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ORIGINAL ARTICLE

Genetic and nongenetic drivers of platelet reactivity in healthy Tanzanian individuals

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Abstract

Background: Platelets play a key role in hemostasis, inflammation, and cardiovascular diseases. Platelet reactivity is highly variable between individuals. The drivers of this variability in populations from Sub-Saharan Africa remain largely unknown.

Objectives: We aimed to investigate the nongenetic and genetic determinants of platelet reactivity in healthy adults living in a rapidly urbanizing area in Northern Tanzania.

Methods: Platelet activation and reactivity were measured by platelet P-selectin expression and the binding of fibrinogen in unstimulated blood and after *ex vivo* stimulation with adenosine diphosphate and PAR-1 and PAR-4 ligands. We then analyzed the associations of platelet parameters with host genetic and nongenetic factors, environmental factors, plasma inflammatory markers, and plasma metabolites.

Results: Only a few associations were found between platelet reactivity parameters and plasma inflammatory markers and nongenetic host and environmental factors. In contrast, untargeted plasma metabolomics revealed a large number of associations with food-derived metabolites, including phytochemicals that were previously reported to inhibit platelet reactivity. Genome-wide single-nucleotide polymorphism genotyping identified 2 novel single-nucleotide polymorphisms (rs903650 and rs4789332) that were associated with platelet reactivity at the genome-wide level ($P < 5 \times 10^{-8}$) as well as a number of variants in the PAR4 gene (*F2RL3*) that were associated with PAR4-induced reactivity.

Conclusion: Our study uncovered factors that determine variation in platelet reactivity in a population in East Africa that is rapidly transitioning to an urban lifestyle, including the importance of genetic ancestry and the gradual abandoning of the traditional East African diet.

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Vesla I. Kullaya, Godfrey S. Temba, and Nadira Vadaq contributed equally to this study.

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KEYWORDS
genetics, platelet activation, platelets, protease-activated receptors, P-selectin

1 | INTRODUCTION

Platelets play an essential role in thrombosis and hemostasis; they are increasingly recognized as important effector cells in host defense mechanisms, inflammation, and atherosclerosis [1–3]. The number of circulating platelets and their reactivity in response to an external stimulus have been associated with an increased risk for cardiovascular disease (CVD) and mortality [4,5].

There is a marked interindividual variation in the number and reactivity of peripheral blood platelets. Different genetic and nongenetic host factors, as well as environmental and dietary factors, have been shown to influence platelet reactivity [6–12]. However, almost all of these data have been derived from individuals living in Europe or North America. The inclusion of underrepresented populations on the African continent in platelet studies is important and timely. Many parts of Sub-Saharan Africa are experiencing a rapid increase in CVDs and other noncommunicable diseases (NCDs) [13]. The adoption of a more sedentary lifestyle and Western type of diet, especially in subjects living in urban areas, may be associated with this rise in NCDs. Lifestyle and dietary changes may impact platelet function. Indeed, many plant-based foods such as vegetables, fruits, and spices contain abundant amounts of phytochemical compounds with modulatory effects on platelet activation and have also been associated with reduced risk for CVD [14]. Traditional African diets are largely plant-based and often contain large quantities of polyphenols and other compounds that inhibit platelet reactivity. In contrast, a Western-type high-fat diet has been shown to increase platelet reactivity [15–17].

Our insight into the genetic regulation of platelet reactivity in populations on the African continent is also limited. We recently showed that healthy Tanzanians have enhanced thrombin generation potential compared to individuals from Western European ancestry [18]. Thrombin is a key enzyme in coagulation and a strong platelet agonist [19]. The genetic regulation of thrombin-induced platelet reactivity is not well understood. Protease-activated receptor (PAR)1 and PAR4 are the main thrombin receptors on platelets. A genetic variant in *PAR4* that is associated with higher PAR4-induced platelet reactivity was found to be common in self-identified African Americans living in the United States [20]. The allele frequency of this variant was subsequently shown to vary widely across Sub-Saharan African populations, highlighting the importance of geographic ancestry [21].

In the present study, we aimed to provide a comprehensive assessment of the genetic and nongenetic host, environmental, and dietary factors that influence platelet reactivity in both a rapidly urbanizing and a more rural community in East Africa. To achieve this aim, platelet activation and reactivity were measured in the first African cohort of the Human Functional Genomics Project (HFGP, humanfunctionagenomics.org). The cohort consists of both healthy urban and rural dwellers living in the Kilimanjaro region in Northern Tanzania. Platelet reactivity data were correlated to demographic and environmental factors, plasma inflammatory cytokines and adipokines, the results of untargeted plasma metabolomics, and genome-wide single-nucleotide polymorphism (SNP) genotypes.

2 | METHODS

2.1 | Study area and population description

The 300 functional genomics (300FG) study is part of the HFGP, which aims to characterize and understand the interindividual variation of human immune responses in healthy and diseased individuals [22]. A total of 319 Tanzanian healthy individuals aged between 18 and 65 years residing in urban or rural areas within the Kilimanjaro region in Northern Tanzania were recruited between March and December 2017 at the Kilimanjaro Christian Medical Center and Lucy Lameck Research Center in Moshi municipality. Detailed information on this cohort, including a map of the study area, has been described earlier [23]. Urban dwellers were categorized as those living in or around the regional capital, Moshi, while the majority of rural participants were situated in villages situated in the foothills of Mount Kilimanjaro. The Kilimanjaro region has significant diversity in ethnic groups, economic status, and lifestyle, whereby most urban inhabitants have adopted a Western lifestyle while rural inhabitants

follow a traditional lifestyle. Exclusion criteria included pregnancy, suffering from any acute or chronic disease, and use of any medication. Participants were also screened for malaria, HIV, random blood glucose, and hypertension. Demographic data were collected using an extensive questionnaire about host factors, lifestyle, dietary habits, and disease history.

2.2 | Measurements of platelet function

Venous blood was collected in citrated tubes (3.2% sodium citrate; Becton Dickinson). Platelet reactivity tests were determined by a whole blood flow cytometry assay within 2 hours as described previously [24]. Briefly, platelet membrane expression of the α-granule protein P-selectin (CD62P) and the binding of fibrinogen to the activated integrin αIIbβ3 were measured in unstimulated whole blood samples and recorded as baseline activation parameters of resting platelets. Platelet reactivity to agonists was determined by using the geometric mean of the median fluorescent intensity of P-selectin expression on platelets after *ex vivo* stimulation with a low or high concentration of adenosine diphosphate (ADP, 1 μM and 125 μM; Sigma-Aldrich), PAR1 agonist thrombin receptor activation peptide-6 (TRAP-6, 5 μM and 125 μM; Sigma-Aldrich), or PAR4 agonist AYPGKF (200 μM and 625 μM; synthesized at the Department of Immunohematology and Blood Transfusion of the Leiden University Medical Center, the Netherlands). The low concentration was chosen to achieve a platelet P-selectin value that falls in the middle of the activation curve and a high concentration was chosen to elicit maximum stimulation. Baseline parameters in unstimulated samples were termed as unstimulated P selectin (Psel) and unstimulated fibrinogen, while induction of platelet P-selectin by ADP and PAR1 and PAR4 agonists were termed as Psel ADP, Psel PAR1, and Psel PAR4, respectively.

The following antibodies were used: PE-labeled anti-CD62P (P-selectin; Sysmex Partec), FITC-labeled antifibrinogen (Dako Ltd), and PE-Cy7–labeled anti-CD61 (Sysmex). Samples were measured on a Sysmex Cube8 flow cytometer, and data were analyzed using Kaluza software (Beckman Coulter). Cells were gated based on their forward and sideward scatter properties and surface expression of their respective identification markers Supplementary Figure S2). Whole blood cell count was performed on EDTA anticoagulated blood using a Sysmex XN450 analyzer.

Details on platelet function measurements in the Framingham Heart Study (FHS) cohort and replication of genome-wide association study (GWAS) signals in FHS are given in the Supplementary Methods.

2.3 | Measurement of circulating mediators

The circulating plasma cytokines IL-6, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, and IL-18–binding protein were measured in EDTA plasma using the Simple Plex cartridges run on the Ella platform (ProteinSimple) following the manufacturer's instructions. Circulating adipokines (leptin, resistin, and adiponectin) and α-1-antitrypsin (AAT) were also measured in EDTA plasma using the R&D Systems DuoSet enzyme-linked immunosorbent assay kits following the manufacturer's standard protocols.

2.4 | Genotyping and imputation

DNA was extracted from whole blood using a DNeasy Blood and Tissue kit. Genotyping was performed using the Global Screening Arrays SNP chip. Default settings of optiCall (version 0.7.0) [25] were used to perform genotype calling. Prior to imputation of the genetic data, filtering was performed by excluding variants with call rate exceeding 0.1, low minor allele frequencies (<0.001), and SNPs deviating from Hardy–Weinberg equilibrium with a P value of $<1 \times 10^{-4}$. Finally, we removed individuals with genetic outliers, which were identified by using multidimensional scaling plots. A total of 409 261 variants and 308 samples passed the quality control procedures. Strand alignment to a reference panel, 1000 genome reference panel dataset, was performed using Genotype Harmonizer [26]. To improve genome coverage, genotype imputation was performed for all chromosomes except for sex chromosomes using the Minimac4 software through the publicly available Michigan Imputation Server [27]. The Human Reference Consortium (HRC) version 1.1 was used as a reference panel and the dataset was phased using Eagle v2.3. We filtered out variants with an imputation quality score (R^2) of <0.3. Individuals with extreme heterozygosity and cryptic relatedness and genetic outliers were excluded. Genotyping and imputation generated a total of 5 271 779 variants from 285 individuals.

2.5 | Analysis of plasma metabolome

Plasma metabolites measurements were performed by General Metabolics as previously described [28]. Briefly, using an untargeted metabolomics workflow, plasma samples were measured using high-throughput mass spectrophotometry using the Agilent Series 1100 LC pump coupled with a Gerstel MPS2 autosampler and the Agilent 6520 Series Quadrupole Time-of-Flight mass spectrometer (Agilent). Obtained data were further analyzed by performing centroiding on high performance computing cluster using bioinformatics function in MATLAB R2010b (MathWorks). A list of putative metabolites (n = 1607) was annotated with a series of analysis strategies, including deisotoping, decluttering, adduct detection, and library matching in the Kyoto Encyclopedia of Genes and Genomes, Human Metabolome Database (HMDB), and Chemical Entities of Biological Interest databases using accurate mass and intensity correlation. Individual metabolites were correlated with the following platelet parameters: platelet number (n = 313), mean platelet volume (n = 314), unstimulated platelet Psel expression (n = 234), unstimulated fibrinogen binding (n = 229), ADP-low Psel (n = 241), ADP-high Psel (n = 236), PAR1-low Psel (n = 235), PAR1-high Psel (n = 232), PAR4-low Psel (n = 235), and PAR4-high Psel (n = 233). Definition and visualization of metabolic clusters were performed by agglomerative hierarchical

TABLE 1 Characteristics of study participants (N = 319).

Variable	All
Location, *n* (%)	
Urban	251 (78.7)
Rural	68 (21.3)
Male, *n* (%)	157 (49.2)
Age (y), median (IQR)	30.2 (23.4-40.2)
Age categories, *n* (%)	
18-30 y	164 (51.4)
31-45 y	96 (30.1)
46-80 y	59 (18.5)
BMI (kg/m^2), median (IQR)	24.0 (21.5-27.3)
BMI categories, *n* (%)	
Underweight, BMI < 18.5	15 (4.7)
Normal weight, BMI = 18.5-24.9	177 (55.5)
Overweight, BMI = 25-30	79 (24.8)
Obese, BMI > 30	48 (15)
Systolic blood pressure (mmHg), median (IQR)	123 (113-136)
Diastolic blood pressure (mmHg), median (IQR)	79 (70-81)
Use of contraceptives, *n* (% of women)	
No	138 (85.2)
Cooking fuel,[a] *n* (%)	
Smoky	115 (36.1)
Nonsmoky	204 (63.9)
Use of tobacco in men, *n* (%)	
Current or past smoker	45 (28.7)
Never smoked	112 (71.3)
Alcohol use, *n* (%)	
No	201 (63.0)
Yes	118 (37.0)
Toilet facility, *n* (%)	
Pit latrine	77 (24.1)
Water closet	242 (75.9)
Exposure to animals, *n* (%)	
No	178 (55.8)
Yes	141 (44.2)
Platelet count (×10^3/μL), mean (SD)	256 (73.9)
Immature platelet fraction (%), mean (SD)	5.2 (3.7)
Mean platelet volume (fl), mean (SD)	10.2 (1.6)
Platelet distribution width (fl), mean (SD)	11.9 (2.7)
Hemoglobin (g/dL), mean (SD)	14.4 (1.8)
Leukocyte count (×103/μL), mean (SD)	5.2 (1.4)

BMI, body mass index.
[a]Smoky fuel includes firewood, charcoal, and kerosene; nonsmoky fuel includes electrical fuel and gas.

clustering on the food-derived metabolome by setting Euclidean distance calculation and using the ward.D2 linkage algorithm in the R package pheatmap (v1.0.12).

2.6 | Statistical analysis

Data on platelet reactivity and cytokines were $\log_{10}$-transformed. The associations between platelet count and platelet reactivity parameters with host and environmental factors, circulating mediators, and *ex vivo* cytokine production were analyzed using Spearman correlation coefficient or linear regression controlling for age, sex, and body mass index (BMI) as indicated. Differences between groups were analyzed using chi-square and Mann–Whitney tests for categorical and continuous variables, respectively. In order to maximize the use of data, missing values were handled on a pairwise basis for each comparison separately. To address multiple testing, P values were corrected according to the Benjamini–Hochberg procedure to decrease the false discovery rate (FDR). Significance was defined by FDR P values <.05. Analyses were performed using SPSS version 25 (IBM Corp.) and GraphPad Prism 7.

For platelet reactivity quantitative trait locus (QTL) analysis, associations between identified SNPs and platelet parameters were analyzed using a linear regression model adjusted for age and sex. All platelet measurements were $\log_2$-transformed prior to analysis. A P value less than 5×10^{-8} was considered the genome-wide significance threshold for significant platelet QTLs.

Analyses of metabolome data were performed by open-source R version 3.5.1. Natural logarithmic transformation was used before analysis. The association between individual plasma metabolites and platelet parameters was done using linear regression with age and sex as covariates. Benjamini and Hochberg FDR was used to account for multiple testing.

3 | RESULTS

3.1 | Cohort characteristics and platelet parameters

The characteristics of the study participants are summarized in Table 1. A total of 319 subjects were enrolled, of whom 251 (78.7%)

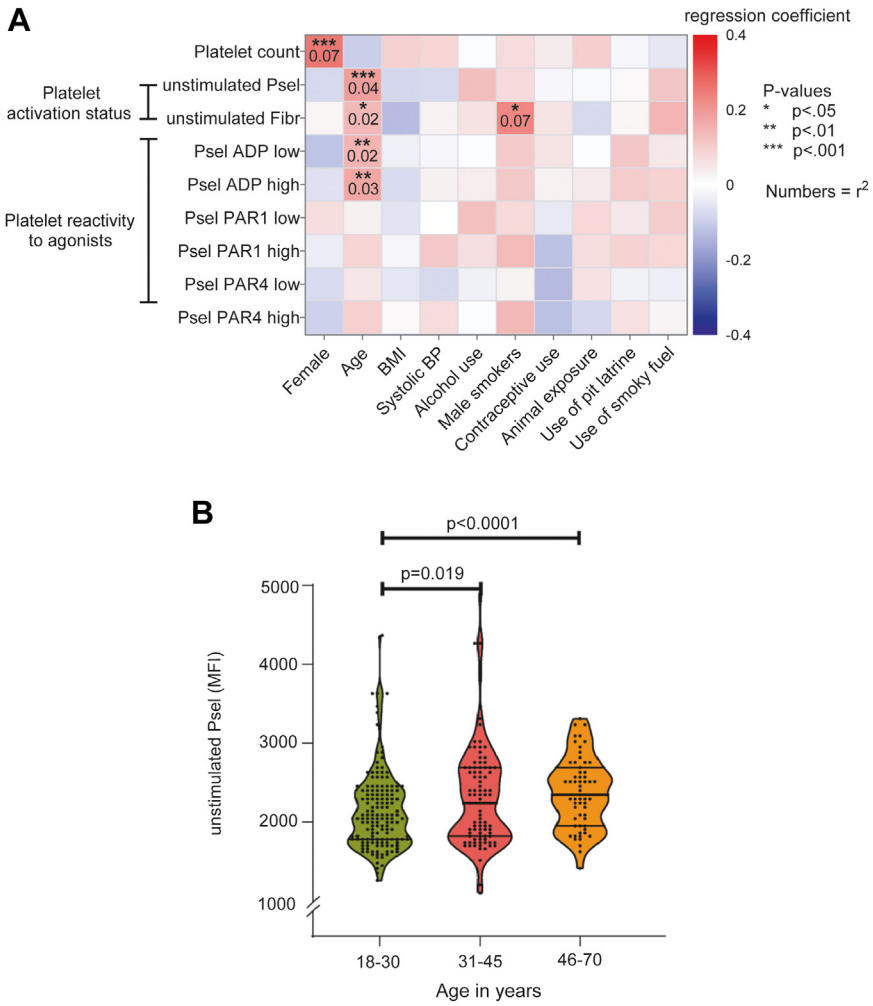


FIGURE 1 Relation between platelet parameters and host and environmental factors. (A) Regression coefficients of associations between platelet parameters and sex (corrected for age) and age (corrected for sex), body mass index (BMI), and other environmental factors corrected for sex and age (n = 319). Red and blue colors indicate positive and negative coefficients, respectively. Analysis was performed using a linear regression model with false discovery rate (FDR) correction. Numbers indicate the squared correlation coefficients. (B) Violin plot showing geometric mean with 95% interval of differences in unstimulated P-selectin across different age categories. Differences were calculated using the Mann–Whitney test. Unstimulated Psel and unstimulated Fibr represent P-selectin and fibrinogen binding to platelets in unstimulated blood samples, respectively. Psel adenosine diphosphate (ADP) low, Psel ADP high, Psel PAR1 low, Psel PAR1 high, Psel PAR4 low, and Psel PAR4 high represent P-selectin expression after stimulation of platelets with a low and high concentration of ADP, PAR1 agonist (TRAP-6), or PAR4 agonists. BP, blood pressure.

were living in an urban area and 68 (21.3%) in a rural area. The median age of the population was 30.2 years (IQR, 23.4-40.2 years), and 50.8% were females. The platelet gating strategy and platelet reactivity plots are shown in Supplementary Figure S1. The distribution of platelet P-selectin (Psel) expression in unstimulated whole blood as well as after *ex vivo* stimulation with 2 concentrations (referred to as "low" and "high") of adenosine diphosphatase (ADP), the PAR1 agonist thrombin receptor agonist-6 (TRAP-6), or the PAR4 agonist are shown in Supplementary Figure S2. Activation of integrin $\alpha_{IIb}\beta_3$ in unstimulated blood was assessed by binding of fibrinogen to platelets. PAR4-induced platelet reactivity yielded the largest interindividual variation of the platelet agonists. Associations between platelet parameters after correcting for age and sex are shown in Supplementary Figure S3. Readouts of platelet activation and reactivity correlated well with each other, except for PAR4-induced Psel response, which correlated only weakly with unstimulated Psel expression.

3.2 | The impact of environmental and nongenetic host factors on platelet function

We first analyzed platelet parameters in relation to a set of host and environmental factors (Figure 1A and Supplementary Figure S4A). Women had a significantly higher mean (SD) platelet count than that in men (272 ± 71 × 10^3/μL vs 239 ± 57 × 10^3/μL; $P < .001$), as also previously reported [29,30]. Age had a positive association with fibrinogen binding and unstimulated Psel expression (Figure 1B) as well as ADP-stimulated Psel expression but not with PAR1- or PAR4-stimulated Psel expression. Neither BMI nor the use of alcohol or tobacco, exposure to animals, or smoke-emitting fuel or the use of a pit latrine (as a measure of socioeconomic status) was associated with platelet reactivity. Overall, there were no differences in platelet activation or reactivity between individuals living in a more urban or rural area.

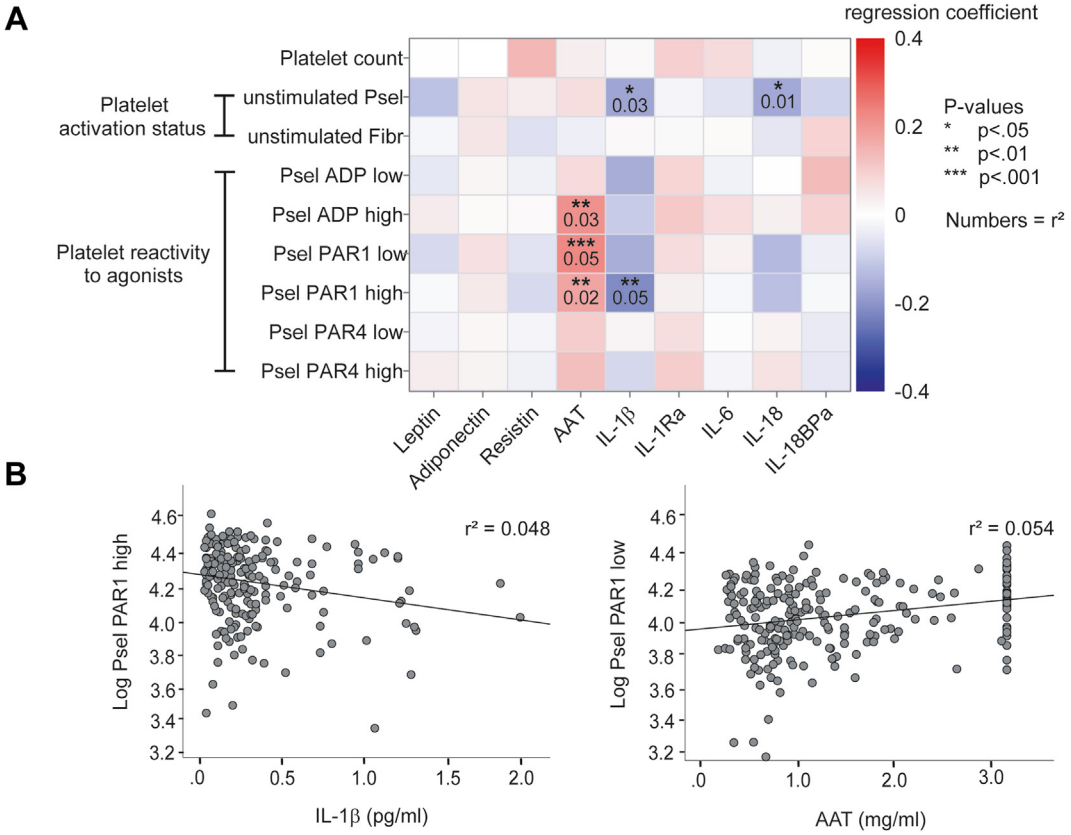


FIGURE 2 Associations between platelet parameters and circulating inflammatory mediators. (A) Heat map showing regression coefficients of associations between platelet parameters and circulating inflammatory mediators corrected for sex, age, and body mass index (n = 241). Red and blue colors indicate positive and negative coefficients, respectively. Analysis was performed using a linear regression model with false discovery rate correction. Numbers indicate the squared correlation coefficients. (B) Scatter plots show significant associations between platelet reactivity with α-1-antitrypsin (AAT) and IL-1β. The r^2 is the squared correlation coefficient. Unstimulated Psel and unstimulated Fibr represent P-selectin and fibrinogen binding to platelets in unstimulated samples, respectively. Psel adenosine diphosphate (ADP) low, Psel ADP high, Psel PAR1 low, Psel PAR1 high, Psel PAR4 low, and Psel PAR4 high represent P-selectin expression after stimulation of platelets with a low and high concentration of ADP, PAR1 agonist, or PAR4 agonists. IL-1Ra, IL-1 receptor antagonist; IL-18BPa, IL-18 binding protein.

3.3 | Association with inflammatory proteins

Next, we evaluated the associations between platelet parameters and the circulating concentrations of inflammation-related proteins. Previous studies have demonstrated a bidirectional relationship between plasma inflammatory cytokines, adipokines, and platelet activation [31,32]. We conducted a regression analysis between platelet parameters and several adipokines, including leptin, adiponectin, and resistin. Additionally, we examined the relationship between platelet parameters and the serine protease inhibitor AAT as well as a selection of plasma inflammatory cytokines and their receptors that have been previously associated with platelet function and thrombopoiesis [33]. These cytokines and receptors included IL-1β, IL-6, IL-1Ra, IL-18, and IL-18 binding protein. Analyses were corrected for sex, age, and BMI as these factors were independently associated with inflammatory mediators (Supplementary Figure S4B). In contrast, urban living was not associated with higher concentrations of inflammatory cytokines except with IL-1β (Supplementary Figure S4B). Overall, only a few associations were found (Figure 2), among which a positive relation between AAT concentrations and ADP- or PAR1-induced Psel expression was the most pronounced. In contrast, IL-1β and IL-18 concentrations were negatively associated with unstimulated- and PAR1-stimulated Psel expressions. We previously reported that platelet count correlated positively with plasma IL-1β concentrations in healthy Dutch adults [34]. In a univariate analysis in the present study, platelet count also correlated positively with IL-1Ra, IL-6, leptin, and resistin (Supplementary Figure S4C), but these associations disappeared after correction for sex and BMI.

3.4 | Association of platelet reactivity with food-derived metabolites

Numerous studies have shown that plant-based foods and their active compounds modulate platelet function [35]. Examples include plant polyphenols and polyunsaturated fatty acids [36–38]. Using untargeted metabolomics, we previously showed in the present cohort that plant-derived polyphenols, such as apigenin, were higher in individuals

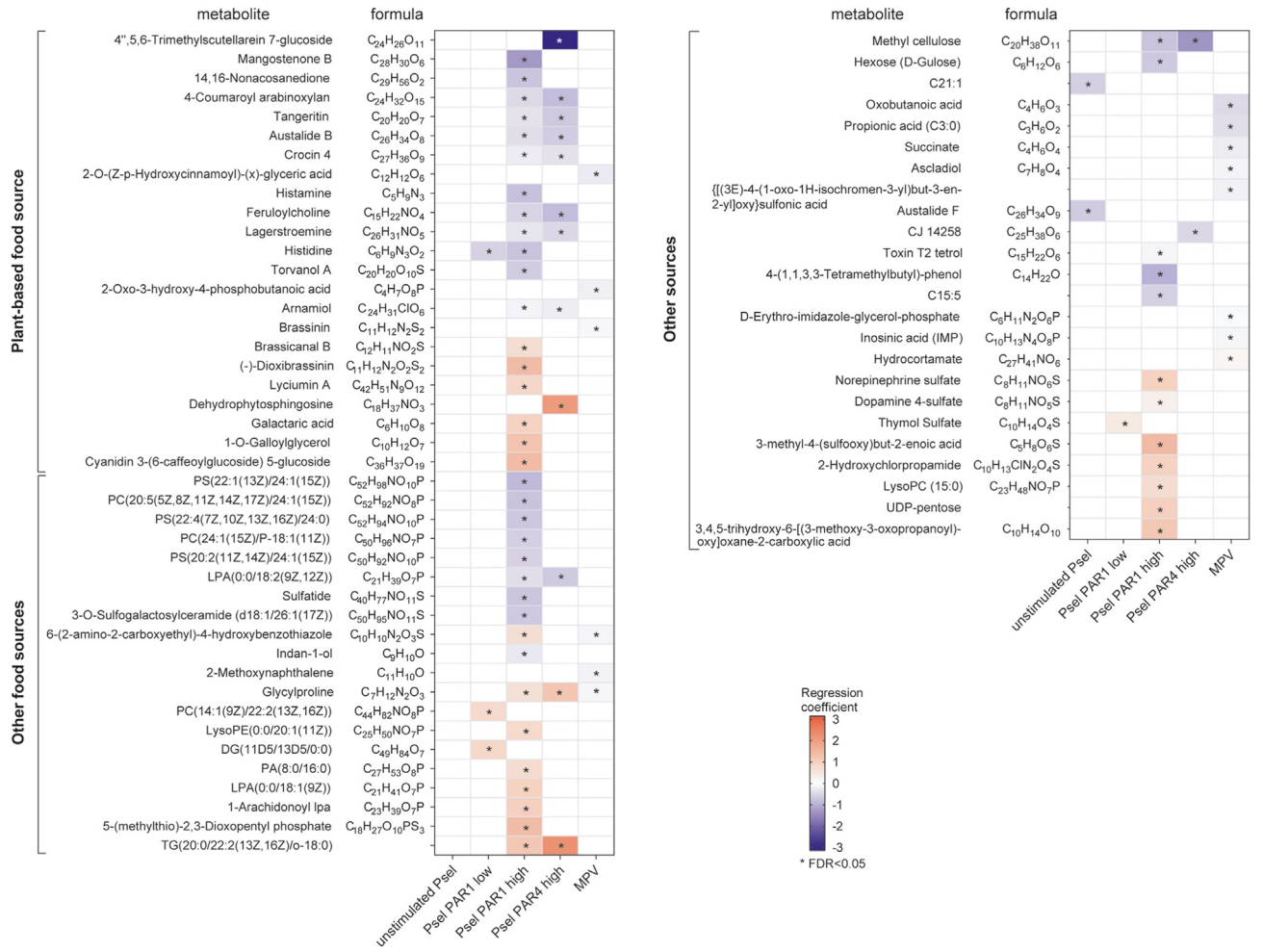
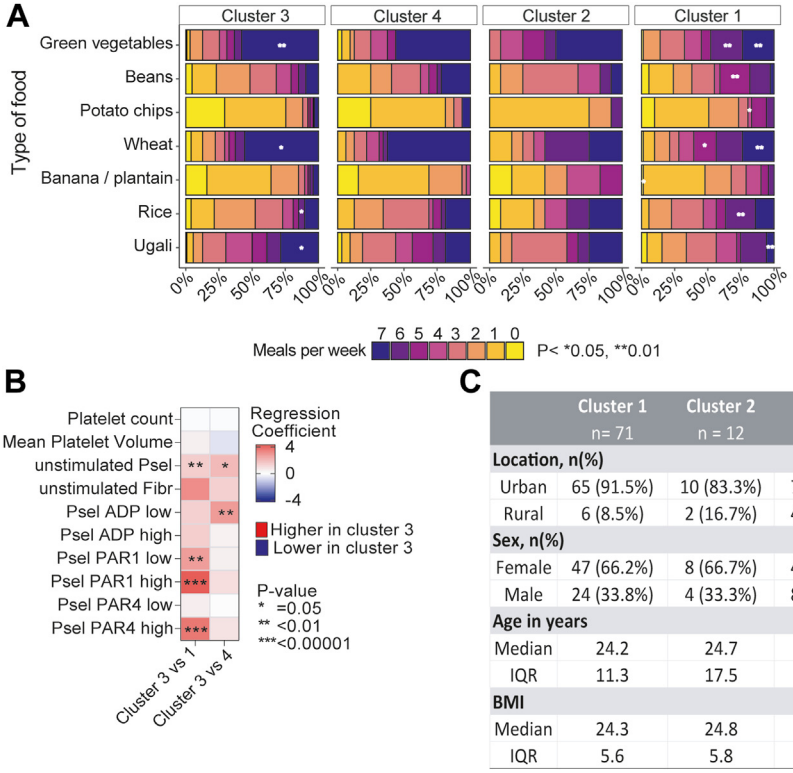


FIGURE 3 Relation between platelet parameters and plasma metabolites. Heat map showing 68 plasma metabolites that had 1 or more significant associations with a platelet parameter. Metabolites were aggregated in primarily plant-derived metabolites and other food- and nonfood-derived metabolites. Colors indicate the regression coefficients. Analysis was performed using Pearson partial correlation corrected for sex and age, followed by false discovery rate (FDR) correction. MPV, mean platelet volume.

consuming a traditional diet [23]. To test the hypothesis that consumption of a traditional plant-based diet and its active compounds modulate platelet function, we first assessed in an unbiased manner the associations between biologically relevant metabolites and platelet parameters. The untargeted plasma metabolomics yielded a total of 1607 annotated compounds in 241 participants. Using a linear regression model corrected for age and sex, a total of 68 metabolites were significantly (FDR corrected $P < .05$) associated with at least 1 platelet parameter—mostly PAR1- or PAR4-induced P-selectin expression (Figure 3). These metabolites were further classified based on the ontology provided by the HMDB (https://hmdb.ca) as having either a dietary source or another source.

These metabolites included different plant-derived compounds that were previously shown to inhibit platelet function. Examples include the flavonoids tangeretin [39], which is present in citrus fruit peels; 4',5,6-trimethylscutellarein 7-glucoside [40]; the carotenoid crocin 4 [41] and lagerstroemine [42]. Other phytochemicals that were inversely correlated with platelet reactivity were mangostenone B, feruloylcholine, and the flavonoid torvanol A. Histamine and histidine, which are present in numerous foods and can be produced endogenously by the gut microbiome, were also negatively associated with platelet reactivity, as also previously shown [43]. In contrast, the metabolites that were positively associated with platelet reactivity belonged predominantly to the class of lipids. Some of these lipids are known to promote platelet activation, including lysophosphatidic acid (LPA(0:0/18:1(9Z)) [44] and triglyceride (TG(20:0/22:2(13Z,16Z)/o-18:0)) [45,46]. Lysophosphatidylcholines and lysophosphatidylethanolamines were also positively associated with reactivity, but it is important to be aware that these phospholipids are also generated during platelet activation [47].

To further explore the contribution of dietary products to the variation in platelet reactivity, we performed hierarchical clustering of food-derived metabolites. This yielded 4 different clusters: cluster 1 (n = 71), cluster 2 (n = 12), cluster 3 (n = 126), and cluster 4 (n = 32). Individuals in these clusters differed significantly in age, sex, and the percentage of urban vs rural dwellers (chi-square test $P < .001$), with cluster 3 having older participants and more males; BMI was equally distributed across the clusters (Figure 4C). Weekly food consumption



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	Cluster 1 n= 71	Cluster 2 n = 12	Cluster 3 n = 126	Cluater 4 n = 32	P-value
Location, n(%)					< 0.001
Urban	65 (91.5%)	10 (83.3%)	78 (61.9%)	29 (90.6%)	
Rural	6 (8.5%)	2 (16.7%)	48 (38.1%)	3 (9.4%)	
Sex, n(%)					< 0.001
Female	47 (66.2%)	8 (66.7%)	46 (36.5%)	20 (62.5%)	
Male	24 (33.8%)	4 (33.3%)	80 (63.5%)	12 (37.5%)	
Age in years					< 0.001
Median	24.2	24.7	36.3	29.3	
IQR	11.3	17.5	14.4	12.8	
BMI					0.12
Median	24.3	24.8	23.2	26.2	
IQR	5.6	5.8	5.7	9.9	

FIGURE 4 Food frequencies and metabolic cluster comparison. (A) Individuals were clustered based on the results of hierarchical clustering of plasma food-derived metabolites, yielding 4 clusters (cluster 1, *n* = 71; cluster 2, *n* = 12; cluster 3, *n* = 126; cluster 4, *n* = 32). Weekly food consumption of ugali (traditional maize porridge), rice, plantain (cooked banana), wheat, potato chips, beans, and green vegetables across 4 clusters. Dependency among the clusters and weekly food-frequency categories (0-7 meals per week) was tested using a chi-square test. (B) Heat map showing regression coefficients of significant associations between platelet parameters and metabolic clusters. Analysis was performed using a linear regression model correcting for sex and age. Unstimulated Psel and unstimulated Fibr represent P-selectin and fibrinogen binding to platelets in unstimulated samples, respectively. Psel adenosine diphosphate (ADP) low, Psel ADP high, Psel PAR1 low, Psel PAR1 high, Psel PAR4 low, and Psel PAR4 high represent P-selectin expression after stimulation of platelets with a low and high concentration of ADP, PAR1 agonist, or PAR4 agonists. (C) Table showing a comparison of age, sex, body mass index (BMI), and residence (urban/rural) between the different metabolic clusters. Continuous variables (age and BMI) were compared using Kruskal–Wallis test, while categorical variables (area and sex) were compared using chi-square test.

habits differed across the clusters (Figure 4A): individuals in cluster 3 consumed more green vegetables, wheat, and ugali (traditional porridge made from maize flour) and less rice, while individuals in cluster 1 consumed more rice, plantain, and beans and less ugali and potato chips (chi-square test; $P < .01$). Differences in platelet parameters between these metabolic clusters were analyzed using a linear regression model, correcting for age and sex (Figure 4B). Cluster 3 had significantly higher platelet Psel expression in unstimulated blood and after PAR1 and PAR4 stimulation compared to that in cluster 1 and higher platelet ADP-stimulated Psel compared to that in cluster 4. Collectively, these observations support that diet is an important driver of platelet reactivity in an East African population.

3.5 | Genetic regulation of platelet reactivity

We subsequently assessed the genetic regulation of platelet reactivity in an unbiased manner. Genome-wide QTL-mapping (correlating platelet parameters with SNP genotypes) identified 2 SNPs that were associated with platelet parameters at a genome-wide significance threshold ($P \leq 5 \times 10^{-8}$) and 1 SNP (rs72958154) that was close to the genome-wide level ($P = 7.2 \times 10^{-8}$) (Table 2). Rs903650 located near the *GPM6A* gene was negatively associated with Psel expression in unstimulated blood ($P = 3.9 \times 10^{-8}$); rs4789332, located near the *ST6GALNAC1* gene, was positively associated with PAR1-stimulated (high concentration TRAP-6) Psel expression ($P = 1.1 \times 10^{-8}$); and rs72958154, located near the *LRRIQ3* gene, was negatively associated with PAR1-stimulated (low concentration TRAP-6) Psel expression ($P = 7.2 \times 10^{-8}$) (Table 2). Regional plots and Manhattan plots are shown in Supplementary Figures S5 and S6, respectively. None of these SNPs have previously been identified in platelet studies. Lowering the threshold to $P = 1 \times 10^{-6}$ yielded an additional 15 independent SNPs that were associated with at least 1 platelet parameter, particularly with reactivity to PAR1 and PAR4 agonists (Table 2).

Previous studies from outside the African continent have shown that individuals of African ancestry exhibit higher platelet reactivity to PAR4 stimulation due to gene variants in the PAR4 gene (*F2RL3*) [20].

TABLE 2 Genome-wide and suggestive loci (*P* value < 1×10^{-6}) for platelet reactivity, platelet count, and mean platelet volume traits.

SNP (allele)	Traits	Chr	bp	Effect allele	Alternative allele	MAF	*P* value	β	Genes
Loci associated with basal platelet activation (unstimulated platelets)									
rs903650	Psel_Un	4	176827467	G	A	0.15	3.9×10^{-8}	−0.25	*GPM6A*[a,b]
rs34812247	Psel_Un	4	132116370	C	T	0.13	1.1×10^{-7}	0.20	*PCDH10*[a]; *U6*[a]
rs56369127	Psel_Un	11	113483442	G	A	0.24	9.1×10^{-7}	−0.15	*TMPRSS5*[a]; *USP28* [b]
rs11199305	Fibr_Un	10	122140667	T	A	0.41	8.9×10^{-7}	0.10	*PPAPDC1A*[a]; *FUT10*[b]
Loci associated with platelet reactivity to ADP									
rs1355857	Psel_ADPhigh	15	66553649	T	C	0.42	2.5×10^{-7}	0.26	*MEGF11*[a]; *DIS3L*[b]; *TIPIN*[b]; *MAP2K1*[b]; *SNAPC5*[b]; *ZWILCH* [b]
Loci associated with platelet reactivity to PAR1 agonist									
rs4789332	Psel_PAR1 high	17	74619503	C	T	0.17	1.1×10^{-8}	0.42	*ST6GALNAC1*[a,b]; *ST6GALNAC2*[b]; *MXRA7*[b]; *RP11-666A8.9*[b]; *RP11-666A8.13*[b]; *AC015802.6*[b]
rs62479830	Psel_PAR1 high	7	151232914	A	G	0.11	3.8×10^{-7}	−0.49	*RHEB*[a,b]; *PRKAG2*[b]
rs72958154	Psel_PAR1 low	1	74447580	T	G	0.14	7.2×10^{-8}	−0.44	*LRRIQ3*[a]
rs541857	Psel_PAR1 low	11	120085469	T	C	0.30	8.5×10^{-7}	−0.29	*POU2F3*[b]; *OAF* [b]
rs720067	Psel_PAR1 low	7	51023364	C	T	0.26	9.1×10^{-7}	−0.33	*COBL*[a]; *RP4-724E13.2*[a]
rs60304319	Psel_PAR1 low	2	208852389	C	T	0.14	9.8×10^{-7}	−0.38	*PLEKHM3*[a]
Loci associated with platelet reactivity to PAR4 agonist									
rs73719337	Psel_PAR4 high	7	119146283	C	T	0.27	5.9×10^{-7}	−0.52	*KCND2*[a]; *AC091320.2*[a]
rs79203246	Psel_PAR4 high	9	129920061	A	G	0.39	9.8×10^{-7}	−0.44	*RALGPS1*[a]
rs3924418	Psel_PAR4 low	4	126004812	C	T	0.33	5.7×10^{-7}	0.47	*FAT4*[a]
rs9920270	Psel_PAR4 low	15	78666288	T	C	0.42	8.2×10^{-7}	0.54	*CRABP1*[a]; *WDR61*[b]; *DNAJA4*[b]; *AC090607.1*[b]
Loci associated with platelet count									
rs9287349	Platelet count	2	143952778	T	C	0.29	6.4×10^{-7}	−0.19	*ARHGAP15*[a,b]; *AC013437.6*[b]
Loci associated with mean platelet volume									
rs2028785	MPV	10	114347161	C	T	0.16	3.0×10^{-7}	0.08	*VTI1A*[a]; *ACSL5*[b]
rs12955310	MPV	18	8216480	A	G	0.11	6.2×10^{-7}	0.09	*PTPRM*[a,b]

This table details the genome-wide association study results of platelet measurements in the Tanzanians. "Psel" is the membrane expression of P-selectin and "Fibr" is the binding of fibrinogen to platelets.

ADP, adenosine diphosphate; β, regression coefficient; bp, base pair; Chr, chromosome; MAF, minor allele frequency; MPV, mean platelet volume; SNP, single-nucleotide polymorphism;.

[a]Genes in close proximity to platelet reactivity–associated single-nucleotide polymorphisms.

[b]Expression quantitative trait loci effect of platelet reactivity trait–associated single-nucleotide polymorphisms based on the publicly available database.

Especially, the A allele of the rs773902 SNP, which encodes a threonine instead of an alanine residue at 120 protein position, was associated with increased PAR4-stimulated platelet aggregation. The allele frequency of this SNP was subsequently shown to vary widely across Sub-Saharan Africa [21]. So far, no studies in populations on the African continent have related rs773902 or other SNPs in *F2RL3* to PAR4-induced reactivity. In our cohort, indeed, the frequency of the A allele of rs773902 SNP was high at 60.9%, and this variant was

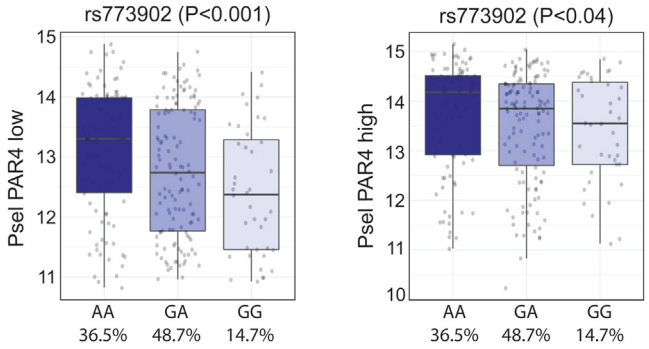

FIGURE 5 The frequency distribution of the A and G alleles of rs773902 single-nucleotide polymorphism and its association with platelet reactivity to both PAR4 low and high doses. Differences in platelet reactivity between different alleles were analyzed using a linear regression model, correcting for age and sex. Rs773902 (A = Thr120 and G = Ala120).

positively associated with platelet reactivity to both PAR4_low ($P < .001$) and PAR4_high ($P = .04$) (Figure 5) and also with PAR1_high ($P = .04$). The intronic rs773904 SNP in the same gene was also associated with PAR4_low ($P < .001$). We could not detect the rs2227346 SNP, which was previously suggested to abolish the enhanced PAR4 responses associated with the PAR4 Thr120 variant [20]. Instead, we identified 3 other SNPs in *F2RL3*—rs2227360 (a noncoding transcript exon variant), rs2227343 (a 5' untranslated region variant), and rs773895 (an intergenic variant)— that showed suggestive associations with platelet reactivity to the PAR4 agonist ($P < .05$).

Next, we tested whether SNPs that were associated ($P < 1 \times 10^{-6}$) with at least 1 platelet parameter in our cohort were also associated in the 500 functional genomics cohort, a HFGP cohort of approximately 500 individuals of Western European ancestry [34]. We were not able to validate any of these SNPs in the 500 functional genomics cohort, arguing for effects specific for African populations, a specificity in line with previous observations for cytokine production capacity [48]. However, it should be acknowledged that PAR1 and PAR4 stimuli were not included in the platelet stimulation in this cohort.

To further validate these results, we chose SNPs that were associated ($P < 1 \times 10^{-6}$) with at least 1 platelet parameter in our cohort and performed a replication analysis using the FHS. Details on platelet function measurements in the FHS cohort and replication of GWAS signals are given in the Supplementary Methods. This replication study comprised the following samples: FHS European ancestry samples (European family–based), OMNI samples (multiethnic), African American samples from OMNI, and FHS European-OMNI merged samples. A total of 23 independent SNPs were selected for replication analysis. Fourteen SNPs were replicated with at least 1 trait across 4 FHS cohorts (Supplementary Figure S7), with the majority of validated SNPs belonging to the African American cohort. It should be noted that this cohort also lacked any PAR1- or PAR4-trait stimulation for this replication effort.

Collectively, our findings suggest that platelet reactivity is controlled by a set of yet-to-be-identified genes that vary remarkably between populations, and pose the need for further investigation into genetic regulators of platelet reactivity in bigger cohorts.

4 | DISCUSSION

In this study, we assessed the nongenetic and genetic drivers of platelet reactivity in a cohort of healthy adults living in Tanzania. Our findings suggest that dietary habits—and especially the intake of products with plant-derived compounds with platelet-modulatory effects—are among the most important drivers of platelet reactivity in East Africa. Our present data also support the concept that genetic regulation of platelet reactivity varies considerably across populations and we confirm the importance of PAR4 gene polymorphisms in this East African population. Finally, we report associations of platelet reactivity with age and with plasma cytokines and inflammatory markers, particularly with AAT and IL-1β.

To the best of our knowledge, this is the first study that investigated comprehensively the genetic and environmental determinants of platelet reactivity in people living in Sub-Saharan Africa. Many communities in Africa are rapidly adopting a more globalized lifestyle, including a more sedentary lifestyle and a change in diet from a traditional, largely plant-based diet to a diet rich in processed foods, refined sugars, and animal fats. The traditional diets in Kilimanjaro districts contain large quantities of phytochemicals that inhibit platelet reactivity. In our present study, untargeted plasma metabolomics identified a large number of such plant-derived compounds, including flavonoids, that were associated with a reduced platelet reactivity. Dietary flavonoids have long been appreciated in reducing CVD risk factors [37], and we speculate that part of this effect is mediated by their effect on platelets. In addition, the gut microbiome may also produce platelet-modulating metabolites such as histamine [49].

We have previously reported in the same cohort that dietary variation is an important determinant of thrombin and plasmin generation and inflammation and host cytokine responses [18,23]. Our present findings offer further support that the gradual abandoning of the traditional diet for a more globalized diet may have important public health consequences, including a further increase in NCDs.

In addition, this study is the first GWAS of agonist-induced platelet reactivity in a population on the African continent. Prior studies have shown that platelet reactivity is highly heritable [7,50]. We have recently shown that the genetic architecture of cytokine responses and thrombin generation in this Tanzanian population share little overlap with that of a Western European population [18,48]. Our current findings are in line with this clear geographical distribution of the genetic factors influencing platelet function in East Africa and Europe, showing that important differences also exist in the genetic architecture of platelet reactivity in individuals of East African ancestry, including variation in the PAR4 gene (*F2RL3*). We were able to validate that the PAR4 Thr120 variant is common in this East African population and is associated with increased PAR4-induced reactivity. Several other variants in the *F2RL3* gene were also identified, as well as new variants associated with other platelet traits. Africa hosts the highest level of genetic diversity in the world, and our present findings illustrate the need for studying common genetic variation in platelet reactivity across populations in Sub-Saharan Africa.

Platelet reactivity and inflammation are intricately linked, but AAT was the only inflammatory protein that was positively associated with platelet reactivity. In contrast, circulating IL-1β concentrations were inversely associated with platelet activation. This is consistent with our previous findings in healthy West European adults that AAT concentrations were positively related to platelet reactivity as well, whereas inflammatory cytokines were not [34]. AAT is an acute phase protein with strong anti-inflammatory and immunomodulatory properties independent of elastase inhibition [51]. AAT has been shown to induce IL-1Ra expression [52,53] and to suppress lipopolysaccharide-induced IL-1β, IL-8, and TNF-α [51]. Whether the association of AAT with platelet reactivity in our study is truly causal remains unknown, also because earlier studies suggested that AAT inhibits platelets [54,55]. Overall, our data indicate that inflammatory cytokines such as IL-1β and adipokines have only a limited influence on platelet reactivity in healthy individuals in East Africa.

Previous studies have related platelet hyperreactivity to classical cardiovascular risk factors, including aging, BMI, use of alcohol or tobacco, and high blood pressure [7,56–58]. In our study, only aging was identified as a predictor of platelet reactivity. Environmental factors that are more specific for low-income regions, such as the use of smoky fuel or the use of pit latrines, were not associated with platelet reactivity. Household air pollution due to biomass fuel is considered a risk factor for cardiovascular events [59], and our present findings are in contrast to a prior study in Indian women that reported that chronic exposure to biomass smoke induces platelet activation and formation of platelet–leukocyte aggregates [60]. We also did not observe a difference in platelet reactivity across urban and rural living individuals. The demarcation between an urban and rural lifestyle is not distinctly delineated; rather, urban-rural living follows a gradual continuum. This gradual distinction may explain our findings of no discernible variance in platelet reactivity between urban and rural dwellers, as well as the absence of clear differences in plasmatic coagulation [18] or plasma inflammatory proteome [61]. In contrast, a significant difference between urban and rural living was observed in whole blood cytokine production and gene transcription [23]. In addition, our finding that BMI was not associated with platelet reactivity underscores the complexity of the relationship between BMI, inflammation, and platelet reactivity. Our previous research involving healthy Western European individuals also found no significant correlation between BMI and platelet reactivity, albeit with a limited number of overweight individuals [34]. In contrast, other studies have observed higher platelet activation or reactivity in overweight people or people living with obesity [62,63]. The effect of BMI on platelet reactivity may also vary among different populations, and factors such as differences in body fat distribution could underlie a weaker effect of BMI on platelets within this East African population. Moreover, it is important to note that the study participants were all in good health, with those having conditions like hypertension or high blood glucose levels being excluded. As a result, even though there was a noted correlation between BMI and inflammatory cytokines, it is possible that the inflammation level did not reach a significant threshold to trigger hyperreactivity in platelets. Furthermore, the platelet reactivity assay used might not have had the sensitivity required to detect subtle differences in platelet function.

A limitation of our observational study is the fact that the observed associations do not imply causality. Nonetheless, many of the food-derived metabolites identified in this study were previously reported to modulate platelet activation in *ex vivo* studies. Dietary intervention studies are required to better define the effects of abandoning the traditional African diet on platelet reactivity. In addition, the sample size of our cohort was small for GWAS analyses. The validation using other cohorts was also hampered by differences in the techniques used to assess platelet function, the used platelet agonists (especially PAR4 agonists), and the fact that cohorts of populations on the African continent are not available. The overall study is also limited to interpretation of the agonist pathways tested as data from other pathways of activation, such as collagen, thromboxanes, and von Willebrand factor, were not collected.

In conclusion, the findings of this study provide novel insights into the drivers of variation in platelet reactivity in healthy subjects living in a rapidly transitioning community in East Africa. It highlights the importance of geographic ancestry and the gradual abandoning of the traditional East African diet.

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ETHICS STATEMENT

The study was performed according to the principles of the Declaration of Helsinki. All participants provided written informed consent. The 300 functional genomics study was approved by the Ethical Committees of Kilimanjaro Christian Medical University College, the National Institute of Medical Research in Tanzania (NIMR/HQ/R.8a/Vol.IX/2290), and the Ethical committee of the Radboud University Medical Center Nijmegen (CMO Arnhem-Nijmegen [CMO:2016-2657]) in the Netherlands.

AUTHOR CONTRIBUTIONS

Conceptualization and study design, Q.d.M., A.v.d.V., M.G.N., V.I.K., G.S.T., and B.T.M. Recruitment, study data collection, and laboratory analysis, G.S.T., V.I.K., J.N., and F.L. Data analysis and interpretation, V.I.K., G.S.T., F.T., M.-H.C., N.V., C.K.B., T.P., B.B.N., Q.d.M., A.v.d.V., and A.D.J. Writing of the original draft, V.I.K and Q.d.M. All authors contributed to the review of the final manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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