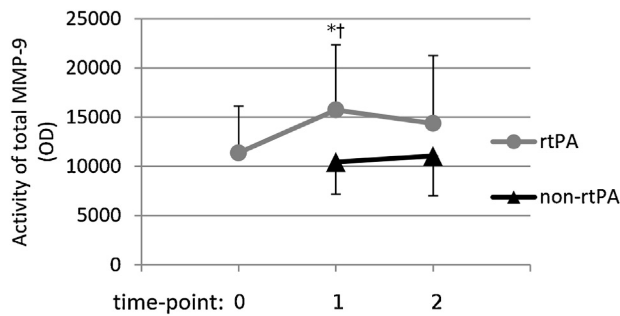

Fig. 2 – Total MMP-9 activity in serum of thrombolytic and non-thrombolytic patients during acute phase of ischaemic stroke. The increase of MMP-9 activity during thrombolysis (time-point 1) in comparison to the baseline (time-point 0, Tukey–Kramer post hoc test, $^{*}p < 0.05$) and corresponding time-point of non-thrombolytic group (t-test, $^{\dagger}p < 0.05$) was noticed.

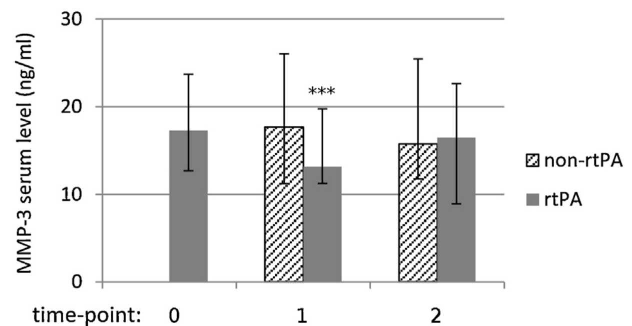

Fig. 4 – MMP-3 serum level in thrombolytic and non-thrombolytic patients during acute phase of ischaemic stroke. The decrease of MMP-3 level during thrombolysis (time-point 1) in comparison to time-point 0 (Tukey–Kramer post hoc test, $^{***}p < 0.001$) was noticed.

Tukey–Kramer post hoc test) as well as compared to the corresponding time-point 1 at non-thrombolytic group ($p < 0.05$, *t*-test) (Fig. 2).

Analysis of variance revealed the difference between TIMP-1 serum levels evaluated in rtPA group ($p = 0.03$, repeated ANOVA). However, the increased TIMP-1 serum level at time-point 2 did not reach statistical significance in post hoc Dunn's test ($p > 0.05$).

The MMP-9/TIMP-1 ratio was insignificantly elevated during rtPA administration in comparison to time-point 0 ($p = 0.07$, repeated ANOVA). The differences between corresponding time-points evaluated in thrombolytic and non-thrombolytic groups were also statistically insignificant ($p = 0.08$ for time-point 1 and 2, *t*-test) (Fig. 3).

3.3. *MMP-3*

MMP-3 serum level significantly decreased during rtPA administration and it is restored on 5 day after stroke onset ($p = 0.0006$, repeated ANOVA). The MMP-3 serum level in rtPA at time-point 1 was insignificantly higher in comparison with

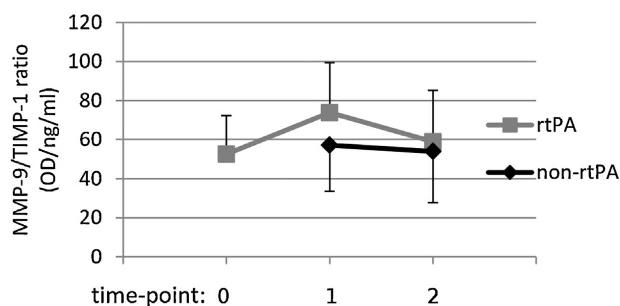

Fig. 3 – MMP-9/TIMP-1 ratio in serum of thrombolytic and non-thrombolytic patients during acute phase of ischaemic stroke. The increase of MMP-9/TIMP-1 ratio at time-point 1 of thrombolytic group did not reach the statistical significance.

corresponding time-point in non-rtPA group ($p = 0.08$, Mann–Whitney test) (Fig. 4). All results were given in Table 2.

3.4. *NIHSS improvement ratio*

Among analyzed relationships between biochemical parameters (MMP-9 activity, MMP-3, TIMP-1, MMP-9/TIMP-1 serum level) vs. neurological status improvement ratio only the correlation with mean value of MMP-3 revealed the negative relationship. However, the result was located on the border of statistical significance ($r = -0.53$, $p = 0.06$, Spearman correlation). The values of remaining correlation coefficients were statistically insignificant ($p > 0.1$, Spearman correlation).

4. Discussion

According to the literature both gelatinases have the ability to destruct the basal membrane of endothelium as well as they digest the proteins located at the tight junctions – occludin and claudin [8]. Despite the similar structures and substrates of MMP-2 and MMP-9 only the expression of MMP-9 is induced by proinflammatory cytokines that are elevated in the blood after acute ischaemia. Therefore, MMP-9 serum activity gradually increases in the course of the first hours and days of IS [16]. Moreover, this enzyme was previously indicated as a risk factor for intracranial bleeding and poorest clinical outcome after IS [17]. Animal and clinical observations revealed that high activity of MMP-9 in the blood of patients treated with rtPA is the risk factor for developing of intracranial bleeding within ischaemic focus [18]. Animal studies indicated that the active form of MMP-9 (86 kDa) can appear in sera of patients treated with rtPA suggesting the intense activation of MMP-9 trigged by plasminogen activator [5,6]. During current study the blood was collected immediately after rtPA administration, however the active form MMP-9 was not observed. Active MMP-9 is formed by cleavage of propeptide covering the active site within the catalytic domain of pro-MMP-9. Under *in vivo* conditions pro-MMP-9 is activated by active MMP-2 and MMP-3 [19]. We did not find the active form of MMP-2 in zymography analysis. In addition, we observed the decrease of MMP-3 serum level

Table 2 – The results of MMP-9 serum activity and MMP-3, TIMP-1, MMP-9/TIMP-1 serum levels at rtPA and non-rtPA groups during acute phase of ischaemic stroke. The mean values (with SD) or medians (with 1st and 3rd quartiles).

rtPA patients	Time-point 0	Time-point 1	Time-point 2	Repeated ANOVA or Friedman test
MMP-9	11,380 ± 4750	15,745 ± 6627*,†	14,373 ± 6899	$p = 0.01$
TIMP-1	213.04 (176.5–233.9)	209.4 (180.5–227.9)	249.05 (221.5–284.2)	$p = 0.03$
MMP-9/TIMP-1	52.54 ± 19.7	73.82 ± 25.4	58.89 ± 26.4	$p = 0.07$
MMP-3	14.9 (10.3–21.3)	11.1 (9.18–17.7)***	15.8 (8.24–21.95)	$p = 0.004$
non-rtPA patients	NA	Time-point 1	Time-point 2	Paired *t*-test or Wilcoxon test
MMP-9		10,439 ± 1032	11,052 ± 4058	$p = 0.7$
TIMP-1		194.1 (168.6–262.4)	234.71 (183.7–282.8)	$p = 0.3$
MMP-9/TIMP-1		57.14 ± 23.64	53.93 ± 26.23	$p = 0.2$
MMP-3		17.7 (11.2–26.0)	15.7 (11.8–25.4)	$p > 0.9$

* $p < 0.05$ the difference in comparison to time-point 0 at rtPA group (Tukey–Kramer).
*** $p < 0.001$ the difference in comparison to time-point 0 at rtPA group (Tukey–Kramer).
† $p < 0.05$ the difference in comparison to corresponding time-point in non-rtPA group (*t*-test).

directly after the thrombolysis. The low MMP-3 serum level after the thrombolysis is confusing cause the previous report revealed the increase the MMP-3 level in the course of IS [20]. Our observation did not find any fluctuation of MMP-3 serum level in non-thrombolytic patients during the acute phase of IS.

The absence of active forms of MMP-2 and MMP-9 as well as the decrease of MMP-3 level after thrombolysis does not support the previous observations that the appearance of active MMP-9 in the plasma during thrombolysis is conducted by MMP-3 [19]. Theoretically, the absence of an active form of MMP-9 can be explanted by immobilization of the enzyme near the place of activation, within basal lamina of brain vessels. Different immunohistochemistry studies showed the intense MMP-9 activity in the vessel walls [21]. First, the rtPA activates plasminogen bounded to the clot, next plasmin activates MMP-3 and MMP-3 activates MMP-9 thereafter. After the recanalization an active MMP-9 attacks and stays attached to its substrate (type-IV collagen) within vessel wall. Further investigations performed on animal models should explain whether an active form of MMP-9 is attached to the basal membranes of brain vessels.

Interesting observation is the elevation of total MMP-9 activity after thrombolysis in comparison to non-thrombolytic patients. Our previous *in vitro* studies also revealed the increase of MMP-9 activity without the presence of an active form (86 kDa) after incubation of serum with different concentrations of rtPA [22]. The potential source of MMP-9 *in vivo* can be either the cells derived from clot destructed by plasmin or the breakdown of MMP-9/MMP-9 homodimer (~200 kDa form) into the 92 kDa form.

Our study showed that MMP-9/TIMP-1 ratio has the trend to be higher after the thrombolysis in comparison with non-thrombolytic patients. The higher activity of 92 kDa form after the thrombolysis supplies the direct precursor of an active MMP-9 and leads finally to BBB destruction.

Last finding was the trend towards the negative correlation between mean of MMP-3 serum level and NIHSS improvement ratio of stroke patients. The role of MMP-3 in the pathogenesis of acute ischaemic stroke is less known in comparison to MMP-9. The PubMed database contains only 28 articles related to phrase ''ischaemic stroke MMP-3'' (''ischaemic stroke MMP-9'' contains over 400 articles). Previously was noticed the increase of MMP-3 serum level after IS and its contribution to pro-MMP-9 activation [14,20,23]. In addition, it was noticed the elevated expression of MMP-3 within the ischaemic focus [24] and its involvement into the intracranial bleeding after animal model of thrombolysis [25]. Our study suggests the possible relationship between high MMP-3 serum level and the worse recovery during acute phase of ischaemic stroke.

5. Limitations of the study

Current study has some limitations. First, the results should be confirmed on larger study group. Second, the first blood collection at non-thrombolytic patients was after the 5th hour from stroke onset. Because it was impossible to obtain the blood earlier than 5th hour of stroke (the patients admitted earlier were enrolled to thrombolytic group according to the qualifying criteria), the activity or concentrations of estimated biochemical parameters within the first 5 h is unknown.

6. Conclusions

Thrombolytic therapy with rtPA increases the MMP-9 activity and decreases the MMP-3 concentration in patients' sera during acute ischaemic stroke.

RtPA does not contribute to the appearance of active form of MMP-9 in the bloodstream.

MMP-3 serum level probably is related with worse recovery of patients during acute phase of ischaemic stroke.

Conflict of interests

None declared.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals.

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