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Profile of reticulated platelets in the early, subacute and late phases after transient ischemic attack or ischemic stroke

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Abstract

Information regarding the profile of reticulated platelets (RP) in ischemic cerebrovascular disease (CVD) patients is limited. Data from two prospective, observational, case-control studies were combined to compare the %RP using whole blood flow cytometry in patients ≤ 4 weeks of TIA/stroke onset (**baseline**, N = 210), and 14 ±7 days (**14d**, N = 182) and ≥ 90 days (**90d**, N = 145) after starting or changing antiplatelet therapy with healthy controls (N = 34). There were no differences in median %RP between the overall CVD patient population at baseline or 14d *vs*. controls (P ≥ 0.2). However, the median %RP was significantly higher in CVD patients overall at 90d (P = .036), and in the subgroup of patients with "lacunar" TIA/ischemic stroke at baseline (P = .04) and at 90d (P = .01), but not at 14d (P = .06) *vs*. controls. There were no significant differences in the median %RP between other TIA/stroke subgroups and controls (P ≥ 0.05). Elevated circulating reticulated platelets, as a marker of increased platelet production/turnover, may occur following an ischemic event in a well-phenotyped TIA/ischemic stroke population overall, but may precede symptom onset at least in the subgroup with small vessel occlusion. These data improve our understanding of the profile of reticulated platelets in CVD patients.

Keywords

Flow cytometry, ischemic stroke, mean platelet volume, platelet distribution width, reticulated platelets, TIA

History

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Introduction

"Reticulated platelets (RP)" are young platelets containing a residual amount of megakaryocyte-derived RNA which are released into the circulation after fragmentation of megakaryocytes in the bone marrow or pulmonary circulation [1]. Mature platelets normally have a lifespan in the circulation of around 7–10 days [2–5], but RP may persist for approximately less than a day based on data from animal studies [6]. Therefore, quantification of the %RP may serve as a measure of increased platelet production and/or turnover, which might occur in subjects with enhanced platelet activation [7]. However, reticulated platelets which tend to be larger in size and more reactive than mature platelets [1], may not increase unless the stimulus to platelet activation also promotes thrombopoiesis [8]. Larger platelets tend to aggregate more easily [9] and are more reactive than mature platelets [9,10], possibly due to the fact that they may contain larger quantities of adenosine triphosphate, glycogen [11], more lactate dehydrogenase activity, serotonin uptake and secretion [12], synthesize more thromboxane B_2 [9,11,13], and have a higher expression of GP1b and integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) [14]. Reticulated platelets have been reported to be elevated in cardiovascular disease patients with myocardial infarction (MI) *vs*. angina [15], and levels reduce significantly over time after a myocardial infarct [16]. Furthermore, the *ex vivo* response to antiplatelet therapy in stable coronary artery disease is strongly associated with the %RP [17,18].

To date, few studies have assessed the %RP in patients following TIA or stroke compared with controls [19–21]. One small study revealed that RP were increased in patients within 3 days of stroke onset compared with controls [19], and a further pilot study showed that RP were only elevated in the subgroup of patients with cardio-embolic stroke *vs*. "neurological controls" [20]. The largest case-control study on this topic to date revealed an increase in the %RP on whole blood flow cytometry in the early (< 28 days) and late (> 78 days) phases after TIA or ischemic stroke only after adjusting for differences in age between patients and controls [21]. More recently, Murphy *et al* reported a higher "% reticulated platelet fraction" in early (5.78%; P <

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.001) and late phase symptomatic (5.11%; P = .01) compared with asymptomatic moderate-severe carotid stenosis patients (3.48%) using an automated assay on a Sysmex XE 2100 haematology analyzer, but there were no significant differences in the % RP between groups using whole blood flow-cytometry [22].

The mean platelet volume (MPV) is an independent predictor of stroke risk in patients with atrial fibrillation, after controlling for age, gender and other $CHADS_2$ criteria [23]. A higher MPV is a predictor of poorer outcome in patients with acute ischemic stroke [24], and MPV may be increased in the early or late phases after non-lacunar ischemic stroke [25]. Preliminary data have also shown a positive correlation between the %RP and the MPV following TIA or ischemic stroke overall [21] and in patients with symptomatic moderate to severe carotid stenosis [22]. Therefore, this topic warranted further study.

The **aims** of this case-control study were to determine whether:

(1) There was convincing evidence of an increase in the % circulating RP in patients in the early or late phases after TIA or ischemic stroke compared with healthy controls despite treatment with commonly-prescribed antiplatelet treatment and other secondary preventive medications;
(2) There were any demographic or vascular risk factors, or TIA/stroke subtypes which influenced the %RP in our CVD patient population;
(3) There was a correlation between the %RP on flow cytometry and the MPV or platelet distribution width (PDW) in CVD patients.

We **hypothesized** that:

(a) Circulating RP would be increased in the early or late phases after TIA or ischemic stroke compared with controls;
(b) Age and TIA/stroke subtype might influence the %RP in CVD patients;
(c) There would be a positive correlation between the %RP and the MPV or PDW, providing further evidence that "younger" RP are larger than more mature platelets in an overall CVD patient population.

Methods

Inclusion Criteria for CVD Patients

Consecutive eligible patients, older than 18 years of age, with a recent **clinical diagnosis** of TIA or ischemic stroke within the preceding 4 weeks, whose treating physician opted to start or change antiplatelet therapy were screened for inclusion in this prospectively-planned, single center, observational analytical study. Patients were participants of one of 2 consecutive observational studies coordinated by our research group: The **TR**inity **A**nti**P**latelet Responsiveness (**TRAP**) study which recruited and followed up patients between September 2007 and February 2010; the **O**ptimal **A**ntiplatelet **T**herapy in TIA and Ischemic **S**troke (**OATS**) Study which intermittently recruited subjects between October 2011 and January 2016. Both studies had the same inclusion criteria and the OATS study design was based on pilot outcome data from the TRAP study. Patients were recruited from the Rapid Access Stroke Prevention service, and from the inpatient population of the Neurology, Age-Related Health Care, Stroke and Vascular Surgery Services at our secondary and tertiary referral university teaching hospital.

Exclusion Criteria

Patients were excluded if they had a MI, DVT, PE or recent surgery within the preceding three months; ongoing unstable angina or unstable symptomatic peripheral vascular disease; platelet count < 100 x 10^9/L; known bleeding or clotting diathesis, including known platelet-related bleeding disorders; active proven vasculitis; active neoplasia; non-steroidal anti-inflammatory drug (NSAID) intake other than aspirin or aspirin in combination with dipyridamole in the preceding 11–14 days [26,27]. We also excluded CVD patients unable to attend for clinical follow-up and repeat testing at 14 ± 7 days, patients with active infection, renal impairment (e.g. urea > 10 mmol/L or GFR < 30 ml/min), or who had a prior history of primary intracranial haemorrhage.

Clinical Assessment

All patients underwent detailed neurovascular assessment by one of the neurology research registrars (WOT or JK in TRAP; STL or SJM in OATS) or supervising consultant vascular neurologist (DJHM) according to ESO guidelines [28] to confirm a clinical diagnosis of TIA or ischemic stroke in all cases. Information regarding vascular risk factors, including hypertension, prior TIA or stroke, ischemic heart disease, atrial fibrillation, valvular heart disease, diabetes mellitus, hyperlipidemia, peripheral vascular disease, migraine, family history of stroke, medication intake (including anti-thrombotic therapy), smoking status, alcohol intake, illicit substance intake, and the method of detection of carotid stenosis and timing of any carotid intervention in patients with large artery atherosclerosis was collected prospectively. Details regarding antiplatelet regimens, dose and duration of therapy were recorded. Results of routine haematological (FBC), coagulation (PT/APTT), biochemical and blood glucose testing were collected prospectively. CT and/or MRI brain and color Doppler ultrasound (CDUS) of neck vessels was performed in all patients, as well as magnetic resonance angiography (MRA) or CT angiography (CTA) to establish concordance between CDUS and another noninvasive imaging modality in recently symptomatic carotid stenosis patients. A chest radiograph, electrocardiograph (ECG), 24-hour ECG recording and transthoracic or transesophageal echocardiograph were obtained in all patients. TIA and stroke subtyping was performed according to the Trial of Org 10 172 in Acute Stroke Treatment (TOAST) classification system [29].

All patients underwent clinical and laboratory assessment before (**baseline**), 14 ±7 days after (**14d**), and at least 90 days after (**90d**) starting or changing their antiplatelet regimen. In the large artery atherosclerotic subgroup of patients who had moderate-severe carotid stenosis, the 90d follow-up visit was performed at least 3 months following carotid surgery or endovascular treatment, unless intervention had been delayed for at least 3 months after symptom onset.

Because most patients in TRAP and OATS at baseline and most patients in the TRAP study at 14d were assessed during their inpatient stay, adherence to antithrombotic therapy in these patients was confirmed by checking the inpatient prescription chart. Adherence in all outpatients was assessed by history taking alone, but all were phoned in the week prior to their scheduled follow-up visit to stress the importance of complete medication adherence before reassessment. If complete adherence was not initially confirmed, any issues potentially affecting adherence were discussed and the follow-up visit postponed for 14 days until full adherence was verbally confirmed.

Controls

Control subjects of similar age and gender were recruited from amongst the staff at AMNCH-TUH and from the local population; spouses of patients and control subjects were also recruited. The exclusion criteria for control subjects were the same as those for patients, with the exception that subjects were also excluded from the control group if they ever had a history of stroke or TIA in the past, if they had evidence of >50% carotid or vertebral artery stenosis on colour Doppler ultrasound screening, or if they were on antiplatelet therapy.

Written informed consent, or "proxy consent" where appropriate, was obtained from all subjects. The TRAP (REC Ref: 2007/07/MA) and OATS (REC Ref: 2011/35/03) studies were approved by the St. James's Hospital/AMNCH Research Ethics Committee.

Blood Sampling and Laboratory Methods

All subjects were rested for at least 20 min, and careful atraumatic venepuncture performed from a free-flowing vein with a 21 G butterfly needle and a Vacutainer® system with a luer adaptor, as outlined previously [22,30]. The first 3 ml was drawn into a sterile tube containing 3.2% sodium citrate and subsequently discarded. Six further 3.2% citrate-anticoagulated samples were subsequently taken, the first of which was used for whole blood flow cytometric analysis to quantify the %RP (see below). The final citrate-anticoagulated sample was used for measurement of the platelet count, MPV and PDW between 2 and 4 hours after venepuncture. Thereafter, three 3 ml K_2EDTA samples were obtained. The first K_2EDTA sample was used to measure the FBC, including the MPV and PDW on a Sysmex XE-2100® Hematology Analyzer (Sysmex UK Ltd, Milton Keynes, UK) between 2 and 4 hours after venepuncture [22] . All MPV measurements were performed in accordance with recent best practice guidelines [22,31].

The whole blood flow cytometry method used in this study was adapted from a previously validated protocol [21]. In brief, a manual gate was positioned around the platelet cloud, identified by its forward and side scatter characteristics. To confirm that the cells within the gate in the "test sample" were platelets, a platelet-specific mouse monoclonal IgG1 antibody to CD42b, conjugated to phycoerythrin (Immunotech, Beckman Coulter, Marseille, France), was used to confirm that > 95% of the cells expressed GpIb in FL2 on our flow cytometer [22,32]. The gating settings were then saved and not repositioned, thus facilitating single-labeling of platelets with Thiazole Orange (Retic-COUNT™, Becton-Dickinson, San Jose, USA) using the "panel set up" on a Beckman Coulter XL MCL flow cytometer [21]. The concentration of the main stock solution of Retic-COUNT was 0.01 mg/ml. One ml of Isoton II® alone had been aliquoted into the "control tube", and a 1:10 dilution of Retic-COUNT™ was performed by adding 900 µl of Isoton II® to 100 µl of Retic-COUNT in the "test sample tube". 5 µl of citrate anticoagulated whole blood was then added to the control and test sample tubes, respectively, between 30 and 60 minutes after venepuncture. The samples were covered and incubated in the dark for exactly 30 minutes, then centrifuged at 1200 x G for 3 minutes. In order to prevent further incubation of the test sample with Retic-COUNT, the supernatant was immediately discarded and the sediment pellet re-suspended in 1 ml of Isoton II®. The nonspecific fluorescence of the control sample was calculated, and the % RP was calculated by measuring the % TO-positive (reticulated) platelets in the test sample in FL1 within an hour of resuspension, as previously described [22]. COULTER EPICS XL-MCL™ Flow Cytometer SYSTEM II™ software was employed to capture and analyze data on the flow cytometer.

Statistical Analysis

The Wilcoxon signed rank test and the Mann–Whitney test were used for comparison of paired and unpaired non-parametric variables, respectively. Paired and unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively. Because there were differences in the demographic and vascular risk profiles between patients and controls that could potentially impact on inter-group comparisons, multiple linear regression analysis was performed to assess the effects of these variables on %RP, using a stepwise regression method. Spearman's rank order correlation was performed to examine the association between %RP and platelet count, MPV and PDW. Statistical analysis of all final-collated data was performed with SPSS, version 22.

Results

Data from 210 eligible patients with recent TIA or ischemic stroke who had baseline data following recruitment to the TRAP and OATS studies were analyzed. The median time interval from TIA or stroke onset to study inclusion was 8 days (minimum-maximum range: 0–28 days). Follow up data were available in 182 patients at 14d, and 145 patients at 90d. Follow-up data were not available in all patients because some had their anti-thrombotic regimen altered by their treating physician (e.g. following detection of paroxysmal atrial fibrillation which warranted a change to anticoagulation or due to intolerance of treatment) and were no longer eligible for follow-up in the TRAP and OATS studies; some were too unwell, unavailable for or lost to follow up. Thirty-four controls were recruited and assessed once.

Table I. Demographic and vascular risk profiles of study participants at enrollment. Values are means [±SD] or absolute values with percentages in parentheses (%), where appropriate. *P* values relate to comparisons between Patients and Controls by Chi-squared or Fisher's Exact tests, where appropriate. Significant *P* values in bold. IHD* = History of ischemic heart disease; **Hyperlipidemia = Total cholesterol > 5.0 mmol/L or LDL > 3.5 mmol/L at the time of the TRAP study design; DVT/PE*** = Deep venous thrombosis/pulmonary embolism.

Parameter	CVD Patients (N = 210)	Controls (N = 34)	*P* Value
Mean Age in years	60 [±13]	51 [±12]	0.04
Sex (M/F)	134/76	20/14	0.7
Index TIA at enrollment	160 (76%)	0	N/A
Index Stroke at enrollment	50 (24%)	0	N/A
Prior Stroke/TIA	49 (23%)	0	N/A
Index event occurred on antiplatelet agent	160 (76%)	0	N/A
IHD *	37 (18%)	0	0.004
Hypertension	100 (48%)	9 (26.5%)	0.03
Diabetes Mellitus	27 (13%)	0	0.02
Hyperlipidemia **	78 (37%)	2 (6%)	< 0.001
Atrial Fibrillation/Flutter at Enrollment	1 (0.5%)	0	1.0
Prior DVT/PE ***	4 (2%)	0	1.0
Peripheral Vascular Disease	10 (5%)	0	0.4
Migraine	39 (19%)	3 (9%)	0.2
Current Smoker	54 (26%)	2 (5.9%)	0.008
Ex-smoker	68 (32%)	6 (17.6%)	0.1
Never smoker	89 (42%)	26 (76.5%)	< 0.001
Statin Therapy	110 (52%)	4 (12%)	< 0.001
Family History of Stroke	70 (33%)	7 (21%)	0.2

Table II. Etiological subtyping of CVD Patients included at baseline according to the TOAST classification (Total N = 210).

TIA/Ischemic Stroke Subtype	Numbers (%)
Large Artery Atherosclerotic	13 (6.2%)
Small Vessel Disease (Lacunar)	36 (17.1%)
Cardioembolic	24 (11.4%)
Other Determined	7 (3.3%)
Undetermined Etiology	130 (62%)

Table III. Prescribed antiplatelet regimens in CVD Patients during the study (Numbers [%]).

Antiplatelet Regimen	Baseline (N = 210)	14d (N = 182)	90d (N = 145)
None	47 [22%]	0	0
Aspirin Monotherapy (75 mg-300 mg daily)	159 [76%]	35 [19%]	21 [15%]
Aspirin (75 mg daily) + Dipyridamole MR (200 mg BD)	4 [2%]	86 [47%]	76 [52%]
Clopidogrel Monotherapy (75 mg daily)	0	61 [34%]	48 [33%]

The baseline demographic and vascular risk factor profiles of study participants (Table I), the TIA/stroke etiological subtypes in CVD patients (Table II) and the prescribed antiplatelet regimens in CVD patients at baseline, 14d and 90d (Table III) are outlined below. One hundred and fifty-nine patients were on aspirin monotherapy at baseline, 47 were on no antiplatelet medication and 4 were on aspirin-dipyridamole combination therapy. Two patients in the subgroup who changed from aspirin to clopidogrel had recurrent TIAs during follow up. One patient had a recurrent TIA due to embolization from an aortic arch atheromatous plaque within a week of recruitment; one patient had a left hemispheric TIA due to possible embolism from a stenosed internal carotid artery within three months of recruitment. No other patients had recurrent TIA, stroke or any other new cardiovascular or venous thrombotic outcome events during follow up in this study.

% Reticulated Platelets on Flow Cytometry in All CVD Patients Vs. Controls

Median % Gp1b expression was similar in CVD patients and controls (99.3 *vs*. 98.9%, P = 1.0), confirming that the vast majority of cells analyzed by whole blood flow cytometry were platelets. The intra-assay co-efficient of variation (CV) for the whole blood flow cytometry assay quantifying the %RP was 6.64%, calculated using 3 separate samples taken from the same patient which were tested between 60 and 90 minutes after venepuncture on the same day.

Table IV. Comparison of % Reticulated Platelets (%RP) between CVD Patients overall at different time points and Controls. Values represent medians (25^{th} – 75^{th} percentiles). Significant *P* values highlighted in bold.

	CVD Patients at baseline	CVD Patients at 14d	CVD Patients at 90d	Controls
%RP	14.2 (9.1– 20.9)	13.7 (8.47– 20.6)	18.0 (10.2– 22.3)	13.0 (10.1– 16.6)
P value	0.2	0.3	**0.036**	

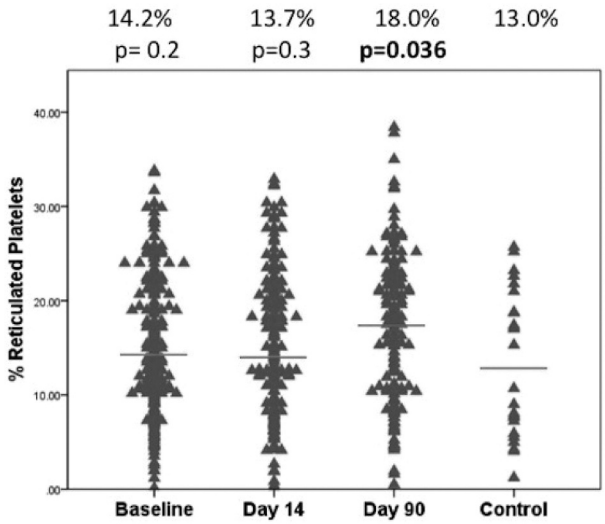

Figure 1. Scatterplot of circulating %RP in CVD Patients overall at Baseline, 14d and 90d *vs*. Controls. Each point represents a single patient. Horizontal bars and numbers above figure indicate median values. *P* values refer to comparison of median %RP between CVD patients at different time points and Controls. Significant *P* value highlighted in bold.

There were no significant differences in the median %RP between the overall TIA/ischemic stroke patient population at baseline or 14d compared with controls (P ≥ 0.2). However, the median %RP was significantly higher in CVD patients overall at 90d than in controls (P = .036) (Table IV and Figure 1). Multiple linear regression analysis revealed that age, a prior history of TIA or stroke, ischemic heart disease, hypertension, diabetes, current smoking, and statin use did not significantly affect the %RP at any stage during the study.

% Reticulated Platelets in CVD TOAST Subgroups versus Controls

The median %RP was significantly elevated in the subgroup of patients with TIA/ischemic stroke due to small vessel disease at baseline (P = .04) and at 90d (P = .01), but was not significantly elevated at 14d (P = .06) compared with controls (Table V). Otherwise, there were no statistically significant differences in the median %RP between other TIA/stroke subgroups and controls (P ≥ 0.05) (Table V).

% Reticulated Platelets in Independent TIA and Stroke Subgroups Vs. Controls

Post hoc subgroup analysis revealed that the median %RP was not significantly increased at baseline or 14d (P = .3) (supplemental figures Ia and Ib)., but was significantly higher in the TIA subgroup alone compared with controls at 90d (P = .04) (supplemental figure Ic). There were no significant differences in the median %RP between the ischemic stroke patient subgroup alone *vs*. controls at baseline, 14d or 90d (P ≥ 0.07) (supplemental figures Ia, Ib, Ic). However, it is important to note that the %RP was similar in TIA and stroke patients at baseline (14.9 *vs*. 13.8%, P = .63), 14d (13.4 *vs*. 17.9%, P = .84) and 90d (17.5 *vs*. 17.9%, P = .87).

Simultaneously-collected FBC Parameters in CVD Patients Vs. Controls

There were no significant differences in median platelet counts (P ≥ 0.07), MPV (P ≥ 0.4) or PDW in EDTA or citrate (P ≥ 0.4), or haemoglobin concentrations in EDTA (P ≥ 0.1) between CVD

Table V. Comparison of %RP between etiological subtypes of CVD Patients at different time points and Controls. Values represent medians (25^{th} – 75^{th} percentiles). "N" in first column refers to the number of patients in each subgroup at baseline. Significant *P* values highlighted in bold.

TOAST Subtypes	CVD Patients at baseline (%RP)	CVD Patients at 14d (%RP)	CVD Patients at 90d (%RP)	Controls (%RP)
Large Artery Atherosclerotic (N = 13)	17 (5.6– 25)	12.8 (6.29– 17.1)	12.5 (8.09– 16.3)	13.0 (10.1–16.6)
P	0.3	0.8	0.8	
Small Vessel Disease (N = 36)	17.8 (10.9– 24)	17.1 (9.17– 24)	20.6 (14.7– 23.5)	13.0 (10.1–16.6)
P	**0.04**	0.06	**0.01**	
Cardioembolic (N = 24)	15 (10.7– 18.1)	17.5 (8.7– 21.4)	18.5 (14.5– 23.1)	13.0 (10.1–16.6)
P	0.4	0.3	0.05	
Other Determined (N = 7)	18.7 (9.1– 24.7)	20.8 (14.9– 24.2)	19.5 (9.2– 25.3)	13.0 (10.1–16.6)
P	0.2	0.06	0.2	
Undetermined (N = 130)	13 (9.3– 20.9)	12.6 (8.9– 19.2)	16.9 (9.9– 22.2)	13.0 (10.1–16.6)
P	0.5	0.6	0.09	

Table VI. FBC parameters in CVD Patients at baseline, 14d and 90d compared with Controls. Values represent medians (25^{th} – 75^{th} percentiles) or means (± SD).

FBC Parameter	CVD Patients at Baseline	CVD Patients at 14d	CVD Patients at 90d	Controls
Platelet Count (x 10^9/L) (EDTA)	242 (198– 282)	241 (201–269)	234 (201– 276)	216.5 (194– 258)
P	0.07	0.08	0.2	
Platelet Count (x 10^9/L) (Citrate)	176 (143– 210)	174 (149– 199)	175 (146– 200)	168 (150– 195)
P	0.8	0.8	0.8	
Mean Platelet Volume (fl) (Citrate)	9.9 (± 0.9)	9.7 (± 0.9)	9.8 (± 0.8)	9.8 (± 0.8)
P	0.96	0.4	0.6	
Mean Platelet Volume (fl) (EDTA)	10.8 (± 0.8)	10.5 (± 0.74)	10.7 (± 0.78)	10.6 (± 0.75)
P	0.23	0.41	0.34	
Platelet Distribution Width (%) (EDTA)	13.2	12.8	12.6	12.4
P	(11.7– 14.3)	(11.4– 14.2)	(11.4– 14.1)	(11.7– 13)
	0.44	0.88	0.98	
Platelet Distribution Width (%) (Citrate)	11.2	11.1	11.0	10.9 (10.3– 11.7)
P	(10.1– 12.4)	(9.8– 12.1)	(9.9– 12.2)	
	0.83	0.40	0.53	
Hemoglobin (g/dl) (EDTA)	14.0 (13.1– 15.3)	13.8 (12.9– 14.7)	13.9 (13.0– 15.0)	13.8 (13.2– 14.3)
P	0.79	0.11	0.31	
WCC (x 10^9/L) (EDTA)	7.4 (6.2– 8.9)	7.1 (5.6– 8.1)	6.9 (5.7– 8.0)	5.36 (4.85– 6.71)
P	< 0.0001	< 0.0001	< 0.0001	
Neutrophils (x 10^9/L) (EDTA)	4.5 (3.2– 5.6)	4.0 (3.0– 4.9)	4.0 (3.1– 5.0)	3.00 (2.54– 3.46)
P	< 0.0001	< 0.0001	< 0.0001	
Monocytes (x 10^9/L) (EDTA)	0.62 (0.48– 0.75)	0.58 (0.46– 0.71)	0.58 (0.48– 0.75)	0.45 (0.37– 0.56)
P	< 0.0001	< 0.0001	< 0.0001	
Lymphocytes (x 10^9/L) (EDTA)	2.0 (1.5– 2.5)	1.90 (1.4– 2.4)	1.90 (1.5– 2.3)	1.96 (1.53– 2.35)
P	0.55	0.79	0.74	
Eosinophils (x 10^9/L) (EDTA)	0.15 (0.09– 0.25)	0.17 (0.11– 0.24)	0.16 (0.09– 0.24)	0.14 (0.07– 0.22)
P	0.33	0.19	0.37	
MCH (fl) (EDTA)	30.6 (29.5– 31.7)	30.5 (29.5– 31.7)	30.7 (29.5– 31.5)	30.8 (29.7– 32.3)
P	0.20	0.11	0.21	
MCV (pg) (EDTA)	90.0 (86.7– 91.9)	89.7 (86.5– 91.7)	90.3 (87.6– 92.6)	90.0 (87.8– 93.9)
P	0.64	0.37	0.81	
HCT (L/L) (EDTA)	0.41 (±0.04)	0.40 (±0.04)	0.41 (±0.04)	0.41 (±0.03)
P	0.77	0.14	0.45	

patients overall and controls at any time point (Table VI). Total white cell count, neutrophil and monocyte counts were higher in CVD patients than in controls at each time point (P < .0001, Table VI).

Correlation Analysis

There was no correlation between the platelet count in either EDTA- or citrate-anticoagulated blood and the %RP on flow cytometry in CVD patients at baseline, 14d or 90d ($P \geq 0.08$). There was no significant correlation between the MPV ($r \leq 0.312$, $P \geq 0.138$) or the PDW ($r \leq 0.244$, $P \geq 0.123$) in citrate or EDTA and the % RP in our control group. However, there was a significant positive correlation between the MPV in citrate and EDTA and the %RP in citrate at baseline ($r \geq 0.29$, $P < .001$), 14d ($r \geq 0.28$, $P < .001$) and 90d ($r \geq 0.3$, $P < .001$) in all CVD patients (supplemental figures IIa, IIb, IIc). There was also a significant positive correlation between the PDW in citrate and EDTA and the %RP in citrate at baseline ($r \geq 0.26$, $P < .001$), 14d ($r \geq 0.22$, $P < .002$) and 90d ($r \geq 0.23$, $P \leq 0.002$) in all CVD patients (supplemental figures IIIa, IIIB, IIIc).

Discussion

To our knowledge, this is the largest case-control study to prospectively assess the % circulating reticulated platelets using whole blood flow cytometry in a highly-phenotyped CVD population in the early, subacute and late phases after TIA/ischemic stroke onset in patients who were starting or changing antiplatelet therapy. The %RP was not significantly increased in the early or subacute phases, but was significantly increased in the late phase after symptom onset in our overall TIA/ischemic stroke population compared with controls. These results are consistent with the %RP data from patients in the late phase after TIA/ischemic stroke after adjustment for age in one prior study [21], but do not confirm the findings of a smaller pilot, case-control study using fixed whole blood which reported an increased %RP in the more acute phase (≤ 3 days) after an ischemic stroke vs controls (N = 18 vs. N = 11) [19]. The fact that our CVD patients were initially studied later, at a median of 8 days after TIA/ischemic stroke onset, and that we did not include demographic and vascular risk factor-matched controls might explain some of the differences between the findings in our study and those of Smith *et al*. [19]. However, Smith *et al*. fixed the samples before the addition of TO, they appear to have used a higher concentration of TO (800 μl) which may well have contributed to non-specific labeling of platelet dense granules and possibly mitochondrial DNA, and they did not assess any patients in the late phase after symptom onset [19].

Prior studies have shown enhanced platelet activation in TIA/stroke patients in the early [32–35] and late [32–34,36] phases following symptom onset. Our data could potentially be interpreted as suggesting that increased platelet production/turnover occurs following symptom onset and in response to the ischemic cerebrovascular event, rather than prior to the onset of TIA/ischemic stroke overall. However, further adequately-sized studies in the early, subacute and late phases after TIA/ischemic stroke are required to test this hypothesis because circulating reticulated platelets have been shown to be increased in certain patients with underlying vascular disease who also have increased platelet activation. For example, the %RP has been shown to be elevated in patients with essential thrombocytosis [37], following renal transplantation [18], the metabolic syndrome [17], peripheral vascular disease [38] or coronary artery disease [18], but not in patients with hypercholesterolemia [39] or the antiphospholipid syndrome [40]. These studies imply that particular risk factors may lead to elevated reticulated platelet formation, whereas others may not. Furthermore, subgroup analysis according to TOAST subtypes revealed that patients with TIA/ischemic stroke due to small vessel disease (SVD) had a significantly higher %RP at baseline and 90d *vs*. controls, but the differences between CVD patients with SVD at 14d and controls did not reach statistical significance (P = .06). The differences in %RP between the other CVD TOAST subtypes and controls were not statistically significant. Because the bulk of patients who had a TIA or ischemic stroke of "known etiology" had underlying SVD, with relatively small numbers of patients in the other subgroups, we acknowledge that the 14d data analysis in this SVD subgroup and the other subgroup analyses could well have been subject to a type II error. In contrast, as alluded to above, the %RP has been reported to be increased in cardio-embolic stroke patients *vs*. "neurological controls", and subgroup analysis from that small pilot study indicated that the %RP in fixed, washed platelet preparations from PRP was increased in the acute (< 7 days, N = 4) compared with the later phase (≥ 31 days, N = 10) after a cardio-embolic stroke [20]. However, the same patients were not assessed at each time point and the number of patients included in the subgroup analysis in that study was very limited. A recent study by members of our group showed that the % reticulated platelet fraction (%RPF), quantified on an automated haematology assay, was higher in the early and late phases after TIA or stroke in patients with recently symptomatic compared with asymptomatic 50–99% carotid stenosis [22]. These data also raised the possibility that there may be an underlying shift toward increased platelet production/turnover in patients with carotid stenosis who become symptomatic and which persists over time [22]. Because only 6% of patients in the combined dataset from the TRAP and OATS studies had TIA or ischemic stroke due to large artery atherosclerosis (Table II), this study was not powered to detect such differences. Further case-control studies with careful phenotyping according to the TOAST and ASCOD [41] classification systems are warranted to quantify reticulated platelets with both flow cytometric and automated analyses in different CVD subgroups.

One should not interpret our *post hoc* data analysis as suggesting that the %RP is only increased following TIA and not after ischemic stroke because the %RP was similar in both TIA and stroke subgroups at each time point. Because only 23% of patients had an ischemic stroke prior to enrollment in the TRAP and OATS studies, these findings most likely reflect a type II error in the comparative analysis of data between the stroke subgroup and controls,

The MPV refers to the average volume or size of circulating platelets in a sample. In normal circumstances, there is an inverse relationship between theplatelet count and MPV that seems to maintain a remarkably tight control on the total platelet mass between individuals [31]. Platelet distribution width refers to the distribution of platelet volume or size in a given blood sample, which provides a measure of platelet anisocytosis. A low PDW indicates that most platelets are the same size, whereas a high PDW indicates that there is a large variation in platelet size within a blood sample. We did not identify an increase in the mean MPV or median PDW in CVD patients *vs*. controls in either EDTA-or citrate-anticoagulated blood. These MPV findings are similar to some prior studies [19,21], but not in agreement with others [42,43]. However, in keeping with prior data, our flow cytometric staining protocol and our correlation analysis did confirm that reticulated platelets are larger than more mature platelets in CVD patients [21], with a highly significant weak positive correlation between both the MPV and PDW and the %RP at baseline, 14d and 90d (P ≤ 0.002).

Platelet counts were not significantly increased in CVD patients at any time point compared with controls, as noted previously [19,21]. These simultaneously-collected data on the %RP, platelet counts and MPV indicate that there is an ongoing stimulus to the formation of larger reticulated platelets, especially in the late phase after TIA or stroke onset in our overall cohort, even though the total platelet count remains stable. This area of platelet biomarker research deserves further study because larger platelets have been reported to be "more reactive", aggregate more rapidly in response to collagen, synthesize more thromboxane B_2, release more granules and serotonin, and express more adhesive surface receptors than smaller platelets [17,22].

The median total white cell count, neutrophil and monocyte counts were higher at baseline, 14d and at 90d in CVD patients than in controls in the absence of any active infection or other "inflammatory diathesis" aside from their recent TIA or ischemic stroke. The duration of the elevated neutrophil and monocyte counts was much longer than the 3 days reported in one prior study [44], but fully in keeping with prior case-control data from a smaller group of CVD patients who were tested in the early and late phases after TIA or stroke onset [21]. These findings indicate that there also appears to be an ongoing stimulus to the release of certain leucocyte subsets

in CVD patients compared with controls which may even predate the onset of their TIA or stroke, but this study was not specifically designed to test that hypothesis. One must stress that the median values for the total white cell count, neutrophil and monocyte counts were still within the overall normal ranges for our laboratory (normal ranges for total white cell count: 4– 11 x 10^9/L; neutrophil counts: 4–7.5 x 10^9/L; monocyte counts: 0.2– 0.8 x 10^9/L). Further prospective studies in healthy controls would be required to determine whether controls with levels of circulating neutrophils or monocytes above a specific threshold have a higher risk of developing a subsequent TIA/stroke than those with lower levels of these leucocyte subsets.

This study had some potential **limitations**. Although one must accept that there is no gold standard method of quantifying the %RP on flow cytometry, we used a low concentration of thiazole orange which specifically stains RNA in reticulated platelets and is considered to be a strength of this study [45–-45–47]. Recent data from our group in patients with symptomatic compared with asymptomatic moderate-severe carotid stenosis have shown that an automated assay on the Sysmex XE-2100® haematology analyzer may be more sensitive than the whole blood flow cytometry assay on our Beckman Coulter flow cytometer at detecting differences in the %RPF between groups [22]. Because the automated assay to quantify the % RPF was not available in our lab at the time of the TRAP study, we could not comment further on the relative sensitivities of the 2 assays in this combined case-control dataset from the TRAP and OATS studies. We did not assess all patients in the hyperacute phase after TIA or stroke onset, with a median time interval from TIA or stroke onset to study inclusion of 8 days (minimum-maximum range: 0–28 days), so we might have missed changes in the %RP very early after TIA or ischemic stroke overall. However, this longitudinal study was performed at 3 timepoints, and provides a very comprehensive analysis of the profile of RPs in CVD patients during follow-up. Because only two patients in the subgroup who changed from aspirin to clopidogrel had recurrent TIAs during follow up, we cannot draw any conclusions from these data regarding the potential value of the % reticulated platelets in predicting outcomes following TIA or ischemic stroke; this would need to be addressed in larger studies with longer-follow up. Our research study might have been subject to some selection bias because the TRAP and OATS studies were initially designed to only recruit CVD patients who could undergo comprehensive longitudinal clinical and laboratory assessment **before and after** starting or changing their antiplatelet regimen. Therefore, we could not include patients who had already been started on their treating physicians' antiplatelet regimen of choice e.g. late at night or at weekends, but this is a limitation commonly encountered in many translational research studies. This case-control analysis of the TRAP-OATS dataset was not designed to look at the impact of commencing or changing to a specific antiplatelet regimen on the %RP expression which is the subject of an ongoing longitudinal study by our research group. There was a high percentage of patients in this study with TIA or stroke of undetermined aetiology (62%). This is likely to be multifactorial, including the exclusion of large artery atherosclerotic patients who needed urgent carotid interventional treatment from the TRAP study and this phase of the OATS study, our comprehensive neurovascular work-up which revealed potential competing etiologies, and our relatively young patient population which often falls into this TOAST subgroup. Furthermore, the majority had a clinical diagnosis of TIA (76%), which can make establishment of the aetiology of the symptoms more difficult if there are no specific acute changes on neurovascular imaging. However, the large, heterogenous population of both TIA and stroke patients ensured that this is a clinically representative study of CVD patients overall.

Conclusions

The percentage of circulating reticulated platelets was increased in the late phase (> 90 days), but not significantly increased in the early phase (≤ 4 weeks) after a TIA or ischemic stroke overall compared with healthy controls in this study. However, higher levels of this important subpopulation of larger, recently-released reticulated platelets were observed in the absence of significant elevations in the total platelet count in patients in the early and late phases after lacunar TIA or ischemic stroke. Further studies are warranted to enhance our understanding of the profile of reticulated platelets in different TIA/ischemic stroke subtypes and their potential impact on the *ex vivo* response to commonly-prescribed antiplatelet therapy in CVD patients.

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Declaration of Interest

The authors have no conflicts of interest to declare.

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