J Mol Med (2016) 94:13–19
DOI 10.1007/s00109-015-1346-3

JMolMed CrossMark

REVIEW

RAP1-GTPase signaling and platelet function

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Received: 31 August 2015 / Revised: 10 September 2015 / Accepted: 17 September 2015 / Published online: 1 October 2015

Abstract Platelets are critical for hemostasis, i.e., the body's ability to prevent blood loss at sites of vascular injury. They patrol the vasculature in a quiescent, non-adhesive state for approximately 10 days, after which they are removed from circulation by phagocytic cells of the reticulo-endothelial system. At sites of vascular injury, they promptly shift to an activated, adhesive state required for the formation of a hemostatic plug. The small GTPase RAP1 is a critical regulator of platelet adhesiveness. Our recent studies demonstrate that the antagonistic balance between the RAP1 regulators, CalDAG-GEFI and RASA3, is critical for the modulation of platelet adhesiveness, both in circulation and at sites of vascular injury. The RAP1 activator CalDAG-GEFI responds to small changes in the cytoplasmic calcium concentration and thus provides sensitivity and speed to the activation response, essential for efficient platelet adhesion under conditions of hemodynamic shear stress. The RAP1 inhibitor RASA3 ensures that circulating platelets remain quiescent by restraining CalDAG-GEFI-dependent RAP1 activation. Upon cellular stimulation, it is turned off by P2Y12 signaling to enable sustained RAP1 activation, required for the formation of a stable hemostatic plug. This review will summarize important studies that elucidated the signaling pathways that control RAP1 activation in platelets.

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Keywords Platelets · GTPase · Signaling · RAP1 · Thrombosis

Introduction

Platelets are highly specialized blood cells evolved to secure the integrity of the cardiovascular system over a broad range of hemodynamic shear conditions [1]. Inhibitory signaling pathways ensure that platelets remain in a quiescent (non-adhesive) state as long as the endothelial lining is physically and biochemically intact. At sites of vascular injury, platelets employ immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors and G protein-coupled receptors (GPCRs) to sense and respond to changes in their environment, such as the exposure of extracellular matrix (ECM) proteins and the activation of the coagulation system. Stimulation of these receptors triggers intracellular signaling cascades [2], including those dependent on elevated cytosolic calcium (Ca^{2+}), which promote dramatic cytoskeletal changes, the secretion of granules, and, most importantly, the conversion of integrins from a low- to a high-affinity state for their ligands (integrin inside-out activation) [3]. Integrins are the main platelet receptors that support platelet-matrix (platelet adhesion) and platelet-platelet interactions (platelet aggregation). αIIbβ3 integrin is by far the most abundant of the β1 integrins and β3 integrins expressed on the platelet surface. It facilitates the binding of various plasma proteins, including fibrinogen and von Willebrand factor (VWF), and it is crucial for platelet adhesion and aggregation. The formation of a stable hemostatic plug or a pathological thrombus requires sustained integrin inside-out activation, provided by co-stimulatory signaling via the autocrine/paracrine agonists thromboxane $(Tx)A_2$ and ADP [4, 5]. ADP is released from dense granules and supports sustained integrin activation by binding to the Gi-coupled receptor, P2Y12, the target of currently used anti-platelet drugs

[6]. Studies by us and others identified a critical role for the small GTPase RAP1B in platelet activation and integrin-mediated cellular adhesion. This review will discuss how RAP1B and its known regulators, CalDAG-GEFI and RASA3, ensure that platelet integrin activation is rapid, sustained, and tightly controlled.

RAP1 GTPases and platelet activation

Approximately 8 % of the known proteins expressed in platelets are small GTPases and their regulators [7, 8]. The most abundant GTPases in platelets are two isoforms of the Ras-related protein (RAP) subfamily, RAP1B (~300,000 copies/platelet) and RAP1A (~125,000 copies/platelet). Like other small GTPases of the Ras superfamily, RAP proteins are molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. Two classes of regulatory proteins control this switch. Guanine nucleotide exchange factors (GEFs) promote the activation by stimulating the exchange of GDP for GTP, and GTPase-activating proteins (GAPs) terminate the activation by catalyzing GTP hydrolysis [9].

In platelets, GTP-loading of RAP1 is stimulated by all known agonists [10, 11]. Upon engagement of agonist receptors, RAP1 translocates from the cytosolic leaflet of intracellular granules, where it is sequestered in resting platelets, to the plasma membrane [12, 13]. Activated RAP1 regulates multiple functional responses in platelets, most notably integrin activation [14, 15]. Genetic deletion in mice of the predominant RAP1 isoform, RAP1B, or inactivation of the main pathways leading to RAP1 activation markedly impaired integrin inside-out [15, 16] and outside-in [17, 18] signaling, granule secretion [18, 19], TxA_2 generation [20], spreading [18, 19], and clot retraction [18, 19]. Consistent with the defective platelet activation response, these mice exhibited significantly prolonged bleeding times and a strong protection from experimental thrombosis [15, 21].

CalDAG-GEFI: a critical RAP-GEF and accelerator of platelet activation

Pharmacological and genetic studies at the turn of the century demonstrated that two kinetically distinct pathways regulate RAP1 activation in platelets. Rapid RAP1 activation is triggered by an increase in intracellular Ca^{2+} concentrations [10], while sustained RAP1 activation requires signaling by protein kinase C (PKC) [22], the Gi-coupled receptor for ADP, P2Y12, and phosphatidylinositol 3-kinases (PI3K) [23–25]. The molecular nature of the GEFs and GAPs regulating RAP1 activity in platelets, however, remained elusive.

The work by Shattil and colleagues was the first to suggest an important role for the calcium-sensing GEF, CalDAG-GEFI (*Rasgrp2*), in RAP1 activity regulation in platelets. Using ES cell-derived megakaryocytes, they demonstrated a good correlation between the expression level of this candidate protein and integrin activation in these cells [26]. Shortly after these pioneering studies, we used *Caldaggef1*-knockout mice to establish a fundamental role for CalDAG-GEFI in Ca^{2+}-dependent RAP1 activation in platelets [27]. Platelets lacking CalDAG-GEFI exhibited a marked aggregation defect to various agonists, including ADP and collagen, while a more robust aggregation response was observed in response to stimulation with thrombin. A very similar aggregation profile was recently described for human platelets isolated from patients with severe bleeding due to a point mutation in the catalytic GEF domain of CalDAG-GEFI [28].

CalDAG-GEFI is the predominant platelet RAP-GEF and the only member of the RASGRP family to be expressed in the platelet/megakaryocytic lineage (Fig. 1) [7, 29]. RASGRPs typically consist of a N-terminal catalytic GEF domain and a C-terminal regulatory region comprising a pair of EF hand domains and a C1 domain (Fig. 2). However, the catalytic specificity and the activity regulation of CalDAG-GEFI are the most divergent within this family, as CalDAG-GEFI has RAP, but not RAS, exchange activity in vivo [30], and structural rearrangements induced by the binding of Ca^{2+} to the EF domains are critical for its activation [31]. The EF hand domains have very high affinity for Ca^{2+} (K_D~80 nM) [31] and thus provide remarkable sensitivity towards minor changes in the cytoplasmic Ca^{2+} concentration, which in resting platelets was measured at ~20–50 nM. The C1 domain, however, is insensitive to the second messenger diacylglycerol (DAG) [32, 33], but it contributes to optimal CalDAG-GEFI-dependent RAP1 activation by a hitherto unknown mechanism [21]. Platelets from *Caldaggef1*−/− mice [27] or from patients expressing an inactive CalDAG-GEFI mutant [28] (a) failed to aggregate in response to calcium ionophore, while they reacted to the DAG mimetic PMA (phorbol 12-myristate 13-acetate); (b) did not respond to threshold doses of physiological agonists (lack of sensitivity); and (c) exhibited a delayed activation response to high doses of strong agonists (lack of speed). Rapid agonist-induced elevation in cytosolic Ca^{2+} concentrations is what drives near-immediate platelet activation, which is essential for efficient platelet adhesion in the presence of hemodynamic shear forces. Thus, platelets lacking the sensitivity and speed provided by CalDAG-GEFI failed to form three-dimensional thrombi particularly at arterial shear rates, and *Caldaggef1*−/− mice were strongly protected from $FeCl_3$-induced carotid artery thrombosis [21] and collagen- [27] or immune complex-induced [34] pulmonary embolism. However, a defective CalDAG-GEFI-dependent pathway does not prevent platelet activation completely. Both mouse and human platelets lacking functional CalDAG-GEFI could undergo a slow but sustained activation that supported the formation of small three-dimensional thrombi at conditions of low shear flow

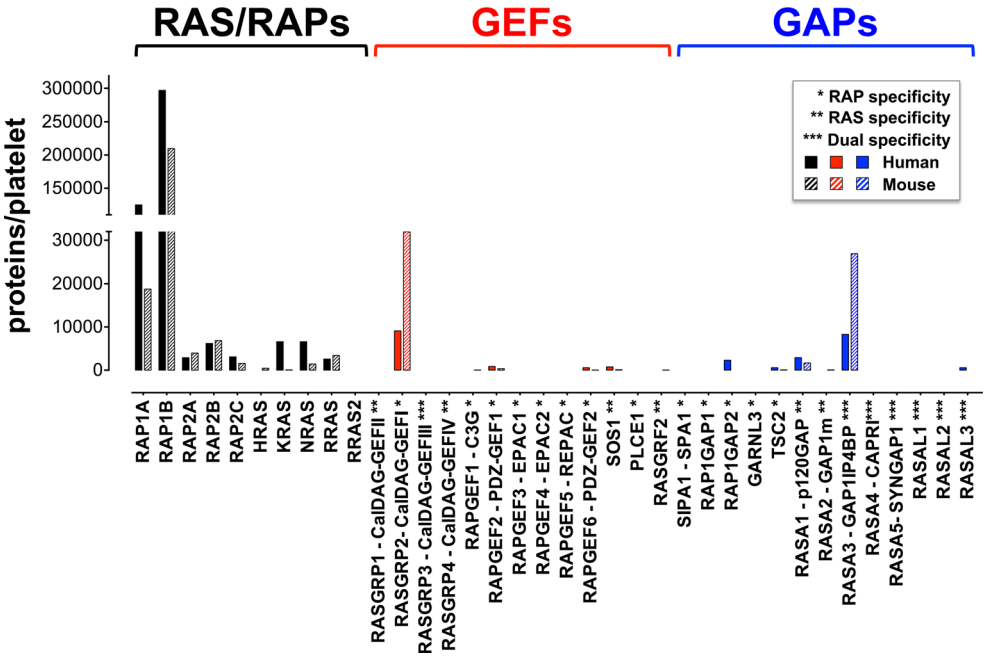

Fig. 1 RAS/RAP, GEF, and GAP protein expression in platelets. Estimated protein levels based on the most comprehensive quantitative proteomic analysis of human [7] (*full bars*) and mouse [29] (*striped bars*) platelets. CalDAG-GEFI and RASA3 are the most abundant RAP regulators expressed in both human and mouse platelets

conditions, such as those found in the venous system [21]. This residual RAP1 activation was inhibited by antagonists of PKC or P2Y12. An important element of this CalDAG-GEFI-independent pathway was discovered very recently (see below).

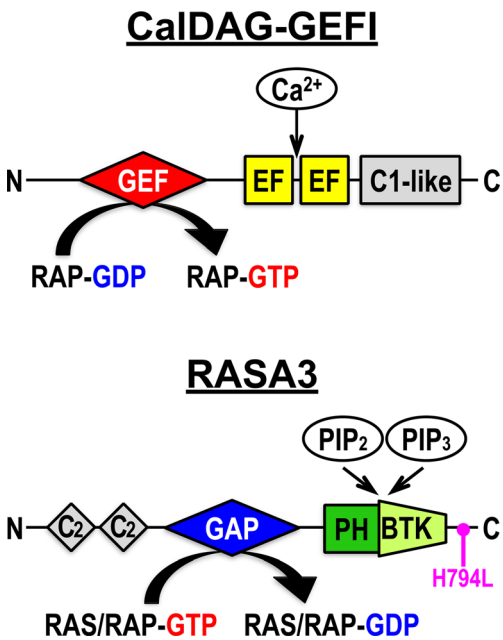

Fig. 2 Domain structure of the RAP1 regulators, CalDAG-GEFI and RASA3. *CalDAG-GEFI* consists of a RAP-specific catalytic guanine nucleotide exchange factor (GEF) domain, a pair of Ca^{2+}-binding EF hand domains (EF), and a C1-like domain of hitherto unknown function that is necessary for optimal RAP-GEF activity. *RASA3* consists of a catalytic GTPase-activating protein (GAP) domain specific for RAS and RAP, flanked by a pair of C2 domains and by a PH/Btk domain that binds phosphatidylinositol 4,5-diphosphate (PIP_2) and phosphatidylinositol 3,4,5-triphosphate (PIP_3). The *Rasa3*^{hlb} mutant mouse strain is characterized by a single point mutation (H794L) in the C-terminal region of RASA3. *GTP* guanosine triphosphate, *GDP* guanosine diphosphate

The RAP-GAP RASA3: a critical inhibitor of platelet activation and the missing link in the P2Y12-RAP1 signaling pathway

Important information on the molecular nature of this missing RAP1 regulator came from unbiased studies of the platelet proteome and transcriptome [7, 29, 35, 36]. These studies demonstrated that the most abundant RAP-GAP in platelets (Fig. 1) is a protein called RASA3 (also known as GAP1^{IP4BP}, GAPIII, R-Ras GAP). RASA3 was originally purified from the plasma membrane of human platelets in 1994 [37], but its role in platelet biology was elucidated only recently with studies in transgenic mouse models [38].

RASA3 belongs to a subfamily [39] of four GAP1 proteins (GAP1^{m}/RASA2, RASA3, RASAL1, and CAPRI/RASA4) structurally characterized by a GAP domain flanked N-terminally by two tandem C2 domains and C-terminally by a pleckstrin homology domain linked to a 26-amino acid Btk motif (PH/Btk) (Fig. 2). The catalytic domain is a conventional RAS-GAP domain, but it is capable of stimulating the GTPase activity of both RAS- and RAP-GTPases depending on the cellular context [40]. This catalytic plasticity is mediated by the N- and C-terminal flanking regions, which orient the RAP-GTP catalytic residues in a way that allows RAP to hijack the RAS-GAP catalytic machinery [41, 42]. The C2 domains of RASA3 are non-canonical and thus do not bind Ca^{2+} or phospholipids [43]. They have currently no other

known function besides their essential role in support of the RAP-GAP activity. On the contrary, the PH/Btk domain, in addition to its steric role in the catalytic reaction, is crucial for RASA3 plasma membrane localization and is presumed to dynamically regulate the GAP activity in response to cellular stimulation [44].

The PH/Btk domain of RASA3 is unique as it binds with similarly high affinity to phosphatidylinositol 4,5-diphosphate (PIP_2) (K_D=0.8±0.5 μM) and phosphatidylinositol 3,4,5-triphosphate (PIP_3) (K_D=0.5±0.2 μM). PIP_2 is abundant in the plasma membrane of both resting and activated cells and mediates RASA3 constitutive localization to this membrane compartment [44]. PIP_3 is the catalytic product of PI3K-mediated phosphorylation of PIP_2. PIP_3 can drive plasma membrane localization of an engineered RASA3 unable to bind PIP_2 [45]. It is not yet clear if and how PIP_3 regulates RASA3 GAP activity. Since RASA3 binds with high affinity also the water-soluble cognate head group of PIP_3, inositol 1,3,4,5-tetrakisphosphate (IP_4), it has been argued that its GAP activity may be regulated by a competition between the binding of IP_4 and membrane phosphoinositides [46]. However, RASA3 has been shown to localize exclusively to the plasma membrane, even in conditions where IP_4 concentrations are high [44]. Thus, the most likely regulatory mechanism of RASA3 involves PIP_3 and does not require membrane detachment. Recent studies, employing two-color super-resolution imaging, have shown that PIP_2 and PIP_3 cluster into distinct membrane microdomains [47]. Future studies need to address if PIP_2/PIP_3-mediated regulation of RASA3 involves compartmentalization into different subregions of the plasma membrane and/or conformational changes of the protein.

To investigate its role in platelet biology, we generated mice with deletion of *Rasa3* in the platelet/megakaryocyte lineage [38]. Unexpectedly, these mice exhibited a high embryonic/neonatal lethality, similar to that observed in germ line-knockout mice [38] or mice expressing a catalytically inactive mutant of the protein (*Rasa3*$^{\Delta GAP}$) [48]. Thus, while studies in the *Rasa3*$^{\Delta GAP}$ mice suggested that the high lethality might be caused by a defect in vascular cells, our studies favor a critical role for RASA3 in platelets/megakaryocytes during embryonic development. Consistent with this conclusion, we found that Rasa3-knockout mice are severely thrombocytopenic and that knockout embryos exhibit a marked vascular mixing phenotype, similar to that observed in other mouse models of severe thrombocytopenia or impaired platelet function [49]. To understand the cause of the thrombocytopenia and the role of RASA3 in platelets, our group employed a mutant mouse strain (*Rasa3*^{hlb}) derived from a forward genetic screen in C57BL/6 mice, which is

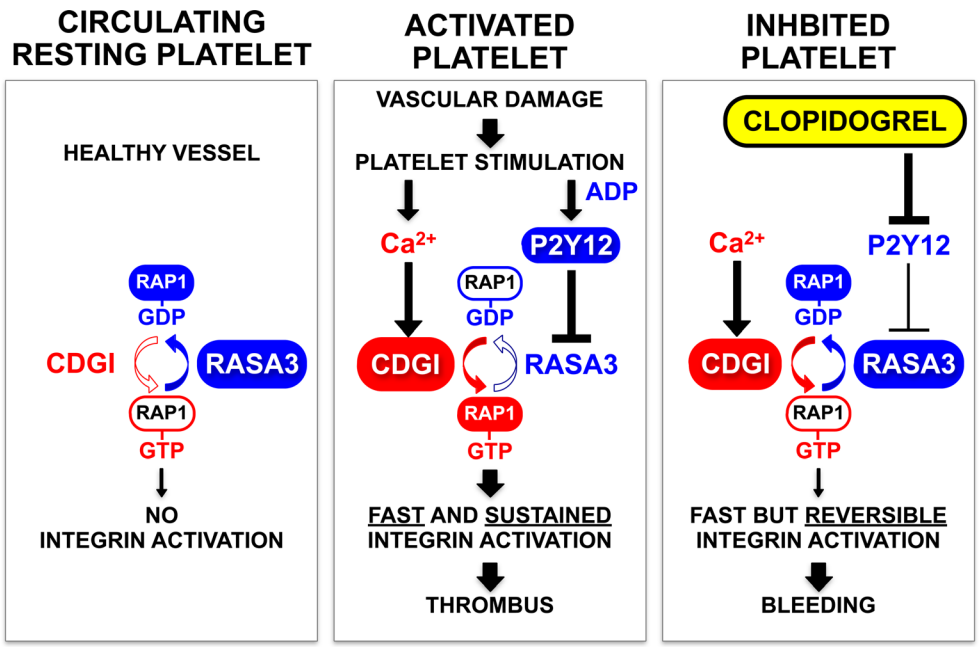

Fig. 3 Schematic model of RAP1-dependent platelet regulation. *Left panel*: In platelets circulating healthy vessels, RASA3 is active to restrain uncontrolled RAP1 activation and maintain a quiescent, non-adhesive state (no integrin activation). *Central panel*: At sites of vascular injury, platelet stimulation results in an increase in the cytosolic Ca^{2+} levels and the release of ADP from dense granules. Ca^{2+} activates CalDAG-GEFI, which mediates rapid GTP loading of RAP1. ADP stimulates the P2Y12 receptor, which leads to decreased RASA3 function and sustained RAP1 signaling. Fast and sustained RAP1/integrin activation results in the formation of stable three-dimensional thrombi over a broad range of hemodynamic shear conditions. *Right panel*: Inhibitors to P2Y12, such as clopidogrel, prevent the inactivation of RASA3, prohibit sustained RAP1 signaling, and destabilize the growing thrombus

characterized by a single point mutation (H794L) in the C-terminal region of RASA3 [38]. Platelets from *Rasa3*^{hlb}-homozygous mice exhibit a markedly reduced RASA3 protein level and a hypomorphic phenotype, characterized by Mendelian birth rates and a severe, but incomplete, thrombocytopenia ($29\pm18\times10^3$ platelets/μl). Compared to controls, the few *Rasa3*^{hlb} mutant platelets present in circulation were younger and bigger in size, had a short half-life, and, consistently with the role of RASA3 as a RAP-GAP, had elevated RAP1 and integrin activation both at baseline and in stimulated conditions. The latter observation prompted us to cross *Rasa3*^{hlb} mice with mice lacking CalDAG-GEFI, a major regulator of RAP1 activation in platelets. Deletion of CalDAG-GEFI reverted the platelet phenotype in *Rasa3*^{hlb} mice, including the pre-activated state, the high turnover, and the severe thrombocytopenia. In contrast to studies in *Rasa3*$^{\Delta GAP}$ mice [50], we did not find any gross abnormalities in the morphology of MKs and in their ability to form pro-platelets.

The studies outlined above suggest that RASA3 is critical to maintain circulating platelets in a quiescent, non-adhesive state. At sites of vascular injury, however, this negative feedback would be a liability for platelets during hemostatic plug formation. Thus, we speculated that RASA3 activity is down-regulated during platelet activation and that the required signal is provided by the CalDAG-GEFI-independent, PKC/P2Y12-dependent signaling pathway (see above). Consistent with this hypothesis, we observed that *Rasa3* mutant platelets do not require feedback activation via the P2Y12 pathway, and that their ability to aggregate is not affected by inhibitors of P2Y12 [38]. Interestingly, RAP1-dependent integrin activation in *Rasa3* mutant platelets was also insensitive to inhibitors of PI3K [38], suggesting that conversion from PIP_2 to PIP_3, mediated by PI3K, is critical in the activity regulation of this important RAP-GAP.

Model of RAP1-dependent platelet regulation

In summary, these studies demonstrate that a tight regulation of the antagonistic balance between the RAP1 regulators, CalDAG-GEFI and RASA3, is critical for both the prevention of premature platelet activation in circulation as well as for hemostatic plug formation at sites of vascular injury. In platelets that patrol healthy vessels, active RASA3 in the plasma membrane is required to antagonize the highly sensitive CalDAG-GEFI/RAP1 signaling module and to maintain the cells in a quiescent state (Fig. 3, left panel). Upon vascular injury (Fig. 3, central panel), platelet stimulation via GPCRs and ITAM-coupled receptors results in increased levels of cytosolic Ca^{2+} and the release of ADP from storage granules. CalDAG-GEFI is activated by small changes in the cytosolic Ca^{2+} concentration, leading to the rapid activation of RAP1 and αIIbβ3 integrin. CalDAG-GEFI signaling, however, is reversible and returns to baseline when cytosolic Ca^{2+} levels normalize. Thus, to sustain integrin activation and to facilitate hemostatic plug formation, RASA3 activity must be reduced as part of the activation response. The required signal is provided upon ADP engagement of the P2Y12 receptor and signaling by PI3K. Finally, our studies suggest that inhibitors of P2Y12, such as clopidogrel bisulfate, protect from thrombosis and impair hemostasis primarily by preventing the inactivation of RASA3 and thus prohibiting sustained RAP1 signaling (Fig. 3, right panel).

There are many unanswered questions concerning RAP1 signaling in platelets. For example, it will be interesting to see if and how platelet function is affected by RAP GEFs and GAPs other than CalDAG-GEFI and RASA3, which are expressed at low levels in these cells. Important information is also missing with regard to the activity regulation of CalDAG-GEFI and RASA3 by engagement of their regulatory domains or posttranslational modifications within these proteins. As for RAP itself, it will be interesting to determine if there are functional redundancies as well as unique contributions of the various RAP isoforms to platelet activation, and which protein effectors link RAP to its downstream platelet responses, especially talin-dependent integrin activation.

In addition to these more fundamental questions, we need more studies on the clinical relevance of alterations in platelet RAP1 signaling. For example, some inherited disorders of platelet function or number may, at least in part, be explained by gene variations in key players of the RAP pathway. Platelet hypo- or hyper-reactivity observed among individuals or as a result of certain disease states may be the result of inter-individual or acquired variability in the antagonistic balance between CalDAG-GEFI and RASA3 (expression and/or activity). If successful, these studies could pave the way to novel and more personalized approaches to anti-platelet therapy.

Acknowledgments This work was supported by the American Heart Association (14EIA18910004) and NIH grants R01 HL121650 and P01 HL120846.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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