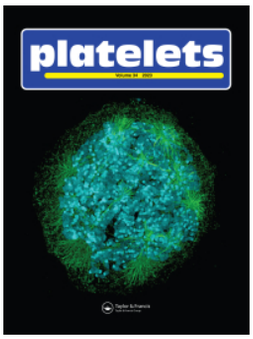

Platelets

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iplt20

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To cite this article: Valentine Robaux, Shakeel Kautbally, Audrey Ginion, Mélanie Dechamps, Sibille Lejeune, Nassiba Menghoum, Luc Bertrand, Anne-Catherine Pouleur, Sandrine Horman & Christophe Beauloye (2023) Dual antiplatelet therapy is associated with high α-tubulin acetylation in circulating platelets from coronary artery disease patients, Platelets, 34:1, 2250002, DOI: 10.1080/09537104.2023.2250002

To link to this article: https://doi.org/10.1080/09537104.2023.2250002

Published online: 12 Sep 2023.

platelets

http://www.tandfonline.com/iplt
ISSN: 0953-7104 (print), 1369-1635 (electronic)

Platelets, 2023; 34(1): 2250002

DOI: https://doi.org/10.1080/09537104.2023.2250002

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RESEARCH ARTICLE

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Dual antiplatelet therapy is associated with high α-tubulin acetylation in circulating platelets from coronary artery disease patients

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Abstract

Platelet inhibition is the main treatment strategy to prevent atherothrombotic complications after acute coronary syndrome or percutaneous coronary intervention. Despite dual antiplatelet therapy (DAPT) combining aspirin and a $P2Y_{12}$ receptor inhibitor, high on-treatment platelet reactivity (HPR) persists in some patients due to poor response to treatment and is associated with ischemic risk. Tubulin acetylation has been pointed out as a hallmark of stable microtubules responsible for the discoid shape of resting platelets. However, the impact of antiplatelet treatments on this post-translational modification has never been studied. This study investigated whether tubulin acetylation differs according to antiplatelet therapy and on-treatment platelet reactivity. Platelets were isolated from arterial blood samples of 240 patients admitted for coronary angiography, and levels of α-tubulin acetylation on lysine 40 (α-tubulin K40 acetylation) were assessed by western blot. We show that platelet α-tubulin K40 acetylation was significantly increased in DAPT-treated patients. In addition, the proportion of patients with high levels of α-tubulin K40 acetylation was drastically reduced among DAPT-treated patients with HPR. Multivariate logistic regression confirmed that DAPT resulting in adequate platelet inhibition was strongly associated with elevated α-tubulin K40 acetylation. In conclusion, our study highlights the role of elevated platelet α-tubulin K40 acetylation as a marker of platelet inhibition in response to DAPT.

Clinical trial registration: https://clinicaltrials.gov - NCT03034148.

Keywords

Antiplatelet monitoring, antiplatelet therapy, aspirin, cardiovascular events, clopidogrel, α-tubulin acetylation

History

Received 7 March 2023
Revised 20 July 2023
Accepted 14 August 2023

Plain Language Summary

What is the context?

- High on-treatment platelet reactivity due to dual antiplatelet therapy poor response is associated with thrombotic risk.
- Acetylation of α-tubulin K40 plays a crucial role in regulating platelet shape.
- High α-tubulin K40 acetylation is a hallmark of stable microtubules.

What is new?

- α-tubulin K40 acetylation is increased in platelets from dual antiplatelet therapy-treated patients.
- High platelet α-tubulin K40 acetylation is mainly observed in clopidogrel-responsive patients.

What is the impact?

- Elevated acetylated K40 α-tubulin could be used as a readout of adequate platelet inhibition in response to dual antiplatelet therapy.
- High α-tubulin K40 acetylation could contribute to maintaining the resting morphology of circulating platelets and therefore modify their capacity to be involved in thrombotic events.

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Introduction

In the context of coronary artery disease (CAD), effective preventive care requires the identification of patients who are at increased risk of atherothrombotic complications. Numerous studies have linked increased ischemic risk to elevated circulating activated platelets, as well as enhanced platelet reactivity. Several platelet activation markers have been found in high-risk CAD patients, including leucocyte-platelet aggregates, P-selectin expression and, more recently, platelet-derived microvesicles.[1–3] Thrombotic events have also been associated with increased reticulated platelets, known to be more reactive than mature platelets.[4] In addition to platelet activation and reactivity, our previous findings identified altered platelet lipid metabolism in high-risk patients. The ACCTHEROMA study revealed a clear relationship between platelet adenosine monophosphate-activated protein kinase (AMPK)-dependent acetyl-coenzyme A (CoA) carboxylase (ACC) phosphorylation on serine 79 and acute coronary events in CAD patients.[5] The AMPK-ACC signaling influences platelet reactivity and thrombus formation by regulating endogenous lipid content.[6] In addition to affecting lipid synthesis, ACC inhibition results in increased platelet α-tubulin K40 acetylation and impaired platelet aggregation.[7]

The tubulin cytoskeleton plays a key role in promoting the shape change of activated platelets, but also in preserving the resting morphology of circulating platelets. The discoid shape of quiescent platelets is maintained by a circumferential bundle of microtubules known as the marginal band.[8] Under resting conditions, these microtubules exhibit high acetylation of α-tubulin K40, which is a hallmark of stable microtubules.[9] Upon platelet activation, circumferential microtubules collapse and depolymerize, supporting the transition to a spherical shape. This cytoskeletal reorganization is associated with massive α-tubulin K40 deacetylation mediated by the histone deacetylase 6 (HDAC6).[10,11] Whether circulating platelets in high-risk patients display modifications of α-tubulin K40 (de)acetylation that could influence their reactivity and prothrombotic potential remains unknown.

Platelet inhibition is the main treatment strategy in the prevention of atherothrombosis. High-risk patients experiencing acute coronary syndrome (ACS) or undergoing percutaneous coronary intervention (PCI) are routinely treated with dual antiplatelet therapy (DAPT) consisting of aspirin and a $P2Y_{12}$ receptor inhibitor (namely clopidogrel, ticagrelor, or prasugrel). Despite antiplatelet medications, especially clopidogrel, high on-treatment platelet reactivity (HPR) can persist due to poor response to treatment. Clopidogrel responsiveness is subject to wide interindividual variability, with up to one-third of patients not achieving adequate platelet inhibition.[12] HPR resulting from poor response to antiplatelet therapy is associated with an increased risk of adverse clinical events including death, myocardial infarction (MI), and stent thrombosis.[13]

The present study reveals that high platelet α-tubulin K40 acetylation is mainly observed in clopidogrel-responsive patients and could be used as a readout of adequate platelet inhibition in response to dual antiplatelet therapy.

Methods

Study design

Discovery cohort

From February to October 2019, 53 patients admitted for coronary angiography were included and made up the discovery cohort. Patients who underwent a coronary angiography were prospectively screened regardless of the angiography's indication. The major exclusion criteria were coagulopathy, anticoagulation therapy, atrial fibrillation, abnormal platelet count, chronic inflammation, and neoplasia, because they may affect platelet function. Based on indication and coronary angiography imaging analyzed by two experienced cardiologists, patients were classified into four groups according to CAD severity and/or clinical presentation. Patients undergoing angiography for chest pain or valvular disease investigation with normal coronary vessels were classified as non-CAD (N-CAD). Patients exhibiting at least one plaque with <50% luminal stenosis were classified as nonsignificant CAD (NS-CAD). Patients showing stable disease with significant stenosis (>50%) were classified as stable CAD (S-CAD). Finally, ACS included unstable angina, non-ST-segment elevation MI, and ST-segment elevation MI.

Confirmation cohort

The ACCTHEROMA (prospective evaluation of acetyl-CoA carboxylase phosphorylation state in platelets as a marker of atherothrombotic coronary and extra-coronary artery disease) study (NCT03034148)[5] enrolled 188 patients between March 2015 and February 2016, as described above. This cohort was used to confirm the results obtained in the discovery cohort and was therefore considered as the confirmation cohort in this paper. The latter comprised 187 out of the 188 patients from the ACCTHEROMA study due to one failed platelet sample. Platelet reactivity to adenosine diphosphate (ADP) was assessed using the Multiplate analyzer (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) to characterize clopidogrel response in the confirmation cohort. HPR was defined as a multiplate ADP value greater than 468 arbitrary units (AU) *min (or 46 U) despite $P2Y_{12}$ inhibitor therapy. This cutoff value was based on consensus recommendation determined by large prospective studies.[14–16]

The study was approved by the institutional ethics committee (2015/08JAN/010) and complied with the Declaration of Helsinki, as well as with the good clinical practice guidelines. All participants provided written informed consent.

Patients were followed prospectively by ambulatory visits and phone calls at 6-months intervals. Clinical and survival status was obtained at follow-up visits and by phone contact with patients, their relatives, or their physician if necessary. The endpoint was defined as major cardiovascular event (MACE), a composite of myocardial infarction, stroke and cardiovascular mortality, as defined in randomized controlled trials.[17]

Blood sampling and α-tubulin K40 acetylation analysis

Blood sampling, platelet isolation, protein extraction, and analysis were carried out as previously described.[5] Briefly, blood samples drawn from the arterial sheath were collected in citrated tubes before any drug administration in the catheterization laboratory, including heparin. All samples were immediately processed for platelet isolation. Platelets were lysed in Laemmli buffer before α-tubulin K40 acetylation, total α-tubulin and gelsolin analysis by western blotting. A standard α-tubulin K40 acetylation positive control was prepared with washed platelets isolated from a healthy volunteer and incubated with tubacin (10 μM) for 1 hour. This standard positive control was used for all the western blots, placed twice on each gel to validate the signal reproducibility. For each patient, band intensities were normalized to corresponding loading controls (gelsolin) on the same gel. The relative acetylated K40 α-tubulin value was normalized by comparison with the standard positive control.

Statistical analysis

Statistical analyses were conducted using SPSS version 27 (IBM Corp., Armonk, NY) and R Version 4.2.2 software (http://www.r-project.org). Categorical variables were expressed as counts (percentages) and continuous variables as mean ±1 standard deviation (SD) if normally distributed, or as median (25^{th}–75^{th} percentiles) if not normally distributed. Glycemia, creatinine, high-sensitivity C-reactive protein (hsCRP), D-dimers, triglycerides (TG), and the ratio of TG to high-density lipoprotein cholesterol (HDL-C) were normalized by log-10 transformation. The confirmation cohort was divided into three equal groups according to tertiles of α-tubulin K40 acetylation level. Statistical comparisons between groups were performed using unpaired Student's t-test, ANOVA, post-hoc Tukey, or Mann-Whitney U test for continuous variables, and chi-square test for categorical variables.

To determine the independent factors associated with high α-tubulin K40 acetylation, significant ($p < .05$) variables identified by univariate ordinal logistic regression model were included in a backward multivariate logistic regression analysis. To avoid a high degree of collinearity or interaction between potentially related covariates, an inflation factor test (VIF) and an interaction test were used. A VIF of a covariate of less than 4 was defined as being weakly correlated with

Table I. Population baseline characteristics of the discovery and confirmation cohorts.

	DISCOVERY COHORT ($n = 53$)	CONFIRMATION COHORT ($n = 187$)	p-value
Clinical characteristics			
Age (years)	69 ± 11	65 ± 12	.023*
Male	41 (77.4)	130 (69.5)	.27
BMI (kg/m^2)	27.6 ± 5.0	27.6 ± 4.9	.99
Hypertension	38 (71.7)	120 (64.2)	.31
Smoking	28 (52.8)	104 (55.6)	.72
Diabetes	12 (22.6)	42 (22.5)	.98
N-CAD	6 (11.3)	27 (14.4)	.56
NS-CAD	18 (34)	38 (20.3)	.038*
S-CAD	25 (47.2)	66 (35.3)	.12
ACS	4 (7.5)	56 (29.9)	.001*
Valvular disease	20 (37.7)	47 (25.1)	.049*
MI	10 (18.9)	35 (18.7)	.98
PCI	14 (26.4)	47 (25.1)	.85
CABG	3 (5.7)	16 (8.6)	.49
Biological parameters			
Hemoglobin (g/dl)	13.8 ± 1.7	14.0 ± 1.6	.38
Fasting glycemia (mg/dl)	106 (92–123)	100 (92–114)	.56
Creatinine (mg/dl)	1.0 (0.8–1.2)	1.0 (0.8–1.1)	.93
CRI	4 (7.5)	22 (11.8)	.36
GFR (ml/kg/min)	74 ± 20.2	85.8 ± 36	.003*
LDL (mg/dl)	95 ± 38	96 ± 41	.88
Non-HDL (mg/dl)	123 ± 41	120 ± 45	.72
HDL (mg/dl)	54.1 ± 18.6	50.3 ± 15.3	.20
TG (mg/dl)	125 (96–182)	102 (76–151)	.034*
TG/HDL	2.6 (1.6–3.8)	2.0 (1.4–3.6)	.28
hsCRP (mg/l)	NA	1.3 (0.6–3.4)	
Platelet count ($10^3/\mu l$)	236 ± 59	244 ± 61	.37
Multiplate analysis			
ASPI test (AU*min)	NA	513 ± 278	
ADP test (AU*min)	NA	638 ± 218	
TRAP test (AU*min)	NA	1101 ± 255	
D-dimers (ng/ml)	NA	413 (284–686)	
Medications			
ACE inhibitor/ARB	27 (50.9)	86 (46)	.52
Beta-blockers	24 (45.3)	97 (51.9)	.40
Lipid-lowering treatment	32 (60.4)	111 (59.4)	.89
Aspirin	36 (67.9)	141 (75.4)	.28
Dual antiplatelet therapy	6 (11.3)	30 (16)	.40
Clopidogrel	5 (9.4)	15 (8)	.74
Ticagrelor	5 (9.4)	13 (7)	.54
Prasugrel	0 (0)	4 (2.1)	.28

Values are expressed as mean ± SD, n (%), or median (interquartile range). * Statistical significance was set at $p < .05$.

Abbreviations: ADP = adenosine diphosphate; ACE = angiotensin-converting enzyme; ACS = acute coronary syndrome; ARB = angiotensin receptor blocker; ASPI = aspirin channel; AU = arbitrary units; BMI = body mass index; CABG = coronary artery bypass grafting; CRI = chronic renal insufficiency; GFR = glomerular filtration rate; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; MI = myocardial infarction; NA = not applicable; N-CAD = non-coronary artery disease; NS-CAD = non-significant coronary artery disease (<50% luminal stenosis); PCI = percutaneous coronary intervention; S-CAD = significant coronary artery disease (>50% luminal stenosis); TG = triglycerides; TRAP = thrombin receptor activating peptide.

other covariates. Odds ratios (OR) were expressed as means and 95% confidence intervals (CI). Event-free survival of patients was estimated using logrank test and Cox regression analysis. Kaplan-Meier curve based on the highest tertile of α-tubulin K40 acetylation level was used to illustrate event-free survival of patients.

Results

Discovery cohort

Baseline characteristics

The discovery cohort consisted of 53 individuals (69 ± 11 years, 77% males) admitted for coronary angiography (Table I). They were classified into four groups according to CAD severity and/or clinical presentation. Overall, six (11%) had normal coronary arteries (N-CAD), whereas 47 (89%) had CAD, including 43 (81%) with stable CAD (with or without significant stenosis) and four (8%) with ACS. Regarding antiplatelet therapies, 36 (68%) patients were taking aspirin, 10 were treated with a $P2Y_{12}$ inhibitor, and six (11%) were under DAPT.

Increased platelet α-tubulin K40 acetylation in $P2Y_{12}$ inhibitor-treated patients

Circulating platelets from patients under $P2Y_{12}$ inhibitor therapy displayed a significantly increased α-tubulin K40 acetylation compared with those not treated with a $P2Y_{12}$ inhibitor (median [IQR] = 0.27 [0.20–0.33] *vs.* 0.07 [0.04–0.16], $p = .0038$) (Figure 1). This observation was made on an aspirin background for most patients. Indeed, 60% and 57% of patients treated with and without $P2Y_{12}$ inhibitor received aspirin, respectively. Positive control consisted of washed platelets from healthy volunteers treated or untreated with tubacin, a selective inhibitor of HDAC6. Total α-tubulin levels did not vary significantly between patients (Figure 1).

Confirmation cohort

Baseline characteristics

The impact of antiplatelet therapy on platelet α-tubulin K40 acetylation was retrospectively investigated in a larger confirmation cohort including 187 patients. This confirmation population was on average younger than the discovery cohort (65 ± 12 *vs.* 69 ± 11 years old, $p = .02$) and included a lower proportion of males (69.5% *vs.* 77.4%, $p = .27$) (Table I). The distribution across the CAD subgroups significantly differed in the confirmation cohort, with less S-CAD (55.6% *vs.* 81.1%, $p = .001$) and more ACS patients (29.9% *vs.* 7.5%, $p = .001$). Fewer patients underwent angiography for valvular disease (25.1% *vs.* 37.7%, $p = .05$). As in the discovery cohort, most patients ($n = 141$, 75%) were on aspirin background. Only two (1%) patients received $P2Y_{12}$ inhibitor without aspirin. A total of 30 (16%) patients were taking DAPT, including 15 (8%) with clopidogrel, 13 (7%) with ticagrelor, and four (2%) with prasugrel. Among the 30 DAPT-treated patients, 11 had S-CAD and 19 ACS.

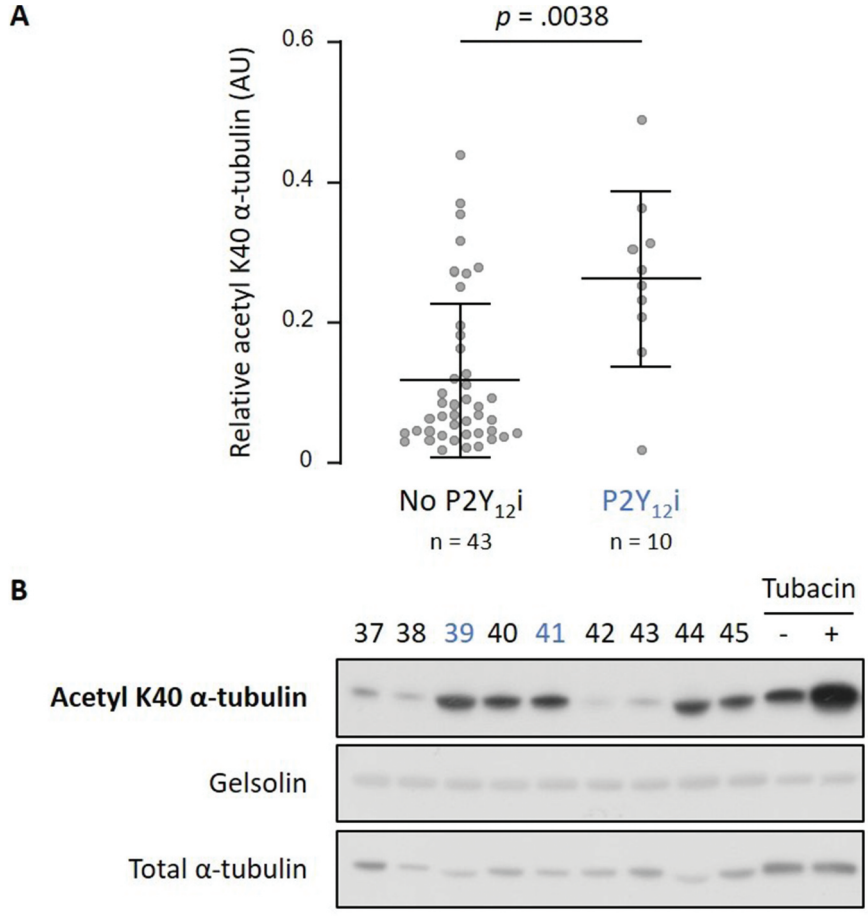

Figure 1. α-tubulin K40 acetylation in platelets from patients of the discovery cohort. (A) Platelet acetylated K40 α-tubulin quantification in patients not treated and treated with a $P2Y_{12}$ inhibitor. Dots and bars represent individual values and means with standard deviations, respectively. *P*-value was

Increased platelet α-tubulin K40 acetylation in P2Y$_{12}$ inhibitor-treated patients

The increase in α-tubulin K40 acetylation in platelets from P2Y$_{12}$ inhibitor-treated patients was confirmed in the confirmation cohort (Figure 2). Indeed, patients taking a P2Y$_{12}$ inhibitor (n = 32, 17%) exhibited higher levels of α-tubulin K40 acetylation than those not treated with a P2Y$_{12}$ inhibitor. The latter group included patients receiving only aspirin (n = 111, 59%) and patients without any antiplatelet therapy (n = 44, 24%) (median [IQR] = 0.15 [0.08–0.25] *vs*. 0.03 [0.01–0.09], $p < .0001$).

Patient characteristics according to platelet α-tubulin K40 acetylation tertiles

Patients of the confirmation cohort were grouped according to α-tubulin K40 acetylation tertiles evaluated by western blot (first tertile < 0.027 AU, second tertile 0.027–0.088 AU, third tertile > 0.088 AU) (Table II). Higher levels of α-tubulin K40 acetylation, compared with low levels, were associated with lower platelet count, less prevalent valvular disease and smoking. More importantly, high platelet α-tubulin K40 acetylation was strongly associated with DAPT treatment combining aspirin and a P2Y$_{12}$ inhibitor (Figure 3A). Consistent with this observation, elevated platelet α-tubulin K40 acetylation was associated with decreased platelet reactivity to arachidonic acid and ADP, as well as the presence of ACS.

On-treatment platelet reactivity and α-tubulin K40 acetylation level

As stated above and illustrated in Figure 3A, DAPT had a significant impact on platelet α-tubulin K40 acetylation levels (chi-square test $p < .001$). Among patients in the lower α-tubulin K40 acetylation tertile, one-third did not take any antiplatelet medication and a minority was on DAPT. In contrast, the highest α-tubulin K40 acetylation tertile included a minority of patients without any antiplatelet therapy and more than one-third of DAPT-treated patients. The patient proportion taking aspirin monotherapy was roughly the same across tertiles.

More interestingly, platelet response to P2Y$_{12}$ inhibitor assessed by ADP multiplate dramatically influenced platelet α-tubulin K40 acetylation (chi-square test $p < .001$) (Figure 3B). The vast majority (83%) of DAPT responders exhibited high α-tubulin K40 acetylation levels (third tertile). By contrast, only 43% of DAPT poor responders belonged to the high α-tubulin K40 acetylation tertile.

Association between antiplatelet therapy and platelet α-tubulin K40 acetylation

Multivariate logistic regression further supported the association between antiplatelet treatment and platelet α-tubulin K40 acetylation levels (Table III). Taking aspirin increased the likelihood of being in the high α-tubulin K40 acetylation tertile (OR: 3.5; 95% CI [1.2, 10.3]; $p = .024$). DAPT treatment without HPR was strongly associated with higher acetylated K40 α-tubulin levels (OR: 33.2; 95% CI [6.7, 165.9]; $p < .001$). In poor responders, DAPT however barely impacted α-tubulin K40 acetylation levels and was similar to aspirin alone (OR: 5.8; 95% CI [0.9, 38.7]; $p = .07$). In addition to antiplatelet therapies, BMI was also independently associated with high α-tubulin K40 acetylation levels (OR: 0.9; 95% CI [0.8, 1.0]; $p = .013$). Interactions between antiplatelet therapy and CAD subgroups were not statistically significant (CAD subgroups and aspirin, $p = .11$; CAD subgroups and DAPT with HPR, $p = .70$; CAD subgroups and DAPT without HPR,

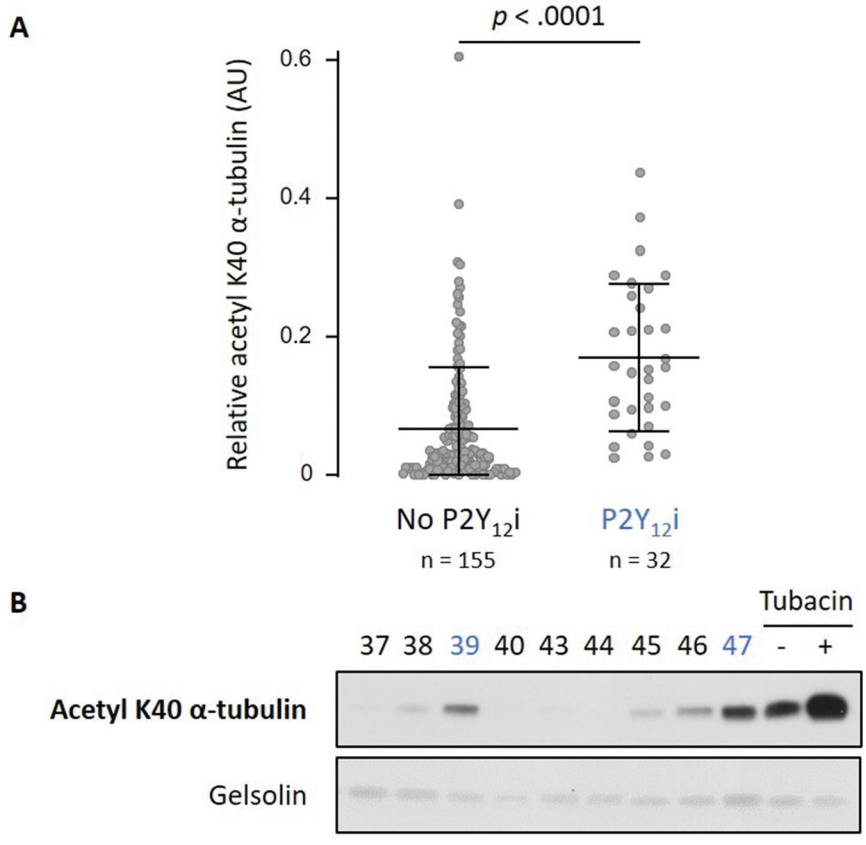

Figure 2. α-tubulin K40 acetylation in platelets from patients of the confirmation cohort. (A) Platelet acetylated K40 α-tubulin quantification in patients not treated and treated with a P2Y$_{12}$ inhibitor. Dots and bars represent individual values and means with standard deviations, respectively. *P*-value was determined using Mann-Whitney U test. (B) Representative western blot of platelet α-tubulin K40 acetylation in nine consecutive patients from the confirmation cohort. Gelsolin was used as a loading control to quantify relative acetylated K40 α-tubulin levels. Standards correspond to washed platelets from a healthy volunteer treated or not with tubacin (10 µM for 1 hour).

Abbreviation: AU = arbitrary units.

Table II. Baseline characteristics according to platelet α-tubulin K40 acetylation tertiles in the confirmation cohort.

	Platelet α-tubulin K40 acetylation tertiles			
	1st tertile (<0.027) (n = 62)	2nd tertile (0.027–0.088) (n = 63)	3rd tertile (>0.088) (n = 62)	p-value
Clinical characteristics				
Age (years)	67 ± 13	65 ± 11	64 ± 13	.24
Male	40 (64.5)	46 (73)	44 (71)	.56
BMI (kg/m^2)	28.4 ± 5.1	28 ± 4.4	26.5 ± 4.9	.081
Hypertension	41 (66.1)	41 (65.1)	38 (61.3)	.84
Smoking	25 (40.3)	37 (58.7)	42 (67.7)*	.007
Diabetes	9 (14.5)	16 (25.4)	17 (27.4)	.18
N-CAD	10 (16.1)	13 (20.6)	4 (6.5)	.071
NS-CAD	22 (35.5)	7 (11.1)	9 (14.5)*	.001
S-CAD	17 (27.4)	28 (44.4)	21 (33.9)	.13
ACS	13 (21)	15 (23.8)	28 (45.2)*†	.006
Valvular disease	22 (35.5)	15 (23.8)	7 (11.3)*	.006
MI	11 (17.7)	8 (12.7)	16 (25.8)	.17
PCI	11 (17.7)	18 (28.6)	18 (29)	.26
CABG	1 (1.6)	7 (11.1)	8 (12.9)	.054
Biological parameters				
Hemoglobin (g/dl)	14.2 ± 1.7	14.2 ± 1.5	13.8 ± 1.5	.16
Fasting glycemia (mg/dl)	100 (91–113)	102 (94–114)	99 (92–114)	.59
Creatinine (mg/dl)	0.9 (0.8–1.1)	1 (0.8–1.2)	0.9 (0.8–1.0)	.65
CRI	6 (9.7)	9 (14.3)	7 (11.3)	.74
GFR (ml/kg/min)	87.5 ± 40.2	83.4 ± 31	86.6 ± 36.6	.89
LDL (mg/dl)	93 ± 40	96 ± 43	100 ± 40	.39
Non-HDL (mg/dl)	117 ± 44	121 ± 47	124 ± 43	.43
HDL (mg/dl)	52.8 ± 17.5	48.6 ± 12.6	49.5 ± 15.3	.23
TG (mg/dl)	103 (78–141)	99 (75–159)	102 (82–148)	.64
TG/HDL	1.9 (1.4–3.3)	2.0 (1.5–4.2)	2.2 (1.5–3.5)	.42
hsCRP (mg/l)	1.2 (0.6–2.7)	1.9 (0.6–3.7)	1.0 (0.5–2.9)	.99
Platelet count (10^3/µl)	263 ± 57	234 ± 53	236 ± 68*	.011
Multiplate analysis				
ASPI test (AU*min)	622 ± 235	513 ± 329	405 ± 214*	<.001
ADP test (AU*min)	682 ± 143	682 ± 208	550 ± 261*†	<.001
TRAP test (AU*min)	1105 ± 224	1105 ± 263	1194 ± 280	.81
D-dimers (ng/ml)	413 (277–697)	401 (288–582)	419 (270–726)	.09
Medications				
ACE inhibitor/ARB	27 (43.5)	34 (54)	25 (40.3)	.28
Beta-blockers	36 (58.1)	30 (47.6)	31 (50)	.47
Lipid-lowering treatment	32 (51.6)	43 (68.3)	36 (58.1)	.16
Aspirin	40 (64.5)	45 (71.4)	56 (90.3)*†	.003
Dual antiplatelet therapy	1 (1.6)	7 (11.1)	22 (35.5)*†	<.001
Clopidogrel	2 (3.2)	3 (4.8)	10 (16.1)	.015
Ticagrelor	0 (0)	3 (4.8)	10 (16.1)*	.001
Prasugrel	0 (0)	1 (1.6)	3 (4.8)	.17

Values are expressed as mean ± SD, n (%), or median (interquartile range). P-values were derived from ANOVA, chi-square or Tukey test as appropriate. * $p < .05$ 3rd tertile *vs.* 1st tertile; † $p < .05$ 3rd tertile *vs.* 2nd tertile.

Abbreviations: ADP = adenosine diphosphate; ACE = angiotensin-converting enzyme; ACS = acute coronary syndrome; ARB = angiotensin receptor blocker; ASPI = aspirin channel; AU = arbitrary units; BMI = body mass index; CABG = coronary artery bypass grafting; CRI = chronic renal insufficiency; GFR = glomerular filtration rate; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; MI = myocardial infarction; N-CAD = non-coronary artery disease; NS-CAD = non-significant coronary artery disease (<50% luminal stenosis); PCI = percutaneous coronary intervention; S-CAD = significant coronary artery disease (>50% luminal stenosis); TG = triglycerides; TRAP = thrombin receptor activating peptide.

$p = .87$). In addition, there was no collinearity between categories of antiplatelet therapy (VIF = 1.24) and CAD subgroups (VIF = 1.18). During a median follow-up of 6.1 years (4.4–6.2 years), 44 patients died (24%) and 17 patients died of cardiovascular causes (17%). A total of 17 patients had a myocardial infarction (9%) and 11 patients a stroke (5%). A total of 37 patients (20%) achieved the MACE composite endpoint (myocardial infarction, stroke and cardiovascular mortality). Figure 4 shows the Kaplan-Meier curve for MACE as a function of the highest tertile of platelet α-tubulin K40 acetylation, demonstrating that patients with the highest tertile of platelet α-tubulin K40 acetylation did not have worse clinical outcome. However, ACS adjusted for the level of platelet α-tubulin K40 acetylation, was independently associated with MACE (Hazard ratio: 1.77 CI [1.3–25.4]; $p = .019$). The onset of MACE is explained more by the initial clinical presentation (ACS) than by the level of acetylation of platelet α-tubulin K40.

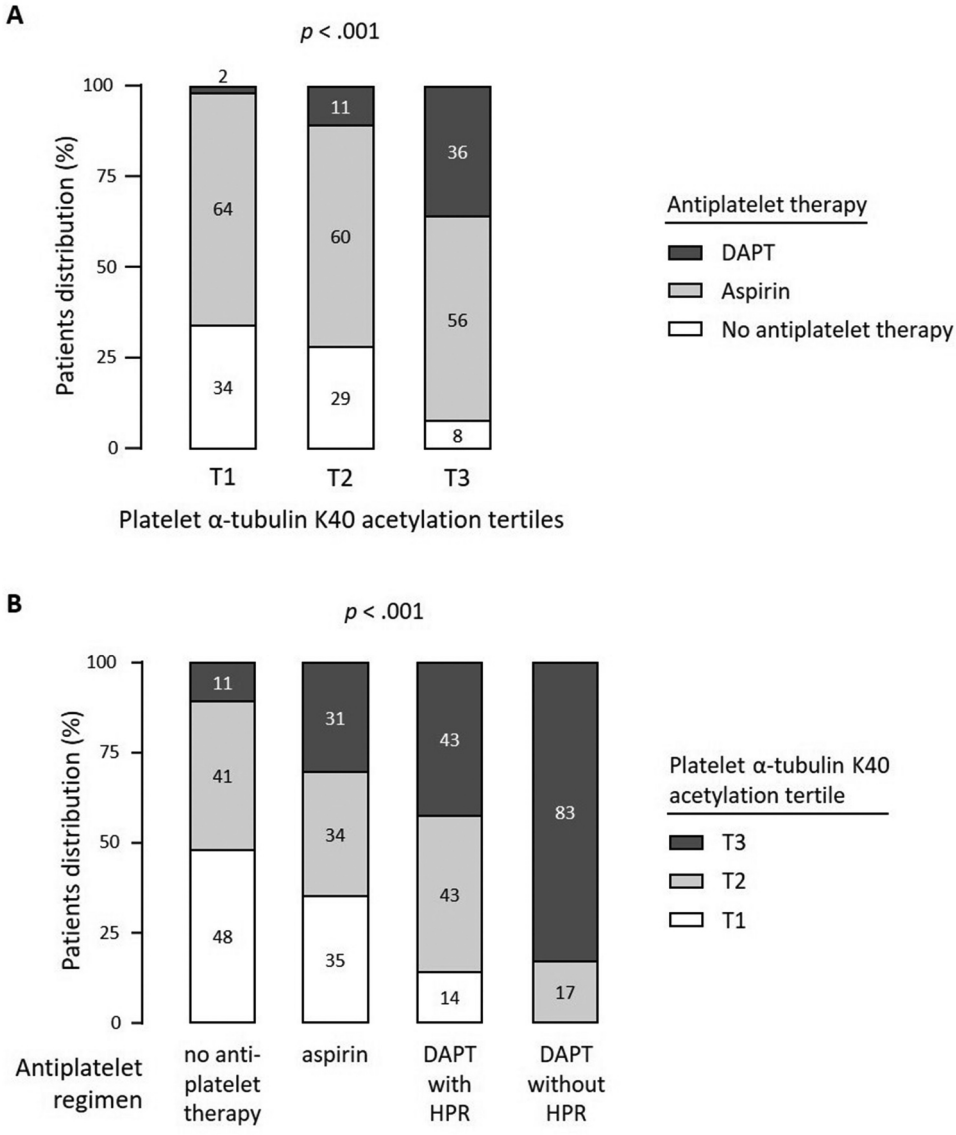

Figure 3. Association between antiplatelet therapy and platelet α-tubulin K40 acetylation in patients from the confirmation cohort. (A) Antiplatelet therapies across platelet α-tubulin K40 acetylation tertiles. First tertile (T1) included 61 patients, second tertile (T2) 63 patients and third tertile (T3) 61 patients. (B) Distribution of platelet α-tubulin K40 acetylation tertiles depending on antiplatelet regimen. Subgroups included 44 patients without any antiplatelet therapy, 111 patients on aspirin alone, 7 patients on DAPT with HPR and 23 patients without HPR. The statistical differences between the groups were determined using the chi-square test.

Abbreviations: DAPT = dual antiplatelet therapy; HPR = high on-treatment platelet reactivity.

Discussion

In the present study, we highlighted an association between antiplatelet therapy and platelet α-tubulin K40 acetylation. Indeed, circulating platelets from DAPT-treated patients exhibited higher acetylated K40 α-tubulin levels, a post-translational modification characterizing stable microtubules. More interestingly, DAPT-treated patients with HPR, *i.e.*, clopidogrel non-responders, were less likely to have increased α-tubulin K40 acetylation levels compared to responders.

Elevated acetylated K40 α-tubulin could therefore be used as a readout of adequate platelet inhibition in response to DAPT, and more particularly to clopidogrel, allowing discriminating between clopidogrel responders and non-responders. α-tubulin K40 acetylation has been evaluated in circulating platelets in the absence of any *ex-vivo* stimulation. This differs from the current tests monitoring platelet reactivity in response to $P2Y_{12}$ inhibitor, which require prior ADP stimulation before measuring either platelet aggregation (Multiplate ADP and VerifyNow $P2Y_{12}$) or vasodilator-stimulated phosphoprotein (VASP) phosphorylation (with an additional incubation with prostaglandin E1). Although platelet function testing (PFT) is helpful in identifying high-risk patients, numerous clinical trials failed to demonstrate any benefit in tailoring antiplatelet therapy.[13,18–21] This limitation of PFT might be due to the tests' restrictive nature to assess the *ex-vivo* platelet response to a single agonist. Here, we have identified a potential biomarker, *i.e.*, platelet acetylated K40 α-tubulin, which reflects DAPT response considering patients' environment and comorbidities. The major limitation is that, at this stage, platelets must be purified before measuring acetylated K40 α-tubulin levels. A simplified test will need to be developed.

Apart from the close relationship between antiplatelet therapy and platelet α-tubulin K40 acetylation, BMI was also an independent factor associated with α-tubulin K40 acetylation change. High BMI has been associated with a reduction in acetylated K40 α-tubulin, which corroborates the results showing enhanced

Table III. Univariate and multivariate analysis of factors associated with high platelet α-tubulin K40 acetylation in patients of the confirmation cohort.

	Univariate		Multivariate	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age	0.99 [0.96, 1.01]	.26		
Gender	1.10 [0.56, 2.14]	.79		
BMI	0.93 [0.86, 0.99]	.026*	0.90 [0.83, 0.98]	.013*
Diabetes	1.41 [0.69, 2.89]	.36		
Smoking	2.28 [1.20, 4.36]	.011*		
Framingham risk score	0.35 [0.02, 6.59]	.35		
hsCRP	0.93 [0.54, 1.62]	.80		
Hemoglobin	0.84 [0.68, 1.02]	.07		
eGFR	1.00 [0.99, 1.01]	.77		
CAD severity	1.77 [1.26, 2.48]	<.001*		
Antiplatelet therapy				
None (reference)				
Aspirin	3.44 [1.25, 9.50]	.017*	3.49 [1.18, 10.31]	.024*
DAPT with HPR	5.85 [1.00, 34.10]	.05	5.78 [0.86, 38.71]	.07
DAPT without HPR	37.05 [8.91, 153.98]	<.001*	33.24 [6.66, 165.88]	<.001*

*Statistical significance was set at $p < .05$.

Abbreviations: BMI = body mass index; CAD = coronary artery disease; CI = confidence interval; DAPT = dual antiplatelet therapy; eGFR = estimated glomerular filtration rate; HPR = high on-treatment platelet reactivity; hsCRP = high-sensitivity C-reactive protein; OR = odds ratio.

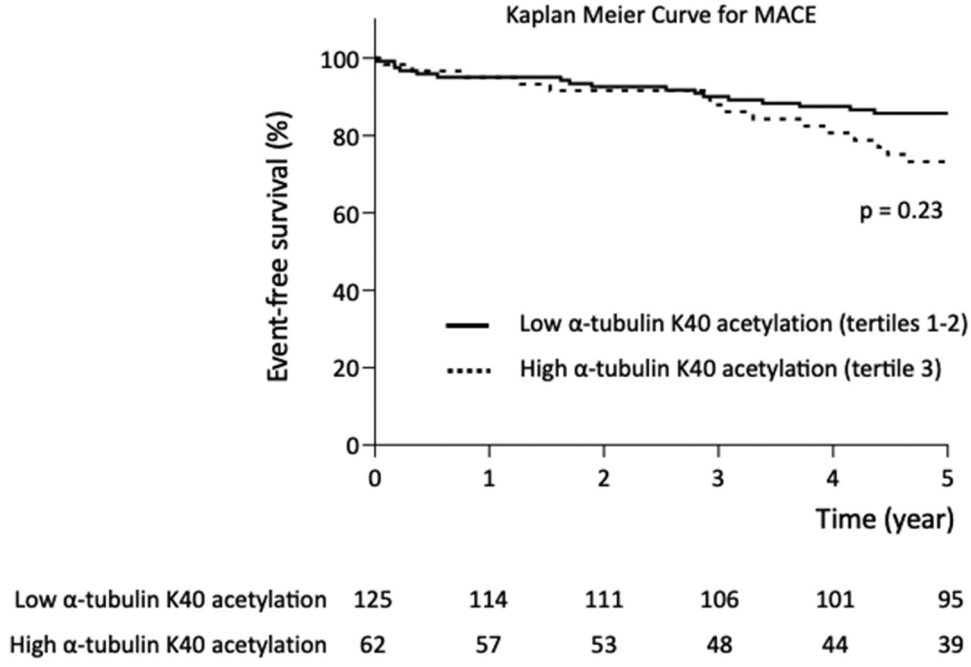

Figure 4. Prognostic impact of high platelet α-tubulin K40 acetylation on event-free survival of patients in the confirmation cohort. Kaplan-Meier curves for the endpoint based on the highest α-tubulin K40 acetylation tertile (3rd tertile) and the lowest tertiles (1st and 2nd tertiles).

Abbreviation: MACE = major adverse cardiovascular events.

platelet activation and reduced platelet response to clopidogrel in obese patients.[22–26] Essentially, α-tubulin K40 acetylation regulates microtubule cytoskeleton dynamics. The latter plays a key role in maintaining the discoid shape of resting platelets, while supporting shape changes during platelet activation.[27] The level of α-tubulin K40 acetylation is dramatically reduced during platelet activation and would result from microtubule depolymerization, as well as from the subsequent exposure of intraluminal acetylated sites to HDAC6, promoting deacetylation.[10,11]

In various cellular models, stabilization of microtubules using taxol leads to their acetylation and, conversely, decrease in α-tubulin K40 acetylation after HDAC6 overexpression reduces microtubule stability, suggesting that both phenomena are reciprocally connected.[28,29] Moreover, several studies support that highly acetylated microtubules are more resistant to depolymerizing drugs, including colchicine.[30–33] More recently, α-tubulin K40 acetylation has been found to protect microtubules from mechanical breakage.[34] One may postulate that DAPT-induced high acetylated K40 α-tubulin is associated with an increase in microtubule stability. Several data, including ours, indicated that α-tubulin K40 hyperacetylation following HDAC6 inhibition impairs platelet aggregation in response to CRP and thrombin,[7,10] but without

affecting platelet morphology and spreading *in vitro*.[7,10,11] Therefore, a high basal α-tubulin K40 acetylation level resulting from antiplatelet therapy is expected to modify the circulating platelets' ability to be activated and involved in atherothrombotic complications. However, the impact of high α-tubulin K40 acetylation induced by DAPT on circulating platelets' resting morphology remains to be determined.

Recent trials revealed a benefit of colchicine in the prevention of ischemic events in CAD.[35–38] This protective cardiovascular effect of colchicine can be explained by both its anti-inflammatory and antiplatelet properties. By disrupting tubulin polymerization, colchicine interferes with platelet cytoskeleton rearrangement and impairs platelet aggregation.[39] Furthermore, colchicine impacts platelet aggregation in DAPT non-responders but not in responders.[40] This difference could be explained by the high α-tubulin K40 acetylation highlighted in DAPT responders. Indeed, one may speculate that a higher level of α-tubulin K40 acetylation protects microtubules against colchicine-induced destabilization. According to this assumption, less acetylated microtubules in DAPT non-responders would be more sensitive to colchicine action.

In summary, our study identified high platelet α-tubulin K40 acetylation as an indicator of adequate platelet inhibition in response to DAPT. In addition to providing an additional diagnostic information, this study speculates on the importance of high acetylated K40 α-tubulin in the circulating platelets' resting morphology stabilization in DAPT responders (Figure 5).

Our study has several limitations. Firstly, it includes a small number of DAPT-treated patients ($n = 30$) with HPR ($n = 7$), and without HPR ($n = 23$). Despite this limited number of patients, we were able to demonstrate an association between the type of antiplatelet therapy and the level of tubulin acetylation, adjusted for BMI, smoking and disease severity. Secondly, we have no evidence at this stage that DAPT-induced high α-tubulin acetylation has an impact on platelet morphology. This remains a speculative hypothesis that should be explored in future work.

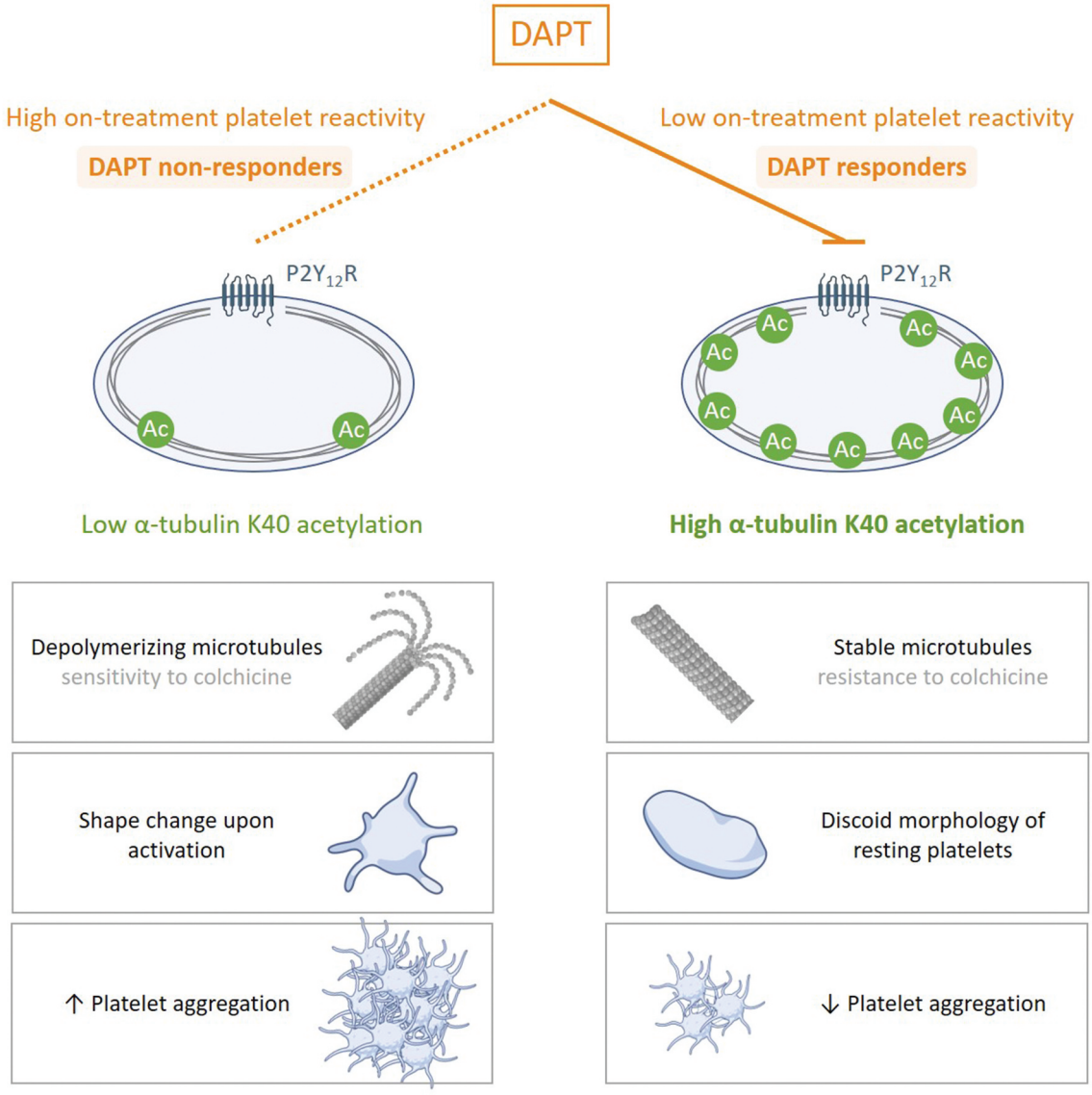

Figure 5. Central figure. Effects of DAPT on platelet α-tubulin K40 acetylation.
Abbreviation: DAPT = dual antiplatelet therapy.

Acknowledgments

The authors wish to thank the clinicians of the coronary angiography department for their support in collecting blood samples.

Author contributions

Conceptualization, V.R., S.H., and C.B.; Analyses, V.R., S.K., S.L., N. M. and A-C.P.; Patients' inclusion, V.R., S.K., A.G and M.D.; Funding acquisition, L.B., S.H., and C.B.; Investigation, V.R. and A.G.; Methodology, V.R., S.K., and A.G.; Project administration, C.B.; Supervision, S.H. and C.B.; Writing-original draft, V.R., S.H., and C. B.; Writing-review & editing, L.B., S.H., and C.B. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No conflict of interest was reported by the author(s).

Funding

This work was supported by grants from the Fonds National de la Recherche Scientifique (FNRS, Belgium, J.0150.18) and the Fondation Louvain, Brussels, Belgium. ACP is a post-doctorate Clinical Master Specialist at FNRS. S.H. works as a Senior Research Associate at FNRS, Belgium.

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