

# Assessment of inflammatory infiltration and angiogenesis in the thrombus and the wall of abdominal aortic aneurysms on the basis of histological parameters and computed tomography angiography study

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**Abstract:** The proliferation of vessels within the aneurysm's wall and the intraluminal thrombus of abdominal aortic aneurysm (AAA) may be the main factor responsible for progression and rupture of AAA. The aim of this study was to compare the parameters of the thrombus (size, density, contrast enhancement) measured by computed tomography (CT) with histological assessment of thrombi removed during surgery. 29 patients with AAA were examined with angio-CT. Post-surgery histopathological evaluation of AAA was performed. Slides were stained with markers of B- (CD20) and T-lymphocytes (CD3), and markers of endothelial cells (CD34). Thrombi were enhanced after contrast media administration in angio-CT ( $p = 0.002$ ). There was a statistically significant correlation between contrast enhancement and the presence of B lymphocytes. Intensity of endothelial cell marker expression significantly correlated with the presence of inflammatory T- and B-cells. No statistical significant correlation was found between contrast enhancement of the thrombus and markers of endothelial cells. The accumulation of inflammatory cells in the wall of AAA thrombus results in the formation of new vessels which participates to the instability of the thrombus and AAA wall. Assessment of the inflammation and neovascularization in the wall and thrombus of the AAA might be an important factor in monitoring the progression and the risk of aneurysm's rupture. (*Folia Histochemica et Cytobiologica* 2012, Vol. 50, No. 4, 547–553)

**Key words:** abdominal aortic aneurysm, thrombus, CD3, CD20, CD34, angiogenesis, CT angiography.

## Introduction

The research on processes occurring in the wall and in the intraluminal thrombus of abdominal aortic aneurysm proves that the disease-affected wall and in-

traluminal thrombus development are the centre of an intense angiogenesis process [1–5].

The proliferation of small vessels within the aneurysm wall and in the intraluminal thrombus of abdominal aortic aneurysm (AAA) may be one of the factors responsible for progression of the AAA and the increased risk of rupture [6, 7]. It has been proved that the course of AAA is connected with neovascularization, inflammatory processes activity, free oxygen radicals activity and also with the width of the mural thrombus [8]. Mayranpaa et al. and Kazi et al. revealed the close link between intraluminal throm-

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bus, inflammation and neovascularization within aneurysmal wall, that may decrease integrity and stability of the aneurysm wall leading to its rupture [9, 10].

The quantitative evaluation of neovascularization in the AAA wall is possible only in the histopathological examination of the preserved tissues sample after AAA surgery [11].

Modern imaging methods, despite the fact of their continual improvement, allow only morphologic and qualitative thrombus assessment.

The aim of this study was to estimate the morphological parameters of the AAA thrombus (size, density, contrast enhancement) obtained by computed tomography angiography (CTA) and correlation with special histological parameters of thrombus removed during surgery.

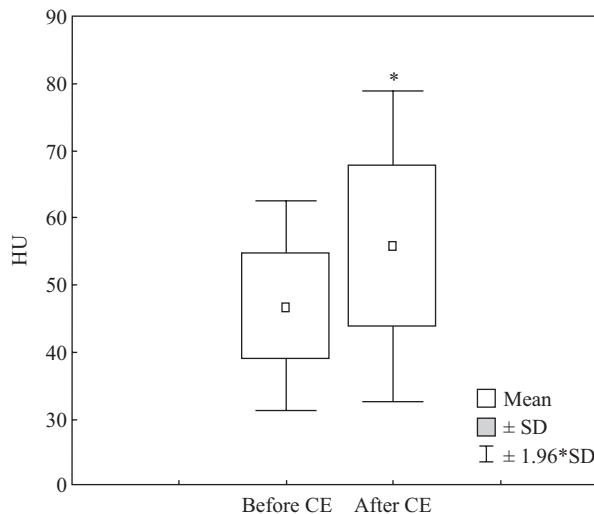
## Material and methods

29 patients with AAA (27 males, 2 female; aged 56–80, average age 70) were examined with CTA and in the next stage, after surgery, histopathological examination was done, including estimation of the inflammation and new vessels proliferation.

All CT examinations were obtained with CT scanner Aquilion 16 (Toshiba Co., Ltd., Tokyo, Japan) equipped with 16 detectors with the following scanning parameters: a gantry rotation speed of 0.4 sec per rotation, detector row configuration of  $16 \times 1$  mm, helical pitch 23 (pitch factor 1.4375). Images were reconstructed with a 1-mm slice thickness at 0, 8 mm intervals. Contrast agent (Iomeron 400, Nycomed) was administered by infusion pump through an antecubital vein to enhance the aorta and iliac arteries followed by saline infusion flush. Contrast volume was estimated according to the rule (time of scanning +  $10 \times 4$ ) and infusion rate of 4 ml/s was set. Coordination between contrast administration and image acquisition was adjusted automatically by the scanner (SUREStart) when 180 HU of contrast concentration was detected at a region of interest located in descending aorta. Axial images were analyzed on available workstation (Vitrea 4.2, Vital Inc.)

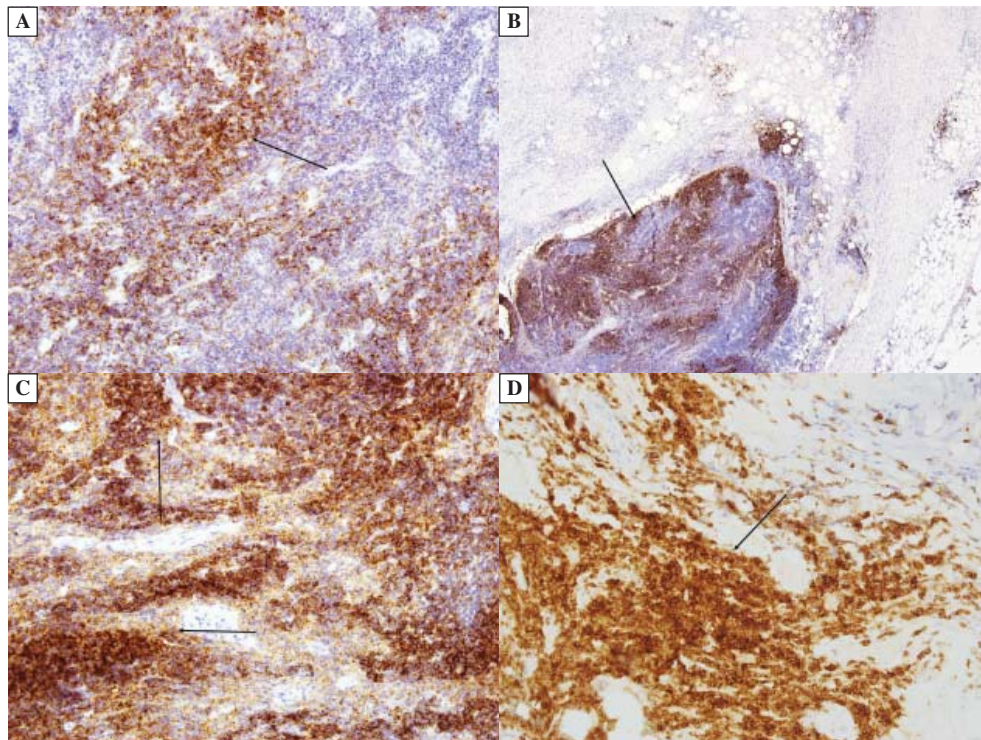
The thrombus density measurements were done by drawing circle or elliptical regions of interest (ROI) in the axial CT scans at several different levels including maximal diameter of the AAA sac and level of maximal thrombus width. The ROIs at each level were set at three localization: close to the lumen, near the wall of aneurysm and in the middle of thrombus. Average densities of ROIs from pre- and post-contrast phase were estimated and then compared (Figure 1).

For the histopathological evaluation the specimen fragments of aorta wall and the thrombus were taken during surgery and were fixed in 10% buffered formalin. Evaluation of inflammatory cells markers expression was per-



**Figure 1.** AAA wall density in Hounsfield units (HU) based on the CT analysis of thrombi, before and after administration of the contrast medium (CE, contrast enhancement). \* $p = 0.002$  ( $n = 29$ )

formed using immunohistochemical method. Following the deparaffinisation and rehydration, epitope retrieval was carried out in the EnVision Flex Target Retrieval Solution (DAKO) in high pH. Endogenous peroxidases were blocked by incubating the sections in methanol and 30% hydrogen peroxidase for 20 minutes. Then slides were incubated with special types of antibodies against following antigens 12): CD3, a common antigen for all types of T lymphocytes cells; CD20, a common receptor of B lymphocytes; CD34, a common endothelial cells marker was used to count a number of new proliferating vessels in the thrombus. CD34 visualisation together with HE examination enabled us to count only small new blood vessels in the thrombus. Monoclonal mouse antibody against CD20 receptor was used (M0755, DAKO) in 1:200 dilution overnight in 4°C and polyclonal rabbit antibody against CD3 receptor (A0452, DAKO) in 1:100 dilution overnight in 4°C. To evaluate new vessels mouse antibody against CD34 receptor was used (IR632, DAKO) in 1:100 dilution incubated overnight in 4°C. Visualisation reagent (LSAB plus, DAKO) was applied for 30 minutes followed by DAB solution for 5 minutes. The slides were then counterstained with hematoxylin and examined under the light microscope Olympus BX45. Immunohistochemical evaluation of antigens' expression was performed by two independent pathologists. The reactions for CD3 and CD20 were estimated as the percentage of positively-stained cells as follows:  $\leq 10\%$  positive cells — negative (-), 11–30% (+), 31–50% (++) and  $> 50\%$  as (+++) of positive cells in random 10 fields under 20x magnification. To measure the numbers of new vessels we counted all of the CD34 positive areas in random 10 fields under 10x magnification. The score was negative (-), if there was no CD34 positive



**Figure 2A and B.** CD3 expression in inflammatory cells (T-cells) within the thrombus. Magnification 200 ×, and 100 ×, respectively. **C and D.** CD20 expression in inflammatory cells (B-cells) within the thrombus, 200 ×, and 100 ×, respectively

cells in all of the examined areas, (+) if less than 10% of the examined fields were CD34 positive; (++) if between 11–50%; and > 50% as (+++) of the examined fields were CD34 positive.

Negative controls were performed using a nonimmunized IgG replacing the primary antibody. Positive controls involved sections of normal lymph node which expressed CD3 and CD20 antigens and angiosarcoma which expressed CD34 molecules.

$\chi^2$  and Pearson's correlation tests and Statistica 8,0 Stat-Soft software were used for statistical analysis. Values of  $p < 0,05$  were considered as statistically significant.

## Results

Microscopic evaluation of the thrombi showed huge percentage of inflammatory cells positive for CD3 or CD20 staining (Figures 2A–D) and new vessels proliferation (Figures 3A–D) in the wall, but also in the thrombus of abdominal aortic aneurysms. CD3, CD20 and CD34 expression were found respectively in 14 (48%), 10 (34%), 9 (31%) cases of thrombus specimens and in 22 (76%), 19 (65,5%), 19 (65,5%) fragments of aortic wall specimens (Table 1).

Significant numbers of inflammatory cells and new vessels were observed both in the wall and the thrombus of abdominal aortic aneurysms (Figures 2 C–D,

3 B–D), which in CT have had morphological signs of inflammatory process surrounding the AAA (visible infiltrations outside the wall of AAA in CT examination).

In CT angiography thrombi were slightly (average 3,5–6,9 HU), but statistically significantly, enhanced after contrast media administration (Figure 1).

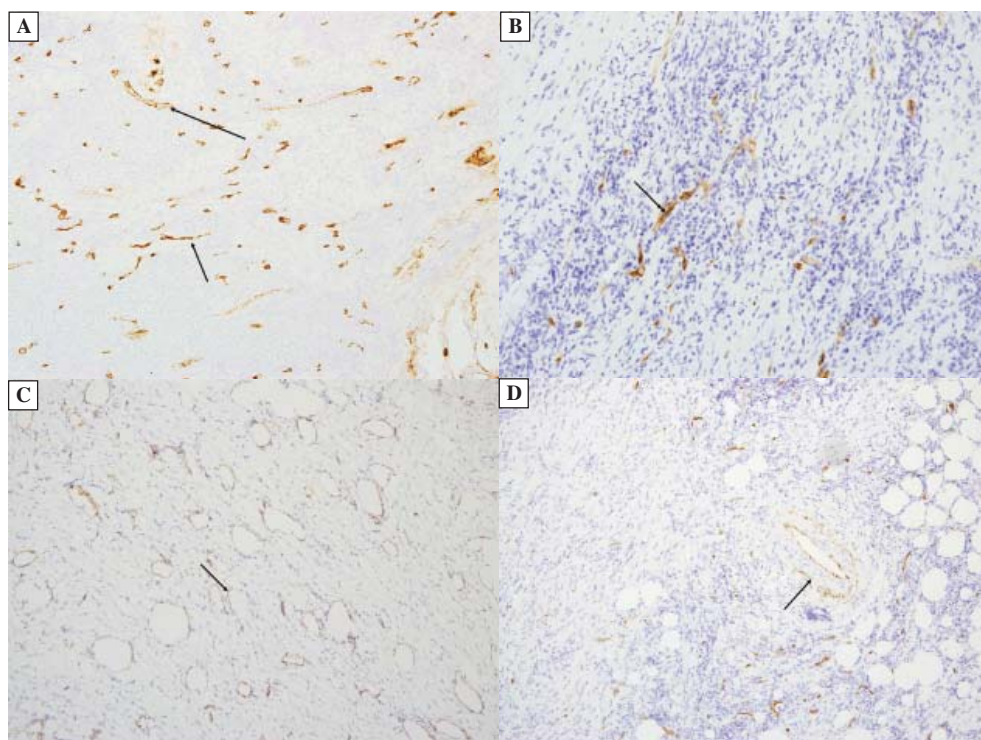
There was also a statistically significant correlation between the presence of infiltrating B-cells and contrast enhancement in CT (Figure 4), although we did not observed significant correlations between contrast enhancement of thrombi with the presence of T-cells ( $r = 0,32$ ,  $p = 0,09$ ) and endothelial cells ( $r = 0,32$ ;  $p = 0,086$ ).

The number of new blood vessels assessed by CD34 expression correlated with the presence of inflammatory cells, both T and B lymphocytes (Figure 5).

Negative correlation was observed between contrast enhancement in CT and the maximum diameter of the aneurysm and width of the thrombus (Figures 6A, B).

## Discussion

Multi-factor etiology of AAA and the latent course of the disease are frequently the causes of the systematical increase in the aorta diameter lasting for years, undiagnosed clinically yet [6, 13, 14]. Frequent-



**Figure 3.** CD34 expression in endothelial cells (arrows). **A.** New small blood vessels within the wall of the AAA thrombus, 100 ×. **B.** Few small blood vessels surrounded by huge inflammatory cells infiltration within the AAA thrombus, 200 ×. **C and D.** Matured blood vessels in the AAA thrombus, 200 × and 100 ×, respectively

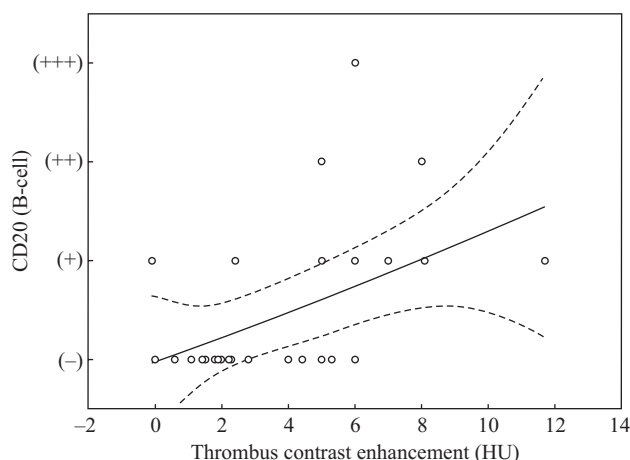
**Table 1.** Expression of cell markers of CD3 (T lymphocytes), CD20 (B lymphocytes), CD 34 (endothelial cells) within the thrombus and the wall of abdominal aortic aneurysm

Intensity of expression*	CD3		CD20		CD34	
	Thrombus	Wall	Thrombus	Wall	Thrombus	Wall
(-)	15	7	19	10	20	10
(+)	7	10	7	15	2	7
(++)	2	5	2	2	2	6
(+++)	5	7	1	2	5	6

Numbers in the table represent number of patients in a given group (-, +, ++, +++). \*expression of CD3, CD20, and CD34 markers was estimated as described in material and methods

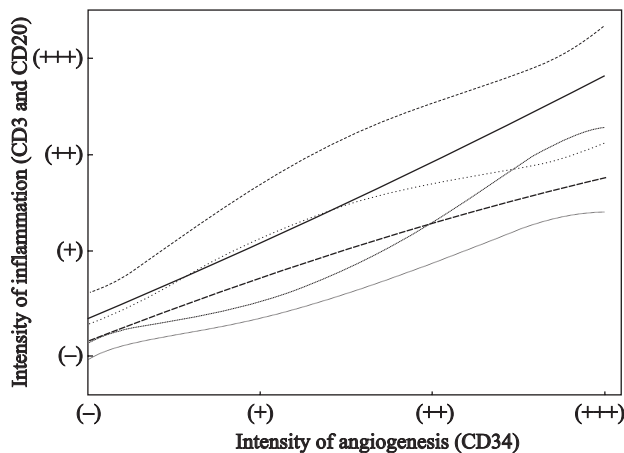
ly, the diagnosis is made as soon as the initial symptoms of the aneurysm rupture will appear [15]. However, the patient is then given little chance of a successful operation, because acute aneurysm rupture results in death up to 90% of cases, regardless of the urgent surgery procedures in such cases.

Nowadays AAA diagnosis is made basing on abdominal ultrasonography examination, often done for other reasons, for instance the growth spread of AAA might be systemically controlled, if is below 5 cm in diameter through about 6 months [15].



**Figure 4.** Correlation between contrast enhancement (HU, Hounsfield unit) with B-cell infiltration ( $r = 0.5, p = 0.005$ ) within aneurysmal thrombus. Y axis: numbers of CD20 positive cells in random 10 fields at the magnification 20 × are denoted as described in material and methods

Randomized studies have shown that the surgery of small AAA (below 5.5 cm in diameter) poses higher death risks compared to monitoring of the disease course. In such case “watchful waiting”, which is a systematical surveillance, yields better results as far



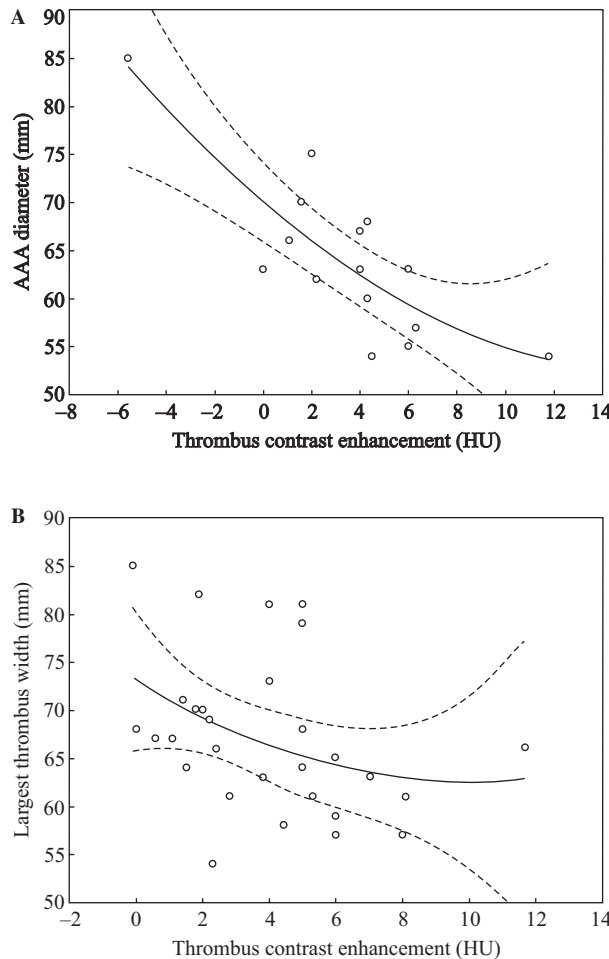
**Figure 5.** Correlation between expression of CD34, endothelial cell marker, and the presence of inflammatory T cells ( $r = 0,608$ ,  $p = 0,027$ ) and B cells ( $r = 0,727$ ,  $p = 0,005$ ). Y axis: numbers of CD34 cells in random 10 fields at the magnification 20x are denoted as described in material and methods

as the long-term survival is concerned [16]. Nevertheless, the diameter above 5.5 cm results in immediate-scheduling for operation on AAA.

The current attempts in the diagnostics path of AAA are to better predict AAA progression to prevent rupture. The basic etiologic factor of the disease is well known: decrease durability of the vessel wall with age due to the degradation of elastin fibers and the transformation of their spatial structure, which results in the vessel widening [17–20].

Damage of the vessel wall and endothelium shortly activate coagulation and thrombosis. Recently, it is admitted that such thrombus may be the main factor leading to further weakening of the vessel wall, disease progression, and in consequence AAA rupture [21, 22]. The growing thrombus impairs the wall nourishing, causing decreased oxygenation and ill-distribution of nutrients from blood to the vessel’s wall. The local hypoxia activates neutrophils and lymphocytes chemotaxis and stimulates angiogenesis by activating growth factors, like Vascular Endothelial Growth Factor (VEGF) [23, 24].

It was discovered, that inflammation and new vessels proliferation within the wall of abdominal aortic aneurysms and within the thrombus might be confirmed by medical imaging technique [23–26]. Our present results show straight correlation between B cells inflammatory infiltration and contrast enhancement in CT. Similarly, Spadaccio et al. observed in AAA subjects four serum inflammatory proteins that show altered expression profile and that could be specifically linked to AAA pathology [27]. Also Houard X et al. indicated neutrophil-derived leukotriene B4



**Figure 6.** Negative correlation between contrast enhancement and the maximal diameter of the aneurysm (A) and the largest thrombus width (B),  $r = -0.515$ ,  $p = 0.004$  and  $r = -0.738$ ,  $p = 0.002$ , respectively

(LTB) as a major neutrophil chemotactic factor released from the intraluminal thrombus of human AAAs, suggesting that targeting LTB receptors may represent a potential therapeutic strategy in the prevention of AAA progression in humans [3]. Kazi et al. suggested that thrombus formation and accumulation of inflammatory cells may perturb the structural integrity and stability of the vessel wall and thereby increase the risk for aneurysm rupture [12]. Also Wolski et al. observed within the AAA thrombus a huge inflammatory cells infiltration together with intensive angiogenesis [28]. Vorp et al. observed localized hypoxia in regions of thicker intraluminal thrombus in AAA, leading to increased, localized mural neovascularization and inflammation, as well as regional wall weakening [29]. They concluded that intraluminal thrombus may play an important role in the pathology and natural history of AAA. Reeps et al. observed that increased 18F-fluorodeoxyglucose

(FDG) uptake was correlated with higher densities of inflammatory infiltrates with reduced content of collagen fiber, suggesting that FDG-PET/CT might be a new diagnostic technique to study AAA disease in vivo and may contribute to improved prediction of individual AAA rupture risk [30].

Local hypoxia and the presence of inflammatory cells might stimulate the secretion of many growth factors such as VEGF, FGF, TGF, EGF or cytokines like interleukin 8 (II-8), resulting in new vessels growth and, in consequence, in instability within the aortic wall and thrombus [31, 32]. Many cytokines and metalloproteinases (MMP-3 or MMP-9), secreted by inflammatory cells, are responsible for further structural proteins like elastin, collagen and laminin degradation of the vessel wall, resulting in a gradual weakening of the wall strength. Therefore, inflammation and angiogenesis may be important events associated with the AAA progression and its rupture [33–35] as was also demonstrated in our present study by showing correlation between CD34 expression and lymphocytic infiltration.

Wiernicki et al. observed increased activity of elastase as well as concentrations of active metalloproteinases like MMP-9, MMP-8 in the thin thrombus-covered wall, demonstrating the differentiation of protease activity within the same AAA wall and its enhancement within the thin thrombus-covered aneurysm wall [36]. Michel et al. observed neoangiogenesis, phagocytosis by mononuclear cells, and a shift from innate to adaptive immunity in the adventitia in the AAA [37]. Similar Mayranpaa et al. presented mRNA increased levels of genes associated with neovascularization (VEGF, FLT1, VE-cadherin, CD31), and mast cells (tryptase, chymase, cathepsin G) in AAA samples than in controls [38].

It might be presumed, that the activation of inflammatory process in the thrombus wall is closely related to angiogenesis and may be directly proportional to the risk of AAA rupture. The best way to demonstrate the new vessels proliferation within the thrombus is microscopic assessment of specimens, sampled during operation, whereas clinical imaging methods give images of living tissues, but their spatial resolution is not enough to directly demonstrate new vessels [39, 40].

However developing new techniques in modern imaging methods for example new contrast media in ultrasonography (US), magnetic resonance imaging (MRI), computed tomography (CT) and in positron emission tomography (PET) increase the opportunity to use these methods for the evaluation of angiogenesis process. Angio-CT and MRI techniques enable measurements of the contrast enhancement of

the thrombus and the AAA wall. Our study presents close link between activation of the inflammatory cells and new vessels proliferation. Inflammatory process may increase thrombus enhancement in CT, what may be an easy way to assess the degree of inflammation and neovascularization used for monitoring the status of AAA and assessing risk of its rupture.

## Conclusions

The accumulation of inflammatory cells in the wall of AAA thrombus results in the formation of new vessels which participates to the instability of the thrombus and AAA wall. Assessment of the inflammation and neovascularization in the wall and thrombus of the AAA might be an important factor in monitoring the progression and the risk of aneurysm's rupture.

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