Published in final edited form as:

Thromb Haemost. 2012 November ; 108(5): 812–823. doi:10.1160/TH12-05-0339.

Local inflammation is associated with aortic thrombus formation in abdominal aortic aneurysms. Relationship to clinical risk factors

Agnieszka Sagan¹, Wojciech Mrowiecki³, Tomasz Mikolajczyk¹, Karol Urbanski¹, Mateusz Siedlinski¹, Ryszard Nosalski¹, Ryszard Korbut², and Tomasz J Guzik¹

¹Translational Medicine Laboratory, Department of Internal and Agricultural Medicine, Jagiellonian University School of Medicine ²Department of Pharmacology, Jagiellonian University School of Medicine, Kraków, Poland ³Department of Surgery, J Grande Hospital, Cracow, Poland

Abstract

Intraluminal thrombus formation in aortic abdominal aneurysms (AAA) is associated with adverse clinical prognosis. Interplay between coagulation and inflammation, characterised by leukocyte infiltration and cytokine production, has been implicated in AAA thrombus formation. We studied leukocyte (CD45+) content by flow cytometry in AAA thrombi from 27 patients undergoing surgical repair. Luminal parts of thrombi were leukocyte-rich, while abluminal segments showed low leukocyte content. CD66b+ granulocytes were the most prevalent, but their content was similar to blood. Monocytes (CD14+) and T cells (CD3+) were also abundant, while content of B lymphocytes (CD19+) and NK cells (CD56+CD16+) were low. Thrombi showed comparable content of CD14highCD16– monocytes and lower CD14highCD16+ and CD14dimCD16+, than blood. Monocytes were activated with high CD11b, CD11c and HLA-DR expression. Total T cell content was decreased in AAA thrombus compared to peripheral blood but CD8 and CD3+CD4–CD8– (double negative T cell) contents were increased in thrombi. CD4+ cells were lower but highly activated (high CD69, CD25 and HLA-DR). No differences in T regulatory (CD4+CD25+FoxP3+) cell or pro-atherogenic CD4+CD28null lymphocyte content were observed between thrombi and blood. Thrombus T cells expressed high levels of CCR5 receptor for chemokine RANTES, commonly released from activated platelets. Leukocyte or T cell content in thrombi was not correlated with aneurysm size. However, CD3+ content was significantly associated with smoking in multivariate analysis taking into account major risk factors for atherosclerosis. In conclusion, intraluminal AAA thrombi are highly inflamed, predominantly with granulocytes, CD14highCD16– monocytes and activated T lymphocytes. Smoking is associated with T cell infiltration in AAA intraluminal thrombi.

Keywords

Thrombus; Aortic Abdominal Aneurysm; Inflammation; Monocyte; T cell

Address for correspondence: Prof. Tomasz J Guzik MD PhD, Department of Internal and Agricultural Medicine, Jagiellonian University School of Medicine, J Dietl Hospital, Ul. Skarbowa 1, 31-121 Cracow, Poland, tguzik@cm-uj.krakow.pl.

Conflicts of interest: None declared.

Introduction

Aortic abdominal aneurysms (AAA) are major causes of morbidity and mortality related to vascular disease [1]. Thrombosis is a very common feature of AAA. The clinical importance of intravascular thrombus is still unclear, but it appears to be related with adverse clinical outcomes, in particular it may be related to increased AAA rupture risk [2]. Thrombus volume is also correlated to the occurrence of major cardiovascular events in AAA patients [3]. Interestingly, this may be related to functional importance of the thrombus as it has been shown that larger thrombi cause higher AAA growth rate, leading to weakening of the AAA wall [4]. Thus, it is very important to clearly understand AAA thrombus structure and function. Recent studies of AAA thrombus architecture indicate that they are very dynamic and undergo constant remodeling including fibrin deposition throughout the thrombus, and degradation principally at the abluminal surface [5]. AAA thrombi have typical “fibrin-rich” composition comparable to intracoronary thrombi [6], in which fibrin accounts for nearly 60% of thrombus [7]. Platelets account for 20%, red blood cells - 10%, crystals of cholesterol - 5%, and leukocytes for merely 2% of intracoronary thrombi during myocardial infarction [7]. Moreover, fibrin content increases with time, and platelet content decreases [7]. The detailed cellular content of chronic AAA thrombus is not clear [8]. While leukocyte content is relatively low - due to their ability to produce numerous mediators and to interact with other cells such as platelets and red blood cells - they may play an important modulatory role. Cells, which are sequestered or actively re-colonize thrombus are an important source of enzymes and factors which may contribute to wall degradation such as elastase or hydrogen peroxide [9, 10]. Increased levels of pro-inflammatory cytokines in the thrombi may indicate significant leukocyte infiltration [11] [12]. Cytokine production as well as CRP levels are linked to intraluminal thrombus size in AAA [12].

Majority of studies looking at inflammatory cell infiltrates in AAA thrombi used immunohistochemistry [8] and focused on the role of polymorphonuclear (PMN) leukocytes, mainly neutrophils. Indeed these cells are relatively abundant in various types of thrombi, and adaptive immunity, through toll-like receptors on PMNs and monocytes may modulate thrombosis and dynamic remodeling of the thrombus. Indeed recent study has shown role for TLR-2 and TLR-4 that were overexpressed on monocytes and granulocytes (TLR-2) in the intracoronary thrombi during acute myocardial infarction (AMI) and their potential functional role for thrombus formation [13].

Much less is known about the role of adaptive immunity in thrombosis, especially in relation to local inflammation in the thrombus. Indeed CD4 and CD8+ T cells were found in the AAA luminal thrombi by immunohistochemistry [8]. In a recent study, T and B cells were shown to be decreased in intracoronary thrombi, when compared to blood but were not further characterized. The exact content and characteristics of T cells in the AAA thrombi remains unknown.

Summarising, numerous studies show that inflammatory cells infiltrating AAA thrombi are not solely innocent bystanders in AAA disease, but may play important regulatory roles. However, the characteristics of inflammatory cells in the thrombus remain unclear.

Accordingly, we aimed to characterise inflammatory cells present in the luminal and abluminal layers of AAA thrombi and to investigate relationships between these characteristics and major risk factors for atherosclerosis and/or AAA.

We find that granulocytes and monocytes are the most predominant cells in the AAA thrombus. T lymphocytes, which are relatively less common, appear to be significantly activated and their content is related to current smoking status.

Materials and methods

Patients and Thrombi

Abdominal aortic aneurysms thrombi were obtained during AAA repair surgery at the site of maximal dilatation from 27 patients (22 males and 5 females). Additionally blood was collected in EDTA containing tubes, (Becton Dickinson) from peripheral access and in selected subpopulation (n=6) from aorta intra-operatively. Detailed clinical data including major risk factors for atherosclerosis and AAA as well as intra-operatively determined size of the AAA were recorded at the time of surgery.

Immediately after harvesting, thrombus sample was placed in ice cold (4°C) phosphate buffered saline (PBS, Gibco) and immediately transported to the laboratory.

Collection of tissues was approved by the Local Research Ethics Committee and informed consent was obtained.

Analysis of cells within the AAA thrombus

Two square specimens (5mmX5mm) from different layers (luminal and abluminal layer; Figure 1A) were taken. Both parts of thrombus were subsequently delicately mechanically disrupted and digested with mix of digestion enzymes (125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-S and 450 U/ml collagenase type I) in PBS containing 20 mM Hepes at 37°C for 20 minutes with gentle agitation to isolate residual cells infiltrating the thrombi. We performed additional experiments using actylise or tPA using protocol described by Wyss et al [13], however we did not observe significantly improved outputs of cell isolation (data not shown). Considering potential effects of actylise or tPA on immune cells and toxic direct effects we used a protocol without the utilization of these compounds.

Resulting cell suspension was then passed through a 70-µm strainer (BD Pharmingen). Erythrocytes were subsequently removed by osmotic lysis using the RBC Lysis Buffer (eBiosciences). Cells were washed twice with ice-cold PBS and incubated with fluorescently labeled antibodies for 20 min at 4°C, to differentiate leukocyte subpopulations. After washing, cells were re-suspended in PBS with 1% FBS (Gibco) and were studied on a FACSCanto II cytometer (BD Biosciences) and analyzed using FACSDiva™ and FlowJo software. The FACS analysis was performed using SSC/FSC scatters, then CD45 positive cells and other subpopulations of leukocytes were identified (see Figure 2A). The same labeling procedures and flow cytometry analysis were applied for blood and thrombus. The following monoclonal antibodies were used (all from BD or BD Pharmingen): anti -CD45-PeCy7, CD45-FITC, CD3-PerCP, CD14-APCH7, HLA-DR-PeCy7, CD66b-FITC, CD19-

APCH7, CD16-PE, CD56-PeCy7, CD4-APC, CD4-PeCy7, CD8-APCH7, CCR5-PE, CD69-FITC, CD25-PE, CD28-APC, CD11b-PB, CD11c-APC.

In separate experiments, we analyzed the number of dead or apoptotic cells in isolates using propidium iodide, which showed over 85% of viable cell content in the thrombus. Majority of non viable cells were in the granulocyte gate. Analysis of individual cellular subpopulations was performed using following gating strategy. The initial selection was performed on FSC/SSC scatter allowing for removal of cellular debris from subsequent analysis. For monocyte analysis monocyte gate was applied at this stage. For subsequent analysis only cells bearing CD45 antigen were selected. Subsequently individual cell populations were defined as indicated in Figure 2A.

Analysis of aortic and peripheral blood

Blood cells were isolated and stained in parallel to the isolation from the thrombus. Erythrocytes were removed by osmotic lysis using the RBC Lysis Buffer (eBiosciences) and cells were stained and processed in parallel to cells isolated from the thrombi. In separate experiments (n=6) we compared cellular content in peripheral and AAA aortic blood but no differences were identified. Similarly we treated cells isolated from circulating blood with digestion cocktail according to identical protocol used to obtain single cell suspensions from thrombi. No significant differences on studied markers were found upon such treatments.

Statistical analysis

Data are presented as mean $\pm$ SEM with n equal to the number of patients. To check the normality Kolmogorov-Smirnov test was performed. Comparisons between thrombus segments or peripheral blood were performed using Wilcoxon matched pairs test. Correlation was measured using Spearman's rank test. Multivariate linear regression analysis was used to assess the effects of risk factors on T cell and leukocyte populations. The data on charts given p-value on alpha error $* < 0,05$ or $** < 0,01$. Values of $p < 0.05$ were considered statistically significant.

Results

Patient characteristics

Fresh abdominal aortic thrombi and blood samples were obtained from 27 patients undergoing surgical repair. The demographic and clinical characteristics were typical for patients undergoing such surgery, with most prevalent risk factors being male sex, hypercholesterolemia, smoking and hypertension (Table 1). AAA patients presented with moderate to large diameters of AAA as determined intra-operatively (Table 1). Ninety-two percent of patients received chronic statin treatment, which is usual in such patients with profound atherosclerosis, while 59% presented with concomitant peripheral arterial disease (PAD). Interestingly only 19 subjects (70%) used regularly low dose aspirin (Table 1).

Leukocyte content in different regions of AAA thrombus

As structure and composition of luminal and abluminal regions of the thrombi were described to differ substantially, we first compared leukocyte content in luminal and

abluminal sections of the thrombus. Respective areas were defined as described in Figure 1A. Importantly, luminal part of the thrombi were significantly infiltrated with CD45+ inflammatory cells, these cells were very rare in abluminal areas of the thrombi, located immediately next to the vessel wall ($41.8 \pm 6.3\%$ vs. $3.8 \pm 1.6\%$; $p < 0.001$) (Figure 1 B-D). There was more than a thousand fold difference in the content of leukocytes in the luminal part of the thrombus (Figure 1). Thus, all subsequent analysis was performed in the luminal part of the thrombus.

Cellular distribution of leukocytes in AAA luminal thrombus in comparison to peripheral blood

All types of leukocytes which are found in peripheral blood were observed in AAA luminal thrombi (Figure 2A). The most prevalent cells in the thrombus were granulocytes, followed by monocytes and T cells (number of which were almost three-fold lower than the respective number of monocytes) (Figure 2B). However, the analysis of cellular composition of individual subpopulations between thrombus and blood, both collected at the same point in time revealed vital differences. In particular, monocytes consisted ca. $7 \pm 0.7\%$ of total leukocytes in peripheral blood, while they represented ca. $18 \pm 2.8\%$ of leukocytes in the thrombus ($p < 0.01$). At the same time the content of T lymphocytes was significantly decreased in the thrombus when compared to peripheral blood ($7 \pm 2.1\%$ vs. $20 \pm 1.8\%$; $p < 0.01$). Content of other leukocyte subpopulations did not differ as dramatically, although the content of B cells and NK cells was also significantly decreased in thrombus as compared to blood (B cells: $2 \pm 0.3\%$ vs. $3 \pm 0.4\%$, $p < 0.05$; NK cells: $1 \pm 0.3\%$ vs. $3 \pm 0.7\%$ $p < 0.05$). Interestingly, the content of granulocytes, the most abundant leukocyte subpopulation, in the thrombi and blood, did not differ significantly between thrombus and blood ($64 \pm 3.9\%$ vs. $66 \pm 2.9\%$; $p > 0.05$) (Figure 2C).

Relationships between leukocyte subpopulations in the peripheral blood and AAA thrombi

We next studied correlations between the presence of leukocyte subpopulations in the peripheral blood and intraluminal thrombus (ILT) in individual patients. Interestingly, none of the studied major leukocyte sub-populations found in the thrombus showed significant relationship to their occurrence in blood using Spearman's rank correlation analysis (coefficients and p-values are as follows: $R_s = 0.07$, $p = 0.8$ for Monocytes; $R_s = -0.34$, $p = 0.1$ for T cells; $R_s = -0.4$, $p = 0.1$ for granulocytes).

Monocyte characteristics in the peripheral blood and AAA thrombi

As monocytes were most significantly preferentially increased in the AAA luminal thrombi, we next sought to characterize their subpopulations (Figure 3A). Interestingly, while classical CD14^{high}CD16⁻ monocyte content was comparable between the AAA luminal thrombi and blood ($81.5 \pm 5\%$ vs. $79 \pm 5\%$; $p > 0.05$), the content of CD14^{high}CD16⁺ and CD14^{dim}CD16⁺ monocytes was significantly lower in the thrombi than in the peripheral blood of AAA patients ($3.5 \pm 0.7\%$ vs. $8.1 \pm 1\%$, $p < 0.05$; $1.6 \pm 0.4\%$ vs. $7.7 \pm 1.4\%$; $p < 0.01$; respectively) (Figure 3B). Further analysis of the expression of monocyte activation markers on the surface of respective monocyte subpopulations, revealed that the most abundant CD14^{high}CD16⁻ monocytes showed significantly more activated phenotype characterized by higher expression of CD11c, CD11b and HLA-DR markers than blood monocytes

(Figure 4A-D). Similar pattern was observed in CD14dimCD16+ monocytes in the thrombus. Interestingly monocyte subpopulation CD14highCD16+, which showed the highest up-regulation of CD11b and HLA-DR in peripheral blood did not show further increase in expression in the AAA luminal thrombi. (Figure 4 A-D).

Lymphocyte T subpopulations in the peripheral blood and AAA thrombi

While T cell content was reduced in AAA luminal thrombi when compared to peripheral blood, they may still play an important regulatory role in thrombus. Therefore we next sought to characterize T lymphocyte subpopulations in AAA luminal thrombi. Although CD4+ cells were predominating population in the AAA luminal thrombus, their percentage content was slightly reduced ($54.2 \pm 3.8\%$ vs. $64.4 \pm 3.1\%$ $p < 0.01$), while CD8+ cell content was significantly increased ($33.2 \pm 2.8\%$ vs. $29.3 \pm 2.4\%$ $p < 0.05$) when compared to peripheral blood (Figure 5A-B). This was associated with significant difference in CD4:CD8 ratio between peripheral blood and thrombus (Figure 5C). The largest, over two-fold increase was observed in CD3+CD4-CD8- cells (double negative; DN T cells) ($11.5 \pm 1.6\%$ vs. $5.8 \pm 1.1\%$) (Figure 5A).

Lymphocyte T activation in AAA thrombi

Next, expression of early activation marker (CD69; Figure 6A); late activation marker (CD25; Figure 6B) and HLA-DR (Figure 6C) on individual subpopulations was studied by flow cytometry in the peripheral blood and AAA intraluminal thrombus. This analysis showed that content of activated CD4+ and double negative cells was significantly increased in AAA thrombi when compared to peripheral blood. In general, this was also reflected by higher MFI of studied markers on cell populations. Interestingly CD8 cells, content of which was increased in AAA thrombi, did not show significantly increased expression of activation markers, apart from higher HLA-DR expression.

Importantly, the expression of both CD69 and CD25 on double negative T cell population, which was particularly strongly increased in AAA thrombi, when compared to circulating blood, was higher than on other classical T lymphocyte subpopulations.

As CD25 expression may indicate either T cell activation or T regulatory (Treg) cell phenotype we investigated CD4+CD25+FoxP3+ cell content in a subgroup of thrombi. However, we did not find any significant differences in T reg content, when compared to peripheral blood of AAA patients, collected at the same time point ($7.7 \pm 0.4\%$ vs. $7.1 \pm 1.1\%$; $p > 0.05$) (Figure 7A).

CD4+CD28 null T cells in AAA thrombus

CD4+CD28 null T cells were identified as particularly pro-inflammatory and pro-atherogenic. We observed that $3.6 \pm 0.9\%$ of circulating T cells had CD4+CD28null phenotype in AAA subjects. An increase in the presence of these cells in AAA thrombi when compared with circulating blood, did not reach statistical significance ($5.1 \pm 0.8\%$ vs. $3.6 \pm 0.9\%$; $p > 0.05$; Figure 7B).

T cell chemokine receptor expression in AAA thrombi

One of important mechanisms for selective activated T cell recruitment to the thrombus can be related to RANTES production in platelets during the formation of thrombus. In line with this RANTES receptor CCR5 expression was significantly increased on CD4+ cells in the AAA thrombus as compared to blood. Interestingly, double negative cells, which were prevalent in the thrombus, presented with almost five-fold higher CCR5 levels than CD4 cells in peripheral blood (Figure 7C).

Inflammation in AAA intraluminal thrombi and risk profile

We next sought to determine relationships between risk factors for abdominal aneurysms and classical risk factors for atherosclerosis and parameters of inflammation in AAA thrombi. Interestingly, no significant relationship was observed between thrombus infiltration with either CD45+, T cells or monocytes and aneurysm size ($R=-0.06$; $p>0.05$ for CD45+; $R=0.08$; $p>0.05$ for CD3+; $R=0.09$; $p>0.05$ for monocytes). Correlations of clinical risk score POSSUM (Physiologic and Operative Severity Score for the enUmeration of Mortality and Morbidity) with either total leukocyte content ($R=0.3$) or T cells ($R=-0.02$) were not significant.

We also analysed the effects of individual major classical risk factors for atherosclerosis on thrombus inflammation. Interestingly a significant and positive association between current smoking and T cell content in the thrombi was observed in a univariate analysis (Table 2). This finding was confirmed with multivariate linear regression analysis (Table 2). Importantly, after log transformation of CD3+ content values, which approximated a normal distribution of the regression residuals, current smoking still remained independently associated with increased T cell content in AAA luminal thrombus ($p=0.006$). Other risk factors showed no significant effect on major inflammation parameters in AAA thrombi. We therefore compared the expression of activation markers on T cells in the thrombi, depending on current smoking status. No major differences were however observed in studied activation markers or chemokine receptor expression (see Suppl. Table 1, available online at www.thrombosis-online.com). The only significant difference was observed in CD25 expression on double negative T cells, although biological importance of this difference remains unclear.

Discussion

Several recent studies have shown functional and clinical importance of intraluminal thrombus formation in patients with AAA [1, 8, 14]. Most studies so far have focused on mechanical properties and physical properties of these predominantly fibrin-rich thrombi [4]. Several authors have however shown that luminal thrombi from patients with AAA are an important source of inflammatory cytokines, reactive oxygen species such as hydrogen peroxide or proteases [9, 10, 15]. While leukocytes, as major source of the above, have been identified in AAA thrombi, their content and detailed characterization remained unclear [8].

In the present study we characterized inflammatory cell content in thrombi formed in the lumen of abdominal aortic aneurysms. We found that luminal part of the thrombus contains

significant leukocyte infiltration, while abluminal part has very sparse leukocyte content. Moreover, we observed that leukocytes infiltrating luminal part of thrombus are viable and activated. Comparison of immune cell composition in circulating blood and AAA thrombi has shown major differences. Polymorphonuclear granulocytes were the most abundant leukocyte subpopulation in both, and their content did not differ between circulating blood and thrombus. However, content of monocytes was significantly increased, while content of T lymphocytes was overall decreased. These results are generally in agreement with studies looking in detail at cellular composition of coronary thrombi [13]. Wyss et al. observed increased percent of monocytes within coronary thrombi compared with monocytes from aortic blood, whereas the percent of granulocytes, T- and B-cells was decreased [13]. We did not observe significant difference in 66b+ granulocyte content between blood and thrombus. However, it is evident that we found significantly higher relative content of granulocytes in both blood and thrombus than was reported for coronary thrombi (60% vs 25%). While this difference is difficult to unequivocally explain, it is possible that different method of isolation of cells from thrombus might play a role. Population of granulocytes reported in our study is also very clearly reflected by SSC/FSC in both aortic blood, peripheral blood and thrombus confirming their identity. The differences in major leukocyte subpopulations distribution in thrombus and blood of patients with acute coronary syndrome and AAA may be related to differences in immunopathogenesis of these diseases [13], which may be, for instance, reflected by increased CD66b expression on granulocytes in AAA. While this has not been focus of the present study, it would be interesting to address this issue in future. Our observation that granulocytes are predominant cells in AAA thrombi is in agreement with previous findings in both abdominal aneurysm thrombi [8, 16, 17], as well as intra-coronary thrombi developing during myocardial infarction [13]. PMN role in AAA has been characterized before, as it has been demonstrated that they are an important source of elastase, which may affect adjacent vessel wall and may affect continuous destruction of the thrombus, which facilitates its turnover [9, 10]. Moreover, recent studies show that PMN cells may be a very important source of oxidative stress [15], which plays an important role in formation of thrombus itself, as well as in modulation of aortic wall destruction, through its effects on metalloproteinases and their tissue inhibitors [18, 19].

Wyss et al. showed particular importance of monocytes and their expression of TLR4 in thrombosis during acute coronary syndrome [13]. In the present study we also focused on detailed characteristics of monocytes infiltrating AAA thrombi, although we did not investigate innate immunity. Monocytes showed predominantly classical CD14^{high}CD16⁻ phenotype, while presence of CD14^{high}CD16⁺ or CD14^{dim}CD16⁺ monocytes was decreased. This was surprising, as numerous inflammatory diseases have been typically linked to increased content of CD14⁺CD16⁺ monocytes in the peripheral blood [20–22]. While these general phenotypic features were unexpected, CD14^{high}CD16⁻ cells showed increased expression of all markers of activation studied i.e. CD11b, CD11c and HLA-DR, which reached levels comparable to CD14⁺CD16⁺ monocytes. Such increased expression of activation markers have been described in allergic and infectious diseases [23]. At the same time, it became apparent that CD14^{high}CD16⁻ monocytes have predictive role in cardiovascular events [24]. Thus, our identification of these cells as an important component

of thrombus may be of particular interest and should be investigated in other, especially intracoronary ACS-related thrombi.

While the presence of granulocytes and monocytes is important, in the present study we focused attention on T lymphocytes, which constitute third largest leukocyte population in AAA thrombus, as adaptive immunity has not been characterized in relation to chronic thrombus formation so far. These cells were generally underestimated in the thrombus, and in contrast to monocytes, their percentage is decreased rather than increased when compared to circulating blood [13].

Moreover, T cell content observed by us was significantly lower than reported for intracoronary thrombi by Wyss et al. However, in our opinion, it is not the number of cells that matters. We noted that T cells present in the luminal part of thrombus are highly activated. Majority of T cells in the thrombus were CD4 positive, but CD4:CD8 ratio was decreased when compared to peripheral blood showing selectively increased presence of CD8 cells. CD4:CD8 ratio is considered a basic marker for adaptive immunity and is changed in autoimmune diseases or viral infections, and has been recently reported in hypertension (25). AAA thrombi contained significantly more CD8+ T cells as compared to peripheral blood. However then most significant difference in the proportion of T cell subsets was observed in relation to double negative CD3+CD4-CD8- cells. This subpopulation of T cells is poorly characterised but received significant attention as pro-inflammatory population. These cells are characteristic for various tissues such as lung or liver, but their genesis remains unknown (26–28). These double negative T cells are very different to thymic DN T cells (29) and are observed in certain inflammatory conditions. For instance, infectious diseases such as cutaneous leishmaniasis or systemic lupus erythematosus (SLE) were associated with increased double negative T cell content (26, 30). Moreover, we have described increased proportion of these cells in hypertension in perivascular adipose tissue and in the kidneys (31). Virtually all subpopulations of T lymphocytes infiltrating the thrombus were strongly activated as indicated by expression of activation markers CD69 and CD25. Moreover, increased expression of HLA-DR was found on the T cells. HLA-DR was related more commonly to aging and may correspond to chronic activation of the immune system. Such activated lymphocytes may proliferate even inside thrombus, which was not studied in our study setting, but is likely to occur, considering the presence of all major components required for local T cell activation. High content of CD25+ cells could indicate that regulatory cells are present in the AAA thrombi, while these cells are characterised by high CD25 levels (32). These cells were shown to be deficient in AAA wall inflammation. Therefore we examined FoxP3 expression to directly assess T regulatory cell content and found no significant differences in their content when compared to peripheral blood. However, these experiments were performed in a relatively small subgroup of patients and should be considered preliminary. Activated T cells, which we identified in the AAA thrombi, may produce extensive amounts of inflammatory cytokines, which were often measured from different types of thrombi. Our findings concerning importance and presence of virtually all subpopulations of T cells, mainly CD4 and CD8+ cells are in agreement with previously published observations (8). Unfortunately, studies of the leukocyte content of fresh thrombi from MI did not include detailed characterization of T cell activation markers so far. Our present study from AAA thrombus

indicates that this might be of interest as well. Moreover, further functional in vitro studies are needed to clearly identify the role of adaptive immunity and T cells in chronic or acute thrombus formation.

Interestingly, we did not see significant differences between blood and thrombus content of CD4+CD28null T cells. These cells were shown to play an important role in atherogenesis and were increased in blood of ACS patients (33), but were not studied so far in AAA or in relation to thrombosis. CD4+CD28 null levels in blood of AAA patients were comparable to those reported for coronary artery disease (ca.5%), higher than in healthy individuals, but were lower than in ACS patients (9%) (33). A major question concerns the mechanism of the presence of studied inflammatory cells in the thrombus. An obvious possibility would be that these cells are trapped in the thrombus during clotting. A question related to this is whether the leukocyte composition of the peripheral blood affects AAA thrombus leukocyte content or vice versa? While this might be the case to some extent in relation to granulocytes, the most abundant cells, monocyte and T cell content and functional characteristics greatly differ between blood and AAA thrombus. Moreover, we did not find excessive apoptosis of these leukocytes or increased numbers of dead cells, which, if present, could indicate that these cells are simply trapped in the thrombus. Interestingly the largest index of apoptotic cells was found among granulocytes, but was very low among monocytes or T cells. Finally, we analysed correlations between major leukocyte subpopulations contents in peripheral blood and thrombi, but no significant correlations were found, which further emphasises that local thrombus microenvironment may be more important for its leukocyte content than composition of leukocyte subpopulations in the peripheral blood. Larger study is, however, warranted to finally confirm these observations.

It is therefore very likely that active recruitment of these cells during the natural history of thrombosis occurs, and it is even possible that there is a dynamic exchange between blood and thrombus of these inflammatory cells. The possibility of efflux of activated inflammatory cells from the thrombi in AAA should be further investigated in experimental models.

One possibility for recruitment of cells, particularly activated T cells, might be related to the production of RANTES chemokine by activated platelets (34). Indeed, intra-thrombus T cells were enriched for CCR5+ cells, which may suggest the role of RANTES chemokine in the regulation of lymphocyte traffic in AAA thrombi.

Finally, an important question raised as a result of our present findings concern possible effects that infiltrating leukocytes might have on thrombus formation (35, 36). These effects may be related to both platelet-leukocyte interactions, as well as to the effects of inflammatory mediators on fibrin generation and clotting (37). Platelet-leukocyte aggregates, involving P-selectin dependent interactions are a reliable marker of a prothrombotic state and accompany numerous inflammatory diseases (38). In the thrombus formation as such, in particular monocyte-platelet interactions appear to be particularly important, which is critical in the light of increased monocyte content in studied thrombi (39). The upregulation of CD11c and CD11b may be particularly important in this process (40). Pro-inflammatory cytokines can activate the coagulation system and down-regulate anticoagulant pathways

ensuing thrombin generation dependent on expression of tissue factor (36). Thus, both coagulation system and platelets are affected by inflammation and leukocytes (41). However, we must keep in mind that both of these processes are under the influence of endothelial function, and dysfunctional endothelium as well as disturbed flow may be equally important in these processes (25, 42).

AAA may itself be related to peripheral leukocyte activation, which might provide a priming point for the participation of activated cells in thrombus formation and dynamics. Studies in humans have focused on increased cytokine levels (43), while cellular markers of T cells and monocytes have not been yet comprehensively compared, apart from T regulatory cells which were found to be decreased in a small cohort AAA patients (32) and CD4CD28null cells which were increased (44).

In preliminary studies we compared activation markers on peripheral blood cells from AAA patients and subjects, matched for age and major risk factors such as smoking, hypertension and hypercholesterolaemia (unpublished data, A. Sagan et al.). We observed that while no disturbances in overall content of monocytes, T cells, granulocytes, B cells or NK cells can be identified, AAA is associated with increased T cell activation markers CD69, CD25 and HLA-DR (unpublished data, A. Sagan et al.). This could indicate that leukocytes are primed in peripheral blood in AAA, which may be an initiating step for their subsequent possible role in thrombus formation. Surprisingly, CCR5 expression did not seem to differ between AAA and non-AAA in our preliminary analysis. These preliminary observations, while potentially interesting, require, however, further elucidation in a larger cohort.

In the present study, we analysed the relationships between several clinical characteristics and inflammatory characteristics of thrombi. In the contrary to our hypothesis we did not observe any relationship between thrombus size or POSSUM clinical risk score with either leukocyte, CD3 or other studied leukocyte subpopulations. This might be related to relatively small sample size, but other studies have previously shown that for example the extent of the haemostatic derangement in AAA does not correlate with AAA sac thrombus volume (14). Smoking was the only risk factor that was significantly associated with the number of CD3+ T cells in the AAA thrombus, which is particularly important taking into account the critical importance of smoking as a risk factor for aneurysm formation. However, subsequent analysis of T cell activation markers and chemokine receptors in the thrombi did not show significant association with smoking.

Our study has certain limitations. We did not analyse medial layer of the thrombus, as we were focusing on the most dynamically remodeled part, in which immune cells are likely to actively repopulate. Moreover, medial part of the thrombus is often thought to contain more apoptotic or dead leukocytes. We focused on comparison between luminal and abluminal sections of thrombi, as they seem to differ most in respect to physical and cellular composition, while medial part has been found to be similar to luminal (5). Physical properties of different layers of thrombi indicate that the luminal part is changing the most dynamically (5).

Moreover, many comparisons shown in the present study relate to peripheral blood. While this choice may be criticised over aortic blood, it must be noted that during AAA surgery obtaining aortic blood would possibly be subject to variability related to differences in local surgical procedures and complications developing during surgery. Moreover, in a subset of patients we compared peripheral and aortic blood and found no significant differences in respect to markers studied.

In summary, present study shows that inflammatory infiltrate, rather than passive entrapment of leukocytes occurs in AAA intraluminal thrombus. Granulocytes, CD14^{high}CD16⁺ monocytes and T lymphocytes are predominant leukocytes in the thrombi.

Monocyte content is increased while T lymphocytes are reduced in AAA intraluminal thrombi when compared to peripheral blood.

However, both populations are highly activated. Moreover, T cell number is correlated with smoking, an important clinical risk factor of AAA. It would be important to bear in mind, while analyzing inflammation in other types of thrombotic material, that it's not the matter of quantity but rather of activation status. These results raise questions about the functional role that intra-thrombus inflammation plays in thrombosis and possible clinical and prognostic implications of these interactions (45).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by Foundation for Polish Science Welcome fellowship (FNP/2009/Welcome02) and by Collegium Medicum to KJ. TJG is supported by International Senior Research Fellowship from the Wellcome Trust and is a member of the Young Investigator Program of the European Molecular Biology Organization.

References

1. Weintraub NL. Understanding abdominal aortic aneurysm. *N Engl J Med*. 2009; 361:1114–1116. [PubMed: 19741234]
2. Satta J, Laara E, Juvonen T. Intraluminal thrombus predicts rupture of an abdominal aortic aneurysm. *J Vasc Surg*. 1996; 23:737–739. [PubMed: 8627917]
3. Parr A, McCann M, Bradshaw B, et al. Thrombus volume is associated with cardiovascular events and aneurysm growth in patients who have abdominal aortic aneurysms. *J Vasc Surg*. 2011; 53:28–35. [PubMed: 20934838]
4. Speelman L, Schurink GW, Bosboom EM, et al. The mechanical role of thrombus on the growth rate of an abdominal aortic aneurysm. *J Vasc Surg*. 2010; 51:19–26. [PubMed: 19944551]
5. Ashton JH, Vande Geest JP, Simon BR, et al. Compressive mechanical properties of the intraluminal thrombus in abdominal aortic aneurysms and fibrin-based thrombus mimics. *J Biomech*. 2009; 42:197–201. [PubMed: 19058807]
6. Matusik P, Mazur P, Stepień E, et al. Architecture of intraluminal thrombus removed from abdominal aortic aneurysm. *J Thromb Thrombolysis*. 2010; 30:7–9. [PubMed: 19943084]
7. Silvain J, Collet JP, Nagaswami C, et al. Composition of coronary thrombus in acute myocardial infarction. *J Am Coll Cardiol*. 2011; 57:1359–1367. [PubMed: 21414532]
8. Adolph R, Vorp DA, Steed DL, et al. Cellular content and permeability of intraluminal thrombus in abdominal aortic aneurysm. *J Vasc Surg*. 1997; 25:916–926. [PubMed: 9152321]

9. Fontaine V, Jacob MP, Houard X, et al. Involvement of the mural thrombus as a site of protease release and activation in human aortic aneurysms. *Am J Pathol.* 2002; 161:1701–1710. [PubMed: 12414517]
10. Fontaine V, Touat Z, Mtairag el M, et al. Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus. *Am J Pathol.* 2004; 164:2077–2087. [PubMed: 15161642]
11. Kato K, Matsubara T, Iida K, et al. Elevated levels of pro-inflammatory cytokines in coronary artery thrombi. *Int J Cardiol.* 1999; 70:267–273. [PubMed: 10501341]
12. Aho PS, Niemi T, Piilonen A, et al. Interplay between coagulation and inflammation in open and endovascular abdominal aortic aneurysm repair--impact of intra-aneurysmal thrombus. *Scand J Surg.* 2007; 96:229–235. [PubMed: 17966749]
13. Wyss CA, Neidhart M, Altwegg L, et al. Cellular actors, Toll-like receptors, and local cytokine profile in acute coronary syndromes. *Eur Heart J.* 2010; 31:1457–1469. [PubMed: 20447947]
14. Davies RS, Abdelhamid M, Vohra RK, et al. The relationship between aortic aneurysm sac thrombus volume on coagulation, fibrinolysis and platelet activity. *Thromb Res.* 2012; 130:463–466. [PubMed: 22497884]
15. Ramos-Mozo P, Madrigal-Matute J, Martinez-Pinna R, et al. Proteomic analysis of polymorphonuclear neutrophils identifies catalase as a novel biomarker of abdominal aortic aneurysm: potential implication of oxidative stress in abdominal aortic aneurysm progression. *Arterioscler Thromb Vasc Biol.* 2011; 31:3011–3019. [PubMed: 21940941]
16. Houard X, Ollivier V, Louedec L, et al. Differential inflammatory activity across human abdominal aortic aneurysms reveals neutrophil-derived leukotriene B4 as a major chemotactic factor released from the intraluminal thrombus. *FASEB J.* 2009; 23:1376–1383. [PubMed: 19136615]
17. Tanaseanu C, Neagu M, Popescu M, et al. Peripheral leukocytes activation in aortic aneurysmal disease. *Rom J Intern Med.* 1998; 36:167–174. [PubMed: 10822513]
18. Davies MJ. Reactive oxygen species, metalloproteinases, and plaque stability. *Circulation.* 1998; 97:2382–2383. [PubMed: 9641687]
19. Rajagopalan S, Meng XP, Ramasamy S, et al. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest.* 1996; 98:2572–2579. [PubMed: 8958220]
20. Ulrich C, Heine GH, Gerhart MK, et al. Proinflammatory CD14+CD16+ monocytes are associated with subclinical atherosclerosis in renal transplant patients. *Am J Transplant.* 2008; 8:103–110. [PubMed: 18021284]
21. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol.* 2007; 81:584–592. [PubMed: 17135573]
22. Schlitt A, Heine GH, Blankenberg S, et al. CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. *Thromb Haemost.* 2004; 92:419–424. [PubMed: 15269840]
23. von Bubnoff D, Scheler M, Hinz T, et al. Comparative immunophenotyping of monocytes from symptomatic and asymptomatic atopic individuals. *Allergy.* 2004; 59:933–939. [PubMed: 15291900]
24. Berg KE, Ljungcrantz I, Andersson L, et al. Elevated CD14++CD16– monocytes predict cardiovascular events. *Circ Cardiovasc Genet.* 2012; 5:122–131. [PubMed: 22238190]
25. Harrison DG, Guzik TJ, Lob HE, et al. Inflammation, immunity, and hypertension. *Hypertension.* 2011; 57:132–140. [PubMed: 21149826]
26. Anand A, Dean GS, Quereshi K, et al. Characterization of CD3+ CD4– CD8–(double negative) T cells in patients with systemic lupus erythematosus: activation markers. *Lupus.* 2002; 11:493–500. [PubMed: 12220103]
27. Ascon DB, Ascon M, Satpute S, et al. Normal mouse kidneys contain activated and CD3+CD4– CD8– double-negative T lymphocytes with a distinct TCR repertoire. *J Leukoc Biol.* 2008; 84:1400–1409. [PubMed: 18765477]
28. D'Acquisto F, Crompton T. CD3+CD4–CD8– (double negative) T cells: saviours or villains of the immune response? *Biochem Pharmacol.* 2011; 82:333–340. [PubMed: 21640713]
29. Papiernik M, Pontoux C. In vivo and in vitro repertoire of CD3+CD4–CD8– thymocytes. *Int Immunol.* 1990; 2:407–412. [PubMed: 2150756]

30. Gollob KJ, Antonelli LR, Faria DR, et al. Immunoregulatory mechanisms and CD4-CD8- (double negative) T cell subpopulations in human cutaneous leishmaniasis: a balancing act between protection and pathology. *Int Immunopharmacol*. 2008; 8:1338–1343. [PubMed: 18687296]
31. Guzik TJ, Hoch NE, Brown KA, et al. Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J Exp Med*. 2007; 204:2449–2460. [PubMed: 17875676]
32. Yin M, Zhang J, Wang Y, et al. Deficient CD4+CD25+ T regulatory cell function in patients with abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2010; 30:1825–1831. [PubMed: 20448211]
33. Liuzzo G, Biasucci LM, Trotta G, et al. Unusual CD4+CD28null T lymphocytes and recurrence of acute coronary events. *J Am Coll Cardiol*. 2007; 50:1450–1458. [PubMed: 17919564]
34. Mause SF, von Hundelshausen P, Zernecke A, et al. Platelet microparticles: a transcellular delivery system for RANTES promoting monocyte recruitment on endothelium. *Arterioscler Thromb Vasc Biol*. 2005; 25:1512–1518. [PubMed: 15890969]
35. Badimon L, Storey RF, Vilahur G. Update on lipids, inflammation and atherothrombosis. *Thromb Haemost*. 2011; 105(Suppl 1):S34–42. [PubMed: 21479344]
36. Levi M, van der Poll T. Inflammation and coagulation. *Crit Care Med*. 2010; 38:S26–34. [PubMed: 20083910]
37. Weber C. Chemokines in atherosclerosis, thrombosis, and vascular biology. *Arterioscler Thromb Vasc Biol*. 2008; 28:1896. [PubMed: 18946056]
38. Cerletti C, Tamburrelli C, Izzi B, et al. Platelet-leukocyte interactions in thrombosis. *Thromb Res*. 2012; 129:263–266. [PubMed: 22075180]
39. Seizer P, Gawaz M, May AE. Platelet-monocyte interactions--a dangerous liaison linking thrombosis, inflammation and atherosclerosis. *Curr Med Chem*. 2008; 15:1976–1980. [PubMed: 18691053]
40. Hirahashi J, Hishikawa K, Kaname S, et al. Mac-1 (CD11b/CD18) links inflammation and thrombosis after glomerular injury. *Circulation*. 2009; 120:1255–1265. [PubMed: 19752320]
41. Shebuski RJ, Kilgore KS. Role of inflammatory mediators in thrombogenesis. *J Pharmacol Exp Ther*. 2002; 300:729–735. [PubMed: 11861775]
42. Marvar PJ, Thabet SR, Guzik TJ, et al. Central and peripheral mechanisms of T-lymphocyte activation and vascular inflammation produced by angiotensin II-induced hypertension. *Circ Res*. 2010; 107:263–270. [PubMed: 20558826]
43. Urbonavicius S, Urbonaviciene G, Honore B, et al. Potential circulating biomarkers for abdominal aortic aneurysm expansion and rupture--a systematic review. *Eur J Vasc Endovasc Surg*. 2008; 36:273–282. [PubMed: 18639476]
44. Duftner C, Seiler R, Klein-Weigel P, et al. High prevalence of circulating CD4+CD28- T-cells in patients with small abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2005; 25:1347–1352. [PubMed: 15845908]
45. Matusik P, Guzik B, Weber C, et al. Do we know enough about the immune pathogenesis of acute coronary syndromes to improve clinical practice? *Thromb Haemost*. 2012; 108:443–456. [PubMed: 22872109]

Figure 1. Aortic abdominal aneurysm thrombus leukocyte infiltration.

Panel A. Anatomical location of luminal and abluminal segments of thrombus which were subsequently analysed. **Panel B.** Example of flow cytometric identification of leukocytes using staining for CD45 pan-leukocyte antigen. Gates were applied based on fluorescence minus one analysis. **Panel C.** Content of leukocytes in cellular fraction of respective segments of thrombus. **Panel D.** Leukocyte content per mg of thrombus sections. Data are presented as mean $\pm$ SEM; n= 20 paired samples; ** - $p < 0.001$ vs luminal thrombus

Figure 2. Cellular composition of inflammatory infiltrates of intraluminal thrombus in AAA in comparison to peripheral blood.

Panel A. Examples of flow cytometric determination of major leukocyte subsets including T lymphocytes (CD3), Monocytes (CD14+HLA-DR+), granulocytes (CD66b) B cells (CD19) and NK cells (CD56+CD16+). **Panel B.** Comparison of leukocyte subpopulations content per mg of AAA intraluminal thrombus. Bars represent means $\pm$ SEM; * – $p < 0.05$ vs T cell.; $n = 13$ –27. **C)** Differences in the % content of individual leukocyte subpopulations in peripheral blood and AAA thrombus; $n = 13$ paired samples; * – $p < 0.05$ vs. blood ** – $p < 0.01$ vs. blood.

Figure 3. Characterization of monocyte subsets in AAA intraluminal thrombus in comparison to peripheral blood.

Panel A. Examples of flow cytometric gating strategy for determination of monocyte subsets into CD14high/CD16- (R2) CD14 high/CD16+ (R3) and CD14dim/CD16+ (R4). Initial gate (R1) was applied to exclude HLA-DR- cells. **Panel B.** Comparison of monocyte subsets in peripheral blood and intraluminal thrombus. **-p<0.05 vs. peripheral blood; *-p<0.01 vs. peripheral blood; n=13; ¶- p<0.05 vs. CD14high/CD16-.

Figure 4. Markers of monocyte activation in AAA intraluminal thrombus in comparison to peripheral blood.

Mean fluorescence intensity was measured for major markers of monocyte activation - CD11c (**Panel B**), CD11b (**Panel C**) and HLA-DR (**Panel D**) in individual monocyte subpopulations. Values of MFI were compared between peripheral blood and AAA thrombus; n=13; *-p<0.05 vs. peripheral blood; ** - p<0.01 vs. peripheral blood. ¶- p<0.05 vs. CD14high/CD16-.

Figure 5. Lymphocytes and their subpopulations in AAA intraluminal thrombus in comparison to peripheral blood.

Panel A. Lymphocyte subpopulation content was compared between peripheral blood and AAA intraluminal thrombus by flow cytometry **Panel B.** Absolute numbers of individual lymphocyte subpopulations per mg of weight of thrombus **Panel C.** CD4:CD8 ratio in peripheral blood as compared to AAA ILT. Bars represent mean $\pm$ SEM; $n=17$; * – $p<0.05$ and **– $p<0.01$ vs. peripheral blood.

Figure 6. Activation of the T cells infiltrating AAA intraluminal thrombus in comparison to peripheral blood.

Expression of early activation marker (CD69; Panel A); late activation marker (CD25; Panel B) and HLA-DR (Panel C) on individual subpopulations was studied by flow cytometry in the peripheral blood and AAA intraluminal thrombus. Data are presented as % of cells expressing individual marker (left panels) and its mean fluorescence intensity (MFI; right panels). Bars indicate means $\pm$ SEM; n=17-25; * - p<0.05 vs peripheral blood; ** - p<0.01 vs peripheral blood

Figure 7. T cell characteristics in the intraluminal thrombus.

A) T regulatory (CD4+CD25+FoxP3+) cells in peripheral blood and ILT (n=5) expressed as % of CD4+. **B)** Pro-inflammatory CD4+CD28null T cells in peripheral blood and ILT (n=25) expressed as % of CD3+. **C)** Expression of RANTES receptor CCR5 on the T cells infiltrating AAA intraluminal thrombus. CCR5 was detected on the surface on individual T cell subpopulations within peripheral blood and AAA ILT. Data are presented as % of cells expressing individual marker (left panel) and its MFI (right panel). Bars indicate mean $\pm$ SEM; n=25; *-p<0.05 vs. peripheral blood; ** – p<0.01 vs. peripheral blood ¶- p<0.05 vs. CD4 cells. NS, non-significant.

Table 1
Clinical characteristics of AAA patients studied.

PAD – peripheral arterial disease; BMI- body mass index; ACE – angiotensin converting enzyme

Mean age (mean; years±SEM)	68±6,77
Gender (M:F)	22:5
<i>Risk factors:</i>	
Hypertension	24 (89%)
Diabetes	2 (7%)
Current smoking	19 (70%)
Hypercholesterolemia	25 (92%)
Obesity (BMI<25)	4 (15%)
<i>Medications:</i>	
Diuretic	11 (41%)
ACE inhibitor	16 (59%)
Acetylsalicylic acid	19 (70%)
Other antithrombotic	3 (11%)
β-blocker	14 (52%)
Ca antagonist	4 (15%)
Statin	25 (92%)
Aneurysms diameter (mean; mm±SEM)	59±2 mm
PAD	16 (59%)

Table 2
Relationship between major clinical risk factors and T cell content in AAA intravascular thrombi.

Univariate analysis was performed by Mann-Whitney U test. Multivariate analysis was performed using linear regression.. RF- risk factor; B=linear regression coefficient

Risk factor	RF n	No RF n	Intrathrombus CD3 content (Mean % $\pm$ SD)		Univariate	Multivariate
			Risk factor	No Risk factor		
Hypertension	24	3	7,0 $\pm$ 7,5	2,2 $\pm$ 1,5	p=0,16	B=-3,0 (p=0,69)
Diabetes	2	25	2,5 $\pm$ 0,2	6,8 $\pm$ 7,4	p=0,46	B=-5,7 (p=0,27)
Current smoking	19	8	8,2$\pm$8,0	2,4$\pm$1,5	p=0,02	B=7,4 (p=0,03)
Obesity	4	23	11,1 $\pm$ 11,9	5,6 $\pm$ 6,1	p=0,41	B =6,6 (p=0,10)
Hypercholesterolemia	25	2	6,9 $\pm$ 7,3	1,4 $\pm$ 0,8	p=0,09	B =6,1 (p=0,47)