The genetics of normal platelet reactivity

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Genetic and environmental factors contribute to a substantial variation in platelet function seen among normal persons. Candidate gene association studies represent a valiant effort to define the genetic component in an era where genetic tools were limited, but the single nucleotide polymorphisms identified in those studies need to be validated by more objective, comprehensive approaches, such as genome-wide association studies (GWASs) of quantitative functional traits in much larger cohorts of more carefully selected normal subjects. During the past year, platelet count and mean platelet volume, which indirectly affect platelet function, were the subjects of GWAS. The majority of the GWAS signals were located to noncoding regions, a consistent outcome of all GWAS to date, suggesting a major role for mechanisms that alter phenotype at the level of transcription or posttranscriptional modifications. Of 15 quantitative trait loci associated with mean platelet volume and platelet count, one located at 12q24 is also a risk locus for coronary artery disease. In most cases, the effect sizes of individual quantitative trait loci are admittedly small, but the results of these studies have led to new insight into regulators of hematopoiesis and megakaryopoiesis that would otherwise be unapparent and difficult to define. (*Blood*. 2010;116(15):2627-2634)

Introduction

Interindividual platelet responsiveness to a variety of agonists is highly variable, as documented in several studies of large cohorts of normal persons.[1-12] At the same time, these and other studies of siblings, twins, and families with a history of coronary artery disease (CAD) have documented that intraindividual responsiveness is highly reproducible over time, regardless of the agonist tested or the chosen method of assessment. These findings strongly suggest that there is a high level of heritability of platelet function, and this has prompted numerous attempts to define the genetic basis for platelet function variability.

A summary of platelet function

To fully appreciate the gene association studies, it is necessary to briefly summarize key aspects of platelet function and establish a molecular and physiologic basis for the selection of genes to study.

When a blood vessel is damaged, circulating platelets interact with components of the extracellular matrix, particularly collagen, and a complex series of receptor-ligand interactions ensues that ultimately leads to the formation of a stable platelet plug or thrombus. This process is a continuum of at least 3 phases that we can describe as initiation, extension, and consolidation, each of which entails the cooperation of a different group of receptors.

In the initiation phase, plasma von Willebrand factor (VWF) binds to collagen via its A3 domain and becomes structurally altered such that its A1 domain then binds to the platelet membrane receptor glycoprotein Ib-IX-V complex (GPIb complex). It is the GPIbα or larger subunit of GPIb that makes direct contact with VWF. This association is a requisite step in the adhesion of platelets to exposed thrombogenic surfaces at sites of vessel wall injury or in regions of atherosclerotic plaque rupture. Concurrently, a more stable platelet monolayer is formed on the collagen surface mediated predominantly by the platelet-specific receptor glycoprotein VI (GPVI) and platelet integrin $\alpha_2\beta_1$.

The engagement of these receptors enhances platelet activation leading to the extension phase, mediated largely by the conversion of prothrombin to thrombin at the activated platelet surface and the secretion of active compounds from platelet granules (α-granules and δ-granules), which can further stimulate platelets. One of these, adenosine diphosphate (ADP), plays a particularly important role in the platelet response, binding to its cognate platelet receptors, the purinogenic receptors P2Y1 or P2Y12, to augment platelet activation. The activated platelet also produces and/or releases additional agonists, including the agonist thromboxane A2 (TXA2), which then binds to the platelet TXA2 receptor. Most of the receptors involved in the events of the extension phase are members of the G-protein–coupled receptor family.

Other agonists are at work during the extension phase, and each has the potential to initiate the involvement of additional platelets. Two prominent agonists are thrombin and epinephrine (EPI); thrombin (also known as factor II) binds to several receptors, but of particular interest to this discussion, thrombin binds the factor II receptor (F2R) that is also known as protease-activated receptor-1. EPI binds to the platelet α-adrenergic receptor 2A (A2AR). EPI can contribute to the initiation phase and, in low doses, is thought to prime platelets for enhanced activation by other agonists. The A2AR is a G-protein–coupled receptor and thus activates heterotrimeric G-proteins, including those containing the β3 subunit (GNB3).[13] Alternative splicing variants of GNB3 appear to enhance G-protein signal transduction[14,15] and could thus alter the effect of EPI on platelets. Thrombin makes a particularly relevant contribution to the augmentation of platelet activation during the extension phase because the activated platelet surface is a nidus for prothrombin conversion. Nonetheless, both thrombin and EPI, as

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well as ADP, are capable of binding to and activating the naive platelet in an alternative initiation phase.

In the consolidation phase, platelet-platelet cohesion (aggregation), mediated by the binding of fibrinogen and/or VWF to the activated platelet integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb-IIIa), together with the assembly of a fibrin network, results in the generation of platelet-rich aggregates or thrombi. Further complexity is inherent in this final phase of platelet plug formation, and current research indicates an essential role for outside-in signaling through integrins and via receptor tyrosine kinases, including members of the Eph kinase family.

Candidate gene association studies

To this point in time, most attempts to identify genetic differences that influence platelet function in normal persons relied on a candidate gene approach. Target genes were rationally selected as candidates (Table 1) based on their established or logical involvement in any of the important molecular events that contribute to normal platelet function.

Most of the candidate gene association studies have focused on platelet surface receptors, for example, those involved in (1) fibrinogen-mediated platelet aggregation via integrin $\alpha IIb\beta3$[16-20]; (2) collagen-induced platelet adhesion and signal transduction via integrin $\alpha2\beta1$[21,22] or GPVI[23,24]; (3) VWF-mediated platelet adhesion via GPIbα[25-28]; or (4) platelet signaling induced by various physiologic agonists.[3-5,9,29]

Many of these gene association studies were limited by small cohort sizes and/or a poor selection of single nucleotide polymorphisms (SNPs). The most appropriate SNP choices, or tagSNPs, are those that take into consideration linkage and reflect haplotype definitions.[30] However, only a few of the foregoing studies used well-defined tagSNPs. Consequently, although the reported associations for *ITGA2* C807T (rs1126643),[22] *GP1BA* T-5C,[27,31] and the *GP6* haplotype b[23] have been replicated, others have not been as reproducible.[32,33]

Genes for the following proteins and their representative SNPs have been included in various gene association studies to assess variation in platelet function among normal persons (Table 1):

1. α-Adrenergic receptor 2A (*ADRA2A* G1780A)[29];
2. *F2R* (A15992T)[3];
3. Immunoglobulin-γ Fc receptor IIA (FCRG2A H131R)[34];
4. GNB3 (C825T)[6];
5. GPIbα (GP1BA T-5C)[27,35];
6. GPVI (GP6 A13039G)[23];
7. Integrin subunit α2 (platelet GPIa) (*ITGA2* C807T,[36,37] C-52T,[38] and G1648A)[39];
8. Integrin subunit αIIb (platelet GPIIb) (*ITGA2B* I843S)[40];
9. Integrin subunit β3 (platelet GPIIIa) (*ITGB3* T196C)[41];
10. ADP receptor P2Y1 (*P2RY1* C893T)[42];
11. ADP receptor P2Y12 (*P2RY12* G52T)[4];
12. Prostaglandin G/H synthase 1 (cyclooxygenase-1) (*PTGS1* C50T)[43]; and
13. TXA2 receptor (*TBXA2R* C795T or C924T or G1686A).[9]

The accuracy of an association study will depend on several factors, and 4 that are considered most relevant are selected here for discussion.

First, the selection of an accurate, reproducible, and sufficiently vetted measure of platelet function is probably the most critical decision. Several assays have been selected that fulfill these criteria, including light transmission aggregometry (LTA),[6] the Platelet Function Analyzer-100 (PFA-100; Siemens Diagnostics),[44] and the measurement of platelet activation events by flow cytometry,[2] but each is used in association studies under carefully considered conditions that may not be identical to those used in the routine clinical laboratory.

Second, cohort sizes must be sufficiently large to provide adequate power to the statistical analyses. Unless adequate cohort sizes are studied, the chance of false-positive association results will increase, particularly when an SNP with a low minor allele frequency (MAF; eg, < 0.2) is studied. Two examples are the SNPs rs5918 and rs6065, which underlie the platelet alloantigen systems HPA-1 and HPA-2, respectively. Initial studies using inadequate cohort sizes concluded that these were novel risk factors for CAD, but a subsequent meta-analysis, with combined cohort sizes, has not confirmed these conclusions.[45]

A third factor that has been largely ignored until recent studies is the ethnic homogeneity of the cohorts. It is now well documented that the presence of "ethnic outliers" between genotype groups can produce false positives if the MAF differs substantially between ethnic groups.[46]

A fourth factor is appropriate SNP selection. Often, a single SNP does not represent the underlying sequence variation within a

Table 1. Candidate gene SNPs used in association studies

Gene	Protein identification	dbSNP or chromosomal location*	Cytoband	MAF
ADRA2A	α-2A-adrenergic receptor	chr10: 112 839 580†	10q24-q26	0.41
F2R	Coagulation factor II (thrombin) receptor; proteinase-activated receptor 1 (PAR-1)	rs168753	5q13	0.14
FCGR2A	IgG Fc receptor type IIa	chr1: 161 479 745‡	1q23	0.44
GNB3	Guanine nucleotide-binding protein beta-3 subunit variant	rs5443	12p13	0.45
GP1BA	Platelet glycoprotein Ib, α subunit	rs6065	17pter-p12	0.10
GP6	Platelet glycoprotein VI	rs1613662	19q13.4	0.16
ITGA2	Integrin subunit-α2	rs1126643 rs28095 rs1801106	5q11.2	0.38 0.36 0.08
ITGA2B	Integrin subunit-αIIb	rs5911	17q21.32	0.41
ITGB3	Integrin subunit-β3	rs5918	17q21.32	0.17
P2RY1	Purinogenic receptor P2Y1	rs1065776	3q25.2	0.05
P2RY12	Purinogenic receptor P2Y12	rs6809699	3q24-q25	0.14
PTGS1	Prostaglandin-endoperoxide synthase 1; cyclooxygenase-1 (COX-1)	rs3842787	9q32-q33.3	0.06
TBXA2R	Thromboxane A2 receptor	rs1131882 rs4523 rs5758	19p13.3	0.13 0.30 0.45

*NCBI SNP database (dbSNP) identification number (Build 131) or flanking nucleotide sequence (when SNP has not been assigned a dbSNP number).
†CATTCCCAACTCTCTCTCTCTTTTT**(G/A)**AAGAAAAATGCTAAGGGCAGCCCTG.
‡AATGGAAAATCCCAGAAATTCTCCC**(A/G)**TTTGGATCCCACCTTCTCCATCCCA.

locus that may result from several haplotype blocks. The careful selection of an adequate number of tagSNPs, as well as a certain degree of resequencing, can ensure that this underlying sequence variation is adequately represented.[2]

Candidate gene association based on LTA

Yee et al[7] have provided a useful paradigm whereby platelet hyperreactivity could be assessed using submaximal concentrations of EPI (0.4μM) or crosslinked collagen-related peptide (CRP-XL; 0.005 μg/mL) as agonists. Persons hyperreactive to one agonist are generally hyperreactive to others, and in the case of EPI-induced aggregation, enhanced reactivity is associated with female sex or higher plasma fibrinogen level. Day-to-day reproducibility of hyperreactivity to EPI under these conditions was very high, probably because of the use of such low agonist concentrations, and could be replicated over an extended period of time (> 2 years).

A substantial minority (14%) exhibited the hyperreactive phenotype (ie, ≥ 60% platelet aggregation induced by 0.04μM EPI). The levels of GPIbα, αIIbβ3, α2β1, or FcγRIIa were not different between the normal and hyperreactive cohorts, after adjustment for sex, fibrinogen level, platelet count, and mean platelet volume (MPV). A2AR mediates platelet activation by EPI,[47,48] yet no association ($P = .654$) was observed between hyperreactivity and the *ADRA2A* G1838A polymorphism, previously linked to variation in EPI-mediated platelet aggregation in blacks.[49] An explanation for the negative findings of Yee et al[6] may be that the study did not take into account ethnic heterogeneity. On the other hand, hyperreactivity was associated with C825T of *GNB3* (rs5443; MAF = 0.45), the gene for the β3 subunit of G-proteins, with a modest degree of statistical significance ($P = .03$).

The major weakness of platelet function testing in previous studies has always been that there is not one ex vivo assay that accurately predicts or reflects the functionality of the same platelet sample in vivo. Thus, a noteworthy finding of Yee et al[6] is that, with the low-dose, EPI defined hyperreactivity by LTA identified persons with a generalized hyperreactivity that was reflected by diverse forms of platelet stimulation and different aspects of platelet function. Thus, hyperreactivity was manifested in LTA using different agonists, in the PFA-100 apparatus, wherein thrombus formation under high shear is induced with collagen plus EPI (CEPI) or collagen plus ADP, and by direct assay of activation events (P-selectin expression or binding of the activation-dependent, αIIbβ3-specific, murine monoclonal antibody PAC-1) using flow cytometry. Hyperreactivity was also consistently observed either in whole blood or platelet-rich plasma and using different anticoagulants, such as sodium citrate or D-phenylalanyl-L-prolyl-arginine chloromethyl ketone.

Yabe et al[29] subsequently established that the *ADRA2A* SNP A1780G (MAF = 0.41) was associated with CEPI CT in the PFA-100, within a cohort of 211 Japanese males. The 1780AA genotype was associated with a shorter CT (ie, platelet hyperreactivity). Even though the SNP designations are different in Yabe et el[29] and Yee et al,[6] it is probable that these represent the same SNPs because the flanking sequence of the major allele in each case (TTTAAA) represents a DraI restriction site. According to the current dbSNP database (Build 131), this polymorphism is located at chr10:112,839,580 but has not been named.

Kondkar et al[50] used an unbiased RNA expression approach to identify genes that are up-regulated or down-regulated in platelets exhibiting hyperresponsiveness to low-dose EPI, as defined by LTA. A total of 290 genes were differentially expressed between subjects of differing platelet reactivity, with generally moderate or small effect sizes, suggesting that several genes contribute to this phenotype. One of the mRNA species that was differentially up-regulated in hyperresponsive platelets is that coding for vesicle-associated membrane protein 8/endobrevin, a member of a group of proteins known as v-SNAREs. This made perfect sense because platelet aggregation in response to EPI is absolutely dependent on the release of granule contents, and release is facilitated by the interaction of integral membrane proteins of the platelet plasma membrane and granule membrane, collectively known as t-SNAREs and v-SNAREs, respectively.[51] Previous studies by others have shown that modulation of platelet SNAREs affects platelet function in vitro.[52]

Candidate gene association based on the PFA-100

The PFA-100 measures platelet function initiated by exposure to collagen and ADP or EPI under high shear stress in citrated whole blood and is uniquely sensitive to genetic differences that modulate the initial stages of platelet activation, namely, those that are involved in the VWF-dependent adhesion of platelets to collagen and the enhanced adhesion mediated by GPVI and integrin α2β1.[53,54] Harrison et al[44] were the first to show that the PFA-100 is capable of detecting platelet hyperfunction, which is influenced by platelet count, hematocrit, and most significantly plasma VWF antigen level.[55-57]

In a cohort of 123 healthy subjects, we measured the combined effects of plasma VWF level, platelet count, hematocrit, sex, and 7 candidate gene polymorphisms on baseline platelet reactivity in the PFA-100.[58] This study was, at the time, the largest gene association study of PFA-100 reactivity in normal donors and confirmed that the PFA-100 can be used to compare normal donors based on their responsiveness to collagen combined with EPI or ADP. We compared the alleles of 7 candidate genes: *ITGA2, ITGB3*, *GP1BA*, *GP6*, *P2RY1, P2RY12*, and *PTGS1* (Table 1).[58] Based on linear and logistic regression models, we found an inverse correlation between baseline CEPI CT and plasma VWF antigen level, *ITGA2* 807T and *P2RY1* 893C. On the other hand, we observed an inverse correlation between baseline collagen plus ADP CT and *P2RY1* 893C or *GP1BA*-5C.

Candidate gene association using a functional genomics approach

Whereas the preceding studies focused on surface receptor genes, others encoding relevant proteins in signaling cascades downstream of these receptors were not studied. The availability of high-throughput, cost-effective genomic sequencing enabled a more objective study of larger cohort sizes and the simultaneous evaluation of many more SNPs.

Through the comparison of a relatively large number of SNPs (n = 1327) in a selected group of several candidate genes (n = 97), Jones et al[2] recently identified 17 independently associated SNPs that account for 48% of the variation in platelet reactivity induced by either ADP or CRP-XL. The success of this study reflects a careful attention to study design that establishes a paradigm for future genomic studies:

Table 2. Loci and genes associated with platelet hyperreactivity as determined by flow cytometric measurement of platelet function and a functional genomics approach

Gene	Protein function	SNP*	Cytoband	Functional trait			
				PA	FA	PC	FC
GP6	Platelet glycoprotein VI	rs1613662	19q13.4	√	—	√	√
FCER1G	Adapter protein for agonist receptor	rs3557	1q23.3	—	—	√	√
PEAR1	Platelet surface receptor PEAR1	rs3737224	1q23.1	—	—	√	√
		rs11264579		√	√	—	—
RAF1	Protein kinase	rs3729931	3p25.1		√	√	√
JAK2	Protein kinase	rs10429491	9p24.1	√	√	—	—
P2RY12	Purinogenic (ADP) receptor P2Y12	rs1472122	3q25.1	√	√	√	—
GNAZ	Adapter protein for agonist receptor	rs3788337	22q11.22	√	—	—	—
VAV3	Intracellular signaling molecule	rs17229705	1p13.3	√	√	√	—
CD36	Platelet surface receptor	rs1049654	7q21.11	—	—	√	√
MAP2K2	Protein kinase	rs350916	19p13.3	—	√	—	—
ITGA2	Integrin subunit-α2	rs41305896	5q11.2	√	√	—	—
AKT2	Protein kinase	rs41275750	19q13.2			√	√
MAPK14	Protein kinase	rs851007	6p21.31	√	√	√	—
MAP2K4	Protein kinase	rs41307923	17p12	—	—	√	√
ITPR1	Intracellular signaling molecule	rs17786144	3p26.2	√	√	—	—

PA indicates P-selectin expression in response to ADP; FA, fibrinogen binding in response to ADP; PC, P-selectin expression in response to CRP-XL; and FC, fibrinogen binding in response to CRP-XL.

Data from Jones et al[2] with permission.

*SNP exhibiting lowest *P* value.

1. The first consideration was cohort selection. The Platelet Function Cohort was established as a group of 500 healthy persons with a similar Northern European ancestry.[8]
2. Second, markers of platelet function were selected. Platelet function was compared by measuring fibrinogen binding to activated αIIbβ3, the requisite step for aggregation, and the expression of P-selectin, a marker of platelet degranulation, after induction with either the GPVI agonist, CRP-XL, or ADP. These agonists represent 2 distinct signaling pathways. GPVI signaling is an early event that acts via an FcRγ/ITAM pathway, and ADP amplifies platelet activation in later stages through the G protein-coupled receptors, P2Y1 and P2Y12.
3. Marker selection. Based on a priori knowledge of the protein components of each of the signaling cascades, 97 candidate genes were selected. The exons of these genes were resequenced in 48 reference DNA samples from the Center d'Etude du Polymorphism Humain European Cohort to maximize the database of sequence variation, leading to the identification of 1327 SNPs that were then genotyped in the Platelet Function Cohort samples.

This association study confirmed that *GP6* is a strong quantitative trait locus (QTL) for platelet response to CRP-XL, but the success of this study lay in the analysis of a large number of genes each exerting a small effect. Using a *P* value less than .005 as a cut-off, to overcome the errors inherent in multiple testing, 68 SNPs encompassed within 15 genes showed an association with the platelet response to CRP-XL, ADP, or both (Table 2).

Variation in *GP6*, as anticipated, accounts for up to 40% of the variation in GPVI signaling.[8] We have determined that the common minor allele of GPVI is associated with decreased Fyn/Lyn binding and enhanced CaM binding to the cytoplasmic tail.[24]

For the remaining genes, 18 independent associations in 15 loci were identified. These included the cell surface receptor genes *CD36*, *GP6*, *ITGA2*, *PEAR1*, and *P2RY12*, adapter proteins *FCERG1* and *GNAZ*, kinases *JAK2*, *MA2K2*, *MAP2K4*, and *MAPK14*, and the intracellular signaling molecules *ITPR1* and *VAV3*.

Of note, an association was not found between *ITGB3* and any of the indices of platelet response. There is a great deal of conflicting evidence in the literature regarding *ITGB3* rs5918, which defines the HPA-1 system.[16-19,32,33] One possible explanation is that the MAF is low (0.15), and the majority of studies were severely underpowered by small cohort sizes. In addition, the diversity of functional assays and platelet agonists used in these studies certainly contributed to the discrepant findings.

Additional major findings resulted from this study. First, *PEAR1* was identified as a regulator of both CRP-XL and ADP signaling, with 2 distinct associations observed. One SNP, rs41299597, was associated with CRP-XL induced pathways, whereas the more common SNP rs11264579 was associated with the ADP pathway. Second, rs170414 in *ITPR1*, which encodes the IP3 receptor, was associated exclusively with the ADP signaling pathway. Lastly, rs17229705 in *VAV3* was associated with P-selectin expression only in response to ADP.

GWAS

At this time, a genome-wide association study (GWAS) using a direct measure of platelet function has not been performed. However, certain quantitative properties of platelets have been reported to significantly influence outcomes in cardiovascular disease and stroke, and these can represent indirect measures of platelet function in normal persons. Examples are mean MPV and platelet count.

MPV is a highly heritable quantitative trait in humans,[59] and the genetic component accounts for approximately 51% of the variation in MPV in a large primate study.[60] MPV was shown to be an independent predictor of negative outcome in CAD and ischemic stroke in 4 of 6 studies,[61-65] and a positive correlation was confirmed in a recent meta-analysis.[66] A biologic explanation for this association with MPV rests on the hypothesis that platelets with increased MPV are more active than smaller platelets, with a greater prothrombotic potential because of higher levels of intracellular TXA2 and an increased procoagulant surface.[67] However, this has been disputed recently in a large cohort of normal persons, where a negative correlation was observed between

Table 3. Loci and genes associated with MPV and platelet count determined by GWAS

Trait/gene	Protein	SNP	Position	Cytoband
MPV				
DNM3	Dynamin-3	rs10914144	170 216 373	1q24.3
TPM1	Tropomyosin α-1 chain	rs11071720	61 129 049	15q22.1
BET1L	Unknown function	rs11602954	192 856	11p15.5
ARHGEF3	Rho guanine nucleotide exchange factor 3	rs12485738	56 840 816	3p21-p13
TMCC2	Unknown function	rs1668873	203 502 613	1q32.1
TAOK1	Serine/threonine-protein kinase TAO1	rs2138852	24 727 475	17q11.2
JMJD1C	JmjC domain-containing histone demethylation protein 2C	rs2393967	64 803 162	10q21.2-q21.3
PIK3CG	Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit γ-isoform	rs342293	106 159 455	7q22.3
SIRPA	CD172a, tyrosine-protein phosphatase nonreceptor type substrate 1	rs6136489	1 871 734	20p13
EHD3	EH-domain containing protein 3	rs647316	31 318 333	2p21
WDR66	WD repeat-containing protein 66	rs7961894	120 849 966	12q24.31
CD226	CD226 antigen	rs893001	65 667 825	18q22.3
PLT				
ATXN2	Unknown function	rs11065987	110 556 807	12q24
SH2B3	SH2B adapter protein 3	rs3184504		12q24
PTPN11	Tyrosine-protein phosphatase nonreceptor type 11	rs11066301	111 355 755	12q24
BAK1	Bcl-2 homologous antagonist/killer function	rs210135	33 648 670	6p21.3
AK3	Adenylate kinase 3	rs385893	4 753 176	9p24.1-p24.3

Data from Meisinger et al[70] and Soranzo et al[68] with permission.

MPV and 2 markers of platelet activation, binding of fibrinogen and annexin V.[68]

Nonetheless, a progression of 3 collaborative European-based GWAS on the effect of MPV has provided relevant information.[68-70] These investigations incorporated subjects from the United Kingdom Blood Services Common Control (UKBS-CC1 and -CC2), the TwinsUK cohort, the Kooperative Gesundheitsforschung in der Region Augsburg (KORA) F3, F4, and S4 cohorts, the Study of Health in Pomerania cohort, and the Cambridge BioResource.

In one study, Soranzo et al[68] analyzed Affymetrix 500K Gene Chip data in the UKBS-CC1 (discovery n = 1221) and replicated these findings in TwinsUK (n = 1050), KORA (n = 1601), UKBS-CC2 (n = 1304), and Cambridge BioResource (n = 3410). This study identified one locus on chromosome 7q22.3 as the first locus to be associated with MPV. The strongest association signal was located at rs342293 within an intergenic region (Table 3). The most proximal hypothetical gene *FLJ36031* is located at a distance of approximately 71 kb, and 5 additional genes are located within a 1-MB interval encompassing rs342293. Intriguingly, all 6 genes are transcribed in megakaryocytes (MKs), *PIK3CG* is robustly transcribed in MKs but not in erythroblasts, and transcripts of 2 of the 6 genes, *PIK3CG* and *PRKAR2B*, are detectable at high levels in platelet RNA.

The minor G allele at rs342293 (MAF = 0.45) is associated with increased MPV ($P = 1.08 \times 10^{-24}$) and with decreased platelet reactivity, based on the level of fibrinogen binding and the proportion of platelets binding annexin V, induced by CRP-XL and measured by flow cytometry. The negative correlation between the minor allele and the proportion of platelets binding annexin V appears to contradict the positive correlation between platelet volume and thrombogenicity, which had been proposed earlier.[67] However, this single association between MPV and annexin V binding, with incorrect directionality, explains only 1.5% of the observed population variation in MPV and therefore does not represent disproof of the probable positive correlation between MPV and prothrombotic tendency.

Both *PICK3CG* and *PRKAR2B* are plausible candidates for genes that would affect MPV and platelet function. *PICK3CG* encodes the γ-chain of PI3/PI4-kinase, responsible for the synthesis of phosphatidylinositol-3,4,5-trisphosphate,[71] which is a key component in the initiation of megakaryopoiesis and proplatelet formation and an essential intermediary in collagen-induced regulation of phospholipase C in megakaryocytes and platelets.[72] PI3K-deficient mice display impaired ADP-induced platelet aggregation and are protected from ADP-induced platelet-dependent thromboembolic vascular occlusion.[73] *PRKAR2B* encodes the β-chain of cAMP-dependent protein kinase, which attenuates the release of intracellular calcium by phosphorylation of ITPR3, the receptor for phosphatidylinositol-3,4,5-trisphosphate,[74,75] and would thus have an antagonsitic effect on megakaryopoiesis and proplatelet formation.

In another GWAS, Meisinger et al[70] evaluated Affymetrix 500K Gene Chip data in KORA F3 (discovery n = 1644) and replicated these findings in the UKBC-CC1 (n = 1203), KORA S4 (n = 4261), and Study of Health in Pomerania (n = 3300) cohorts. They found that MPV is strongly associated with 3 common SNPs in 3 other loci (Table 3): rs7961894 located within intron 3 of *WDR66* on chromosome 12q24.31, rs12485738 upstream of the *ARHGEF3* on chromosome 3p13-p21, and rs2138852 located upstream of *TAOK1* on chromosome 17q11.2. These SNPs had *P* values of 7.24×10^{-48} for rs7961894, 3.81×10^{-27} for rs12485738, and 7.19×10^{-28} for rs2138852.

Each of these 3 genes is also a plausible candidate gene involved in regulation of MPV. In the case of *WDR66*, previous studies have shown that WD-repeat proteins are involved in the regulation of various cellular functions, cell-cycle regulation, and apoptosis.[76] Moreover, in the study of Meisinger et al,[70] expression analysis indicated a direct correlation of WDR66 transcripts and MPV. *ARHGEF3* encodes the rho guanine-nucleotide exchange factor 3 (RhoGEF3), which activates RhoGTPases, which play an important role in the regulation of cell morphology, cell aggregation, cytoskeletal rearrangements, and transcriptional activation.[77,78] *TAOK1* encodes the TAO kinase 1 peptide (hTAOK1, also known as MARKK or PSK2), a microtubule affinity-regulating kinase important in regulation of mitotic progression, chromosome congression, and checkpoint-induced anaphase delay.[79]

The 7q22.3 locus discovered by Soranzo et al[68] explains 1.5% of the variance in MPV attributable to genetic factors, and the 3 QTL identified by together account for only 4% to 5% of the variance in MPV. Clearly, MPV is regulated by the cumulative

effects of many more genes, so it was not unexpected that additional QTLs might be discovered that are associated with this quantitative trait. The discovery sample sizes in these 2 GWAS (n ~ 1200 in Soranzo et al[68] and ~ 1600 in Meisinger et al[70]) are relatively modest. As noted by Weedon et al[80] in a GWAS of adult height, the power for the discovery of QTLs is very limited in samples sizes less than 10 000.

The third study, a meta-analysis by Soranzo et al,[69] analyzed larger numbers of persons, from essentially the same population cohorts. Eighty-eight SNPs that showed a nominal association with MPV in the discovery phase (n ~ 4600) were taken forward in a replication study of additional healthy subjects (n ~ 9300). This identified 12 QTLs for MPV, including the aforementioned 4 and 8 new loci (Table 3). In addition, they reported the first 3 loci associated with platelet count. Nine of the 12 MPV loci were also associated with platelet count, and in all cases, those alleles that were associated with an increase in MPV were associated with a decrease in platelet count. Overall, the fraction of genetic variance explained by each locus in regression models adjusted for sex and age was 8.6% for MPV traits and 0.5% for platelet count traits.

Two candidate genes within the newly identified MPV-associated loci have a known role in MK development and are expressed at higher levels in MK relative to erythroblasts: *DNM3* on chromosome 1q24.3 and *CD226* on 18q22.3.[81,82] Four of the other loci map in or near 4 genes that are indirectly involved in hematopoiesis in humans: jumonji gene *JMJDIC*, which encodes a probable histone demethylase[83]; *TPM1*, which encodes tropomyosin I[84]; *SIRPA*, which encodes a protein that is involved in cell adhesion[85]; and *EHD3*, which encodes a mediator of protein transport and endocytosis.[86]

Three other independent loci contain genes with a known influence on platelet count: *BAK1* within the 6p21.3 locus encodes a proapoptotic protein that controls platelet life span[87]; the locus at 12q24.12 harbors *PTPN11*, *SH2B3*, and *BRAP*,[88,89] which play a regulatory role in a wide array of cell-signaling events important in MK and other cells; and the locus at 9p24.1-p24.3 may regulate the transcription of the proximal gene *JAK2*, a key regulator of MK maturation, but this remains to be proved.[90]

The possibility always exists that heterogeneity between population cohorts can obscure real association signals or even create false positives in GWAS that include moderate samples sizes (eg, n < 10 000). For population-based studies of quantitative traits, discovery sample sizes in excess of 20 000 are often needed. Nonetheless, the findings made in a statistically robust meta-analysis with adequate power should confirm the authenticity of true associations, and this was apparently the case in this series of studies culminating in the meta-analysis of Soranzo et al.[69]

Concluding remarks

In conclusion, it must be acknowledged that none of the SNPs claimed to be associated with platelet function in normal subjects has been proven to be risk loci for CAD.[91-94] However, of the 15 QTLs associated with MPV and platelet count, one located at 12q24 is also a risk locus for CAD.[69]

In the case of MPV and platelet count, the majority of the GWAS signals are in noncoding regions, supporting the contention that the principle mechanisms by which sequence variation alters phenotype are at the level of transcription or splicing events. This has been a major outcome of all GWAS to date.[95]

The effect sizes of individual QTL are small, and this might lead one to question the value in conducting such massive GWAS of platelet function. But the results of those studies already completed have led to new insight into regulators of hematopoiesis and megakaryopoiesis that would otherwise remained unexplored. It also represents a means to identify *cis*-acting regulatory motifs, of which the chr7 MPV-QTL is an excellent example. In addition, platelet function QTL studies have identified key regulators of signaling pathways. Further meta-analysis of MPV and platelet count in tens of thousands of subjects will obviously increase the number of MPV/platelet count QTLs, and the completion of the ongoing 1000 genomes initiative (tens of thousands by 2014) will serve to identify rare coding and noncoding variants. Some of these may exert a large effect on platelet phenotypes.

We caution the reader that knowledge of platelet QTLs for function, MPV, or platelet count should not be used in clinical practice to infer risk of thrombotic or bleeding events.

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