Higher leukocyte count is associated with lower presence of carotid lipid-rich necrotic core: a sub-study in the Plaque At RISK (PARISK) study

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Abstract

Background Increasing evidence suggests that inflammation inside the vessel wall has a prominent role in atherosclerosis. In particular in carotid atherosclerosis, vulnerable plaque characteristics are strongly linked to an increased stroke risk. An association between leukocytes and plaque characteristics has not been investigated before and could help with gaining knowledge of inflammation in plaque vulnerability, which could contribute to a new target for intervention. In this study, we investigated the association of leukocyte count with carotid vulnerable plaque characteristics.

Methods All patients from the Plaque At RISK (PARISK) study whom had complete data on leukocyte count and CTA- and MRI-based vulnerable plaque characteristics, were included. Univariable logistic regression was used to detect associations of leukocyte count with the separate vulnerable plaque characteristics (intra-plaque haemorrhage (IPH), lipid-rich-necrotic core (LRNC), thin or ruptured fibrous cap (TRFC, plaque ulceration and plaque calcifications). Subsequently, other known risk factors for stroke were included as covariates in a multivariable logistic regression model.

Results 161 patients were eligible for inclusion in this study. Forty six (28.6%) of these patients were female with a mean age of 70 [IQR 64-74]. An association was found between higher leukocyte count and lower prevalence of LRNC (OR 0.818 (95% CI 0.687-0.975), while adjusting for covariates. No associations were found between leucocyte count and presence of IPH, TRFC, plaque ulceration or calcifications.

Conclusions Leukocyte count is inversely associated with presence of LRNC in the atherosclerotic carotid plaque in patients with a recently symptomatic carotid stenosis. The exact role of leukocytes and inflammation in plaque vulnerability deserves further attention.

Background

Internal carotid artery stenosis (ICAS) is the known cause of stroke in approximately 15% of ischemic stroke patients (1). More importantly, ICAS is the cause of recurrent stroke in 37% of all patients that suffer from recurrent stroke (2). In the last decade, imaging-based research on carotid atherosclerotic plaques has identified certain plaque components and morphological characteristics of the plaque that strongly relate to increased risks of stroke and recurrent stroke (3, 4). As such, the presence of intra-plaque haemorrhage (IPH), a large lipid rich necrotic core (LRNC), and a thin or ruptured fibrous cap (TRFC) all showed to be significant predictors for ischemic stroke on the ipsilateral side of ICAS (3). Of these vulnerable plaque characteristics, IPH and TRFC show the highest risk for first or recurrent ischemic stroke (5). For carotid plaque calcifications, conflicting results are shown, with no clear results in role in stroke prediction (6).

During the past years, the topic of inflammation has gained much interest in the etiological framework of atherosclerosis. In particular inflow of leukocytes into the vessel wall,(7) that target encapsulated cholesterol, may, in a substantial number of patients, result in a second auto-immune reaction which

encompasses activation of a surplus of macrophages after the endothelial damage has occurred. This activation causes phagocytosis of necrotic cells and damaged cells within the vessel wall (7). It is thought that this second auto-immune reaction can be one of the causes of plaque vulnerability and subsequent ischemic stroke.

To our knowledge, no research has been done to investigate a potential association between leukocyte count and (vulnerable) plaque characteristics in ICAS, while there is a role for leukocytes in the initiation of atherosclerosis itself. The aim of this study, which is a sub-study of the Plaque At RISK study (PARISK), is to investigate if there is such an association between each plaque characteristic individually with the leukocyte count in patients with symptomatic ICAS of < 70%. This could potentially help further individualisation of risk prediction in ICAS patients and a potential better risk model. We hypothesize that an increase in leukocyte count is related to the presence of vulnerable plaque characteristics, out of the hypothesis that inflammatory active plaques are more vulnerable. This inflammatory activity could be reflected in high leukocyte count and presence of vulnerable plaque characteristics.

Methods

Setting

Patients for this sub study were derived from the Plaque At RISK (PARISK) study, a diagnostic multicentre cohort study carried out in four academic medical centres in the Netherlands between September 2010 and December 2014 (8). In the PARISK study, patients with an ischemic neurological event in the last three months before inclusion having ipsilateral ICAS < 70% were included. The following exclusion criteria were used in PARISK: any contraindication for MRI or MRI contrast, a clotting disorder, a likely cardiac source of the neurological event, patients in which surgical intervention of the carotid stenosis was planned or performed or patients with multi comorbidity.

These patients underwent carotid magnetic resonance imaging (MRI) and multi detector-row computed tomography angiography (MDCTA) to assess vulnerable plaque characteristics and general plaque properties. The focus of this sub study is the association of the individual plaque characteristics, i.e. presence of IPH, LRNC, TRFC, plaque ulceration or plaque calcifications (as assessed in PARISK) and leukocyte count.

Ethical approval

was acquired before the start of this study and all patients signed informed consent before inclusion in PARISK.

Assessment of leukocyte count

Blood leukocytes determined within two weeks from ischemic stroke or TIA onset were post-hoc collected in the PARISK patients. Patients with leukocyte count ≥ 50 x 10^9/L were excluded, since this

hyperleukocytosis could only be explained by a malignancy. Only if data regarding (vulnerable) plaque characteristics and leukocyte count were available patients were eligible for inclusion in this sub study.

Assessment of carotid atherosclerosis

Plaque characteristics were assessed as described in the PARISK protocol (8). The plaque assessment was performed by blinded assessors and according to PARISK study protocol. In brief, all ipsilateral (symptomatic) carotid arteries were assessed for the presence, and size or volume of (vulnerable) plaque characteristics. Specifically, for the assessment of IPH, LRNC, TRFC and plaque calcifications, MRI scans were used. For the assessment of plaque ulceration CTA scans were evaluated.

Assessment of covariables

Cardiovascular risk factors were assessed using interview (age, current smoking, medical history of hypertension and/or hypercholesterolemia), sex and kind of event (amaurosis fugax, TIA or ischemic stroke) and physical examination (weight and length). Body mass index was calculated as the square of length (metres) divided by weight. Age in years at baseline was used. Smoking was scored if patient reported to smoke during baseline visit. A stroke was defined as TIA if all the symptoms disappeared within 24 hours.

Statistical analyses

We investigated the association of leukocyte count with carotid plaque characteristics using the following strategy. First, univariable logistic regression was used to evaluate the association between leukocyte count and the separate plaque characteristics (IPH, LRNC, TRFC, plaque ulceration or plaque calcifications). Additionally, we investigated the association for all the covariates individually described above and the plaque characteristics. Second, multivariable logistic regression models were run to investigate the association of leukocyte count with the separate plaque characteristics, while adjusting for the covariates. The covariates were based on expert opinion and assumed pathophysiology, since it is unknown which factors could predict presence of these vulnerable plaque characteristics. SPSS (version 26, IBM) was used for the statistical analyses.

Results

Study population

Between September 2010 and December 2014, 244 patients were included in the PARISK study and in 238 of these patients plaque imaging has been performed. Out of these 238 patients, 161 were eligible for inclusion in this sub study. In all of the 67 excluded patients no usable leukocyte count was available. Forty-six patients were female (28.6%) and the median age was 70 (interquartile range [IQR] 64–74). Seventy-six (47.2%) patients suffered from ischemic stroke, 72 (44.7%) from a TIA and thirteen (8.1%) from amaurosis fugax. The mean leukocyte count was 7.36 x 10^9/L (standard deviation [SD] 2.20). IPH was present in 61 (37.9%) patients. LRNC was found in 101 (62.7%) and TRFC was present in 65 (40.4%)

of all patients. A calcified plaque was found in 148 (91.9%) of all patients. Finally, an ulcerated plaque was determined in 35 (25.7%) patients. The other baseline characteristics are presented in Table 1, the imaging biomarkers in Table 2.

Table 1
Baseline characteristics – Median values with inter quartile range (IQR), means with standard deviation (SD) or number with percentage when appropriate.

Number of included patients	**161**
Baseline Characteristics	
Age, years (median [IQR])	70.00 [64.00, 74.00]
Women (%)	46 (28.6)
Classification event (%)	
TIA (%)	72/161 (44.7)
Stroke (%)	76/161 (47.2)
Amaurosis fugax (%)	13/161 (8.1)
Hypercholesterolemia (%)	81/158 (51.3)
Hypertension (%)	98/161 (60.9)
Diabetes mellitus (%)	35/161 (21.7)
History of TIA or ischemic stroke (%)	43/161(26.7)
History of ischemic heart disease (%)	28/161 (17.4)
History of peripheral artery disease(%)	17/161 (10.6)
Current smoking (%)	38/161 (23.6)
Body mass index (BMI), kg/m^2 (mean (SD))	26.72/158 (4.11)
Use of cholesterol lowering medication (%)	74/160 (46.2)
Use of antihypertensive drugs (%)	91/161 (56.5)
Use of anti-platelets (%)	60/160(37.5)
Use of anticoagulants (%)	3/161 (1.9)
Leukocyte count	
Leukocyte count x 10^9 /L (mean (SD))	7.36 (2.20)
Time interval between index event and leukocyte count measurement, days (median [IQR])	1.00 [0.00–4.00]

Table 2
Imaging biomarkers – number/total number of patients with percentage of total

Imaging biomarkers	
Intra-plaque hemorrhage (IPH) (%)	61/161 (37.9)
Lipid-rich-necrotic core (LRNC) (%)	101/161 (62.7)
Thin or ruptured fibrous cap (TRFC) (%)	65/161 (40.4)
Ulcerated plaque (%)	35/136 (25.7)
Plaque calcifications (%)	148/161 (91.9)

Leukocyte count and plaque characteristics

In the univariable regression analyses we found that higher leukocyte counts were associated with a lower prevalence of LRNC (OR 0.859 (95%CI 0.738-1.000) ($p = 0.049$), but no association was found with the other plaque characteristics (see Table 3). Out of the other variables that were used for univariate regression analyses with LRNC, sex showed a significant association with a *p*-value of 0.002. An overview of the other univariate regression analyses can be found in Table 4.

Table 3
Univariate logistic regression analyses for leukocyte count and individual vulnerable plaque characteristics.

Leukocytes and plaque characteristic	OR	95% CI	*p*-value
IPH	0.910	0.778–1.065	0.242
LRNC	0.859	0.738-1.000	0.049
TRFC	0.891	0.760–1.043	0.152
Plaque ulceration	1.008	0.850–1.195	0.931
Plaque calcifications	1.257	0.888–1.779	0.196

Table 4
Univariate logistic regression for LRNC and several baseline variables.

Variable and LRNC	OR	95% CI	*p*-value
BMI	0.934	0.862–1.012	0.094
Age (year)	0.986	0.946–1.028	0.522
Smoking (current)	1.189	0.555–2.549	0.656
Sex (female)	0.323	0.159–0.655	0.002
Hypertension	1.055	0.548–2.032	0.873
Hypercholesterolemia	1.143	0.601–2.175	0.684
Type of event (ischemic stroke or TIA/amaurosis fugax)	1.428	0.749–2.721	0.279

Multivariable regression

When multivariable logistic regression for LRNC was performed, with inclusion of BMI, age, current smoking, sex, hypertension, hypercholesterolemia and kind of ischemic event as covariates, the association between leukocyte count and LRNC remained statistically significant with an odds ratio (OR) of 0.818 (95% CI 0.687–0.975, p = 0.025). The complete results of the multivariable regression analysis can be found in Table 5.

Table 5
Multivariate logistic regression analysis for LRNC with leukocyte count, corrected for other variables.

	p-value	OR	95% CI
BMI	0.096	0.926	0.846–1.014
Age (year)	0.805	1.006	0.959–1.055
Smoking (current)	0.270	1.699	0.663–4.355
Sex (female)	0.001	0.252	0.114–0.556
Hypertension	0.848	0.927	0.430–2.002
Hypercholesterolemia	0.826	1.091	0.502–2.368
Variety of event (ischemic stroke or TIA/amaurosis fugax)	0.217	1.593	0.761–3.337
Leukocyte count	0.025	0.818	0.687–0.975

Discussion

The precise role for inflammation in plaque vulnerability remains enigmatic. This sub-study in the PARISK study shows an independent inverse association between an increase in leukocyte count (within fourteen

days after the ischemic event) and presence of LRNC on plaque MRI in the sub-acute moment after an ischemic event in patients with symptomatic ICAS < 70%. This finding is not in line with the current hypothesis on the vulnerable plaque. However, to our understanding this is the first study that investigated the possible association between leukocyte count and vulnerable plaque characteristics.

Since the beginning of this millennium a lot of papers were published regarding plaque vulnerability and the specific vulnerable plaque characteristics on MRI in the carotid plaque. Some of these plaque characteristics were associated with a higher risk for either recurrent or first stroke (5, 9). Gupta *et al.* published one of the first systematic reviews on ischemic stroke risk for some of these vulnerable plaque characteristics with a meta-analysis that did show an OR for ischemic stroke or TIA combined for IPH of 4.59 (95% CI 2.91–7.24), 3.00 (95% CI 1.51-5-95) for LRNC and 5.93 (95% CI 2.65–13.20) for TRFC (9). Recently, Schindler *et al.* reported a hazard ratio (HR) of 11.0 for IPH specifically for ipsilateral stroke (5). These high OR/HR fuelled the interest in researching the a potential role of inflammation by investigating leukocytes and plaque characteristics.

For a long time, inflammation has been regarded a key factor in initiation and progression of atherosclerosis. Yet, recently studies focussing on treatment of this inflammatory reaction published their results (10–12). In the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) patients with recent myocardial infarction and elevated high sensitivity C-reactive protein were randomized between several doses of canakinumab (a monoclonal antibody against interleukin-1β) and placebo. They showed a HR of 0.85 (95% CI 0.74–0.98) with $p = 0.021$ for combined cardiovascular outcome measure (recurrent myocardial infarction, ischemic stroke or other cardiovascular death) (11). Alike CANTOS, in the Low-dose colchicine for secondary prevention of cardiovascular disease 2 (LoDoCo-2 trial) they tried to reduce recurrent myocardial infarction, ischemic stroke and other vascular death, but then with 0.5g colchicine once daily in patients with chronic coronary disease. They found a HR of 0.69 (95% CI 0.57–0.83) with $p < 0.001$ for the primary outcome (12). In contrast to canakinumab, colchicine is an old and inexpensive drug that has been used for a long time in treatment of gout. These studies show promising results for treatment of inflammation in cardiovascular disease.

All these studies were done in patients after myocardial infarction. In stroke patients the COlchiciNe for prevention of Vascular Inflammation in Non-CardioEmbolic stroke (CONVINCE) trial is currently including patients with recent non-cardio embolic TIA or ischemic stroke. In CONVINCE patients are randomised between standard stroke treatment or standard treatment plus low dose colchicine (13). It will nonetheless take some more years before these results are published.

Even though, the studies mentioned above, did show a treatment effect towards prevention of recurrent cerebrovascular events in general and most evidently in a decrease of ischemic stroke cases, no evident blood biomarkers for recurrent stroke have been identified. Leukocyte count could be considered as a potential biomarker, since we found an inverse association between leukocyte count as determined in the sub-acute moment and LRNC. However, the found association was the opposite of what was

hypothesized, with a lower prevalence for LRNC in ICAS when a higher leukocyte count is found. The role of leukocytes in predicting recurrent cardiovascular events should be tested in the future.

A potential explanation for the inverse relation that was found, is the general nature of leukocyte count as a mix of several types of leukocytes. Fani *et al.* recently investigated the association between blood immunity markers, both from the innate and adaptive immune system, and vulnerable plaque characteristics in the Rotterdam Study (14). The Rotterdam Study is a prospective population-based cohort study that started in 1989. Fani *et al.* used 1602 patients with subclinical carotid atherosclerotic disease. They found that the leukocytes that are part of the innate immune system (macrophages, granulocytes and platelets) were associated with larger plaque and greater plaque thickness and that the monocyte-to-lymphocyte ratio was associated with higher incidence of LRNC. On the other hand the lymphocytes were associated with smaller carotid plaques and a lower prevalence of IPH. This could be an important reason to assess the leukocyte differential count and calculate the ratios used in their study and make a distinction between the innate and the adaptive immune system in future research. In the investigated setting of the presented study, this association shows the opposite relation as expected, this could be due to a higher proportion of lymphocytes in the researched population. This could however not be investigated since only the leukocyte count was available.

This study has some limitations. Firstly, the relatively low number of patients included in the analyses. This was mainly due to the fact that this sub study was not planned when the study was designed and so leukocyte counts were not available in all patients.

Secondly, there is a risk of confounding because of a possibly higher leukocyte count through necrotic brain tissue after the ischemic event. Correcting for this potential confounder was difficult, since NIH Stroke Scale scores (NIHSS) were not available for the included patients. Nonetheless, in this patient population the estimated effect will probably be minimal, because more than half of the patients suffered from a TIA or amaurosis fugax; and only minor stroke patients could be included (modified Rankin scale ≤ 3). In these patients the volume of necrotic brain tissue is generally low and thus the effect on leukocyte count. Finally, the last limitation of our study is the cross-sectional study design. Due to this study design we were only able to show any present associations and were unable to investigate the prediction of stroke risk.

The strengths of this study are evident as well. PARISK was a prospective, well defined, diagnostic cohort study with very complete baseline data. Moreover, it was performed in Dutch comprehensive stroke centres with large experience and specific expertise in MRI plaque imaging. The first strength of this study is the state of the art MR imaging and MDCTA that were performed in these patients (8). Both imaging modalities used in this sub study have shown high specificity and sensitivity for detecting the vulnerable plaque characteristics they are used for and so create high quality data (15–17).

Secondly, the patients included in PARISK all suffer from mild to moderate carotid stenosis, with a minimum of 30% measured with ECST criteria. This is the range in which prior research on ICAS showed the least benefit of revascularisation through carotid endarterectomy or carotid stenting (18, 19). The

investigated patient population could therefore be the group in which carotid revascularisation is not performed and thus be of great interest in prevention of recurrent stroke. Potentially, patients in which the cause of stroke is stated to be cryptogenic, could be caused by vulnerable plaques in the carotid artery but with a percentage stenosis < 50%. Since these patients were included in the PARISK study, it could give a more accurate depiction of the actual situation within the overall patient population at risk of stroke out of ICAS. This is a valuable inclusion criterion of PARISK.

The final strength of this study is the use of leukocytes that were determined within two weeks around the ischemic event. With a median of one day (IQR 0.00–4.00), it decreases the risk of confounding due to necrotic brain (or eye) tissue. Furthermore, this sub-acute leukocyte count could be a valuable and easy to detect value of the general inflammatory activity within these patients, may be before the occurrence of the ischemic event as well. This hypothesis deserves future attention.

As stated above, this sub study did find an association between leukocyte count and LRNC. The direction of the relation is opposite from what was expected. This could be due to an increase in inflammatory cell count because of the cytokines that are excreted and on the plaque surface that is in contact with the streaming blood. These cytokines could increase the production of leukocytes. It could be hypothesized that plaques that excrete more cytokines are in another phase of plaque progression and thus have less LRNC compared to plaques that show LRNC more frequently and in parallel these patients could show less circulatory leukocytes. This statement is highly hypothetical and deserves further attention in future research, both in clinical as in more translational or fundamental research. At least it could be established that more research into inflammatory biomarkers seems a feasible but unexplored research field and is highly needed.

Future research should determine if the found association is useful in further risk determination. Additionally, the PARISK study could be used to determine recurrent stroke risk on basis of multiple vulnerable plaque characteristics in the form of a new risk model for ICAS. Next to these analyses, a new prospective study should be set up in which patients get blood drawn at pre-specified moments within the (sub-)acute setting (for example on the emergency department, 24 hours, three days, one week and two weeks after admission), preferably with leukocyte differential count, instead of the general leukocyte count that was used in this study. Several studies showed usefulness of using ratios between some of the components of the differential count. For example, the value of neutrophil-to-lymphocyte ratio was investigated for predicting presence of carotid atherosclerosis.(20) This use seems impractical since most patients get carotid imaging quickly, either during presentation on the emergency department or in the upcoming days, but it could be seen as indirect evidence of the importance of the inflammatory reaction inside the carotid vessel wall. This could be helpful in initiation of future studies into treatment of the inflammatory reaction in atherosclerosis. Besides this, the leukocyte differential count has been evaluated for use of predicting outcome of cardiovascular events, both stroke and myocardial infarction (21, 22).

Finally, a future prospective study should, next to leukocyte differential count and other inflammatory biomarkers, at least include carotid MRI and ideally one or several forms of imaging parameters that could detect plaque inflammation, in particular positron emission tomography (PET) CT or dynamic contrast enhanced (DCE-)MRI. In that way we could learn more on plaque inflammation in relation to time. It seems valuable to assess the course of the investigated potential biomarkers over time as well.

Conclusions

In conclusion, this study showed the independent inverse association between leukocyte count, as determined within fourteen days of the ischemic event on MRI, and lipid-rich necrotic core. Future research projects should aim on identification of more (inflammatory) blood biomarkers for both presence of vulnerable plaque characteristics as well as recurrent stroke in patients with ICAS. This could increase the speed in risk determination and potentially decrease time until revascularisation. Additionally, the assessment of biomarkers could be less expensive than MRI. A future study with both leukocyte differential count and inflammatory imaging is highly wanted to aid in better risk prediction in ICAS patients.

Abbreviations

BMI Body Mass Index

CI Confidence interval

CANTOS Canakinumab Anti-Inflammatory Thrombosis Outcomes Study

CONVINCE COlchiciNe for prevention of Vascular Inflammation in Non-CardioEmbolic stroke

DCE Dynamic contrast enhanced

ECST European Carotid Surgery Trial

HR Hazard ratio

ICAS Internal carotid artery stenosis

IPH Intra-plaque haemorrhage

IQR Inter quartile range

LRNC Lipid-rich-necrotic core

MDCTA Multi detector computed tomographic angiography

NIHSS NIH Stroke Scale scores

PARISK Plaque At RISK

PET Positron emission tomography

TIA Transient ischemic attack

TRFC Thin or ruptured fibrous cap

Declarations

Ethics approval and consent to participate

Written informed consent from each patient or representative, and ethics approval by the Dutch ethics committee on research on humans were obtained through *Medisch Ethische Toetsings Commissie* of the participating centres. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors report no conflicts of interests and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential competing interests.

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Author contributions:

TJV, JS and PJN researched literature and conceived the study. TJV wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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