Journal of Thrombosis and Haemostasis

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--Manuscript Draft--

Manuscript Number:	
Full Title:	Evaluating plasma vesicle signatures in chronic coronary artery disease patients for tailored dual therapy with low-dose rivaroxaban and aspirin
Article Type:	JTH Commentary - Invited
Section/Category:	Thrombosis
Keywords:	Aspirin, Rivaroxaban, atherothrombosis, extracellular vesicle, plateletes, inflammation, proteomics
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Invited Commentary to JTHA-D-24-00829R1

Evaluating plasma vesicle signatures in chronic coronary artery disease patients for tailored dual therapy with low-dose rivaroxaban and aspirin

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Most ischemic events in patients with chronic coronary artery disease (CAD), peripheral artery disease (PAD), or cerebrovascular disease result from thrombosis or embolism arising from disrupted atherosclerotic plaques [1,2]. Dual antiplatelet therapy lowers the risk of thromboembolic events in the initial 90 days after an acute ischemic stroke or transient ischemic attack, as well as in the first 12 months after an acute coronary syndrome [3]. However, the advantages of long-term treatment are not as clearly defined. In the COMPASS (Cardiovascular Outcomes for People Using Anticoagulant Strategies) trial, Eikelboom et al. [4] addressed the safety and efficacy of low-intensity dual pathway inhibition of both thrombin activation and platelet aggregation with 2.5 mg BID of rivaroxaban, a direct FXa inhibitor [5], and 100 mg/d of aspirin, an irreversible inhibitor of cyclooxygenase (COX)-1-dependent thromboxane (TX)A_2 generation [6], respectively, in patients with CAD or PAD. They were eligible if they were less than 65 years of age, with clinically stable atherosclerosis involving at least two vascular beds, or those with risk factors including current smoking, diabetes, renal impairment, heart failure, or recent non-lacunar ischemic stroke. The combination of rivaroxaban 2.5 mg BID and aspirin 100 mg/d compared with 100 mg/d of aspirin alone significantly reduced the primary outcome, a composite of cardiovascular (CV) death, stroke, or myocardial infarction (MI), by 24% [4]. Based on the COMPASS trial, Eikelboom et al. [7] further explored the effects of the combination of rivaroxaban 2.5 mg BID and aspirin 100 mg/d compared with aspirin 100 mg/d on mortality by cause and in risk subgroups. Higher baseline risk features (i.e., poly-vascular disease, chronic kidney disease, mild or moderate heart failure, and diabetes) in CAD and PAD patients were associated with a greater reduction in overall CV mortality by the combination of rivaroxaban and aspirin versus aspirin alone. However, the concurrent blockade of both the thrombin and TXA_2 pathways may not be appropriate for all patients with atherosclerosis due to the enhanced risk of bleeding, particularly gastrointestinal, compared to aspirin monotherapy. The combination of low-dose rivaroxaban and aspirin is preferred in clinical practice for patients with multivessel disease, diabetes, or heart failure who have no history of bleeding complications.

The long-term open-label extension (LTOLE) study [8] assessed the outcomes of patients with CAD and/or PAD who participated in the COMPASS trial and received the combination treatment of rivaroxaban 2.5 mg BID alongside aspirin 100 mg/d for extended treatment periods (a median of 1 year and a maximum of 3 years). The study showed incidence rates for efficacy and bleeding that were similar to or lower than those observed during the randomized treatment phase.

To summarize, these studies indicated that using aspirin alongside low-dose rivaroxaban (known as the COMPASS regimen) significantly reduces the occurrence of major adverse cardiovascular events(MACE) compared to using aspirin alone. However, the mechanisms contributing to these potential synergistic and non-antithrombotic effects remain unclear. Additionally, biomarkers for

identifying patients who could benefit from the combined therapy of low-dose rivaroxaban and aspirin still need to be determined.

Rivaroxaban exhibits several non-hemostatic effects that could account for the enhanced cardiovascular outcomes observed in the COMPASS trial [9-11]. It has been shown to slow the progression of atherosclerosis, to lower pro-inflammatory gene expression in models of atrial fibrillation and reduce FXa-mediated inflammatory signaling in acute lung injury [10,11]. A study demonstrated that FXa activates the acute inflammatory response [12]. The COMPASS trial results indicate that the enhanced cardiovascular benefits are linked to the coadministration of aspirin with rivaroxaban [3]. This suggests a potential increase in FXa-mediated inflammatory signaling due to platelet biosynthesis of TXA_2. Several lines of evidence support the role of TXA_2 beyond thrombosis by promoting an inflammatory milieu in different tissues, thus fostering atherosclerosis and colorectal cancer [13, 14].

Given the key role of circulating extracellular vesicles(EVs) in atherosclerosis [15], the study by Weiss et al. [16] published in this issue of the Journal, aimed to test the hypothesis that the molecular signatures of plasma EVs significantly change in CAD patients after starting the treatment with low-dose aspirin and rivaroxaban. They utilized advanced methods, such as single-vesicle analysis and proteomic profiling, to evaluate the vesicular signatures of CAD patients initially while on low-dose aspirin and after six months of low-dose aspirin and rivaroxaban.

EVs are a diverse group of small membrane-bound vesicles released from cells *in vivo* and *in vitro*. They transport various molecules, with classical types of EVs including exosomes, microvesicles (also referred to as microparticles), and apoptotic bodies [17]. These vesicles are categorized based on their biogenesis mechanisms and size. EVs that are pelleted at intermediate centrifugation speeds (lower than 20,000 g) are known as “medium-sized EVs” (mEVs), which include microvesicles/microparticles and ectosomes [18].

The molecular cargo contained within EVs can be delivered to recipient cells, thereby influencing their phenotype and functions. Several lines of evidence support the role of platelet-derived EVs (PEVs) in hemostasis, thrombosis, inflammation, tumorigenesis, angiogenesis, and immunity. PEVs may contribute to an increased risk of thrombosis and multiple malignancies, particularly in individuals with obesity [19].

Additionally, analyzing EVs, whether focusing on the proteome or transcriptome, offers valuable insights into specific clinical conditions and serves as a pathophysiological biomarker [19]. This analysis is particularly important for PEVs because platelets can absorb various molecules from plasma. As a result, these vesicles gain unique molecular characteristics that reflect an individual's specific health status.

Weiss et al. [16] studied a cohort of CAD patients (n=40) who participated in the COMPASS trial and were previously randomized to receive aspirin (100 mg/d) and were prospectively recruited and assigned a revised regimen of open-label aspirin plus rivaroxaban (2.5 mg BID). Blood samples were obtained at baseline (aspirin only) and 6-month follow-up. Plasma EV concentration, size, and origin were analyzed using a nanoparticle tracking analyzer (NTA) and flow cytometry. EVs were enriched by ultracentrifugation for proteomic analysis. The main findings of the study are summarized in Table 1. The treatment with aspirin and rivaroxaban was found to significantly change the concentration and size of small (<200 nm) and large (200-1000 nm) EVs compared to aspirin alone (Table 1A). Notably, levels of PEVs were markedly reduced at follow-up, indicating inhibition of platelet activation. Likewise, myeloperoxidase (MPO)-positive EVs decreased significantly at follow-up. Since MPO is a key element of neutrophil extracellular traps (NETs), these findings may suggest decreased neutrophil activation (Table 1A). No significant differences were found in the large EVs derived from endothelial cells.

Weiss et al. [16] found that circulating EV profiles were modulated, potentially contributing to the pleiotropic effects of rivaroxaban. In contrast, other studies examining the impacts of antiplatelet therapies, such as rivaroxaban or aspirin as single therapies, show conflicting results regarding changes in total EV. These findings can be reconciled by hypothesizing the critical requirement of the

concurrent actions of thrombin and TXA_2 signaling in releasing EVs from various cell types implicated in inflammation-driven atherothrombosis. Additional studies need to be conducted to clarify this possibility.

Table 1 B summarizes the results from the study's proteomics analysis. A comparative proteomics characterization of enriched plasma EVs revealed profound changes in protein expression associated with inflammatory processes and endothelial dysfunction. Two highly cytotoxic histone proteins that can augment coagulation in vitro and in vivo were uniquely found only in the baseline aspirin cohort. There was a significant improvement in the systemic inflammatory status of patients receiving treatment with aspirin and rivaroxaban, as demonstrated by the decrease in plasma levels of high-sensitivity C-reactive protein (hs-CRP).

The data of Weiss et al. [16] indicate that patients treated with aspirin and rivaroxaban exhibit an anti-inflammatory phenotype associated with cardioprotection. This finding is consistent with the results of the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) trial, which evaluated Canakinumab, a human monoclonal antibody targeting IL-1β, in patients with atherosclerotic disease [20]. The trial demonstrated a significant reduction in MACE, suggesting that the underlying inflammatory state of these patients may play a crucial role. Additionally, the study by Weiss et al. [16] highlights a new pathway of cardiovascular inflammation involving the interplay of platelet-derived TXA_2 and thrombin signaling, which work together to develop atherothrombosis. This study presents some limitations, as highlighted in Table 1C. However, the results show the usefulness of evaluating EV subpopulations alongside the differential protein expression in clinical studies exploring the cardioprotective effects of rivaroxaban and aspirin. Assessing these biomarkers and the previously mentioned risk factors can aid in identifying CAD patients most likely to benefit from dual antiplatelet therapy using low-dose aspirin and rivaroxaban. To this aim, integrating diverse data sets—including clinical characteristics, EV features and proteomics data, and plasma inflammatory protein levels in response to dual antiplatelet therapy— is necessary. This strategy will allow the identification of individuals likely to derive the greatest benefit from treatment with rivaroxaban and aspirin while minimizing the potential risk of bleeding. Machine learning techniques should be utilized to effectively address the complexities associated with predicting drug responses within this integrated framework.

Author Contributions

Write the commentary: PP

Review the commentary critically: ST and ADM

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Table 1A. Main findings of circulating EV signatures

	Aspirin + Rivaroxaban (COMPASS regimen) *versus* Aspirin alone
Total small (<200nm) EV count	↔
Total large (200-1000nm) EV count	↔ (600-800nm↑)
Small EV mode size	↑
CD41/CD42b/CD62P-triple positive large (Annexin V^+) EVs	↓
MPO^+ large (Annexin V^+) EVs	↓

Table 1B. Main findings of circulating EV proteomics signatures

A. A total of 233 proteins were identified in at least 50% of at least one treatment group, with two proteins exclusive for the low-dose aspirin cohort [HIST1H4A (Histone H4) and HIST2HAA3 (Histone H2A type 2-A)]

B. 31 differentially expressed proteins were found among the 231 shared proteins:

Upregulated (n=18)	**Downregulated (n=13)**
AGT (Angiotensinogen)	IGKV3D-11 (Ig kappa chain V-III region VG)
APOC2 (Apolipoprotein C-II)	ACTB (Actin, cytoplasmic 1)
IGFBP3 (Insulin-like growth factor binding protein 3)	S100A9 (Protein S100-A9)
MBD1 (Methyl-CpG-binding domain protein 1)	ANG (Angiogenin)
MASP2 (Mannan-binding lectin serine protease 2)	HABP2 (Hyaluronan-binding protein 2)
CLEC3B (Tetranectin)	SERPINA7 (Thyroxine-binding globulin)
IGKV3-20, IGKV2-24, IGHV3-21, IGHV3-38, and N/A (Ig kappa chain V-III region B6, Ig kappa variable 2-24, Ig heavy chain V-III region JON, Ig heavy variable 3-38 and Ig kappa chain V-I region HK101)	IGHG4 and IGHV3-9 (Ig gamma-4 chain C region and Ig heavy chain V-III region DOB)
GP1BA (Platelet glycoprotein Ib alpha chain)	RBP4 (Retinol-binding protein 4)
PROC and PROZ (Vitamin K-dependent protein C and Vitamin K-dependent protein Z)	SEPP1 (Selenoprotein P)
HGFAC (Hepatocyte growth factor activator)	APOD (Apolipoprotein D)
PI16 (Peptidase inhibitor 16)	TGFB1 (Transforming growth factor beta-induced protein ig-h3)
SAA1 (Serum amyloid A-1 protein)	ALB (Serum Albumin)
C4A (Complement C4-A)	

Table 1C. Overview of study limitations

A. Insufficient statistical power prevents linking clinical outcomes (MACE or bleeding) to changes in EV profiles; relevant outcome data is not accessible to the study's authors
B. As the LTOLE trial is uncontrolled, caution is needed when interpreting the observed changes as treatment effects
C. Differential proteins identified in the proteomics analysis were not validated using independent methods
D. Analyses were conducted at only one follow-up time point, making it impossible to predict long-term anti-inflammatory effects
E. The concentration, size, and origin of large, enriched plasma EVs were analyzed using flow cytometry; small-size EV concentrations were not analyzed
F. The ultracentrifugation method, designed for mass spectrometry, primarily enriches small EVs
G. Recruiting patients from a single site and only including those randomized to low-dose aspirin in the COMPASS trial