Implication de la voie de l'AMP cyclique plaquettaire dans la modulation de l'inflammation

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Implication de la voie de l'AMP cyclique plaquettaire dans la modulation de l'inflammation

Application à l'étude de l'inhibition du récepteur P2Y12 au cours du sepsis et de l'inflammation liée aux circulations extracorporelles

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Abréviations

ADP: adénosine diphosphate

ALP: amas leuco-plaquettaire

AMPc: adénosine mono-phosphate cyclique

ATP: adénosine triphosphate

CCR: chemokine receptor

CD62P : P-Sélectine

CEC: circulation extracorporelle

RSPO: recuperation sanguine peropératoire

CLP: Cecal Ligation and Puncture

COVID-19: coronavirus disease 2019

CRP: C-reactive protein

CS: cecal slurry

DAMP: Damage Associated Molecular Pattern

ECMO: extracorporeal membrane oxygenation

FVW: facteur von Willebrand

GMPc: Guanosine monophosphate cyclique

IL: interleukine

LPS: lipopolysaccharide

NET: neutrophil extracellular trap

OCS: Organ Care System

PAMP: Pathogen Associated Molecular Pattern

PAR: Protease-activated receptor

PDE: phosphodiestérase

PF4: Platelet-factor 4

PRR: Pattern recognition receptor

SARS-CoV2: severe acute respiratory syndrome coronavirus 2

SOFA: Sequential Organ Failure Assessment score

TLR: Toll-like receptor

TNF: tumor necrosis factor

TRALI: Transfusion-related acute lung injury

TXA2: thromboxane A2

Avant-Propos

Le travail de thèse d'Université présenté dans ce manuscrit s'inscrit pleinement dans le parcours hospitalier et universitaire d'Anesthésie-Réanimation cardiothoracique et vasculaire que je poursuis. Les plaquettes sanguines, par leur importance cruciale dans l'hémostase, la thrombose et la gestion périopératoire du risque hémorragique, font l'objet de nombreux travaux cliniques et fondamentaux. De même, l'inflammation occupe une place majeure dans les processus physiopathologiques qui affectent nos patients, qu'ils soient infectieux ou liés à l'utilisation de suppléances d'organe et de circulations extracorporelles.

Néanmoins, l'implication des plaquettes dans la pathogénie du sepsis et des états inflammatoires liés à l'utilisation de circulations extracorporelles est à ce jour peu étudiée et non élucidée. En pratique clinique, le rôle fondamental joué par les plaquettes dans la modulation de la réponse inflammatoire reste mal connu, ce qui contraste avec l'importance des moyens pharmacologiques d'inhibition de l'activation plaquettaire à notre disposition.

Ce constat nous a permis de formuler au sein de l'équipe les deux hypothèses suivantes :

1) L'activation plaquettaire pourrait jouer un rôle dans la réponse inflammatoire aiguë observée en réanimation cardiothoracique et qui impacte le devenir des patients
2) L'inhibition de l'activation plaquettaire par la voie de l'AMP cyclique et en particulier par l'inhibition de $P2Y_{12}$ pourrait permettre de moduler cette réponse inflammatoire

Ce travail de thèse d'Université, conduit en parallèle de mon activité clinique en Anesthésie-Réanimation cardiothoracique et vasculaire, s'est ainsi construit autour de deux objectifs :

1) Mettre au point des modèles précliniques permettant d'étudier l'effet de l'inhibition de $P2Y_{12}$ sur la réponse inflammatoire aiguë, septique ou non
2) Mettre en évidence l'activation plaquettaire et son association avec la réponse dans des conditions cliniques de réanimation et leur association avec la réponse inflammatoire

Nous avons choisi de centrer ce travail sur l'inhibition du récepteur $P2Y_{12}$ du fait de la disponibilité en pratique clinique d'inhibiteurs pharmacologiques, permettant d'envisager secondairement des applications cliniques en anesthésie et réanimation.

Après une introduction permettant de comprendre le rôle inflammatoire des plaquettes et l'implication de la voie du $P2Y_{12}$ dans la régulation de l'activation plaquettaire, ce travail s'articule autour de deux parties.

La première partie rapporte le travail collaboratif conduit à l'Université de Rennes 1 et à l'Université de Paris, au sein de l'unité INSERM UMR_S 1085 IRSET (Professeur Olivier Fardel, Docteur Valérie Lecureur-Rolland) et de l'unité INSERM UMR_S1140 IThEM (Professeur Pascale Gaussem, Docteur Christilla Bachelot-Loza). La mise au point et l'application de deux modèles précliniques y sont développées, ainsi que la mise au point à Rennes de la technique de mesure des amas leuco-plaquettaires. Ces travaux sont toujours en cours au sein de l'IRSET (encadrement d'un étudiant en Master 1 puis en Master 2, en cours) et feront l'objet d'une publication.

La seconde partie rapporte les résultats de trois travaux ayant pour objectif d'évaluer l'activation plaquettaire dans des conditions cliniques de réanimation et leur association avec la réponse inflammatoire. Ces travaux ont fait l'objet de trois articles (deux acceptés, un soumis).

Enfin, l'Annexe comporte trois articles (deux publiés, un en cours de révision) portant sur deux travaux de l'unité INSERM UMR_S1140 IThem auxquels j'ai participé, ainsi que sur l'évaluation de l'hémostase dans la situation clinique de forte réponse inflammatoire des patients atteints de formes sévères de COVID-19 sous circulation extracorporelle

Introduction

Physiologie de l'inflammation aiguë : une vue d'ensemble

L'inflammation répond historiquement à une définition clinique, décrite depuis l'antiquité, associant rougeur, chaleur, douleur, œdème et perte de fonction[1]. Même si cette définition persiste de nos jours, les connaissances sur l'origine évolutive, la physiologie et l'impact pathologique de l'inflammation sont aujourd'hui extrêmement importantes. L'inflammation peut être définie comme un ensemble de processus physiologiques complexes et adaptatifs, hautement conservés au cours de l'évolution, survenant en réponse à une agression exogène ou un processus pathologique endogène, et ayant pour finalité la défense de l'organisme, la restauration d'un état d'homéostasie et l'initiation de la réparation tissulaire[2,3]. Les mécanismes inflammatoires sont non seulement impliqués dans la genèse de pathologies aiguës (sepsis, syndrome de déstresse respiratoire aiguë, défaillance d'organes post-traumatique ou périopératoire), mais également dans la physiopathologie de maladies chroniques (cancer, athéromatose, dépression, neuropathies dégénératives) et dans la régulation physiologique des principales fonctions de tissus et organes[3–5]. On distingue classiquement l'inflammation aiguë de la réponse inflammatoire chronique d'une part, et de la phase de résolution d'autre part[5]. Nous nous intéresserons dans la suite cet exposé uniquement à la phase inflammatoire aiguë et au rôle exercé par les plaquettes.

La réponse inflammatoire aiguë est initiée et régulée par des réseaux extrêmement complexes de médiateurs et d'effecteurs que l'on peut, dans un objectif de simplification, organiser en circuits inflammatoires selon le schéma suivant (Figure 1).

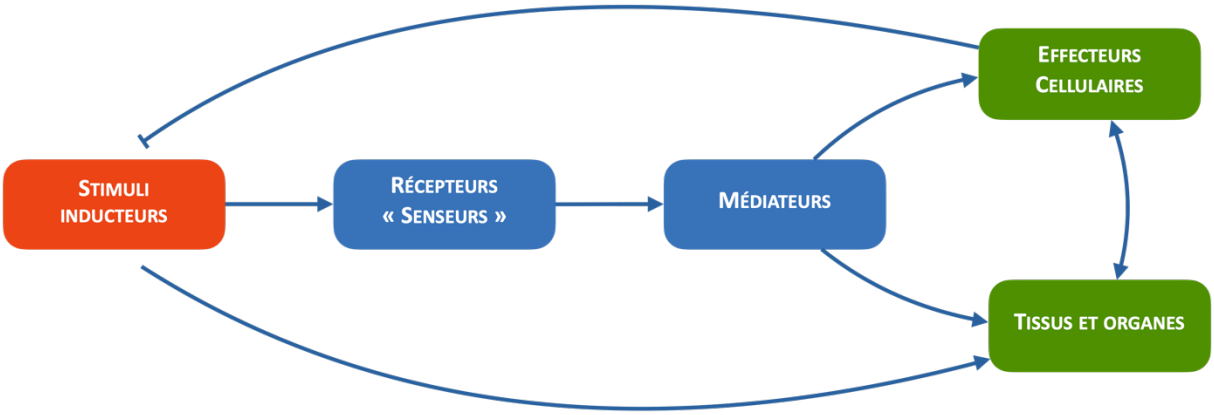

Figure 1. Schéma générique des circuits inflammatoires

(adapté de [3])

De manière générale, les signaux inducteurs sont reconnus par des récepteurs spécialisés dont l'activation entraîne la génération de médiateurs, qui à leur tour modifient le comportement des organes et tissus et des cellules effectrices de l'inflammation. Il est important de souligner à ce stade que, sans préjuger du mécanisme inducteur, c'est l'intensité de la réponse inflammatoire et son caractère systémique qui conditionnera l'impact pour le patient en termes de dysfonctions d'organe et, in fine, son admission en Réanimation[1,6–8].

Les inducteurs de la réponse inflammatoire aiguë, très nombreux, peuvent être d'origine exogène ou endogène et sont fréquemment associés de manière concomitante ou séquentielle. Ils peuvent initier une réponse inflammatoire en étant reconnus par des récepteurs spécifiques, ou bien en induisant des perturbations tissulaires (perte de structure, perte de fonction, perte de régulation) qui à leur tour engendrent l'inflammation[3,9].

Les micro-organismes (bactéries, virus, parasites, champignons) forment une classe importante d'inducteurs exogènes principalement via les PAMPs (pathogen-associated molecular patterns). Ces molécules inductrices sont reconnues par le système immunitaire inné grâce à des récepteurs spécifiques ou PRRs (pattern-recognition receptors)[1–3,10]. Les microbes peuvent aussi induire une réponse inflammatoire de type rétrospective par l'action de leur facteurs de virulence (porines, lysines, lipases, hyaluronidases, …) sur les tissus. Les inducteurs exogènes non-microbiens incluent, en autres, les allergènes, les toxiques et irritants chimiques, les particules non métabolisables et les corps étrangers[2]. Enfin les inducteurs endogènes correspondent à des signaux produits ou libérés par les cellules et/ou la matrice extracellulaire, en réponse à un traumatisme, un stress (métabolique, hypoxique) ou à l'induction de l'apoptose. Ces inducteurs sont plus généralement regroupés sous la dénomination DAMPs (damage-associated molecular patterns)[5,11].

Parmi les nombreux PRRs décrits, la famille des TLRs (Toll-like receptors) occupe une place centrale, hautement conservée dans l'évolution[10,12]. Ces récepteurs de PAMPs/DAMPs sont exprimés sur un grand nombre de cellules effectrices immunitaires (macrophages, cellules dendritiques, lymphocytes) mais également non-immunes (plaquettes, fibroblastes, cellules épithéliales, cellules endothéliales)[12–15]. La reconnaissance des PAMPs/DAMPs par les TLRs peut ainsi induire des modifications cellulaires majeures, participant à la réponse inflammatoire, incluant en particulier des modulations transcriptionnelles, des modifications du métabolisme et du cytosquelette ainsi que l'activation cellulaire et la libération de DAMPs. $

L'interaction entre les LPS (lipopolysaccharides) des bactéries à Gram négatif et le TLR4 en est un parfait exemple, sur lequel repose la mise au point du modèle in vitro qui sera présenté au point I.2. La paroi des bactéries à Gram négatif est constituée d'une membrane plasmique, de

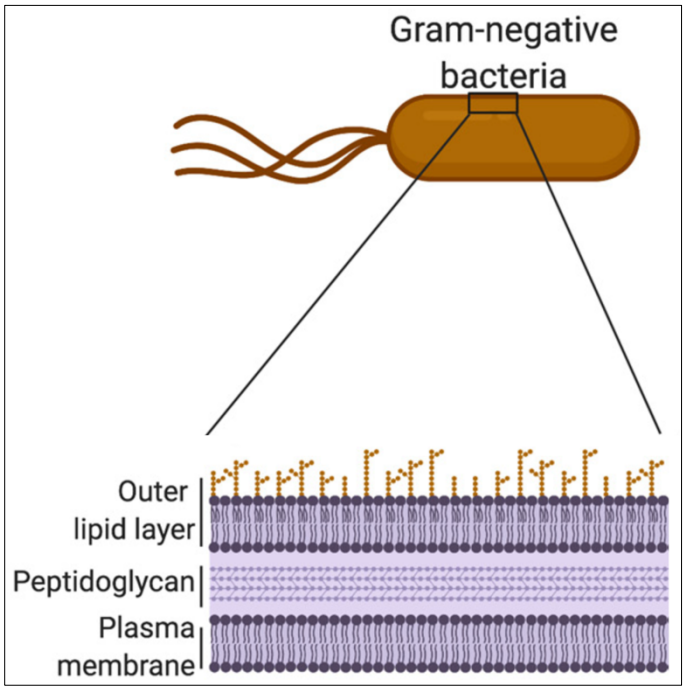

Figure 2. Structure de la membrane des bactéries à Gram négatif
La paroi des bactéries à Gram négatif est constituée d'une membrane plasmique, de peptidoglycanes et d'une membrane externe contenant des LPS à la face externe (adapté de [16]).

peptidoglycanes et d'une membrane externe contenant des LPS à la face externe (Figure 2). Le LPS comporte trois parties : la chaine O, le noyau et le lipide A[16]. Dans le milieu extracellulaire, le LPS est capté par la protéine LBP (LPS-binding protein), permettant de potentialiser son interaction avec CD14, une glycoprotéine exprimée notamment par les leucocytes, sous forme soluble (sCD14) ou membranaire [10,12,16]. Le complexe CD14/LPS peut ainsi se fixer à la membrane plasmatique et interagir avec TLR4 associé à son corécepteur MD-2 (Figure 3). La formation de ce complexe et son endocytose enclenchent deux voies de signalisation aboutissant notamment à l'activation des facteurs de transcription NF-κB, AP- 1 et IRF3, et à la production de cytokines pro-inflammatoires (IL-6, IL-1β, IFN-α, TNF-α, IL-8, MCP-1).

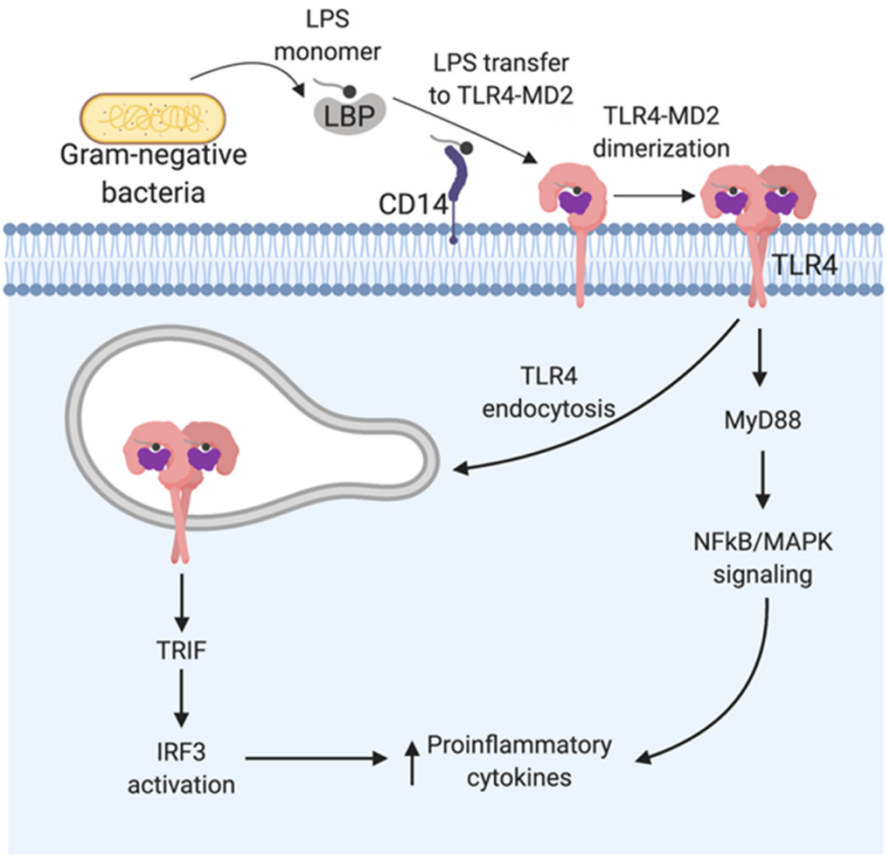

Figure 3. Liaison du LPS au TLR-4
Le LPS est capté par la protéine LBP (LPS-binding protein), permettant de potentialiser son interaction avec CD14, sous forme soluble (sCD14) ou membranaire. Le complexe CD14/LPS peut ainsi se fixer à la membrane plasmatique et interagir avec TLR4 associé à son corécepteur MD-2 (adapté de [16])

Comme nous l'avons vu, l'interaction inducteur/récepteur déclenche, en autre, la libération de nombreux médiateurs inflammatoires, qui a leur tour sont capables d'induire des modifications de fonction des cellules effectrices de l'inflammation d'un part, et des organes et tissus de l'organisme d'autre part. Ces médiateurs sont communément classés en sept catégories, incluant : les cytokines (IL-1, IL-6, TNF-α, …), les chémokines (MCP-1, MIP, RANTES, PF4, …) ; le complément (C3a, C4a, C5a), les amines vasoactives (histamine, sérotonine), les peptides vasoactifs (substance P, kinines, …), les médiateurs lipidique (leucotriènes, prostaglandines, prostacyclines, thromboxanes, …) ainsi que les enzymes protéolytiques (métalloprotéinases matricielles, cathepsines, …)[2,3]. Les cytokines pro-inflammatoires, dont la mesure plasmatique est aisée, font l'objet d'une recherche intense, tant sur le plan physiopathologique que dans la recherche de biomarqueurs prédictifs[7][17].

Les médiateurs inflammatoires exercent des effets pléiotropes et sont capables d'induire des modulations de fonctions des organes et tissus à l'échelle locale mais aussi de façon systémique. Ainsi les cytokines pro-inflammatoires sont capables de réguler la fonction endothéliale, le métabolisme adipocytaire et hépatique, l'hématopoïèse ou encore les fonctions endocrines et neurologiques centrales [4,9]. De façon concomitante, les médiateurs inflammatoires activent les cellules immunitaires effectrices, en particulier les macrophages, les neutrophiles et les lymphocytes T[1,9].

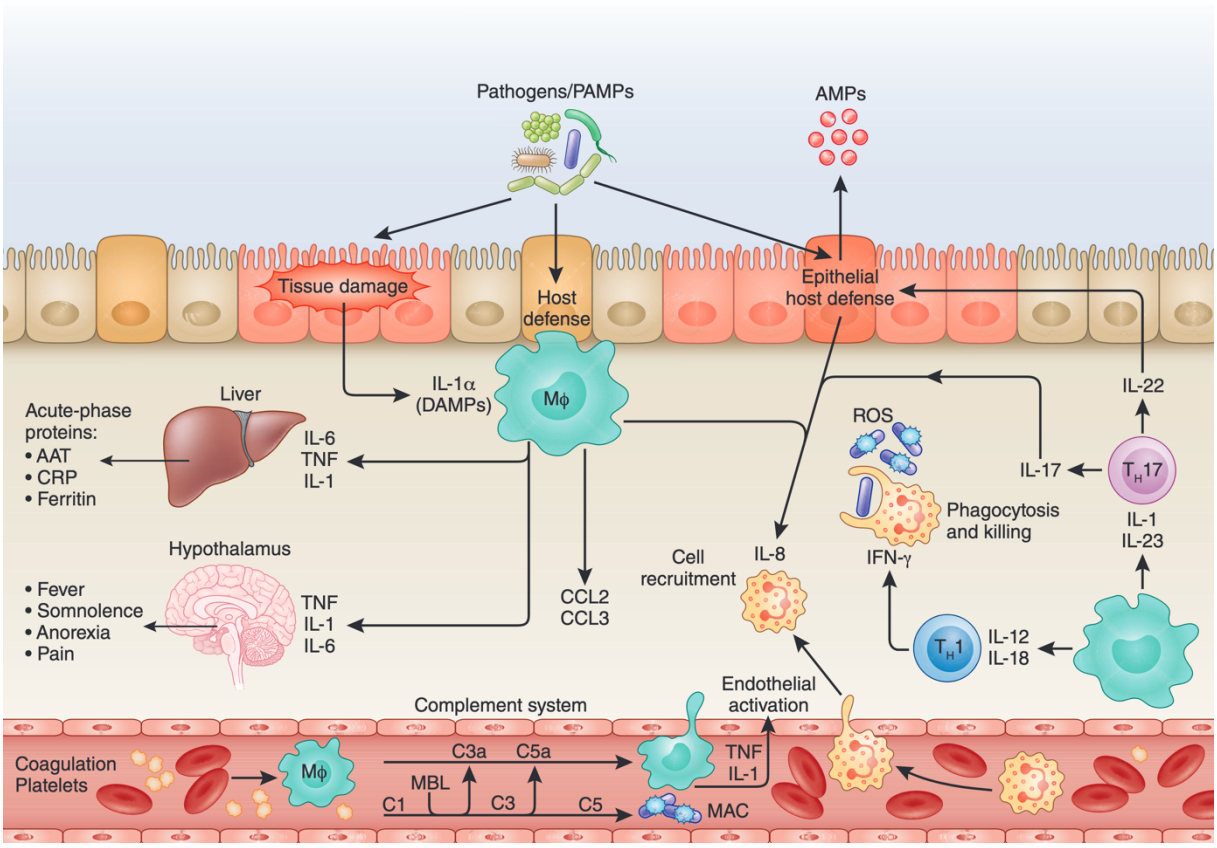

Figure 4. Principaux mécanismes de l'inflammation aiguë induite par les pathogènes
AAT: α1-antitrypsin; PAMPs: pathogen-associated molecular patterns; AMPs: peptides antimicrobiens; DAMPs: damage-associated molecular patterns; MAC: complexe d'attaque membranaire; ROS: espèces réactives de l'oxygène; CRP: protéine C-réactive; MBL: mannose-binding lectin; Mf: macrophage ou monocyte. (d'après [1]).

Les données de la littérature ont récemment fait émerger trois effecteurs inflammatoires majeurs, qui étaient jusqu'ici très peu associés aux descriptions classiques de la réponse inflammatoire aiguë : la NETose, l'activation de la coagulation et l'activation plaquettaire.

La NETose est un mécanisme d'immunité innée, correspondant à la sécrétion par les neutrophiles de NETs (Neutrophils Extracellular Traps), structures extracellulaires en forme de toile composées de chromatine décondensée et de nombreuses protéines issues des granules

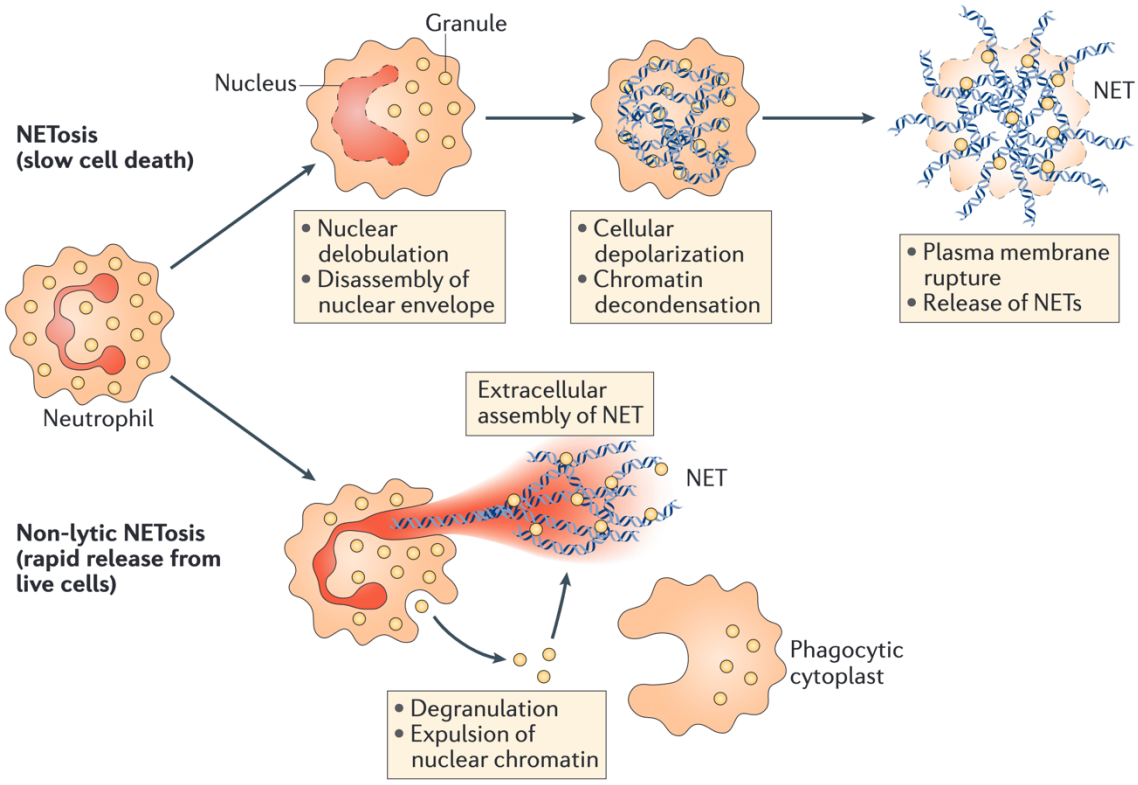

Figure 5. Formation des NETs
La génération de NETs (Neutrophil extracellular traps) par les neutrophiles peut se dérouler selon une voie de mort cellulaire associée à une rupture de la membrane plasmatique ou selon une voie non lytique associant dégranulation et expulsion de chromatine décondensée et laissant un phagocyte anucléé fonctionnel. (d'après [18]).

neutrophiles (myeloperoxydase, élastase neutrophile) (Figure 5) [18,19]. La génération de NETs peut être initiée par de nombreuses voies d'activation, notamment associées aux PRRs du neutrophile. Les NETs peuvent piéger, neutraliser et détruire les micro-organismes pathogènes, mais également réguler positivement la sécrétion de cytokines pro-inflammatoires, en particulier via l'activation des macrophages[18,20]. Enfin ils forment une surface très favorable à la thrombose, capable de lier la fibrine, le facteur von Willebrand, le facteur XII et les plaquettes, et de protéger les facteurs de coagulation activés de la protéolyse et des inhibiteurs circulants[18,19,21]. Cette interaction forte entre le système immunitaire inné et la thrombose et l'exemple même des concepts récents d'immunothrombose et de thrombo-inflammation[19,22,23]. L'activation de la coagulation, qui a pour finalité la génération de

thrombine (facteur IIa) et la constitution d'un réseau de fibrino-plaquettaire stable, est en effet fortement impliquée dans la réponse inflammatoire à la fois comme inducteur et comme effecteur[19,22]. Ainsi, le facteur tissulaire et la thrombine, notamment via l'activation des récepteurs PAR (protease-activated receptor), sont au cœur d'une boucle de rétrocontrôle positif pro-inflammatoire impliquant les plaquettes, l'endothélium, les cellules musculaires lisses vasculaires et les leucocytes[24]. De même le système fibrinolytique (tPA/uPA/plasmine) et la phase contact (Facteur XI, Facteur XII, prékallikreine, kininogène de haut poids moléculaire) exercent de puissants effets pro-inflammatoires[19].

L'activation plaquettaire constitue enfin le dernier effecteur que nous aborderons. Il est particulièrement intéressant de noter que les revues de littérature présentées dans cette première partie n'abordaient que très succinctement le rôle des plaquettes sanguines[1–4]. Ce rôle inflammatoire, pourtant majeur, fait l'objet du chapitre suivant.

Rôle des plaquettes dans l'inflammation aiguë

Vue d'ensemble de la physiologie hémostatique plaquettaire

Les plaquettes sanguines sont de petites cellules anucléées, principalement distribuées dans le compartiment sanguin. Leur structure est représentée à la Figure 6. Elle est contenue par la membrane plasmique, bicouche phospholipidique, dont les invaginations forment le système canaliculaire ouvert. Le système tubulaire dense assure la régulation du calcium ionisé intra-plaquettaire et ainsi l'activation plaquettaire. Le cytosquelette composé de microtubules et d'actine permet les changements de conformation plaquettaire lors de l'activation. Les granules plaquettaires, dont le contenu peut être libéré par fusion avec la membrane plasmique, ont un rôle clé dans les fonctions hémostatiques et inflammatoires plaquettaires. Les granules- α contiennent de nombreuses molécules pro-thrombotiques et inflammatoires, incluant le

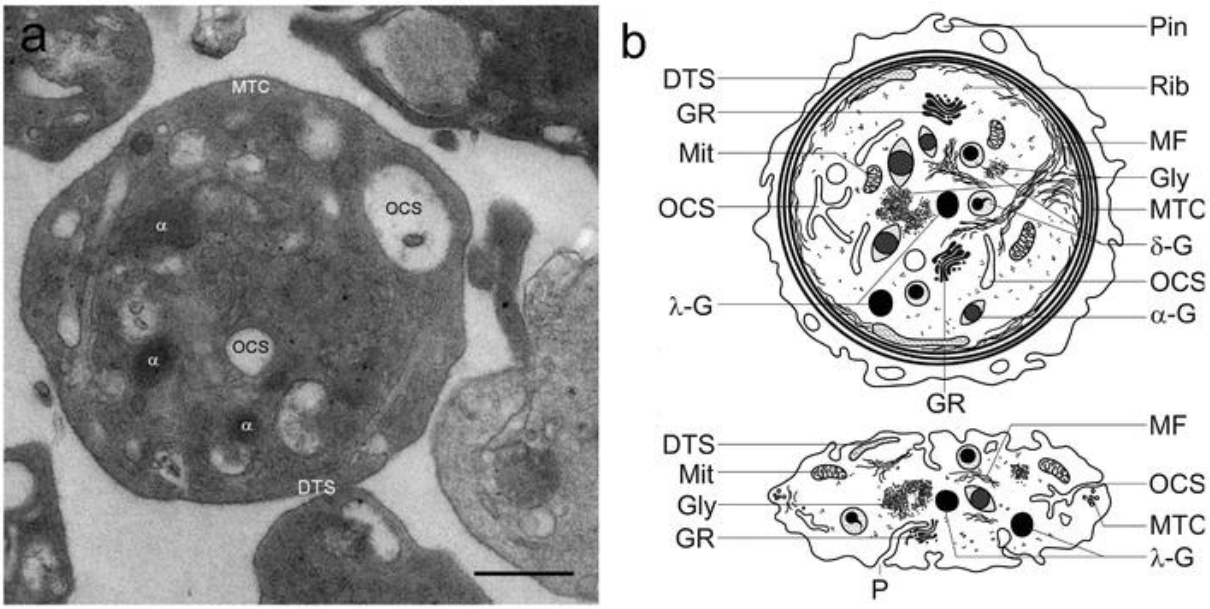

Figure 6. Structure d'une plaquette au repos
(a) Microscopie électronique en transmission. (b) Vue schématique. Abréviations: DTS: système tubulaire dense; Gly: glycogène; α: granules alpha; δ: granules denses; λ: lysosomes; GR: résidus du Golgi; MF: microfilaments; Mit: mitochondrie; OCS: système canaliculaire ouvert; P: pores du système canaliculaire ouvert; Rib: ribosomes. Adapté de [129]

fibrinogène, le facteur von Willebrand (FVW), la thrombospondine, des facteurs de coagulation, des chémokines, les fractions C3 et C4 du complément ainsi que des facteurs de croissance et angiogéniques. Leur membrane contient également la P-sélectine (CD62P) et l'intégrine αIIbβ3. Les granules denses permettent le stockage de l'ADP, de l'ATP, des polyphosphates ainsi que des amines vasoactives [25,26].

Loin d'être une simple bicouche phospholipidique, la membrane plaquettaire porte de très nombreux récepteurs, associés aux voies de signalisation intra-plaquettaires, qui lui confèrent ses fonctions hémostatiques, mais également des capacités de modulation de la réponse inflammatoire et immune[27].

Les plaquettes jouent un rôle central dans l'hémostase primaire, tout d'abord en adhérant à l'endothélium pathologique ou lésé [26,28]. Cette adhésion, dépendante des contraintes de flux, est permise d'une part par l'interaction du FVW au complexe glycoprotéique plaquettaire GPIb-IX-V, et d'autre par les récepteurs au collagène : l'intégrine α2β1 (GPIaIIa) et le complexe GPVI-FcRγ.

Cette adhésion initie l'activation plaquettaire, marquée par la sécrétion du contenu granulaire et de molécules néo-synthétisées (thromboxane A2), des modifications de composition membranaire (externalisation de la phosphatidyl sérine) et de forme (émission de pseudopodes).

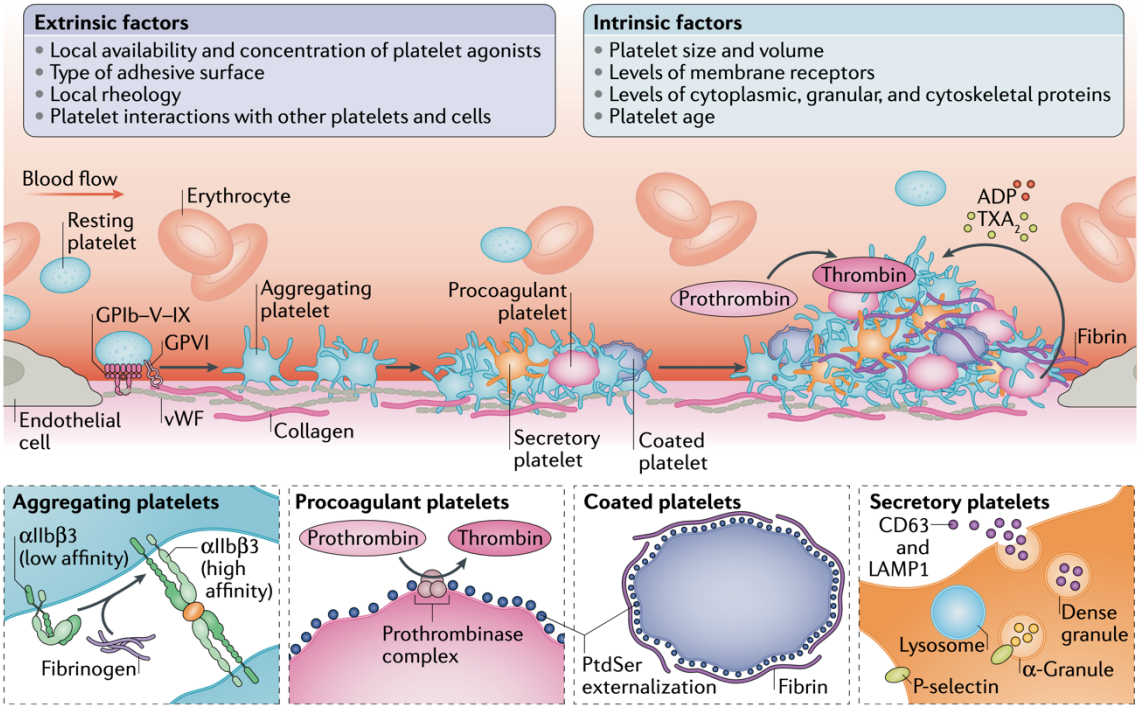

Figure 7. Rôle des différents phénotypes palquettaires dans l'hémostase
.vWF : facteur von willebrand, TXA: thromboxane. Adapté de [26]

Cette sécrétion engendre une boucle de rétrocontrôle positif incluant le recrutement d'autres plaquettes, la génération de thrombine et l'activation de l'intégrine αIIbβ3 (GPIIbIIIa) par la voie de signalisation "inside-out". Cette dernière étape permet l'agrégation plaquettaire grâce

au fibrinogène formant des ponts inter-plaquettaires entre deux αIIbβ3, induisant secondairement une signalisation "outside-in" importante pour la croissance du thrombus et sa stabilité[28,29]. Plusieurs phénotypes d'activation plaquettaire ont été décrits, favorisés par l'exposition différentielle aux contraintes de flux, aux surfaces adhésives, à la thrombine et autres plaquettes et cellules (Figure 7). Ainsi, des phénotypes plaquettaires procoagulants ont pu être mis en évidence (plaquettes procoagulantes, COAT-platelets) dont l'exposition de phosphatidylsérine à la membrane externe fournit une surface idéale pour la génération de thrombine[26,30–32].

Les voies d'activation plaquettaires et leur régulation seront abordées dans le chapitre suivant.

Rôle inflammatoire des plaquettes : mécanismes physiopathologiques

En plus de ce rôle hémostatique, les plaquettes sont désormais reconnues comme des acteurs importants de la réponse inflammatoire aiguë, pouvant agir au sein des circuits inflammatoires à la fois comme récepteurs, comme médiateurs mais aussi comme effecteurs (Figure 1). Plus encore, elles agissent comme un outil de médiation réciproque entre système immunitaire et hémostase, leur conférant un rôle potentiellement majeur dans l'inflammation aiguë.

Les plaquettes : récepteurs inflammatoires

Les plaquettes expriment différentes classes de récepteurs inflammatoires, de surface ou cytosoliques, incluant des PRRs, ainsi que des récepteurs aux cytokines et aux chémokines (Figure 8).

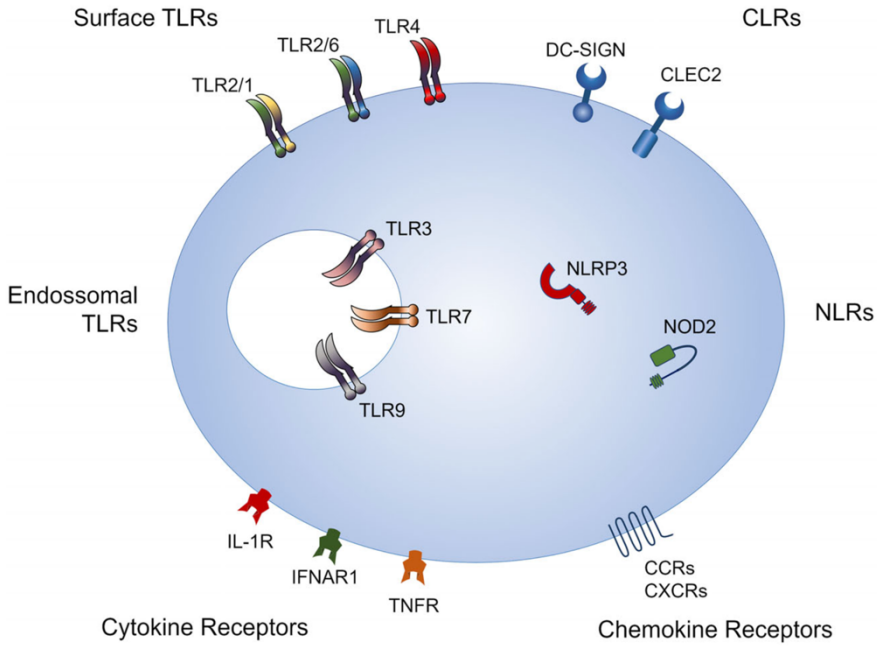

Figure 8. Expression plaquettaire des PRRs, des récepteurs aux cytokines et aux chémokines
Les plaquettes expriment des TLRs à leur surface (TLR1,2,4,et 6) ou dans les endosomes (TLR3,7, et 9) des récepteurs CLR (CLEC-2 et DC-SIGN); et NLR cytosoliques (NOD2 et NLRP3). Elles expriment également des récepteurs aux cytokines (IL-1R, IFNAR1 et TNFR) ainsi que des récepteurs aux chémokines (CCRs et CXCRs) Adapté de [33]

Elles expriment ainsi à leur surface des récepteurs à l'IL-1, au TNF-α, aux interférons de type 1, de même que des récepteurs aux chémokines de type CCRs et CXCRs, qui participent donc à l'activation et à l'agrégation plaquettaires [27,33,34]. Les plaquettes expriment également des PRRs de type NOD-like receptors (NLRs) cytosoliques et C-type lectin receptors (CLRs),

impliqués notamment dans la reconnaissance des micro-organismes pathogènes. Mais surtout, les plaquettes expriment à leur surface ainsi qu'en intracellulaire des TLRs, capables de reconnaitre de nombreux PAMPs et DAMPs[15,27,31,33–35]. L'interaction LPS TLR4 plaquettaire en est un exemple parfait. Les plaquettes sont en effet capables de reconnaitre le LPS via TLR4, en présence de CD14 soluble, induisant une augmentation de l'expression de P-Sélectine et de la sécrétion de CD40L soluble (sCD40L) et cytokines pro-inflammatoires[33,36,37]. Cependant, malgré une abondante littérature, de nombreuses questions subsistent concernant la régulation de ces PRRs, leur impact sur les fonctions hémostatiques et immunitaires plaquettaires ainsi que dans la physiopathologie des états septiques et inflammatoires non septiques[15].

En dehors des PRRs, les plaquettes peuvent interagir avec divers pathogènes incluant des virus, des parasites et mais surtout les bactéries[31,33,34,38–40]. Les principaux récepteurs plaquettaires impliqués dans l'interaction plaquettes-bactéries, la GpIbα, la GpIIbIIIa, le récepteur du complément C1q et le récepteur FcγRIIa, permettent une liaison directe ou indirecte aux bactéries via le fibrinogène, le FVW, des immunoglobulines ou le complément. Certaines toxines bactériennes ou facteurs de virulence peuvent également induire une stimulation plaquettaire, via les récepteurs plaquettaires ou en ciblant directement la membrane plaquettaire. Ces interactions permettent ainsi secondairement d'amplifier la boucle activation plaquettaire-thrombose-immunité, mais également comme nous le verrons par la suite de favoriser la neutralisation directe des pathogènes et la présentation d'antigènes au système immunitaire adaptatif.

Les plaquettes : médiateurs et effecteurs inflammatoires

Les plaquettes sont capables de jouer le rôle de médiateurs inflammatoires selon deux mécanismes : 1) en interagissant directement avec les cellules effectrices (leucocytes et cellules endothéliales) 2) indirectement en libérant des médiateurs circulants (thrombine, cytokines, chémokines, vésicules extracellulaires).

L’interaction directe des plaquettes avec les leucocytes se manifeste par la formation d’agrégats ou amas leucoplaquettaires (ALP). Outre leur possible intérêt comme biomarqueur inflammatoire, les ALP ont un rôle physiologique et probablement pathologique important, au croisement de la thrombose, de l'inflammation et de l'immunité. Les mécanismes de formation

des ALP seront repris dans les deux revues de littérature présentés plus loin dans cette introduction (Mansour et al J. Clin. Med. 2020, 9, 2361, et Mansour et al. Int. J. Mol. Sci. 2020, 21, 1391). Brièvement, les plaquettes interagissent directement et préférentiellement avec les monocytes et les neutrophiles, ainsi qu'avec les lymphocytes et les cellules dendritiques[31,33,34,41,42]. Trois mécanismes principaux sous-tendent ces interactions : la liaison P-Sélectine/PSGL-1, la liaison (s)CD40L/CD40 et la liaison indirecte αIIbβ3/Mac-1 via le fibrinogène (Figure 10). La formation des ALP induit une activation leucocytaire favorisant ainsi la génération de médiateurs pro-inflammatoires, la NETose, la différenciation macrophagique et la diapédèse leucocytaire. Les plaquettes sont également capables de stimuler la réponse immunitaire adaptative par leur interaction avec les lymphocytes et les cellules dendritiques qui les phagocytent[33]. Ce circuit inflammatoire, appelé immunothrombose, aboutit à la constitution de réseaux intravasculaires capables de ralentir la dissémination systémique bactérienne.[22,23,40]

En situation physiologique, l'intégrité physique de l'endothélium et sa production d'inhibiteurs plaquettaires (monoxyde d'azote, prostaglandine I2) prévient l'interaction plaquette-cellule endothéliale. En situation de stress, les plaquettes interagissent avec l'endothélium par

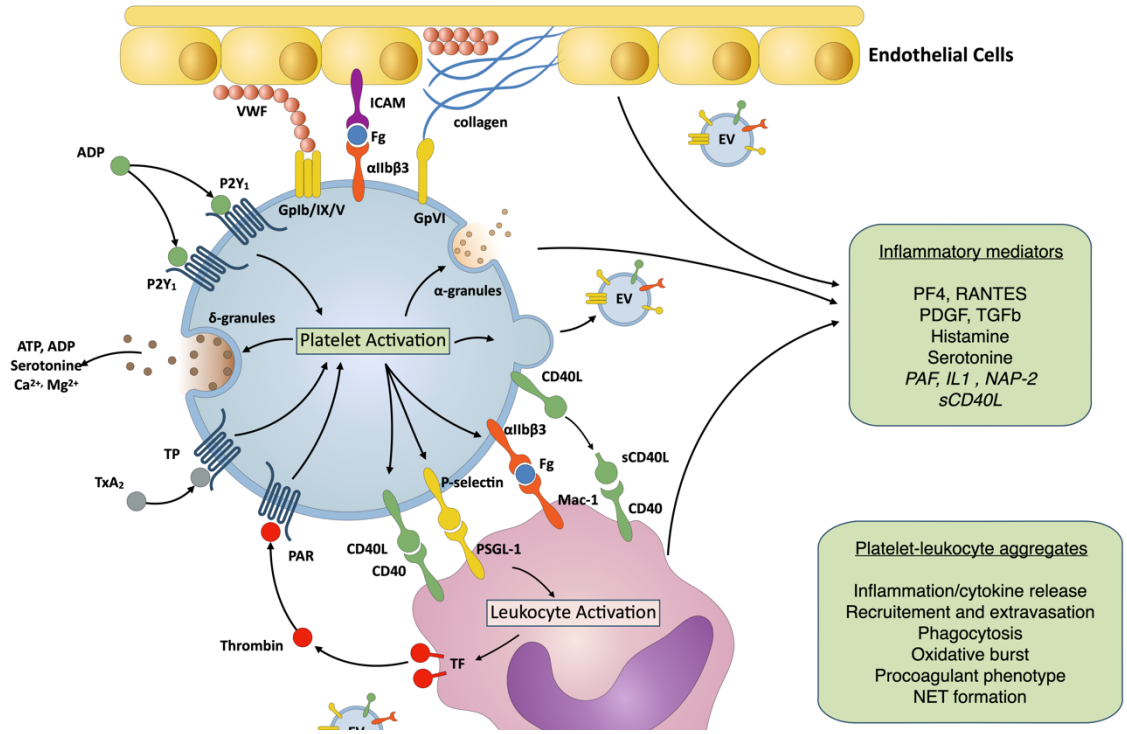

Figure 9. Mécanismes de l'interaction des plaquettes avec l'endothélium et les leucocytes
EC: cellules endothéliales; EV: vésicules extracellulaires; IL1: Interleukin 1; NAP-2: Neutrophil Activating Peptide-2; NET: neutrophil extracellular trap; PAF: platelet activating factor; PAR: protease activated receptor; PF4: platelet factor 4; PDGF: Platelet Derived Growth factor; TF: Facteur Tissulaire; TGF β: Transforming Growth Factor β; VWF: facteur von Willebrand.

l'intermédiaire du FVW et du complexe GPIb-IX-V, par la liaison P-Sélectine/PSGL-1 mais également de façon plus stable par la liaison ICAM-1/αIIbβ3 dépendante du fibrinogène[31,43]. Cette interaction favorise à nouveau une activation réciproque pro-inflammatoire (Figure 9).
Enfin, les plaquettes agissent comme médiateurs inflammatoires indirects en libérant localement et de manière systémique leur contenu granulaire (PF4, RANTES, Sérotonine) ainsi que des médiateurs néo-synthétisés (TxA2, IL-1β)[31,34,35]. De plus, les plaquettes sont considérées comme la source principale de CD40L soluble (sCD40L) et favorisent la génération de thrombine, puissants médiateurs pro-inflammatoires [42,44]. Enfin, la génération de vésicules extracellulaires plaquettaires, exprimant la phosphatidylsérine et portant les molécules membranaires plaquettaires, outre son rôle de biomarqueur d'activation, pourrait agir comme médiateur inflammatoire[45].

En plus des rôles précédemment décrits, les plaquettes peuvent agir comme des effecteurs directs de l'inflammation. Elles sont en effet capables de neutraliser les pathogènes en les internalisant, ce qui put être démontré pour des bactéries (E. coli, S. aureus, L. monocytogenes) ainsi que pour des parasites comme Plasmodium Falciparum[31,38,39]. Le devenir de ces pathogènes internalisés reste cependant sujet à des controverses. En effet, même si les plaquettes disposent de molécules microbicides dans leurs endosomes, l'internalisation des pathogènes pourrait favoriser leurs capacités de dissémination en échappant au système immunitaire, comme cela a été décrit pour certains virus[38].

Implications dans l'inflammation septique

Définition du sepsis

Le sepsis une pathologie complexe définie comme une réaction dérégulée de l'hôte à une infection aboutissant à des défaillances d'organes menaçant le pronostic vital[46]. Il représente 20 à 30% des admissions en réanimation et est à l'origine de plusieurs millions de morts par ans dans le monde[47]. L'infection bactérienne en est la principale étiologie, en particulier d'origine respiratoire et abdominale. La physiopathologie du sepsis fait intervenir des relations réciproques complexes entre inflammation, coagulation et immunité adaptative, secondaire à la reconnaissance de pathogènes par le système immunitaire (Figure 10)[47,48]. Les plaquettes, comme vu précédemment, occupent une place centrale dans ces interactions[49,50].

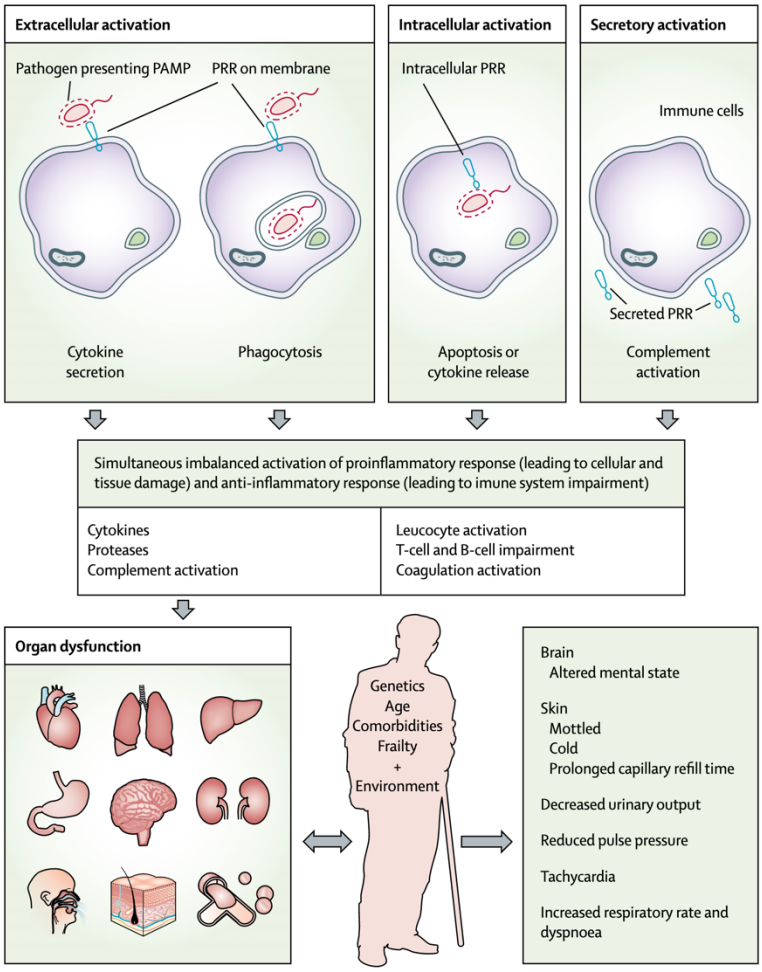

Figure 10. Principaux mécanismes impliqués dans la physiopathologie du sepsis
(adapté de [47])

Cytokines pro-inflammatoires

Comme vu précédemment, les cytokines occupent un rôle clé dans la physiologie de l'inflammation, et en particulier dans le sepsis. De par leur solubilité plasmatique, la recherche clinique et académique s'est logiquement tournée vers la recherche de biomarqueurs diagnostiques et pronostiques du sepsis. Depuis près de 30 ans, plusieurs centaines de biomarqueurs ont ainsi été évalués[7]. Parmi eux, de nombreuses cytokines et chémokines pro-inflammatoires ont démontré leur valeur pronostique, en particulier IL-6, IL-8, IL-10, TNF, MIF (Macrophage migration inhibitory factor) et MCP-1 (Monocyte chemotactic protein).

Thrombopénie et sepsis

La thrombopénie est définie par un compte plaquettaire <150 G/L et qualifiée de sévère en dessous de 50 G/L. Elle survient chez plus de 50% des patients présentant un sepsis avec plus de 10% de thrombopénies sévères[49–52]. La thrombopénie septique présente fréquemment un profil biphasique avec un nadir autour du 4ème jour puis une ascension progressive pouvant être suivie d'une thrombocytose secondaire. La profondeur de la thrombopénie et sa persistance au-delà du 4ème jour sont des marqueurs de mauvais pronostique du sepsis, associés à la mortalité[51,53]. A ce titre, le compte plaquettaire fait partie de la définition du score SOFA (Sequential Organ Failure Assessment Score) utilisé à la fois pour le diagnostic positif et l'évaluation du pronostic des patients septiques[46].

Il faut toutefois souligner que cette association, bien qu'indépendante, ne préjuge pas d'un lien de causalité. De nombreux mécanismes, souvent concomitants, peuvent induire une thrombopénie septique : consommation associée aux coagulopathies disséminées, augmentation de la clairance par désialylation plaquettaire, hémodilution, infections virales, inhibition septique ou médicamenteuse de la thrombopoïèse, thrombopénies auto-immunes.

Marqueurs d'activation plaquettaires au cours du sepsis

Parmi les biomarqueurs plasmatiques d'activation plaquettaire évalués au cours du sepsis, les concentrations de sCD40L, P-Sélectine soluble et PF4 apparaissent corrélées à la survenue d'états septiques graves, aux défaillances d'organes et à la mortalité[54–59]. A l'inverse, les amas leucoplaquettaires (ALP) circulants ont été peu évalués au cours du sepsis[49]. Les

conditions pré-analytiques et analytiques nécessaires à leur mesure sont détaillés dans la revue de littérature présentée au chapitre suivant (Mansour et al J. Clin. Med. 2020).

Modèles précliniques d'étude du rôle des plaquettes dans le sepsis

De nombreux modèles précliniques d'étude du sepsis ont été développés ou testés, en particulier pour l'évaluation du rôle des plaquettes. Les modèles in vitro, reproductibles et de mise en œuvre relativement aisée sont cependant rapidement limités et ne permettent pas d'analyser les régulations complexes entre les systèmes précédemment développés. Ils permettent cependant d'évaluer rapidement des techniques analytiques et de nombreux modulateurs, y compris sur sang total humain, et sont à ce titre indispensables au développement de stratégies thérapeutiques novatrices. Les modèles murins, plus complexes, peuvent utiliser différents inducteurs : des PAMPs (en particulier le LPS), des lignées de pathogènes uniques (E. Coli, S. Aureus, Candida Albicans, …) ou des infections polymicrobiennes (ponction ligature caecale CLP, Injection intrapéritonéale de matière fécales - Cecal Slurry)[49]. Ces différents modèles impactent la réponse inflammatoire, les défaillances d'organes, ainsi que la participation plaquettaire à l'inflammation (Figure 11). Les modèles septiques polymicrobiens sont considérés comme des modèles de référence.

Évaluation clinique des thérapeutiques antiplaquettaires dans le sepsis

Des travaux observationnels, parfois de grande ampleur, suggèrent que l'utilisation de médicaments antiplaquettaires utilisés dans la prise en charge du risque thrombotique artériel (aspirine et inhibiteurs du récepteur $P2Y_{12}$) pourrait être associée à une amélioration du pronostic des patients septiques, en particulier d'origine respiratoire[60–62].

Peu d'études prospectives interventionnelles ont évalué l'effet des antiplaquettaires dans le sepsis. A ce jour l'étude ANTISEPSIS est la seule étude randomisée contrôlée de grande ampleur à avoir comparé l'aspirine (100mg par jour) à un placebo dans la prise en charge du sepsis chez le patient de plus de 70 ans. L'inclusion de 16703 patients n'a pas permis de mettre en évidence un bénéfice sur la mortalité[63]. L'étude XANTHIPPE a évalué le ticagrelor contre placebo sur un effectif de 60 patients souffrant de pneumopathie infectieuse et a démontré une réduction des taux d'IL-6 et le taux d'ALP[64]. Enfin plus récemment, l'étude REMAP-CAP n'a

pas permis de mettre en évidence de bénéfice à associer un antiplaquettaire ($P2Y_{12}$ ou aspirine) à une anticoagulation chez le patient COVID-19 en réanimation[65].

L'évaluation clinique des anti-$P2Y_{12}$ sera détaillée dans la revue de littérature présentée au chapitre suivant Mansour et al. Int. J. Mol. Sci. 2020, 21, 1391)

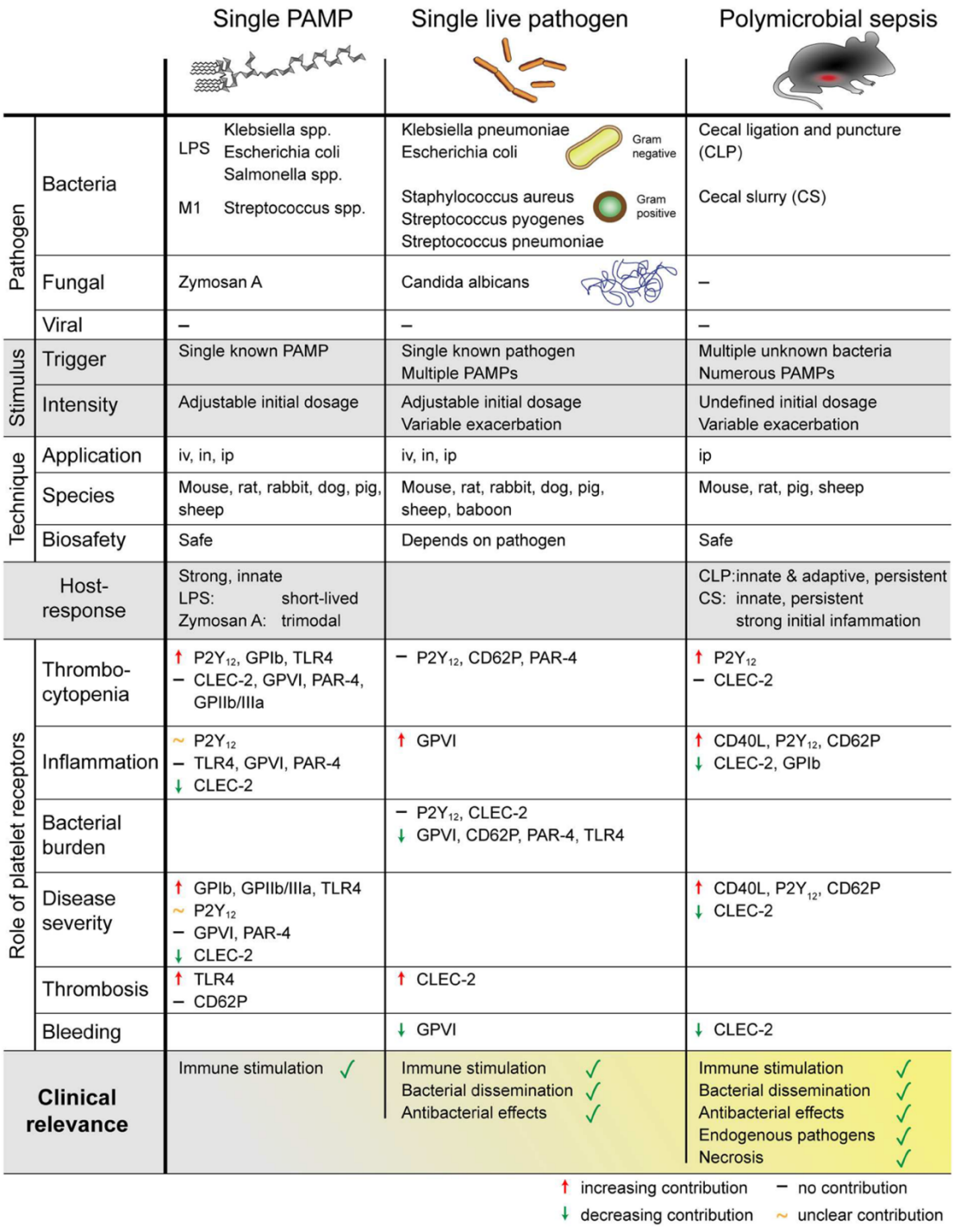

Figure 11. Types de modèles pré-cliniques murins de sepsis
Adapté de [49]

Implications dans l'inflammation liée aux circulations extracorporelles

Les techniques de suppléance d'organe font partie intégrante de l'arsenal thérapeutique en anesthésie et en réanimation. Plusieurs d'entre elles font appel à des circuits de circulation extracorporelle. C'est le cas des assistances circulatoires (circulation extracorporelle de chirurgie cardiaque, ECMO ou extracorporeal membrane oxygenation)[66,67], des dispositifs d'épuration extra-rénale (dialyse) ou encore de l'épuration extracorporelle hépatique (Système MARS)[66–70]. Les dispositifs de récupération sanguine peropératoire (RSPO), utilisés au bloc opératoire, partagent des caractères communs avec ces dispositifs de suppléance d'organe, à la fois sur les caractéristiques des patients mais également du fait des contraintes d'hémocompatibilité qui s'appliquent au traitement du sang total récupéré[71]. De même, de nouveaux dispositifs permettent la perfusion normothermique ex-vivo d'organes (Organ Care System, TransMedics) dans l'objectif d'améliorer les performances des greffons, en particulier cardiaques et pulmonaires[72,73]. Utilisant du sang total humain du donneur, ces dispositifs sont exposés de la même façon aux contraintes d'hémocompatibilité et d'inflammation induite que les autres dispositifs d'assistance.

Du fait des pathologies ou des procédures subies par le patient (sepsis, chirurgie, traumatisme) mais également des conséquences propres des interactions sang-surfaces exogènes, les différentes circulations extracorporelles sont associées à une réponse inflammatoire aiguë, à une activation des cellules circulantes et de la coagulation[23,67,68,74–80]. C'est principalement le cas des assistance circulatoires (ECMO, CEC de chirurgie cardiaque). Cette réponse inflammatoire, liée à l'hémocompatibilité des dispositifs, impacte le devenir des patients en réanimation. Plusieurs facteurs sont susceptibles de moduler ce phénomène : a) Le terrain du patient et son motif d'admission en réanimation, b) Le contact du sang avec des surfaces exogènes (canules, tubulures, membranes) susceptibles d'activer la phase contact de la coagulation, c) Les contraintes de cisaillement exercées par les pompes et les membranes d) Les thérapeutiques médicamenteuses en cours, y compris antithrombotiques et e) L'exposition aux transfusions allogéniques.

L'impact de ces circulations extracorporelles sur l'activation plaquettaire et sur le risque thrombotique, h®morragique et inflammatoire qui pourraient en d®couler est d®crit dans la revue de litt®rature suivante, au travers de l'exemple de l'assistance circulatoire par ECMO.

Mansour A, Roussel M, Gaussem P, Nédelec-Gac F, Pontis A, Flécher E, Bachelot-Loza C, Gouin-Thibault I. Platelet Functions During Extracorporeal Membrane Oxygenation. Platelet-Leukocyte Aggregates Analyzed by Flow Cytometry as a Promising Tool to Monitor Platelet Activation. J Clin Med. 2020 Jul 23;9(8):2361. doi: 10.3390/jcm9082361. PMID: 32718096; PMCID: PMC7464627.

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Review

Platelet Functions During Extracorporeal Membrane Oxygenation. Platelet–Leukocyte Aggregates Analyzed by Flow Cytometry as a Promising Tool to Monitor Platelet Activation

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Abstract: Extracorporeal membrane oxygenation (ECMO) is an extracorporeal circulation used to manage patients with severe circulatory or respiratory failure. It is associated with both high bleeding and thrombosis risks, mainly as a result of biomaterial/blood interface phenomena, high shear stress, and complex inflammatory response involving the activation of coagulation and complement systems, endothelial cells, leukocytes, and platelets. Besides their critical role in hemostasis, platelets are important players in inflammatory reactions, especially due to their ability to bind and activate leukocytes. Hence, we reviewed studies on platelet function of ECMO patients. Moreover, we addressed the issue of platelet–leukocyte aggregates (PLAs), which is a key step in both platelet and leukocyte activation, and deserves to be investigated in these patients. A reduced expression of GPIb and GPVI was found under ECMO therapy, due to the shedding processes. However, defective platelet aggregation is inconsistently reported and is still not clearly defined. Due to the high susceptibility of PLAs to pre-analytical conditions, defining and strictly adhering to a rigorous laboratory methodology is essential for reliable and reproducible results, especially in the setting of complex inflammatory situations like ECMO. We provide results on sample preparation and flow cytometric whole blood evaluation of circulating PLAs.

Keywords: platelet activation; platelet aggregation; von Willebrand factor; leukocytes; platelet-leukocyte aggregates

1. Introduction

Extracorporeal circulation is used to manage patients with severe organ dysfunction. Extracorporeal membrane oxygenation (ECMO) provides gas exchange during severe respiratory failure with veno-venous (VV-ECMO) circuits [1]. Veno-arterial ECMO (VA-ECMO), also called extracorporeal life support (ECLS), has been increasingly used in life-threatening circulatory failure refractory in conventional treatments, with the aim of maintaining satisfactory tissue perfusion pending myocardial recovery or heart transplantation. In all these devices, blood comes into sustained contact with a large artificial material surface area and a pump, leading to a complex inflammatory response involving activation of the coagulation system, complement system, endothelial cells, leukocytes, and platelets [1]. The pump can be either centrifugal or pulsatile with different rotational speeds and variable effects on hemostasis and bleeding [2]. ECMO is associated with bleeding and thrombotic complications, which remain a leading cause of morbidity and mortality of such fragile patients. Therefore, managing hemostasis to avoid clot formation without increasing bleeding risk in patients with ECMO, remains a major challenge. According to the recent review by Doyle et al. the rate of ECMO-associated venous thromboembolism (VTE) widely varies from 18% to 85%, depending on the studies and the anticoagulation regimens, with oxygenator clotting thrombosis in around 10–16% of patients, depending on the circuit type and patient age [1]. Severe hemorrhage is reported in nearly 40% of patients, with intracranial hemorrhage in 16–21% of them [1]. The pathogenesis of these complications, which can be related to the underlying disease or to the ECMO circuit, is therefore multifactorial and is not fully elucidated. Vessel damage and low blood flow at cannula sites, immobility, disseminated intravascular coagulation, elevated factor VIII, procoagulant hydrophobic artificial surfaces, hemolysis, circulating extracellular vesicles, and monocyte tissue factor (TF) expression, all contribute to the prothrombotic changes in patients receiving ECMO [1]. Beyond TF-based activation of coagulation, contact activation through artificial surfaces involving notably FXII and FXI, might also play an important role in clot formation.

Concerning the bleeding risk, acquired von Willebrand syndrome (AVWS) was reported in ECMO patients [3–5]. AVWS is characterized by a defect in the multimeric von Willebrand factor composition, which is a key factor during primary hemostasis. Upon exposition onto artificial surfaces and high shear stress, von Willebrand factor unfolds, which facilitates subsequent proteolysis and cleavage of large multimers by the ADAMTS-13 protease. The loss of the high-molecular weight multimers results in a decreased binding of von Willebrand factor to collagen and platelets. AVWS rapidly occurs, within 24 h after ECMO implantation, and is reversed few hours after explantation [3]. Thrombocytopenia, which is frequently observed in these patients, with a median platelet count between 50 and 100 $\times$ 10^9/L (see below), might contribute to the hemorrhagic risk. It was shown that ECMO patients who experienced bleeding events had a significantly lower platelet nadir than patients without bleeding [6]. A platelet dysfunction was also reported but not clearly established, with some studies showing platelet glycoprotein (GP) Ibα and GPIV shedding, as detailed below.

In addition to promoting thrombus, platelets are involved in inflammatory reactions by their ability to release stored and newly formed mediators. They also interact with leukocytes and play an essential role for leukocyte recruitment and initiation and propagation of inflammation. The purpose of this review was, therefore, to summarize the various roles of platelets in the context of ECMO in adult patients, with a special focus on the potential usefulness of studying platelet–leukocyte interactions, by measuring the so-called platelet–leukocyte aggregates (PLAs) through flow cytometry.

2. Platelet Activation, Aggregation, and ECMO

In a systematic recent review, Balle et al. reported that there are only a limited number of published studies investigating platelet functions in patients during ECMO therapy. These studies suggest a reduced potential for platelet adhesion, activation, and aggregation during ECMO [7].

Shear stress plays an essential role in regulating platelet activation and aggregation through VWF (von Willebrand Factor) binding to platelet GPIb within the platelet GPIb–IX–V complex, with limited

binding under the low shear condition. Above a critical shear rate the structural change of VWF enables its binding to GPIbα with increased affinity. This GPIbα–VWF interaction, leading to platelet translocation and rolling, enables stable platelet adhesion on collagen and subsequent GPVI-mediated activation of $\alpha_{IIb}\beta_3$ and other platelet integrins [8]. GPIbα and GPVI shedding occurs via activation of the constitutively present platelet membrane-bound ADAM-17 and ADAM-10 metalloproteases, and as a consequence downregulates platelet adhesion and signaling [8]. The activation mechanism of ADAM-17 and ADAM-10 is not clearly defined but the trans-membrane protein tetraspanins and iRhoms could be involved in the regulation of their activity [9]. Whether high shear rate leads to the direct activation of ADAM metalloproteases and triggers platelet receptor shedding, needs to be further investigated.

In ECMO, shear-induced shedding might not require platelet signaling pathways or activation of $\alpha_{IIb}\beta_3$ and could be a direct effect of exposure to fluid shear stress [9]. The analysis of the level of adhesion/activation receptors on circulating platelets in patients receiving ventricular assist devices or ECMO therapy were first reported by Lukito et al. in 2016 [10]. This single-center observational study enrolled 20 patients (14 VA-ECMO and 6 VV-ECMO, Medtronic centrifugal pumps), with a median time post-ECMO initiation of four days (range: 1–11 days). The authors reported that platelet receptor shedding was demonstrated by a significantly reduced surface GPIbα and GPVI levels, and a concomitant increase in plasma soluble GPVI in patients, compared to healthy donors. However, since the level of glycocalicin, which is a large proteolytic extracellular fragment of the GPIbαreceptor released upon GPIbα proteolysis was not measured, shedding of GPIbα could not be distinguished from internalization. By contrast, there was no significant loss of $_{IIb}$ subunit of integrin $\alpha_{IIb}\beta_3$ (also named GPIIb/IIIa), which is a platelet-specific receptor subunit that is not susceptible to proteolysis [10].

In another cohort of 20 VA-ECMO patients who had a mean platelet count of three time-points comprised between 84×10^9/L to 108×10^9/L, the authors demonstrated a severely reduced platelet adhesion and aggregation, under a high shear rate of 2000 s^{-1}, in a flow chamber with collagen-coated channels. Adhesion and aggregation improved but did not normalize with in vitro addition of VWF concentrate. The circulating glycocalicin level was found to be elevated, consistent with an increased GPIb proteolysis [11].

Concerning platelet aggregation, most published studies used whole blood impedance aggregometry with the Multiplate® analyzer and adenosine diphosphate (ADP), thrombin-receptor-agonist-peptide-6 (TRAP), and arachidonic acid (AA) as activators, or ristocetin (Table 1).

Table 1. Main results of platelet aggregation studies with Multiplate®.

Type of ECMO and Pump Number of Patients Study Reference	Blood Collection	Results
Centrifugal pump Jostra pumphead, Maquet® VA $n = 7$; VV $n = 3$ Nair et al. [12]	During ECMO. Citrated whole blood.	Patients with platelet count > 100×10^9/L 50% to 72% of patients within low range of ADP, TRAP, collagen aggregation, and ristocetin agglutination
VA $n = 26$; VV $n = 12$ Tauber et al. [13]	Before, after 24 h and 48 h initiation of ECMO therapy and 24 h after ECMO termination. Hirudin anticoagulated whole blood.	30% to 40% decrease in aggregation with ADP, TRAP, AA after 24 h/baseline. After 48 h, aggregation with TRAP = baseline, ADP and AA lower/baseline Return to baseline after 24 h
ROTAFLOW® or CARDIOHELP® centrifugal pumps. VV $n = 20$ Wand et al. [14]	Before, 6 h, 1, 2, 3 and 7 days after the start of ECMO therapy. Whole blood sample on heparin-anticoagulated and calcium-balanced tubes.	Patients with platelet count > 70×10^9/L. Reduced platelet aggregation with ADP, TRAP, AA 6 h after ECMO initiation/before, spontaneous recovery on day 2 with values exceeding baseline afterwards
VA $n = 23$; VV $n = 10$ Balle et al. [15]	Every day from day 1 to day 7 in 33 patients. Hirudin anticoagulated whole blood.	Platelet aggregation with ADP, TRAP, AA: lower compared to healthy volunteers from day 1 up to day 7 but similar when analyzed relative to platelet count

TRAP—thrombin receptor activating peptide-6. AA—arachidonic acid. ADP—adenosine diphosphate.

Multiplate® tests were performed along with thromboelastometry (ROTEM®) on citrated whole blood of seven patients, over a 110 day-period. Thrombocytopenia was found in 76% of patients. Thromboelastometry maximum clot firmness (MCF), which is very sensitive to fibrinogen and to a lesser extent to platelet count, was highly specific, but not very sensitive in the prediction of bleeding

(MCF-intrinsic rotational thromboelastometry, specificity: 91% and sensitivity: 53%). Unfortunately, details on the timing of the test and the bleeding events were lacking. Aggregation tests were performed only in samples with a platelet count above 100×10^9/L. They were inconsistently abnormal with low platelet function in 72% of tests performed with ADP, 50% with collagen and TRAP, and 61% with ristocetin [12].

Platelet aggregation in hirudin anticoagulated whole blood of 38 patients, activated with TRAP, ADP or AA, and analyzed with the Multiplate® analyzer, decreased by 30% to 40%, after 24 h on ECMO, compared to baseline, and was significantly associated with transfusion requirement. After 48 h on ECMO, aggregation induced by TRAP did not differ from baseline, while those induced by ADP and AA were still lower than baseline values. Within 24 h of weaning from ECMO, the platelet count and function returned to the baseline values [13].

In a recent study, platelet aggregometry was assessed on whole blood samples of 20 patients collected in heparin calcium-balanced tubes, provided that platelet count was $\geq 70 \times 10^9$/L. It should be mentioned that median aggregation results using AA, ADP, and TRAP as activators were below the normal reference range given by the manufacturer, before VV-ECMO initiation. Platelet count significantly dropped six hours after implementation of ECMO therapy to a median level of 126×10^9/L and continuously decreased thereafter with a median level of 102×10^9/L on day 4. Platelet aggregation was significantly reduced six hours after VV-ECMO initiation, compared to before, and spontaneously recovered and exceeded baseline values on day 2 and after, in contrast to the continuously decreasing platelet counts. There was no difference in platelet aggregation results between patients with and without signs of bleeding. As discussed by the authors, the recovery of platelet aggregation after day two, might be due to a decreasing effect of factors like the initial hemodilution under extracorporeal circulation [14].

Finally, only in one study, platelet count was taken into consideration and results were compared with the 95% prediction interval of platelet aggregation obtained in healthy whole blood at various platelet counts. Platelet activation was also measured by flow cytometry as the increase in expression of platelet surface P-selectin (CD62P, alpha granule membrane protein) and CD63 (lysosome and dense granule membrane protein), following activation, as well as bound fibrinogen to $\alpha_{IIb}\beta_3$. Hirudin anticoagulated whole blood was used for impedance aggregometry analyses, and citrated whole blood for flow cytometry analysis. Following ECMO initiation, platelet count significantly decreased and remained low during ECMO therapy, despite transfusion of platelet concentrates to 20 patients to maintain platelet count above 50×10^9/L. The findings indicated that platelet aggregation assessed with the Multiplate® during ECMO therapy was not impaired when interpreted relative to platelet count. When using flow cytometry, no association was found between platelet count and activation. With this method less dependent on platelet count, they found that platelets demonstrated a reduced ability to become activated. The authors suggested that this could actually be consecutive to the vast activation of platelets in the ECMO circuit, related to the high shear stress, consistent with the increased surface expression of the activated fibrinogen receptor at rest, and a decreased expression of P-selectin, probably due to its shedding [15]. This was in agreement with previous data from our group in the setting of cardiopulmonary bypass in children [16].

In the study of Kalbhenn et al., the reference method, i.e., light transmission aggregometry, was performed in a subgroup of patients with VV-ECMO treatment. All patients had a platelet count $> 100 \times 10^9$/L and platelet-rich plasma count was adjusted to 250×10^9/L, however, contrary to what is currently recommended. After stimulation with ADP, collagen, and epinephrine, hypoaggregability and altered agglutination induced with ristocetin were found, with incomplete recovery of platelet function on day 3 after explantation. Flow-cytometric platelet analysis revealed severely reduced expression of CD62P and CD63 during ECMO (impaired and dense granules secretion), when platelets were stimulated with various thrombin concentrations, as compared to healthy subjects, while CD41 (α_{IIb}), CD42a (GPIX), and CD42b (GPIb) expressions, as well as ADP-induced fibrinogen-binding were normal [17].

To summarize, concerning the effect of ECMO on platelet adhesion receptors and membrane proteins, discrepancies between studies exist. Some studies report a reduced level of GPIb and GPVI

on circulating platelets that were exposed to abnormal shears. This finding supports the hypothesis that the association of a reduced level of platelet surface receptors with thrombocytopenia and AVWS could contribute to bleeding events in this situation of elevated shear rates. However, the design of these studies and the different preanalytical conditions cannot allow drawing firm conclusions on the effect of ECMO therapy on platelet function. Moreover, the type of device as well as their rotational speed was sparsely reported, while they could have different effects on platelets. To add to the complexity, patients who require VA-ECMO or VV-ECMO, have different underlying diseases with various resulting effects on hemostasis. Studies specifically taking into account these variables would be informative. Moreover, the studies were not powered to establish relationships between bleeding events and platelet dysfunction. Concerning platelet aggregation, abnormalities were inconsistently reported. The use of multiple electrode aggregometry analyzer in most studies presents the advantage to assess platelet function in whole blood, but does not allow to specifically investigate platelet dysfunction per se. While low hematocrit is not thought to have a major influence on Multiplate® analyzer results, platelet count $\leq 100 \times 10^9$/L, which is frequently encountered in patients with ECMO treatment, leads to reduced platelet aggregation with Multiplate® analyzer system, and therefore, could render the interpretation of the findings to be complicated. Platelet count should, therefore, be taken into account in analyzing the results of the studies (Table 1).

3. Inflammation in ECMO Patients

One of the relevant complications of ECMO is the inflammatory response. Following ECMO initiation, the association of an increase in pro-inflammatory cytokines with immune system-activation might contribute to end-organ dysfunction and death. However, as with bleeding and thrombotic complications, it is difficult to evaluate the respective part of the ECMO circuit per se in the inflammatory response and that of the underlying disease and its management. Unlike the endothelium, artificial surfaces have no regulatory molecules of the activation of the complement system. Hence, artificial surfaces contribute to the propagation and positive feedback of the complement cascade, leading to an excessive inflammatory response and capillary leak syndrome, which is a complication of extracorporeal circuits [1,18]. Following ECMO initiation, the contact system of coagulation becomes also activated and activated FXII (FXIIa) triggers the kallikrein–kinin pathway leading to the release of the proinflammatory peptide hormone bradykinin. In line with this, molecules inhibiting FXIIa were shown to reduce inflammation in ECMO models [19].

4. Inflammation and Platelet–Leukocyte Interactions

It is likely that the platelet-related inflammatory and immune functions that are triggered in host defense and in inflammatory syndromes or diseases are also set off in ECMO patients. Hemostasis and inflammation are intimately linked, and induce and amplify one another with beneficial or deleterious consequences, depending on the clinical situation [20,21]. The different mechanisms sustaining the interaction between platelets and inflammation were extensively described in recent reviews [20,21]. In brief, platelets recognize and respond to pathogens through the expression of multiple Toll-like receptors and other receptor classes that mediate inflammatory and immune signaling. Platelets are involved in endothelial barrier function and vascular permeability, through various cell adhesion molecules acting at the interface between platelets and endothelial cells. Upon activation, the platelet release stored inflammatory mediators and immunomodulators (RANTES, CD40, Platelet Factor 4 (PF4), Neutrophil-Activating Peptide-2, Platelet Activating Factor, Transforming Growth Factor- ...). They synthesize reactive oxygen species, inflammatory proteins (Interleukin-1β ...), and produce extracellular vesicles with inflammatory, immune, and thrombogenic activities. They induce neutrophil extracellular traps (NETs) and interact with leukocytes forming PLAs, thereby inducing new gene expression and synthesis of inflammatory mediators in those cells [20,22].

Different subsets of leukocytes can interact with platelets to form PLAs, leading to various effects. Among all leukocytes, monocytes show the highest affinity for platelet P-selectin, followed by

neutrophils; lymphocytes have the lowest affinity [23]. It was shown that among monocytes, different subclasses are involved in PLA, in normal subjects and in patients with myocardial infarction as well [24]. The formation of PLAs involves the release of mediators and mutual activation of both cell types. Platelets are the predominant source of both P-selectin and CD40L, which are initially located in the membrane of α granules, and become expressed on the plasma membrane upon platelet activation, enabling the interaction between platelets and leukocytes, which is a critical step in platelet-mediated inflammation. P-selectin cross-links platelets and leukocytes through its corresponding ligand P-selectin glycoprotein ligand-1 (PSGL-1), expressed on the surface of neutrophils, monocytes, dendritic cells, subclasses of lymphocytes, and endothelial cells [21]. Platelet P-selectin binding to PSGL-1 promotes the activation of Mac-1 ($\alpha_M\beta_2$) and LFA-1 ($\alpha_L\beta_2$) on neutrophils and β_1 and β_2 integrins on monocytes and lymphocytes. The main role of Mac-1 is then to mediate the firm adhesion of neutrophils, monocytes and of some subclasses of lymphocytes to activated platelets and, therefore, to stabilize PLAs [25] (Figure 1). While the resting platelets do not interact with resting leukocytes, they are able to interplay with activated leukocytes, through the interaction of platelet GPIbα with the activated Mac-1. Integrins expressed on platelet plasma membrane also play a role in the interaction with the subendothelial, extracellular matrix, leukocytes, and endothelial cells, and are capable of transducing activating signals [22]. The $_{IIb3}$ integrin is crucially involved in the initiation and regulation of interactions of platelets with leukocytes, under inflammatory conditions. Platelet $_{IIb3}$ serves as a binding partner of the integrin Mac-1 via a fibrinogen bridge, which initiates outside-in signaling into leukocytes and is necessary for leukocyte recruitment, NET formation, and ROS production [26]. Platelets CD40L also interact with CD40 on endothelial cells to promote secretion of chemokines and expression of adhesion molecules [26]. This facilitates migration of leukocytes to the site of vascular injury and subsequent adhesion [27]. Platelet–leukocyte interaction has a functionally important role in leukocyte rolling and adhesion to platelets and endothelium, which are critical steps in the process of transendothelial migration, leukocyte extravasation to the site of inflammation and initiation of vascular inflammation [27–29]. During the interaction of platelets with monocytes, expression of tissue factor by the latter initiates coagulation and contributes to thrombus formation. Moreover, after recruitment to the vessel wall, monocytes extravasate into the surrounding tissues and differentiate into macrophages [23].

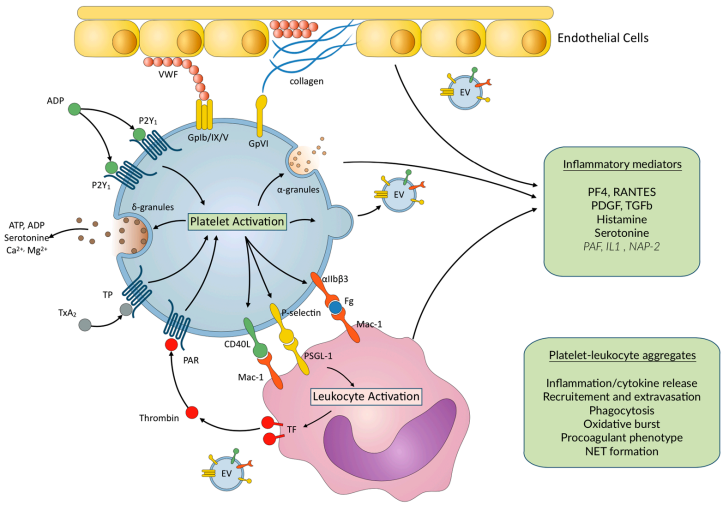

Figure 1. Main platelet–leukocyte interactions and inflammatory mediators released upon platelet activation. EC—endothelial cells; EV—extracellular vesicles; IL1—Interleukin 1; NAP-2—Neutrophil Activating Peptide-2; NET—neutrophil extracellular trap; PAF—platelet activating factor; PAR—protease activated receptor; PF4—platelet factor 4; PDGF—Platelet Derived Growth factor; TF—Tissue factor; TGFβ—Transforming Growth Factor β. VWF—von Willebrand factor. Mediators are stored in platelets. In italics—synthetized mediators/proteolytic cleavage of stored precursors upon platelet activation.

The effect of platelet–leukocyte interplays on the modulation of monocyte, neutrophil, and lymphocyte activation and function, and the subsequent effect of the different types of PLAs in inflammatory disorders were recently thoroughly reviewed [23,30]. While the prominent role of platelets in pulmonary neutrophil migration and in the recruitment and activation of neutrophils was specifically shown during the pathogenesis of acute lung injury in other settings than ECMO [22,31,32], neutrophil infiltration was described to lead to ECMO-associated lung injury and end-organ damage [19].

Due to the involvement of platelets in the inflammatory process, platelet inhibition was evaluated in some studies to reduce sepsis-associated inflammation. As a receptor of ADP, an agonist originating mainly from dense granule secretion, the platelet $P2Y_{12}$ receptor has a central role in the amplification of platelet activation, in response to a number of agonists. $P2Y_{12}$ is coupled to the Gi protein, the activation of which leads to platelet aggregation, potentiation of granule release, procoagulant activity, and PLA formation. By inhibiting platelet reactivity to ADP and a broad range of other agonists, $P2Y_{12}$ inhibitors reduce the release of pro-inflammatory mediators by platelets. The inhibition of platelet $P2Y_{12}$-mediated platelet-leukocyte interactions is thought to be one of the main mechanisms through which $P2Y_{12}$ inhibitors affect inflammation [28,33]. Using a mouse model of intra-abdominal sepsis and acute lung injury, it was shown that inhibiting $P2Y_{12}$ led to a decrease in platelet activation, platelet–leukocyte interactions, and lung injury, suggesting a key role for activated platelets and the $P2Y_{12}$ receptor during sepsis [34,35]. In the PLATelet inhibition and patient Outcomes (PLATO) trial comparing ticagrelor with clopidogrel, in patients with acute coronary syndromes, there were fewer deaths attributed to sepsis in the ticagrelor group. The more potent anti-$P2Y_{12}$ effect of ticagrelor, combined with its inhibition of adenosine reuptake by red blood cells, might account for the difference in clopidogrel and ticagrelor in this trial [36]. Indeed, adenosine is formed from ATP and ADP released locally at the sites of ischemia, tissue damage, and inflammation with a very short half-life in circulation, due to its rapid cellular uptake and intracellular metabolism. Ticagrelor inhibits the ENT1 transporter and thereby reduces the cellular uptake of adenosine, resulting in increased adenosine-induced responses such as platelet inhibition and neutrophil chemotaxis and phagocytosis [37,38].

The investigation of the interaction between leukocytes and platelets was facilitated by the use of flow cytometric analysis and advanced microscopy techniques [30]. However, characteristics of PLAs in terms of size and number of platelets bound per leukocytes are not well defined. In vitro experiments reported that after platelet activation with thrombin, the semi-quantitative estimate of the number of platelets bound per leukocyte was 2 and 22 for inactivated and activated platelets, respectively [39]. Images showing PLAs as investigated by flow cytometry, tissue section or live cell imaging were presented by Finsterbush et al. in their review on platelet–leukocyte interactions in acute ischemic stroke, renal diseases, and hepatic as well as lung inflammation and infection. They suggested that PLAs within the circulation or locally at the sites of inflammation might represent markers of many thrombo-inflammatory diseases and could be used for the assessment of both thrombotic risk and disease progression [30]. Moreover, PLAs could be of great interest as markers of the modulation of thrombo-inflammation in patients receiving antithrombotic drugs in the setting of acute inflammation.

Despite the potential implication of platelets in inflammation and hemostasis complications of ECMO, to the best of our knowledge, no data are currently available on circulating PLA levels in these patients. Just one recent study was performed on membrane oxygenators taken from 21 patients with VV-ECMO. Membrane oxygenators were collected after termination of ECMO therapy or after replacement during therapy. They were found to be loaded with VWF, activated platelets and leukocytes, and co-localization of nucleated cells (DAPI-positive) and P-selectin-positive structures, consistent with locally formed PLAs. The clinical relevance of these results needs further investigation [40].

At present, much of what is known about PLAs and artificial devices comes from studies in patients undergoing cardiac surgery under cardiopulmonary bypass, but the effects of short-term extracorporeal circulation on platelet function might not be fully translatable to long-term extracorporeal circulation such as in ECMO therapy. Li et al. showed that coronary artery bypass grafting (CABG) induced marked pro-thrombotic and inflammatory responses, which persisted for at least one week.

Platelet activation, platelet reactivity, PLA formation, thrombin generation, and TNF-release showed a second peak one week after surgery. These findings suggested that intensified and prolonged antithrombotic/inflammatory treatment should be considered after CABG surgery [41]. In a recent work from the same team, platelet-monocyte and platelet–neutrophil levels were lower three months after CABG than before, suggesting an improvement in wound healing and inflammation, whereas signs of mildly increased platelet reactivity persisted up to three months after surgery. The authors concluded that their data supported the need for efficient antiplatelet therapy during the first three month after CABG [29].

As pointed out by Finsterbush et al. in their review of the different methods to measure PLAs, to allow validating PLAs as a biomarker in the future, protocols for standardized sample preparation and robust reference values need to be established first. Histochemical and immunofluorescent imaging, coupled with advanced confocal or electron microscopy, provide high-resolution images to visualize platelet–leukocyte interactions within tissues [30]. Finsterbush et al. also described different methods to visualize PLAs in vivo—intravital microscopy, which allows tracking cells in live animals over longer time periods in organs; intravenous administration of fluorochrome-conjugated monoclonal antibodies, and microfluidic assays to analyze platelet–leukocyte interaction dynamics under well-defined conditions, including investigation of human cells [30]. Nevertheless, flow cytometry remains the method of choice for measuring circulating PLAs, representing a reliable method that enables concomitant analysis of platelet activation and PLA formation, within the same blood sample [30]. However, the relationship between tissue or in vivo PLAs and circulating PLAs measured ex-vivo through flow cytometry is not yet established.

Moreover, the measurement of PLAs requires specific conditions to avoid ex vivo platelet activation and subsequent neoformation of PLAs; pre-analytical conditions need to be strictly defined and standardization of data analysis are also required.

5. Flow Cytometric Whole Blood Measurement of Platelet–Leukocyte Aggregates

5.1. General Considerations

Flow cytometry allows rapid and sensitive analysis of fluorescent-labeled cells passing through a flow cell and hit by a focused beam of a laser light. Detectors analyze the light scattering and the emitted fluorescence of each cell or aggregate. Forward-scattered light (FS) and side-scattered light (SS) give physical information regarding cell dimensions and structural complexity. Fluorescence measurement of fluorophore-conjugated monoclonal antibodies allows the analysis of presence and cell density of specific targeted cell antigens, hence permitting study of cell phenotype, function, and interaction with other cells [42].

PLA measurement with whole blood flow cytometry requires at least two fluorescent-labeled antibodies, targeting platelets and leukocytes, respectively. PLAs are then expressed as a percentage of total leukocytes positive for a platelet-specific marker [41,43]. Hence, being independent of absolute leukocyte number and considering the abundance of circulating platelets over leukocytes, PLAs can be measured in both leukopenic and thrombocytopenic patients.

Leukocytes are usually labeled using anti-CD45 (leukocyte common antigen) pan-leukocyte antibody and platelet-specific labeling that typically includes CD41 (GPIIb, α_{IIb}), the non-platelet specific CD61 (GPIIIa, β_3), CD42a, or CD42b. Among these, the platelet $\alpha_{IIb}\beta_3$ integrin is often used as the platelet-specific antibody target because of the high copy number of this receptor, i.e., a mean of around 50,000 copies per platelet at rest. Similarly, as specific platelet glycoproteins, CD42a and CD42b are potential targets but should be used with caution, as platelet activation downregulates their expression [44].

Upon activation, platelet extracellular vesicles, measuring from 100 to 1000 nm, are released and circulated, and it cannot be ruled out that they form complexes with leukocytes [24]. Since these

complexes are CD41-positive, they can hardly be distinguished from PLAs with platelets. However, both result from platelet activation and reflect interactions with leukocytes.

It should always be kept in mind that PLA formation is a dynamic process and that formation and dissociation of aggregates can also occur in vitro. Therefore, potential influence of platelet-specific antibodies on PLAs in vitro must be considered, especially using CD42b, giving its role in PLA stabilization [45].

Neutrophils, monocytes, and lymphocytes can be distinguished, based on light-scattering properties, allowing basic discrimination of PLA subtypes (platelet–neutrophil, platelet–monocyte, and platelet–lymphocyte aggregates) [30]. Platelet–platelet aggregates that might be present in samples can also interfere with light-scatter gating. Thus, identification of PLA subtypes, while avoiding detection of platelet–platelet aggregates, is improved by using leukocyte subtype-specific antibodies (e.g., CD66b for neutrophils; CD14 for monocytes; CD3, CD4, and CD8 for T-cells; CD19 for B-cells; CD56 for NK-cells) [44].

5.2. Pre-Analytical Requirements—Choice of Anticoagulant, Delay between Sampling and Immunolabeling, Sample Stability after Immunolabeling, Effect of Strong Vortex Agitation prior to Immunolabeling

Given the high sensitivity of the flow cytometry assay and the high reactivity of human platelets, PLA measurement is extremely susceptible to in vitro activation and pre-analytical conditions [30]. Defining a rigorous laboratory methodology is essential to provide reliable and comparable results, especially in the setting of complex inflammatory situations like ECMO.

We sought to determine the effect of blood drawing and sample preparation on PLA measurement. Peripheral venous blood was obtained from seven healthy volunteers who were not taking any anti-platelet medication. Blood was drawn by clean venipuncture of a large antecubital vein, after applying a tourniquet, using a 21-gauge needle, and was collected into vacuum tubes containing 3.2% buffered sodium citrate or CTAD (sodium buffered citrate, theophylline, adenosine, and dipyridamole). Hirudin was not tested in this experiment because of its limited application in hemostasis laboratory, except for the whole-blood impedance aggregometry method. The first milliliters were discarded. The tubes were gently inverted once, immediately after blood collection. Blood was labeled using CD45-FITC and CD41-PE antibodies, with 15 min incubation time in darkness, without lysis or fixation. Samples were then diluted in phosphate buffer saline (final blood dilution 1:44) and analyzed, the events being acquired on Navios (Beckman Coulter) or Lyric (Becton Dickinson) (Figure 2). We evaluated (i) the type of anticoagulant solution, (ii) the delay between sampling and immunolabeling (15, 60, and 150 min), (iii) the sample stability after immunolabeling (0 and 90 min), and (iv) the effect of strong vortex agitation (10 s at 3200 rpm), prior to immunolabeling.

The results were as follows—(i) compared to sodium citrate, CTAD had a protective effect by reducing the rate of in vitro PLA formation prior to immunolabeling ($p = 0.037$) (Figure 2c); (ii) the time-interval between blood collection and immunolabeling had a marked effect on PLA levels; indeed, considering a mean level of 17% PLAs, an increase in PLAs by 1.7% was noticed every 10 min of delay with sodium citrate as anticoagulant (95% CI 1.1 to 2.3, $p < 0.0001$) and by 0.8% with CTAD (95% CI 0.2 to 1.4, $p = 0.005$); (iii) in vitro PLA formation still occurred after immunolabeling with an increase in PLA levels from baseline (0 min) to 90 min, using sodium citrate (15.7 ± 4.0% to 37.5 ± 7.9 %; $p = 0.0003$) and CTAD (16.5 ± 5.6% to 42.0 ± 10.4%; $p = 0.0004$); (iv) finally, strong vortex agitation prior to immunolabeling had no significant effect on PLA levels, whatever the anticoagulant ($p = 0.87$). Using conditions that minimize in vitro PLA formation (15 min delay between blood collection and immunolabeling and no delay between immunolabeling and flow cytometric assay), mean PLA levels with sodium citrate and CTAD were, respectively, 17.8 ± 7.4% and 16.9 ± 6.2% ($p = 0.7$).

These results demonstrate that sample preparation before flow cytometry assay is critical, as it induces a significant variability in the PLA levels.

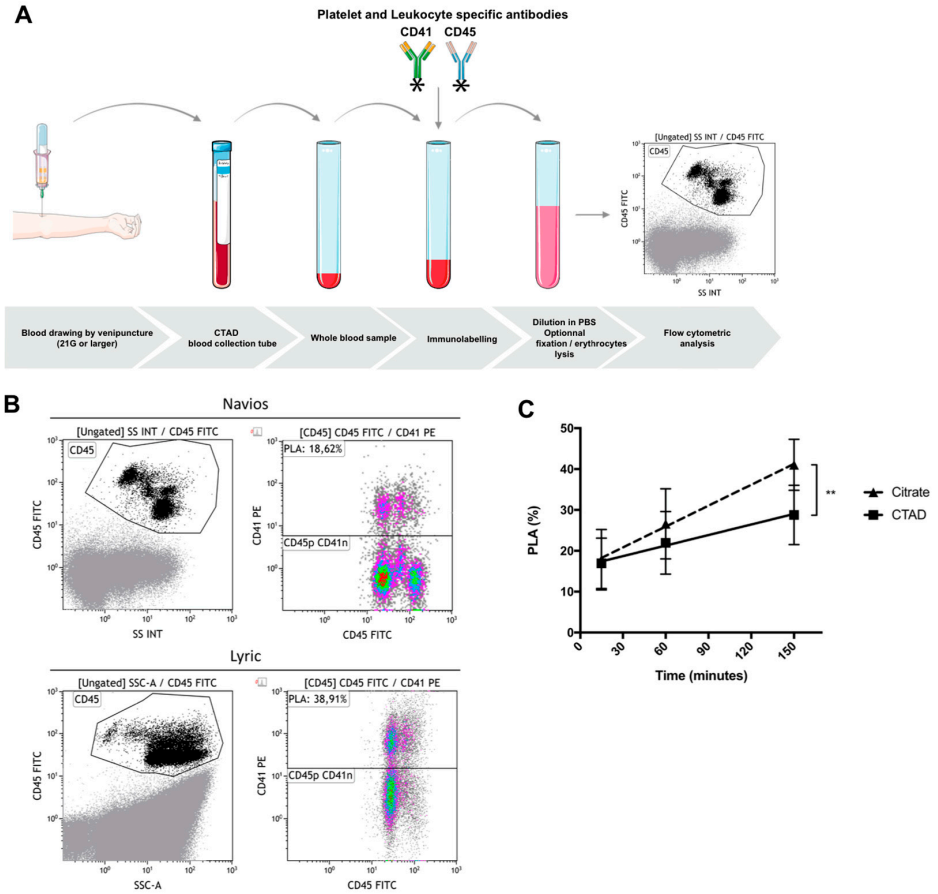

Figure 2. Platelet–leukocyte aggregates (PLAs) analyzed with flow cytometry (**A**). Schematic representation of sample preparation for PLA measurement in whole blood. Venous blood was drawn by puncture of a large vein using a 21-gauge needle and collected into tubes containing CTAD (sodium buffered citrate, theophylline, adenosine, and dipyridamole). The first milliliters were discarded. Blood sample was labeled using antibodies against CD45 and CD41 (20 µL of blood + 20 µL of PBS buffer + 10 µL of each antibody), with fixed incubation time (<1 h), followed by dilution (≥1/40) in PBS or with lysis and fixation buffer. (**B**) Example of samples acquired on Navios (top) or Lyric (bottom) flow cytometer. Blood was labeled using antibodies against CD45-FITC and CD41-PE, with 15 min of incubation (no lysis, no wash protocol). Final blood dilution was 1:44. Flow rate was at 70 events/sec and 10,000 events were acquired. Leukocytes (CD45) were defined as CD45pos side scatter high [CD45] (left panels). Then PLAs in [CD45] gated dot plots, were identified with the co-expression of CD45 and CD41 (right panels). (**C**) Effect of time prior to immunolabeling on PLAs. Blood was drawn into tubes containing 3.2% buffered sodium citrate or CTAD. Immunolabeling and processing of whole blood samples were performed 15, 60, and 150 min, following blood collection. Blood was labeled using CD45-FITC and CD41-PE antibodies (cf above), without lysis or fixation. Events were acquired on the Lyric system (Becton Dickinson). For every 10 min of time-interval prior to immunolabeling, PLA levels increased by 1.7% (95% CI 1.1 to 2.3, $p < 0.0001$) in citrate-anticoagulated blood (closed triangles, dashed line) and 0.8% (95% CI 0.2 to 1.4, $p = 0.005$) in CTAD-anticoagulated blood (closed squares, plain line). Linear regression analysis demonstrated a reduced rate of in vitro PLA formation, prior to immunolabeling with CTAD (solid line), compared to sodium citrate (dashed line) ($p = 0.037$). Data are reported as mean ± SEM ($n = 7$). SS INT and SSC-A—side scatter.

5.3. Optimal Conditions for Whole Blood Cytometric Measurement of PLAs: A Proposal

Our study was consistent with previous results published by Harding et al. regarding whole blood flow cytometric analysis of circulating platelet–monocyte aggregates (PMA) [46]. They reported a significant effect of anticoagulant type on PMA levels, in vitro PMA formation being better prevented

with EDTA and sodium citrate than with heparin, hirudin, and PPACK. Indeed, platelet activation is likely best prevented by calcium chelators than with anticoagulant agents. Venipuncture led to lower PMA levels, compared to intravenous cannulas. Delay prior to immunolabelling induced significant in vitro PMA formation. Since red blood cell lysis could lead to platelet activation by ADP released from erythrocytes, immediate fixation should be performed along with red cell lysis, to avoid increase in PLA levels. Hence, Harding et al. demonstrated that erythrocyte lysis together with fixation after immunolabelling did not affect PMA and that PMA remained stable over 24 h when fixed and stored at 4 °C [46]. In line with these results, we found similar levels of total PLAs after dilution of the labeled blood samples with either phosphate buffer saline or FACS-lysing solution®.

Regarding the type of anticoagulant solution, CTAD seemed to offer the best protection from in vitro spontaneous PLA formation [44]. EDTA should be avoided as it could cause in vitro dissociation of platelets from leukocytes, notably through an αIIbβ3 integrin dissociation [44,47].

The fixation with paraformaldehyde along with erythrocyte lysis after immunolabeling seems to offer acceptable sample stability and reproducibility up to 24 h at 4 °C, without any effect on the PLA levels [43,44,46]. On the contrary, prefixation and red cell lysis before immunolabeling should not be used, as it can induce considerable change in the PLA formation dynamic, with an important increase in the PLA levels [41]. Additionally, since most PLAs are formed with monocytes, LPS-containing solutions should not be used.

Finally, due to a high number of platelets in the whole blood (10- to 100-fold higher than leukocytes), PLA analysis can be greatly altered by coincidence, i.e., double positivity occurring from non-interacting coinciding leukocytes and platelets or platelet-derived extracellular vesicles [48–50]. Coincidence is exacerbated by small dilution of samples and a high cytometric flow rate. Dilution and flow rates impact should be determined in each laboratory and should be stated in PLA cytometric protocols. Although final blood dilution should be above 1:40 to minimize coincident events detection [48], increasing blood dilution might induce PLA dissociation. Several approaches need to be considered to reduce coincident events measurement, including doublet-discriminator strategy [48,51] and imaging flow cytometry [52].

The optimized conditions we propose for whole blood cytometric measurement of PLAs, in order to minimize artefactual in vitro platelet activation and PLAs formation, are summarized in Table 2.

Table 2. Sample preparation and flow cytometric whole blood measurement of circulating platelet–leukocyte aggregates (PLAs).

Sample Handling and Preparation	
Anticoagulant in blood sampling tube	Preferably CTAD (other option: sodium citrate)
Storage temperature	Room temperature
Centrifugation before immunolabeling	None (whole blood protocol)
Prefixation before immunolabeling	None
Erythrocyte lysis before immunolabeling	None
Processing time before immunolabeling	Fixed time to allow comparative analysis As short as possible, preferably < 1 h after blood withdrawal
Fixation after immunolabeling	Optional, paraformaldehyde fixation (0.5 to 1%)
Final blood dilution	≥1:40
Erythrocyte lysis after immunolabeling	Optional
Processing time after immunolabeling	Without delay if no fixation 24 h at 4 °C, after fixation
Cytometric flow rates	Low to medium, determined in each laboratory
Immunolabeling	
Platelet-specific antibody	Preferably: CD41 Other option: CD61
Leukocyte-specific antibody	CD45
Leukocyte subtype-specific antibody	Neutrophils: CD66b
	Monocytes: CD14
	T-cells: CD3, CD4 and CD8
	B-cells: CD19
	NK-cells: CD56

6. Conclusions

In the setting of ECMO, platelets could be involved in both bleeding and thrombotic complications. Indeed, ex-vivo studies showed either no effect or decreased platelet reactivity, which could be consecutive to vast in vivo activation of platelets in the ECMO circuit and, therefore, consumption and clearance of activated platelets and loss of reactivity. Upon activation, platelets might also interact with the inflammation system, resulting in pro-thrombotic effects. It is of utmost importance to better understand these mechanisms, in order to prevent and treat bleeding and thrombosis in the setting of ECMO. In this context, it would be valuable to study PLA formation to better assess the involvement of platelets in the inflammatory complications of ECMO. Moreover, PLAs could serve as markers of the modulation of thrombo-inflammation in patients receiving antithrombotic treatment in this setting of acute inflammation. Owing to the high reactivity of human platelets and the dynamic nature of platelet–leukocyte interaction, flow cytometric measurement of PLAs could be challenging and requires strictly defined pre-analytical conditions. International standardization is crucial to ensure comparability and reproducibility of future clinical trials.

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AMP cyclique plaquettaire : rôle dans l'activation plaquettaire et régulation

Comme nous avons pu le décrire dans les chapitres précédents, de nombreux agonistes sont capables d'induire une activation plaquettaire, au travers de la liaison à leur récepteurs spécifiques[25,26,28,81–83].

Trois glycoprotéines de surface, le complexe GPIb-IX-V, la GPVI et l'intégrine αIIbβ3 (GPIIbIIIa), jouent un rôle majeur dans l'adhésion, l'activation et l'agrégation plaquettaires. Le complexe GPIb-IX-V est le récepteur principal du FVW, mais peut également se lier à la thrombospondine, à la thrombine et au facteur XI. Elle permet d'initier, en association avec d'autre agonistes l'activation de l'intégrine αIIbβ3. La liaison du collagène à la GPVI initie des voies de signalisation aboutissant à l'élévation de la concentration cytosolique de calcium plaquettaire provoquant le remaniement du cytosquelette, la libération du contenu granulaire et l'activation de l'intégrine αIIbβ3. Après activation "inside-out", l'intégrine αIIbβ3 devient le médiateur de l'agrégation plaquettaire, responsable d'une amplification de l'activation plaquettaire. Elle est à ce titre une cible pharmacologique d'utilisation clinique.

Les plaquettes expriment par ailleurs plusieurs récepteurs à sept domaines transmembranaires couplés au niveau intracellulaire à des protéines G qui permettent la transduction du signal. Plusieurs récepteurs se lient à des agonistes induisant l'activation plaquettaire (thrombine, ADP, TXA2, catécholamines). A l'inverse les récepteurs de l'adénosine et de la prostacycline (PGI2) ont un effet inhibiteur de l'activation médié par une protéine Gs qui stimule la synthèse d'AMPc.

Les plaquettes expriment deux récepteurs principaux à la thrombine (PAR-1 et PAR-4) lui permettant d'exercer un puissant effet agoniste sur l'activation plaquettaire. L'ADP exerce un rôle agoniste faible mais crucial en tant que cofacteur de l'activation plaquettaire, par sa fixation aux récepteurs P2Y1 et $P2Y_{12}$. Les mécanismes de signalisation de la voie du de l'ADP sont détaillées dans la revue de littérature présentée au chapitre suivant (Mansour et al. Int. J. Mol. Sci. 2020, 21, 1391). Enfin le thromboxane A2 issu du métabolisme de l'acide arachidonique, ainsi que les catécholamines (adrénaline et noradrénaline) exercent également des fonctions agonistes de l'activation plaquettaire respectivement médiées par le récepteur TP et les adrénorécepteurs α2.

En situation physiologique, il existe un équilibre entre les signaux activateurs et inhibiteurs plaquettaires. Ce rôle est en grande partie joué par l'endothélium qui secrète des substances inhibitrices de l'activation plaquettaire, en particulier la prostacycline (PGI2) et le monoxyde d'azote (NO)[26,84,85]. Elles sont en effet responsables de la production intraplaquettaire de nucléotides cycliques, puissants inhibiteurs de l'activation et de l'agrégation plaquettaire : l'adénosine monophosphate cyclique (AMPc) et la guanosine monophosphate cyclique (GMPc). La voie de l'AMPc est principalement activée par la PGI2 et celle du GMPc par le NO (Figure 12).

L'AMPc est synthétisée par l'adénylate cyclase à partir de l'ATP. Elle exerce son rôle inhibiteur en activant la protéine kinase A (PKA) qui peut alors phosphoryler de nombreux substrats, et en particulier le récepteur IP_3 induisant une diminution des concentrations cytosoliques de calcium. Ce mécanisme aboutit notamment à l'inhibition de la voie "inside-out" et au maintien au repos de l'intégrine αIIbβ3.

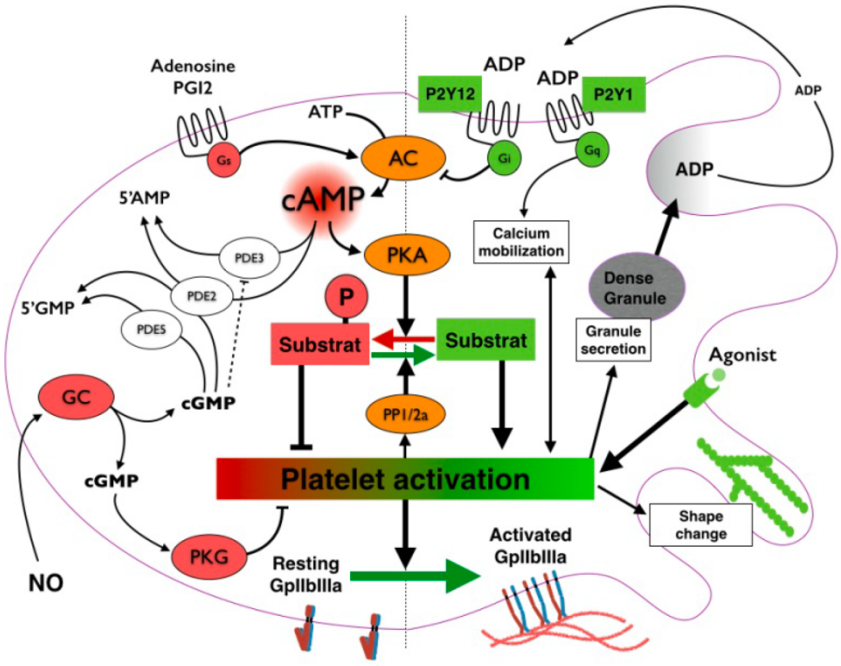

Figure 12: Rôle de l'AMPc dans la régulation de l'activation plaquettaire
Dans la plaquette au repos (à gauche) : la PGI2 (ou la PGE2) en se fixant sur son récepteur IP entrainent, via la protéine Gs, une activation de l'adénate cyclase (AC) à l'origine d'une augmentation de la concentration en AMPc. Ce second messager active la protéine kinase A qui peut alors phosphoryler de nombreux substrats à l'origine du maintien au repos de l'intégrine αIIbβ3. Le NO diffuse dans la plaquette et induit la synthèse de GMPc. Les phosphodiesterases régulent la concentration en nucléotides cycliques en hydrolysant l'AMPc et le GMPc. Lors de l'activation plaquettaire (à droite), l'ADP secrété potentialise l'activation en se fixant sur ses récepteurs, P2Y1 et P2Y»12» qui entraine l'inhibition de l'AC. Cette inhibition est responsable de la diminution de la concentration en AMPc, ce qui supprime l'activation de la PKA et permet l'activation d'αIIbβ3, la fixation du fibrinogène et finalement l'agrégation plaquettaire (Reproduit avec autorisation [85])

Plusieurs voies de régulation des taux d'AMPc plaquettaires ont été mis en évidence. Premièrement, les phosphodiestérases (PDE) hydrolysent les nucléotides cycliques. Deuxièmement, l'adénylate cyclase est sous le contrôle agoniste des voies de signalisation de l'adénosine et de la prostacycline d'une part, et sous le contrôle antagoniste de la voie de signalisation ADP/$P2Y_{12}$, d'autre part (voir chapitre suivant). Enfin, notre équipe a publié plus récemment le rôle de la protéine d'efflux MRP4 qui permet la déplétion de l'AMPc du cytosol[86].

Inhibition de la voie du $P2Y_{12}$: effets sur l'inflammation

Comme nous l'avons vu précédemment, l'AMPc plaquettaire joue un rôle prépondérant dans le contrôle de l'activation plaquettaire. A ce titre la voie ADP/$P2Y_{12}$ joue un rôle crucial en tant que cofacteur de l'activation plaquettaire.
La disponibilité et l'utilisation courante des inhibiteurs de $P2Y_{12}$ en pratique clinique a justifié de centrer ce travail de thèse sur le rôle de la modulation de $P2Y_{12}$ sur les fonctions inflammatoires des plaquettes.

Ceci a fait l'objet d'une revue de littérature publiée et présentée ici.

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Review

$P2Y_{12}$ Inhibition beyond Thrombosis: Effects on Inflammation

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Abstract: The $P2Y_{12}$ receptor is a key player in platelet activation and a major target for antithrombotic drugs. The beneficial effects of $P2Y_{12}$ receptor antagonists might, however, not be restricted to the primary and secondary prevention of arterial thrombosis. Indeed, it has been established that platelet activation also has an essential role in inflammation. Additionally, nonplatelet $P2Y_{12}$ receptors present in immune cells and vascular smooth muscle cells might be effective players in the inflammatory response. This review will investigate the biological and clinical impact of $P2Y_{12}$ receptor inhibition beyond its platelet-driven antithrombotic effects, focusing on its anti-inflammatory role. We will discuss the potential molecular and cellular mechanisms of $P2Y_{12}$-mediated inflammation, including cytokine release, platelet–leukocyte interactions and neutrophil extracellular trap formation. Then we will summarize the current evidence on the beneficial effects of $P2Y_{12}$ antagonists during various clinical inflammatory diseases, especially during sepsis, acute lung injury, asthma, atherosclerosis, and cancer.

Keywords: platelets; $P2Y_{12}$; inflammation; hemostasis; sepsis; cancer; leukocytes; antiplatelet agents; asthma; atherosclerosis

1. Introduction

Nucleotides are universal extracellular signaling molecules, acting as intercellular or autocrine messengers that can be passively released by injured cells or secreted by specific mechanisms. Among their countless physiological or pathological functions, purinergic signaling can regulate hemostasis, thrombosis, and inflammation through the costimulation of various cell types, including platelets, leukocytes, endothelial, and vascular smooth muscle cells [1].

The specific plasma membrane receptors for nucleotides are called P2 receptors and are divided into two subgroups: P2X ligand-gated ion channels and P2Y G-protein (guanine nucleotide-binding protein)-coupled receptors. Eight P2Y receptors have been identified and divided according to their preferred agonist: adenine nucleotides (ADP and ATP) for P2Y1, P2Y11, $P2Y_{12}$, and P2Y13 receptors;

uracil nucleotides (UDP and UTP) for P2Y4 and P2Y6; adenine and uracil nucleotides for P2Y2; and UDP and UDP-glucose for P2Y14 [2–5].

Adenine nucleotide mediated platelet activation is a critical mechanism in both physiological and pathological hemostasis (including thrombosis), and it involves three platelet P2 receptors (P2Y1, $P2Y_{12}$, and P2X1, the latter one being an ATP channel). Among the P2Y ADP receptors, $P2Y_{12}$ is a key receptor and the unique P2 target for clinically approved antiplatelet drugs (herein called $P2Y_{12}$ inhibitors) [6].

Besides their hemostatic capacities, platelets play an emerging and significant role in regulating inflammatory and immune response. Indeed, they are able to interact with immune cells through membrane exposure of P-selectin and CD40 (cluster of differentiation 40) ligand, and to release inflammatory mediators (cytokines, chemokines) [7,8]. Platelets are also involved in tumorigenesis and in the modulation of tumor microenvironment [9]. Moreover, $P2Y_{12}$ is expressed in other cell types than platelets, including cancer, immune and vascular cells, and binding of ADP to $P2Y_{12}$ might activate leukocytes and dendritic cells [2].

Therefore, together with antithrombotic effects, $P2Y_{12}$ inhibitors might also have a role in modulation of inflammation.

After a brief description of $P2Y_{12}$ function and its current clinical inhibitors, we will review the effects of $P2Y_{12}$ inhibition on molecular and cellular mechanisms of inflammation and the current evidence for $P2Y_{12}$ antagonism in clinical inflammatory diseases and syndromes.

2. $P2Y_{12}$ Receptors

2.1. Structure of $P2Y_{12}$ Receptors

The $P2Y_{12}$ receptor was originally described as a platelet ADP receptor inhibited by thienopyridine antiplatelet agents ticlopidine and clopidogrel. It was further identified by cloning in 2001 by Hollopeter and Zhang and maps to chromosome 3q25.1 [10,11].

$P2Y_{12}$ is a Gi-coupled seven-transmembrane domain receptor composed of 342 amino acids with a molecular weight of 39kDa. Its structure consists of a seven-transmembrane (7-TM) bundle of α-helices connected by three intracellular and three extracellular loops (EL), and a carboxy-terminal helix (H8) that is parallel to the membrane bilayer on the cytoplasmic side [12]. It contains four extracellular cysteines at positions 17, 97, 175, and 270 that form two disulfide bridges (between the *N*-terminal domain and EL3 and between EL1 and EL2) which are important sites for receptor expression and potential target for active metabolites of thienopyridines [2,13,14].

2.2. Expression of $P2Y_{12}$ Receptor

The $P2Y_{12}$ receptor was originally sought to be exclusively expressed on platelets, with about 400 copies of the $P2Y_{12}$ receptor per cell, and to a lesser extent in subregions of the brain [11,15]. However, further studies demonstrated that $P2Y_{12}$ has a wider tissue distribution, being expressed and functional in microglial cells [16], vascular smooth muscle cells [17,18], and on several immune cells including dendritic cells [19], mast cells [20], eosinophils [21], monocytes [22], lymphocytes [23,24] and macrophages [25]. The $P2Y_{12}$ receptor expression has been also recently reported in osteoclasts [26] as well as in brain and breast cancer cell lines [27]. $P2Y_{12}$ is not expressed by human endothelial cells of aortic, cerebral or cardiac origin [28]. Still, the expression and function of $P2Y_{12}$ in other cell types remain poorly investigated. Although $P2Y_{12}$ is expressed on the plasma membrane of resting platelets, an inducible pool of $P2Y_{12}$ can also be exposed upon platelet activation by strong agonists [29].

2.3. Role of $P2Y_{12}$ Rreceptor in Platelet Activation Pathways

Among extracellular nucleotides, ADP (adenosine 5′-diphosphate) plays a key role in platelet function and thrombus formation (Figure 1). It is a weak platelet agonist as it only induces reversible responses, including shape change and reversible aggregation. Platelets express two G protein-coupled

receptors for ADP: P2Y1, which is coupled to Gq (G-protein q), and $P2Y_{12}$, which is coupled to Gi (G-protein i) [3]. Despite the fact that ADP alone is not able to induce the release of platelet dense granules, binding of ADP to its platelet receptors amplifies and sustains the secretion and aggregation induced by other strong agonists [3]. Hence, $P2Y_{12}$ plays an important role in the stabilization of platelet aggregates induced by other agonists such as thrombin and thromboxane A2 (TXA2), as well as in thrombogenesis in vivo [3,13,18,30,31]. This central position explains both the clinical benefit of $P2Y_{12}$ antagonists and the bleeding diathesis observed in $P2Y_{12}$ deficient patients [32,33].

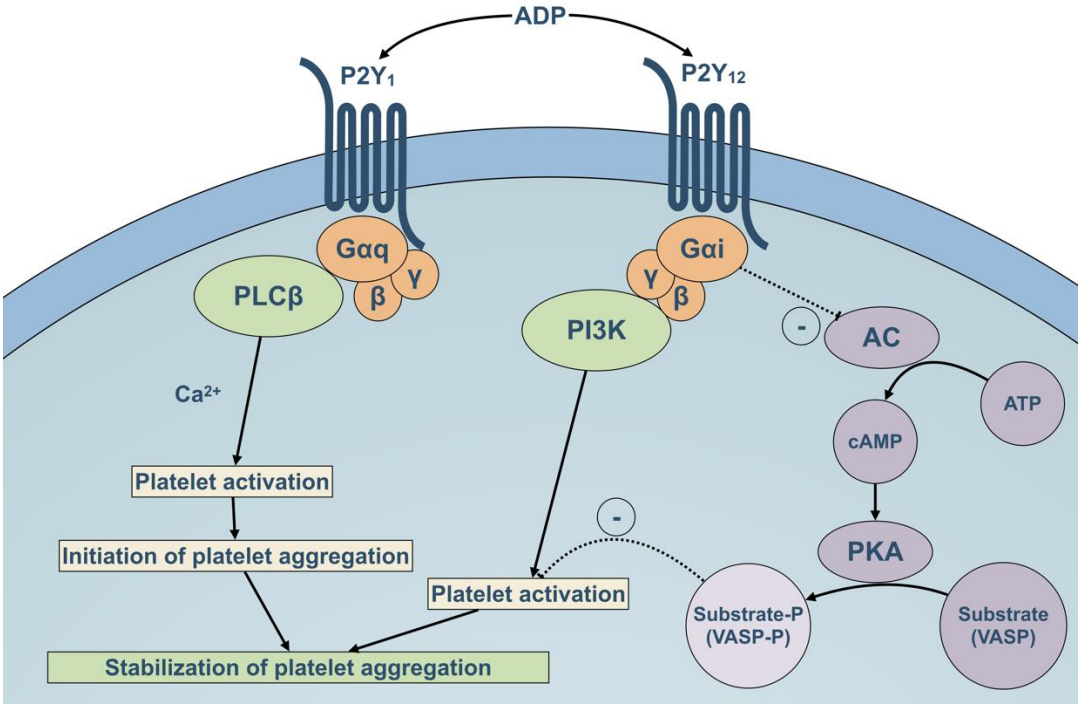

Figure 1. Schematic illustration of the platelet P2-mediated ADP signaling pathway and its role in platelet activation. ADP can induce platelet activation by interacting with two G-coupled platelet receptors, $P2Y_1$ and $P2Y_{12}$, and contributes to the stabilization of platelet aggregation induced by other strong agonists such as thrombin and TXA2. ADP binding to $P2Y_1$ induces a mobilization of calcium ions through the stimulation of phospholipase C and therefore initiates early reversible aggregation. ADP binding to $P2Y_{12}$ induces PI3K activation through the recruitment of $\beta\gamma$ subunits of Gi and inhibition of adenylate cyclase through the recruitment of αi subunit, therefore decreasing cAMP levels and leading to impaired PKA activation. These pathways ultimately lead to platelet activation, αIIbβ3 activation and stabilization of platelet aggregates. AC: adenylate cyclase; ADP: adenosine diphosphate; ATP: adenosine triphosphate; PI3K: phosphoinositide-3-kinase; PKA: protein kinase A; PLC: phospholipase C; VASP: vasodilator-stimulated phosphoprotein.

Concomitant stimulation of $P2Y_1$ and $P2Y_{12}$ receptors by ADP is required to generate normal ADP-induced platelet aggregation [2,4]. Binding of ADP to $P2Y_1$ initiates shape change and early reversible aggregation by mobilization of calcium ions from internal stores through the stimulation of phospholipase C and formation of inositol 1,4,5-trisphosphate (IP3) [5,13,34]. $P2Y_{12}$ activates a G αi2 G protein subtype that mediates the inhibition of adenylate cyclase and therefore cyclic adenosine monophosphate (cAMP) production, leading to impaired protein kinase A (PKA) activation and a subsequent inhibition of downstream effectors such as vasodilator-stimulated phosphoprotein (VASP) [5,13,35]. This cAMP inhibition is not sufficient to induce platelet aggregation by itself but can promote it [36]. VASP phosphorylation state can be used to monitor the effects of anti $P2Y_{12}$ therapy [37,38]. Binding of ADP to $P2Y_{12}$ also induces an activation of two isoforms of phosphoinositide-3-kinase-(PI3K) - p110β and p110γ - through the recruitment of $\beta\gamma$ subunits of

Gi, which is critical for integrin αIIbβ3 (GpIIbIIIa) activation and stabilization of platelet aggregates, especially induced by thrombin or TXA2 [13,39–42]. This PI3K activation also amplifies platelet secretion induced by other agonists [13,43]. Finally, $P2Y_{12}$ supports thrombin generation by amplifying membrane exposition of phosphatidylserine, platelet-derived microparticle formation and collagen-induced exposure of tissue factor (TF). Moreover, it contributes to leukocyte activation induced by surface P-selectin exposure and formation of platelet-leukocyte aggregates [13,44–48]. Formation of coated-platelets—which are a subpopulation of platelets characterized by surface retention of procoagulant proteins, expression of phosphatidylserine and strong prothrombinase activity—is dependent on ADP-induced $P2Y_{12}$ activation [49–51].

2.4. Role of Non-Platelet $P2Y_{12}$ Receptors

Vascular smooth muscle cells (VSMCs) play a key role in the physiological functions of blood vessels, such as vasoconstriction, vasodilatation and extracellular matrix production. They are also involved in the pathogenesis of vascular diseases, especially atherosclerosis, vascular inflammation and restenosis following angioplasty [52]. $P2Y_{12}$ receptor expression and function in ADP-induced vessel contraction was first described in human internal mammary artery SMCs in 2004 [18]. Activation of $P2Y_{12}$ is thought to induce an inflammatory state in VSMCs and correlates with atherosclerotic plaque instability [17,52–54]. It is associated with increased monocyte chemoattractant protein 1 (MCP-1) expression and monocyte adhesion [54]. $P2Y_{12}$ is also implicated in the migration VSMCs through cAMP/PKA signaling pathway associated with actin disassembly and therefore an increase in VSMC motility and migration [55].

$P2Y_{12}$ is also functionally expressed in microglial cells and can play a role in their activation [16] and in microglia-neuron communications and microglial neuroprotection [56]. Thus, as microglial cells are the primary innate immune cells of the brain and play an important role in the pathophysiology of many brain-based conditions, an implication of $P2Y_{12}$ may be expected in various diseases including multiple sclerosis, Alzheimer's disease, traumatic brain and spinal cord injury, and brain cancers [55].

Dendritic cells (DC) are considered the most efficient antigen presenting cells and are able to regulate adaptive immunity by inducing naïve T cell activation and effector differentiation [57]. $P2Y_{12}$ receptor is expressed in murine DCs and its activation enhances specific T cell activation by increasing antigen endocytosis [19]. $P2Y_{12}$ is also expressed in human DCs and it has been demonstrated that inhibition of $P2Y_{12}$ mediated PI3K activation induces an immunosuppressive effect on DCs by decreasing antigen uptake [58].

To date, only few studies have evaluated the role of $P2Y_{12}$ receptor in the purinergic signaling in leukocytes. High amounts of the $P2Y_{12}$ receptor mRNA were previously described in lymphocytes as well as in CD34+ progenitor cells [24]. In a clinical study involving cardiologic patients, Diehl et al. showed that $P2Y_{12}$ mRNA was expressed in leukocytes obtained from leukapheresis and that $P2Y_{12}$ inhibition by clopidogrel decreased leukocyte CD11b (Mac-1) expression [59]. The $P2Y_{12}$ expression was also reported in human eosinophils in which $P2Y_{12}$ activation induced the release of eosinophil peroxidase [21] that could be prevented by clopidogrel [60]. Activation of $P2Y_{12}$ in macrophages induced cell spreading with formation of lamellipodia, and $P2Y_{12}$ inhibition alleviated chemotaxis [25]. Micklewright et al. demonstrated in human THP-1 monocytic cells that $P2Y_{12}$ was expressed and positively regulated P2Y6-mediated intracellular Ca^{2+} signaling through suppression of adenylate cyclase activity [22]. Finally, Vemulapalli et al. recently showed that functional $P2Y_{12}$ was expressed by T lymphocytes and that it exerted effects on biological responses of T cells when stimulated [23].

Taken together, these results suggest that $P2Y_{12}$ inhibitors might target immune cell function and contribute to the modulation of inflammatory and immune responses.

Even though $P2Y_{12}$ inhibitors are being explored as potential anti-cancer agents, expression of $P2Y_{12}$ receptor in cancer cells has only been reported by a few studies. $P2Y_{12}$ has been found in glioma and astrocytoma cells [61,62]. $P2Y_{12}$ expression has been also described in breast cancer cell lines

and was upregulated by cell treatment with cisplatin [63]. In those cells, co-administration of $P2Y_{12}$ inhibitor with cisplatin resulted in significantly higher cytotoxic response [63].

3. $P2Y_{12}$ Antagonists

3.1. Thienopyridines

As previously described, $P2Y_{12}$ activation by ADP has a fundamental role in thrombus formation and stabilization. Hence, antiplatelet agents inhibiting $P2Y_{12}$ form a cornerstone of therapy for patients at risk of major adverse cardiovascular events (MACE), especially those with acute coronary syndrome undergoing percutaneous coronary intervention, as well as in the secondary prevention of cardiovascular events [33]. Besides their antithrombotic benefits, $P2Y_{12}$ inhibitors also carry a bleeding risk, in both procedural and non-procedural settings [64–66].

Pharmacological description of $P2Y_{12}$ inhibitors is essential to better understand their potential role in the modulation of inflammation, whether by a platelet dependent and/or an independent mechanism.

The $P2Y_{12}$ inhibitors involve two classes of drugs: the thienotetrahydropyridines or thienopyridines (clopidogrel, prasugrel, and ticlopidine) and the nucleoside–nucleotide derivatives (cangrelor and ticagrelor) [33,67–69]. Pharmacological properties of $P2Y_{12}$ inhibitors are summarized in Table 1.

Table 1. Pharmacological properties of $P2Y_{12}$ receptor inhibitors.

Drug	Ticlopidine	Clopidogrel	Prasugrel	Ticagrelor	Cangrelor
Target	$P2Y_{12}$	$P2Y_{12}$	$P2Y_{12}$	$P2Y_{12}$ ENT-1	$P2Y_{12}$
$P2Y_{12}$ receptor binding	Irreversible	Irreversible	Irreversible	Reversible	Reversible
Route of administration	Oral	Oral	Oral	Oral	Intravenous
Metabolism	Prodrug CYP450	Prodrug Esterase CYP450	Prodrug Intestinal Esterase CYP450	Direct-Acting and CYP450	Direct-Acting Dephosphorylation
Time to maximum IPA [1]	3–4 days	4–5 h	2–4h	2–4 h	2 min
Steady-state IPA [2]	20–30%	33–64%	43–73%	82–95%	>80%
Offset of action [3]	11–13 days	5–7 days	7–10 days	3–5 days	30–60 min

CYP450: cytochrome P450; IPA: inhibition of platelet aggregation; [1] after a loading dose; [2] percentage inhibition of platelet aggregation measured by light transmission aggregometry; [3] based on return of platelet aggregation and/or bleeding time to baseline values; [67–76].

Thienopyridines are orally administered inactive prodrugs that develop their antiplatelet effects only after in vivo metabolism to active metabolites [6]. Active metabolites of thienopyridines have a very short half-life and selectively interact with $P2Y_{12}$ by forming a disulfide bond with cysteine-97 and then irreversibly inhibit ADP-induced activation [6]. Consequently, the inhibitory effect of thienopyridines on platelets lasts for the lifespan of a circulating platelet, which is 8 to 12 days on average [6].

The active metabolites of thienopyridines, and particularly for clopidogrel, are produced by a complex multistep hepatic cytochrome P450 (CYP450) dependent activation [6,77]. Although clopidogrel was shown to exhibit a beneficial effect in the prevention of MACE, it suffered from many drawbacks including slow onset of action, high variability of response, drug resistance due to CYP450 polymorphisms, moderate inhibition of platelet aggregation (33–64%) and drug interactions [6,67,68,77,78]. This led to the development of third generation thienopyridine prasugrel. Unlike ticlopidine and clopidogrel, prasugrel is first metabolized by an intestinal esterase and then

undergoes a single step CYP450 dependent activation [6,79]. This allows a faster onset of action, more potent inhibition of platelet aggregation (50–80%), fewer resistance and drug interactions [68,77,80,81]. Consequently, prasugrel is associated with a higher bleeding risk [67].

3.2. Direct $P2Y_{12}$ Inhibitors

By contrast, several reversible direct-acting $P2Y_{12}$ receptor antagonists were developed using adenine-nucleotides (ADP or ATP) as the primary structure, that do not require hepatic metabolism [6,33,67–69]. Altogether, this pharmacological approach allows a faster, more potent and more predictable platelet inhibitory effect than thienopyridines [6,33].

Ticagrelor is an oral direct-acting $P2Y_{12}$ inhibitor belonging to the cyclopentyl-triazolo-pyrimidines class. It has a rapid onset of action (2h) and produces a dose-dependent and important inhibition of platelet aggregation (60–90%) [68,71,73]. However, whether ticagrelor exerts a competitive or noncompetitive inhibition of ADP-induced $P2Y_{12}$ activation is still debated [82,83]. Ticagrelor has one active metabolite (AR–C124910XX) produced by hepatic metabolism, which is at least equipotent and accounts for a third of its antiplatelet effects [73,84]. Even if the offset of action is significantly faster than prasugrel, the more potent platelet inhibitory effect induces a rate of offset still equivalent to clopidogrel, with platelet aggregation returning to pretreatment values after 5 days [68,71].

Ticagrelor exhibits PY12-independent pleiotropic effects. In addition to anti-$P2Y_{12}$ activity, it has been shown to inhibit equilibrative nucleoside transporter 1 (ENT-1) which is a ubiquitous membrane transport protein, notably responsible for adenine uptake by erythrocytes and platelets [85–87]. Ticagrelor inhibits adenine uptake in vitro, inducing increased biological effect of exogenous adenosine and increased plasma levels and biological effects of endogenous adenosine [87]. High plasma levels of adenosine can induce platelet inhibition and coronary vasodilatation, reduce inflammatory response and improve ischemia/reperfusion injuries [87]. Thus, ENT-1 inhibition by ticagrelor may account for some of its biological effect, including inhibition of platelet activation. However, ticagrelor-induced increase in adenosine plasma levels in vivo remains very controversial [87–89].

It was also established that, together with an inhibition of platelet ENT1, ticagrelor can act as an inverse agonist at the $P2Y_{12}$ receptor [86]. This inverse agonist effect was further investigated in the study of Garcia, supporting a new concept of $P2Y_{12}$ receptor constitutive Gi/o-dependent signaling [90]. This effect was inhibited by ticagrelor but not by the thienopyridine inhibitors and led to an increase of cAMP-dependent signaling pathway compared to resting condition [90]. Moreover, Reiner et al. showed that ticagrelor exhibited endothelial-specific antithrombotic properties by reducing tissue factor expression and activity, independently of $P2Y_{12}$ and ENT-1 [28]. Finally, a recent study by Lancellotti et al. demonstrated that ticagrelor had a bactericidal activity against antibiotic-resistant gram-positive bacteria in vitro and inhibited biofilm growth and dissemination of bacteria on a mouse model of implanted Staphylococcus aureus–preinfected disks [91].

Cangrelor is the only intravenous $P2Y_{12}$ inhibitor. It is a direct-acting ATP analogue that selectively and reversibly binds to the $P2Y_{12}$ receptor in a dose-dependent manner, without requiring metabolism. It achieves a high inhibition of platelet aggregation (>80%) with a rapid onset of action (2 min) and a very fast offset of action (30 to 60 min) due to a half-life of 3 to 6 minutes [76]. It is therefore a therapeutic option for patients with coronary artery disease (CAD) undergoing percutaneous coronary intervention (PCI), and to maintain $P2Y_{12}$ inhibition when oral therapy is interrupted for any reason [92,93].

4. Effects of $P2Y_{12}$ Inhibitors on Inflammation: Possible Molecular and Cellular Mechanisms

As described above, ADP-mediated activation of $P2Y_{12}$ seems to be a common activating pathway in many inflammatory and immune cell types including platelets, leukocytes and dendritic cells.

Hemostasis and inflammation are intimately linked, inducing and amplifying each other and this interconnection contributes to many pathological situations including sepsis, acute lung injury, autoimmune diseases, tumorigenesis and metastasis.

Hence, $P2Y_{12}$ inhibitors might modulate inflammation through many different cellular and molecular mechanisms, summarized in Figure 2 and further detailed in this section.

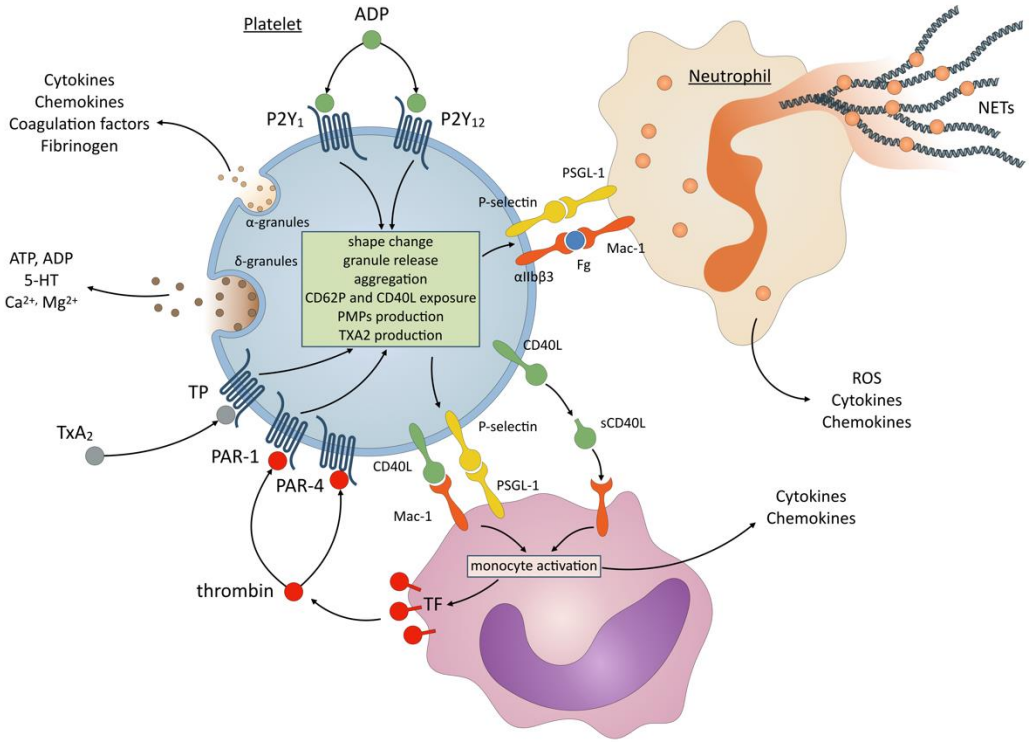

Figure 2. Schematic overview of the role of platelet $P2Y_{12}$ activation in inflammation. ADP binding to $P2Y_{12}$ initiates platelet activation and amplifies granule secretion and aggregation induced by ADP-mediated $P2Y_1$ stimulation and by strong agonists such as thrombin (PAR-1 and PAR-4 receptors) and TXA_2 (TP receptor). Activated platelets release alpha and dense granules contents, including cytokines, chemokines, coagulation factors and platelet agonists. Cytokines, chemokines and soluble CD40 ligand (sCD40L) can recruit and activate leukocytes, especially neutrophils and monocytes. Expression of P-selectin and CD40L at the surface of activated platelets allow interaction between platelets and leukocytes which is a critical step in platelet-mediated inflammation. P-selectin cross-links platelets and leukocytes through its corresponding ligand P-selectin glycoprotein ligand-1 (PSGL-1) present on monocytes and neutrophils. Platelet-leukocyte aggregates are further stabilized by numerous additional receptor/ligand pairs, especially leukocyte Mac-1 that recognizes platelet CD40L and fibrinogen (Fg) bound to platelet αIIbβ3. Activation by platelet interaction or soluble mediators stimulates monocyte cytokine and chemokine production, expression of tissue factor and thrombin generation. Platelet-bound neutrophils produce inflammatory mediators including reactive oxygen species (ROS), and release neutrophil extracellular traps (NETs) that play a critical role in many inflammatory conditions, especially sepsis-induced organ injuries, autoimmunity, tumorigenesis and metastasis.

4.1. Thrombin Generation

Thrombin generation is a critical step during in vivo thrombogenesis that initiates the formation of fibrin clots and platelet activation. Besides, thrombin can mediate direct effects on inflammatory and immune cells through activation of protease-activated receptors (PARs), and notably PAR-1 [94,95]. Thrombin has been shown to induce the secretion of proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and monocyte chemotactic protein MCP-1 from different cell types including vascular endothelial cells and monocytes [96–98]. It also enhances angiogenesis by stimulating the expression of angiogenic growth factors including platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [99,100]. Finally, thrombin

has been shown to support T-cell proliferation and to induce leukocyte recruitment through PAR-1 activation [94,97].

Interplay between thrombin generation and inflammation involves positive feedback as leukocyte activation can also trigger thrombin generation, mainly through the release of neutrophil extracellular traps (NETs) and the expression of tissue factor by monocytes, macrophages and leukocyte-derived microparticles [95,101].

As described above, $P2Y_{12}$ plays a role in thrombin generation and in thrombus formation in vitro. Accordingly, $P2Y_{12}$ antagonism has been shown to inhibit thrombin generation through the decreased formation of coated-platelet in vitro and in vivo [50,51,102,103]. Clopidogrel can reduce ex vivo thrombin generation triggered by low concentrations of TF in rodent platelet rich plasma [104]. In a murine experimental endotoxemic model, clopidogrel was recently shown to reduce TF expression on leukocytes [105]. Finally, ticagrelor inhibited TF expression and activity in human aortic endothelial cells, independently of $P2Y_{12}$ and ENT-1, and decreased thrombosis and endothelial TF expression in a photochemical mouse model of arterial thrombosis [28].

4.2. Release of Inflammatory Mediators

Inflammatory mediators, including cytokines, chemokines and growth factors are key modulators of acute and chronic inflammatory processes. Most of them, including IL-1, IL-6, IL-8 and TNFα are essentially produced by macrophages, monocytes and lymphocytes. They play a critical role in the positive feedback between hemostasis and inflammation [95,106]. Indeed, they induce proliferation, activation and recruitment of leukocytes, but also TF expression on cell surface of mononuclear and endothelial cells, as well as platelet activation [95,106–108].

Upon activation, platelets secrete a great amount of soluble mediators from their granules, with potent procoagulant and proinflammatory effects [109,110]. Cytokines and chemokines released by platelets include IL-1β, CD40L, platelet factor 4 (PF4, CXCL4), RANTES (CCL5), βTG (CXCL7), and IL-8 (CXCL8) [106,109,110]. These mediators play an important role in paracrine activation of platelets and in immune cell activation, proliferation and chemotaxis [106]. Notably, soluble CD40L activates platelets, induces IL-6 of and monocyte chemoattractant protein (MCP-1) secretion and enhances TF expression by monocytes, leading to increased thrombin generation [111,112].

$P2Y_{12}$ inhibition by clopidogrel or by gene knockout in murine models of abdominal sepsis or lipopolysaccharide (LPS)-induced inflammation has been shown to decrease proinflammatory mediator levels in plasma, notably IL-6, TNFα, CCL4 (MIP-1β) and IL-1β [48,105,113]. This is consistent with the reduction in IL-6 and TNFα levels, upon clopidogrel treatment, in a rat model of LPS-induced inflammation [114]. In a human experimental model of intravenous LPS-induced inflammation, ticagrelor and clopidogrel were associated with a significant reduction of IL-6, TNFα and CCL2, and an additional reduction of IL-8 was obtained with ticagrelor [115]. However, anti-inflammatory effect of clopidogrel is inconsistently reported in clinical trials enrolling patients with acute coronary syndromes or stable coronary artery disease, with a reduction of CRP and TNFα [116]. Still, the recently published double-blinded randomized XANTHIPPE study comparing ticagrelor to placebo in pneumonia, demonstrated a significant reduction in plasma IL-6 levels in the ticagrelor group [113].

4.3. Platelet-Leukocyte Interactions and Formation of Neutrophil Extracellular Traps (NETs)

The pathophysiology of platelet-leukocyte interaction has recently been extensively reviewed by Rossaint et al. [117]. Resting circulating platelets act as sentinels in the blood. Upon activation, degranulation and membrane expression of platelet receptors enable physical interactions between platelets and leukocytes, especially neutrophils, forming so-called platelet-leukocyte aggregates (PLAs) [117–119]. This interaction is critical in both thrombosis and inflammatory situations. It allows leukocyte activation and recruitment to sites of inflammation and induces the release of proinflammatory mediators, reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) by neutrophils [117–119].

The first interaction between platelets and leukocytes is established between P-selectin (CD62P) on activated platelets and PSGL-1, constitutively expressed on the surface of neutrophils, monocytes, dendritic cells, and subclasses of lymphocytes. CD62P/PSGL-1 interaction induces the activation of αMβ2 (Mac-1) integrin on leukocyte surface. Platelet leukocyte interaction is then stabilized by direct-binding of Mac-1 to platelet GPIbα and indirect binding to activated platelet αIIbβ3 (GPIIbIIIa) via fibrinogen [117–119].

NETs are large, web-like extracellular chromatin structures that are released by neutrophils upon various activating stimuli, including exogenous microorganisms (bacteria, fungi, virus, and parasites), immune complexes and activated platelets [120]. NETs can trap, neutralize and kill microorganisms, but also activate platelets and promote coagulation [121]. They are considered as critical players in many inflammatory conditions, especially sepsis-induced organ injuries, autoimmunity, tumorigenesis and metastasis [120]. NETs also contribute to thrombogenesis by forming a mesh with platelets and fibrin and accumulating coagulation factors such as TF [118,120,122].

The crosstalk between activated platelets and neutrophils is a critical regulator of NETosis [123,124]. Platelet-neutrophil direct binding is an essential step in platelet-driven NETosis and is mainly mediated by P-selectin/PSGL-1 and GPIbα/Mac-1 interactions [120,123–125]. NET formation is further stimulated by platelet-released soluble mediators including high mobility group box 1 (HMGB1), PF4 and RANTES [120,123–125].

$P2Y_{12}$ inhibition by clopidogrel, prasugrel, ticagrelor and cangrelor has been consistently associated with inhibition of platelet-leukocyte interaction and platelet P-selectin expression in animal models of inflammation and in vitro studies [48,105,126–129]. In a human experimental model of intravenous LPS-induced inflammation, both ticagrelor and clopidogrel were associated with a significant reduction of platelet-monocyte aggregates [115]. Moreover, reduction of PLAs and P-selectin expression by clopidogrel was reported in clinical studies of patients with acute coronary syndromes and atherosclerotic vascular disease [130,131]. Finally, the recent XANTHIPPE study demonstrated a reduction in PLAs in the ticagrelor group compared to placebo [113]. To date, no study reported a significant modulation of NETosis by $P2Y_{12}$ inhibitors, although ticagrelor was recently shown to reduce in vitro NET-induced platelet aggregation, secretion and expression of P-selectin [113,121].

4.4. Adenosine Mediated Effects

The mechanisms of adenosine signaling on immune cells has been extensively reviewed in a recent work by Vigano et al. [132]. Adenosine exerts a powerful modulatory effect on inflammation and innate immune responses through the activation of four different receptors (A1, A2A, A2B and A3) which are expressed in the majority of immune cells, including neutrophils, lymphocytes, macrophages, mast cells and dendritic cells. Adenosine principally induces an anti-inflammatory phenotype characterized by a decreased ability to release inflammatory mediators, an inhibition of immune cell activation, proliferation and chemotaxis. Therefore, adenosine acts as a regulator of immune cells that aims at preserving host integrity and promotes the resolution of inflammation. Moreover, adenosine is a potent inhibitor of platelet aggregation and adenosine receptor agonists have been shown to potentiate antiplatelet effects of $P2Y_{12}$ antagonists [87,133,134]. Thus, adenosine might have a beneficial effect in the management of severe inflammatory disorders including sepsis.

As described above, ticagrelor has been shown to increase plasma levels of adenosine by inhibiting ENT-1. To date, no study demonstrated an adenosine-mediated anti-inflammatory effect of ticagrelor, although ticagrelor was shown to increase the in vitro inhibitory effect of exogenous adenosine on platelet aggregation [87].

5. Current Evidence for $P2Y_{12}$ Inhibition in Clinical Inflammatory Diseases and Syndromes

$P2Y_{12}$ is now considered as a potential target in several inflammatory diseases, including sepsis, asthma, atherosclerosis and cancer. Main clinical studies evaluating the effects of $P2Y_{12}$ inhibition in those inflammatory diseases are reported in Table 2 and will be further discussed in this section.

Table 2. Clinical studies investigating anti-inflammatory effects of $P2Y_{12}$ antagonists.

Study	Type of Study	Condition	Antiplatelet Drugs Evaluated	Effects of $P2Y_{12}$ Antagonists
Winning et al. [135]	Observational 224 patients	CAP	ASA and thienopyridines	Lower use of ICU and shorter stay in hospital (thienopyridines plus ASA or thienopyridines alone
Storey et al. [136]	Observational post hoc analysis18,421 patients	ACS	Ticagrelor vs clopidogrel	Lower mortality risk following pulmonary events and sepsis
Tsai et al. [137]	Observational 683,421 patients	Sepsis	ASA and thienopyridines	Lower risk of mortality
XANTHIPPE Sexton et al. [113]	RCT 60 patients	Pneumonia (CAP, HAP)	Ticagrelor vs placebo	Reduced PLAs and IL-6 levels improved. Decreased supplemental oxygen requirements
Laidlaw et al. [138]	Crossover RCT 40 patients	AERD	Prasugrel vs placebo	No effect on clinical or inflammatory parameters
PRINA Lussana et al. [139]	Crossover RCT 26 patients	Chronic asthma	Prasugrel vs placebo	Decreased airway hyperresponsiveness
Raposeiras-Roubin et al. [140]	Observational 4229 patients	ACS	Ticagrelor, Clopidogrel and Prasugrel	Lower cancer risk ticagrelor vs clopidogrelNS clopidogrel vs prasugrel
Leader et al. [141]	Observational 3479 patients	ACS	ASA and clopidogrel	Lower cancer risk (clopidogrel plus ASA or clopidogrel alone)
Hicks et al. [142]	Observational 41,403 patients	Cancer (breast, colorectal, prostate)	Clopidogrel	No increase in cancer risk
Elmariah et al. [143]	Meta-analysis 48,000 patients	Cardiovascular and cerebrovascular disease	ASA and clopidogrel	No increase in cancer risk
Rodriguez-Miguel et al. [144]	Observational 75,491 patients	CRC	ASA and clopidogrel	Lower cancer risk (clopidogrel plus ASA or clopidogrel alone)

CAP: community acquired pneumonia, HAP: hospital acquired pneumonia, ASA: acetylsalicylic acid=aspirin, ICU: intensive care unit, ACS: acute coronary syndrome, RCT: randomized control trial, AERD: aspirin-exacerbated respiratory disease, NS: non-significant difference, CRC: colorectal cancer.

5.1. Sepsis and Sepsis-Induced Acute Lung Injury (ALI)

Sepsis is defined as a life-threatening organ dysfunction that is caused by a dysregulated systemic inflammatory and immune host response to infection [145]. The mechanisms underlying the excessive inflammation in sepsis have been extensively reviewed by van der Poll et al. [146]. To summarize, sepsis-induced organ dysfunctions result from the interplay between uncontrolled activation of the complement, coagulation, and inflammatory systems. Platelet-mediated inflammatory response is now recognized as a critical player in the pathophysiology of sepsis, and is especially involved in sepsis-induced experimental acute lung injury (ALI) and clinical life-threatening acute respiratory distress syndrome (ARDS) through several mechanisms, including pathogen sensing, release of inflammatory mediators, recruitment and activation of immune cells and thrombosis [109,147–151].

First report of the potential benefits of $P2Y_{12}$ inhibition in sepsis came from observational studies of patients receiving antiplatelet agents. A study of 224 consecutive patients admitted for community acquired pneumonia showed lower use of intensive care unit and shorter stay in hospital in patients receiving antiplatelet agents (aspirin and/or thienopyridines) for at least 6 months compared with age-matched controls [135]. More evidence came from a post hoc analysis of the PLATO trial, in which ticagrelor was shown to significantly reduce death from vascular causes, myocardial infarction, or stroke compared to clopidogrel in 18,624 patients with ACS [152]. This analysis on 18,421 patients revealed that, compared to clopidogrel, ticagrelor was associated with a lower mortality risk following pulmonary events and sepsis in acute coronary syndrome [136]. Finally, a recent observational study including 683,421 patients hospitalized for sepsis, showed a lower risk of mortality in patients receiving antiplatelet agents before admission [137]. Using a nested-control study design in 372,748 patients, they showed that both aspirin and $P2Y_{12}$ inhibitors were associated with a lower adjusted risk of mortality [137].

Those findings were further confirmed in experimental animal models of endotoxemia and abdominal sepsis. In a rat model of LPS-induced endotoxemia, clopidogrel inhibited IL-6 and TNFα secretion and reduced lung and liver histological injuries [114]. Liverani et al. demonstrated in a mouse model of intra-abdominal sepsis that clopidogrel-treated and $P2Y_{12}$ null mice were refractory to sepsis-induced lung injury and exhibited a decrease in platelet activation, platelet-leukocyte aggregation and release of inflammatory cytokines [48]. Those results were confirmed in another mouse model of intra-abdominal sepsis in which ticagrelor reduced histological findings of sepsis-induced lung injury, pulmonary infiltration of neutrophils, formation of PLAs and platelet activation [126]. To our knowledge, only one experimental study on LPS-treated mice demonstrated a benefit of ticagrelor on mortality [113].

The XANTHIPPE trial (Examining the Effect of Ticagrelor on Platelet Activation, Platelet-Leukocyte Aggregates, and Acute Lung Injury in Pneumonia), was the first double-blinded placebo-controlled randomized study to evaluate the effect of ticagrelor on inflammation, platelet activation and lung function in 60 patients with community or hospital-acquired pneumonia [113]. Ticagrelor administration to patients within 48h of pneumonia diagnosis demonstrated anti-inflammatory effect with reduced PLAs in circulation, lowered IL-6 levels and improved lung function with a decrease in supplemental oxygen requirements [113].

5.2. Asthma

Platelet activation is strongly involved in the pathogenesis of allergic asthma, including bronchial hyperresponsiveness and airway wall inflammation and remodeling [153–155]. Among proposed mechanisms, platelet $P2Y_{12}$ pathway seems to play an important role. In a study of murine leukotriene E4-induced asthma, clopidogrel treatment and $P2Y_{12}$ gene knock-out reduced IL-13 levels and airway eosinophilia and inflammation [20]. The underlying mechanism remains unknown. Two randomized trials evaluated the effect of $P2Y_{12}$ inhibition in patients with asthma. In a double-blind placebo-controlled crossover trial of prasugrel in 40 patients with aspirin-exacerbated respiratory disease, prasugrel did not attenuate aspirin-induced symptoms and failed to decrease PLA levels and

mast cell activation [138]. Finally, the PRINA (effect of prasugrel on bronchial hyperreactivity and on markers of inflammation in patients with chronic asthma) trial, in which 26 asthmatic patients were randomly and blindly allocated to prasugrel or placebo for 15 days followed by a 15-day wash-out and a cross-over, showed that $P2Y_{12}$ inhibition by prasugrel significantly decreased airway hyperresponsiveness with improvement of forced expiratory volume [139].

5.3. Atherosclerosis

Atherosclerosis is a chronic inflammatory vascular disease involving cellular and molecular interactions between platelets, endothelial cells, VSMCs and monocytes, interactions that deeply involve the $P2Y_{12}$ receptor [156–158].

The role of $P2Y_{12}$ in atherosclerosis has been well documented in experimental models. In a mouse model of atherosclerosis by Apolipoprotein E (ApoE) gene knock-out, genetic $P2Y_{12}$ deletion was associated with reduced lesion area, increased fibrous content at the plaque site and decreased inflammatory cell infiltration [159]. Likewise, clopidogrel and ticagrelor limited the progression of late atherosclerotic lesion and promoted plaque stability in ApoE knockout mice [160–163]. Unfortunately, differential analysis of the role of platelet and non-platelet $P2Y_{12}$ receptors was not possible with these studies. Therefore, a study using $P2Y_{12}$ gene knockout and bone marrow transplantation in ApoE null mice demonstrated that, compared to vessel wall $P2Y_{12}$, platelet $P2Y_{12}$ had no effect on early atheroma formation, implying additional role of VSMC $P2Y_{12}$ receptors [164]. Further studies are necessary to clarify the extent to which vessel wall and platelet $P2Y_{12}$ influence early and late atherogenesis.

5.4. Cancer—Tumor Growth and Metastasis

The recent advances in the knowledge of cancer have shown that the biology of cancer has evolved from a tumor-centered view to a concept that places cancer cells within a cell network, including fibroblasts, vascular cells and inflammatory immune cells, that form the tumor microenvironment (TME) [165]. Modulation of inflammation within the TME is a critical player during all stages of tumorigenesis and involves reciprocal interactions between cancer cells and surrounding inflammatory cells. These interactions can promote tumor growth by direct proliferative effect on tumor cells and by inducing immunosuppression [165].

The role of platelets in all steps of tumorigenesis including tumor growth, tumor cell extravasation and metastasis has been extensively studied and is now well-recognized [9,166]. Activated platelets not only secrete PF4 but also lipids, microRNAs and numerous growth factors, including TGFβ (transforming growth factor β) and VEGF, which favor tumor cell proliferation, metastasis and angiogenesis. Moreover, the interaction between platelets and cancer cells is largely described in the literature as a mechanism favoring tumor- cell induced platelet activation (TCIPA), and inducing a "shield" of platelets coating the tumor cells and protecting them from the immune response [9].

5.4.1. In Vitro and Preclinical Studies on $P2Y_{12}$ Inhibition in Cancer

Consequently, most of published data have evidenced a net anti tumoral effect of antiplatelet agents, including aspirin, cilostazole and $P2Y_{12}$ inhibitors. The rationale to focus on anti-$P2Y_{12}$ agents came recently first from the discovery of ADP secretion by tumor cells. For instance, Cho et al. nicely showed the secretion of ADP by ovarian cancer cells [167]. Therefore, platelets can be activated directly by tumor cells via cell-to-cell interactions, and indirectly via ADP secreted by tumor cell. Second is the expression of $P2Y_{12}$ receptors in cells other than platelets, such as VSMCs and tumor cells such as breast cancer cell lines [27]. In Cho's study, the use of ticagrelor reduced tumor growth by 60% compared to aspirin and 75% versus placebo, inhibited proliferation as assessed by Ki67 positivity, and increased apoptosis of ovarian cancer cells. In $P2Y_{12}-/-$ mice, the growth of ovarian tumors was reduced by over 85% compared to wild-type animals, but not in $P2Y_1-/-$ mice, pointing to the essential role of $P2Y_{12}$ between the two ADP receptors. Finally, the invalidation of ectopyrase gene (CD39, which catabolizes ADP) in cancer cells increased platelet-related cancer cell proliferation.

This finding is not restricted to ovarian tumor cell lines. Gareau et al. showed the beneficial effect of ticagrelor in limiting the interaction between platelets and human mammary carcinoma cells [168].

In other preclinical studies, ticagrelor used at clinical dose (10 mg/kg) inhibited metastasis and improved survival in a mouse model of melanoma metastasis [169].

In a Lewis lung carcinoma spontaneous metastatic mouse model, $P2Y_{12}$ deficiency reduced pulmonary metastasis and reduced the ability of platelets to secrete active TGFβ1, thus limiting epithelial to mesenchymal transition and invasiveness of tumor cells [170]. Same findings were observed with B16 melanoma metastasis model.

Consequently, targeted therapies start to emerge in the aim to prevent platelet-tumor cell interactions. The tumor-homing peptide Cys-Arg-Glu-Lys-Ala (CREKA) that targets fibrin-fibronectin complexes found on the tumor stroma and vessel wall was linked to ticagrelor. CREKA-ticagrelor inhibited platelet-induced migration of 4T1 tumor cells and prevented tumor-platelet interaction. In vivo, it suppressed lung metastasis in a mouse model injected with breast cancer cells [171]. This finding opens the way to innovative antimetastatic agents.

5.4.2. Are anti $P2Y_{12}$ Agents Protective or at Risk for Increased Cancer-Related Events?

Numerous studies analyzing the potential effect of antiplatelet agents on cancer converge to a protective effect of low dose aspirin on cancer-related mortality. For instance, in the meta-analysis of Algra and Rothwell including 17 studies, regular use of aspirin was protective from colorectal cancer ([OR] 0.62, 95% CI 0.58–0.67, $p < 0.0001$) [172]. Given that COX1 inhibition is predominant over COX2 inhibition at low dose aspirin, this effect is likely to result primarily from the antiplatelet effect of aspirin.

By contrast, results from randomized clinical trials on anti $P2Y_{12}$ agents are conflicting. Whereas CAPRIE and CHARISMA studies of clopidogrel versus aspirin did not report increased cancer development, some data from trials using prolonged anti $P2Y_{12}$ treatment showed increased rates of cancer-related mortality [173,174]. TRITON-TIMI 38 trial of prasugrel compared to clopidogrel on top of aspirin for 6 to 15 months showed a significantly accelerated cancer progression and increased risk of cancer death in the prasugrel group, particularly with breast, colorectal and prostate cancers [175]. One explanation for this apparent paradoxical effect was that the more potent antiplatelet effect of prasugrel brought more events to medical attention and to an increased number of diagnosed cancers. However, results were different in the TRILOGY trial with no difference in cancer frequency between clopidogrel and prasugrel groups after a median follow-up of 17 months [176]. Clopidogrel and ticagrelor given more than 12 months after drug-eluting stenting in the DAPT trial showed a significant increase in cancer-related deaths [177]. However, deaths related to cancer in this study were relatively low in number. Also concerning ticagrelor, PEGASUS-TIMI 54 trial showed an enhanced cancer risk of ticagrelor administered beyond 1 year, whereas PLATO was negative [152,178,179]. Interestingly Raposeiras-Roubin et al. performed a retrospective study on 4229 consecutive acute coronary syndrome patients with a median follow up of 46 months [140]. They found that ticagrelor resulted in a lower cancer risk than clopidogrel without difference between clopidogrel and prasugrel. Noteworthy, only 311 patients were diagnosed with cancer during the follow up (incidence 2.1 per 100 people per year) and ticagrelor-receiving population was 459 versus 3530 with clopidogrel.

Overall, these clinical randomized trials do not include an untreated comparator arm, and are not powered to detect differences in cancer-related events or mortality. Consequently, the Food and Drug Administration (FDA) reported a two trial-level that "rejected the hypothesis of cancer association in patients on dual anti platelet therapy with clopidogrel, that is, the adverse mortality findings in the DAPT trial were not confirmed" [180]. Moreover, the FDA Adverse Event Reporting system is probably unreliable for adequate assessment of cancer risk during antiplatelet treatment as associated cancers might be unreported and/or missed [181].

The evidence for no cancer risk with $P2Y_{12}$ inhibitors mostly stems from meta-analysis and cohort studies. The meta-analysis of Kotronias et al included nine studies with more than 282,000

participants [182]. When compared with standard aspirin or placebo, the thienopyridines clopidogrel and prasugrel were not associated with cancer mortality and event rate. The study concluded that there was insufficient evidence to suggest an association between thienopyridine exposure and increased risk of cancer event rate or mortality.

The question of the duration of treatment was also addressed in cohort studies. Leader et al showed a lower risk of cancer in subjects exposed to aspirin compared to non-users, with or without clopidogrel, on long-term follow-up [141]. In a large cohort of 10,359 colorectal cancer, 17,889 breast cancer, and 13,155 prostate cancer patients, Hicks et al evaluated the post-diagnostic use of clopidogrel and cancer-specific mortality during an average follow-up of 5 years [142]. Overall, there was no increase in the rate of cancers in patients receiving clopidogrel, after adjustment for potential confounders. Finally, the meta-analysis of Elmariah et al including more than 48,000 patients from six randomized trials confirmed the absence of impact of prolonged clopidogrel on top of aspirin on mortality or cancer [143]. More recently, Rodriguez-Miguel et al showed in 15,491 cases of colorectal cancer versus 60,000 controls, that low-dose aspirin was associated with a reduced risk of colorectal cancer incidence in patients receiving treatment for more than one year [144]. Same reduction of 20 to 30% was found for clopidogrel alone or in combination with aspirin. In short-term users, there was on the contrary an increased risk for patients on clopidogrel and aspirin. Again, the hypothesis raised was an increased incidence of gastro-intestinal bleedings that led to a greater number of colonoscopies and early diagnosis.

Altogether, if it is challenging to compare the effects of antiplatelet agents on cancer-related death in studies designed to analyze adverse cardiovascular-related events, a head-to-head comparison between molecules is also questionable because their pharmacology differs. The thienopyridine clopidogrel has a less predictable effect than prasugrel or ticagrelor. Clopidogrel is indeed less effective in a subset of around 30% of patients, especially those carrying loss-of-function 2C19*2 variant [78]. Besides, ticagrelor not only has more predictable pharmacokinetics than clopidogrel but has been shown to exert an anti-inflammatory effect due to an increased adenosine extracellular concentration. Finally, most studies have investigated the effect of anti P2Y12 agents on top of aspirin and it is difficult to conclude for one drug over the others.

6. Conclusions

$P2Y_{12}$-mediated nucleotide signaling is now considered to be a critical player in inflammatory response. Besides platelets, $P2Y_{12}$ is expressed in many immune and vascular cells and has been shown to modulate inflammatory processes, including the release of inflammatory mediators, platelet–leukocyte interactions, and thrombosis. In addition to their antithrombotic properties, $P2Y_{12}$ inhibitors can therefore be considered to have valuable pharmacological targets for inflammation, and the beneficial effects of anti-$P2Y_{12}$ drugs have been reported for several experimental and clinical inflammatory diseases, including sepsis, acute lung injury, asthma, atherosclerosis, and cancer. Still, evidence is missing on the exact role of nonplatelet $P2Y_{12}$ receptors and on how to distinguish between the $P2Y_{12}$-mediated and adenosine-mediated effects of ticagrelor. More prospective controlled clinical studies are warranted to determine the potential benefits of $P2Y_{12}$ inhibition therapy for inflammatory diseases, and pharmacological strategies should be further developed to overcome potential $P2Y_{12}$-associated bleeding risks.

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Abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ALI	Acute lung injury
APC	Antigen-presenting cells
ApoE	Apolipoprotein E
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CD40	Cluster of differentiation 40
COX	Cyclooxygenase
CREKA	Tumor-homing peptide Cys-Arg-Glu-Lys-Ala
CRP	C-reactive protein
CYP450	Cytochrome P450
DC	Dendritic cells
EL	Extracellular loop
ENT-1	Equilibrative nucleoside transporter 1
FDA	Food and Drug Administration
Gi	G-protein i
G-protein	Guanine nucleotide-binding protein
Gq	G-protein q
GPIIbIIIa	Glycoprotein αIIbβ3
HMGB1	High-mobility group box 1
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphate
LPS	Lipopolysaccharide
MACE	Major adverse cardiovascular event
MCP-1	Monocyte chemoattractant protein 1
NETs	Neutrophil extracellular traps
PAR	Protease-activated receptors
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PF4	Platelet factor 4
PI3K	Phosphoinositide-3-kinase
PKA	Protein kinase A
PLAs	Platelet leukocyte aggregates
RANTES	Regulated on activation, normal T-cell-expressed and -secreted
ROS	Reactive oxygen species
TCIPA	Tumor-cell-induced platelet activation
TF	Tissue factor
TGF	Transforming growth factor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TXA2	Thromboxane A2
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
VSCMs	Vascular smooth muscle cells

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Partie 1: Étude de la modulation de $P2Y_{12}$ dans des modèles précliniques d'inflammation

Mise au point d'une méthode d'évaluation par cytométrie en flux de l'interaction leuco-plaquettaire : amas leuco-plaquettaires

L'introduction de ce travail a permis de mettre en évidence le rôle fondamental de l'interaction réciproque plaquettes-leucocytes dans la réponse inflammatoire. La formation des agrégats ou amas leuco-plaquettaires (ALP) en est le témoin direct.

La formation des ALP est un phénomène sensible, dynamique et potentiellement réversible, dépendant de plusieurs liaisons ligand/récepteur[87,88]. De plus, l'affinité des plaquettes pour les différentes sous-populations leucocytaires (monocytes, neutrophiles, lymphocytes) est variable et semble plus importante pour les monocytes et les neutrophiles que pour les lymphocytes[87,89].

De nombreuses méthodes d'analyse des ALP ont été décrites, en circulation ou dans les tissus, in vivo ou in vitro, en utilisant des techniques de cytométrie en flux ou de microscopie optique[88]. Ces techniques reposent principalement sur le marquage des plaquettes et des leucocytes à l'aide d'anticorps spécifiques.

Dans un objectif de recherche translationnelle, nous avons choisi de mettre au point une technique simple de mesure des ALP en circulation par cytométrie en flux. Cette technique permet en effet des mesures rapides, à la fois chez l'homme et chez la souris. Elle permet également de répondre au double objectif d'analyse du rôle inflammatoire des plaquettaires et de recherche de biomarqueurs applicable à la recherche clinique.

Du fait de la grande sensibilité des ALP à l'activation cellulaire, leur mesure nécessite un contrôle strict des conditions pré-analytiques et analytiques[88,90–94]. Nous avons mis au point ces conditions d'analyse chez l'homme au CHU de Rennes, en collaboration avec le service d'hématologie biologique de l'HEGP (Pr Pascale Gaussem), incluant l'évaluation des conditions pré-analytiques et des mesures de répétabilité. Cette mise au point a fait l'objet d'une publication en étant associée à la revue de littérature présentée dans l'introduction (Mansour et al. J Clin Med. 2020 Jul 23;9(8):2361). Nous avons ainsi confirmé l'importance des conditions de prélèvement, du temps entre prélèvement et marquage ainsi que de la durée entre marquage

et passage au cytomètre (formation d'ALP in vitro). La nécessité de pouvoir analyser plusieurs échantillons simultanément nous a conduit à associer une fixation post-marquage permettant de maintenir les taux d'ALP au minimum dans les 6 suivants le marquage.

Le protocole actuellement en place au CHU de Rennes utilise un marqueur pan-leucocytaire (CD45) et plaquettaire (CD41, GPIIb) ainsi défini :

- Prélèvement de sang périphérique (ou sur cathéter artériel) sur citrate de sodium (3,2%)
- Techniquage dans l'heure
- Marquage de 20 µL de sang total par 2 µL d'anti-CD45-APC (Miltenyi Biotec) et 2µL d'anti-CD41-PE (Miltenyi Biotec)
- Homogénéisation manuelle sans vortexage
- Incubation de 20 minutes à température ambiante à l'abri de la lumière
- Fixation et lyse des globules rouges par ajout de 400 µL de BD FACS Lysing (BD Biosciences)
- Stockage à +4°C avant passage au cytomètre (maximum 6 heures)
- Acquisition au cytomètre LSRFortessa (BD Biosciences) (medium flow rate)
- Leucocytes totaux ou sous-populations (neutrophiles, lymphocytes, monocytes) définis par gating CD45/SSC
- Définition de la positivité CD41+ par contrôle isotypique (IgG1 PE, Miltenyi Biotec)
- Amas leuco-plaquettaire exprimés comme le pourcentage d'évènements CD41+/CD45+ dans la gate leucocytes (ou sous-populations).

Mise au point d'un modèle de sepsis murin par injection intrapéritonéale de matières fécales adapté à l'étude des interactions leuco-plaquettaires et application à la modulation de $P2Y_{12}$

Nous avons décrit précédemment la place de l'activation plaquettaire dans l'inflammation et le l'implication de la voie du $P2Y_{12}$ dans sa modulation.

Des travaux initiaux ont suggéré un bénéfice de l'inhibition de $P2Y_{12}$ sur l'inflammation et les défaillances d'organes au cours du sepsis[95–97]. Cependant, ces études ont principalement évalué le clopidogrel et une étude récente rapporte des résultats contradictoires[98]. Ces résultats pourraient, en autre, être expliqués par une variabilité de la réponse inhibitrice plaquettaire induite par le clopidogrel dans le contexte septique. Le clopidogrel est en effet une prodrogue nécessitant un métabolisme hépatique par le cytochrome P450[99]. Dans la population générale, la réponse au clopidogrel est variable et il a été montré que l'inhibition plaquettaire induite par le clopidogrel était diminuée au cours du sepsis, chez l'homme et chez la souris[100,101]. A l'inverse, le ticagrelor, inhibiteur direct de $P2Y_{12}$, a démontré une inhibition plus forte et plus constante chez l'homme[102], ainsi qu'au cours du sepsis murin[101]. De plus, plusieurs travaux cliniques et précliniques murins, ont rapporté un effet bénéfique du ticagrelor sur la réponse inflammatoire et les défaillances d'organes induites par le sepsis[62,64,103–107].

De plus, les travaux précliniques reposaient principalement sur des modèles d'inflammation stérile (injection de LPS) ou sur des modèles de ponction-ligature caecale (CLP). Le modèle CLP, classiquement considéré comme un modèle de référence, est cependant sujet à des variabilités de sévérité clinique et biologique dues aux modalités de chirurgie et de ponction caecale[108,109]. Cette variabilité du modèle pourrait également être à l'origine des résultats contradictoires observés jusqu'ici.

Par conséquent, nous avons fait le choix d'évaluer l'impact du ticagrelor dans un modèle murin de sepsis polymicrobien par injection intrapéritonéale de matières fécales ou cecal slurry (CS). Ce modèle, récemment décrit par une équipe américaine, permet grâce à la cryopréservation du contenu caecal en PBS-glycérol d'induire un sepsis de sévérité et de variabilité inter-individuelle contrôlées [110,111].

Nous avons mis au point ce modèle au sein de l'unité INSERM UMR_S1140 IThEM (Faculté de Pharmacie, Université de Paris, Professeur Pascale Gaussem, Docteur Christilla Bachelot-Loza), et en collaboration avec le Docteur Nicolas Nesseler (CHU de Rennes) au cours de son post-doctorat (Professeur Michael Matthay, Cardiovascular Research Institute, UCSF, San Francisco, USA).

Nous présentons ici ces résultats sous la forme d'un article, qui fera secondairement l'objet d'une publication (premier auteur Alexandre Mansour).

Impact of ticagrelor pretreatment in a cecal slurry mouse model of polymicrobial abdominal sepsis

Introduction

Sepsis is a life-threatening caused by an inappropriate host response to an infection, by means of dysregulated systemic inflammatory and immune response [1–3]. During sepsis, organ failure results from a complex interplay between innate immunity, coagulation but also platelet and endothelium activation [3–6]. Indeed, in addition to their critical role in hemostasis, blood platelets are now recognized as key players in the innate immune response and inflammation [7–10]. Upon activation, platelets synthesize and release numbers of pro-inflammatory mediators such as soluble CD40L, sCD62P (soluble P-Selectin), PF4 and thromboxane A2 which play a role in paracrine and systemic activation of leucocytes and trigger further platelet activation [11–13]. Also, during acute inflammation, especially sepsis, platelets can directly interact with leukocytes forming platelet-leukocyte aggregates (PLAs) [8,14–16]. This interaction is a critical step in platelet-mediated inflammation, inducing leukocyte activation, release of inflammatory mediators and formation of Neutrophils Extracellular Traps (NETs) by neutrophils [7,17,18]. Indeed, platelet–leukocyte interactions mediate the process of transendothelial migration, leukocyte recruitment to the site of inflammation and vascular inflammation [14]. While platelets seem to play an important role in host-defense, especially against pathogens, their unregulated activation might participate in the pathogenesis of sepsis-induced multiple organ failure [4,7,11,19]. Indeed, thrombocytopenia is frequent during sepsis and correlates with prognosis [10,20].

Platelet activation is driven by numbers of receptors and subsequent signaling pathways [21,22]. Among them, ADP receptor $P2Y_{12}$ plays a critical role in platelet aggregation and activation and its pharmacological inhibition represents a cornerstone of therapy for patients at risk of major adverse cardiovascular events [15,23]. In addition, $P2Y_{12}$ receptor is also expressed by immune cells and vascular smooth muscle cells, which might also impact acute inflammatory response [24].

First studies have suggested a potential beneficial effect of $P2Y_{12}$ inhibition during sepsis [25–27]. However, conflicting results emerged from recent studies, especially regarding the effects

of clopidogrel [28,29]. Clopidogrel, an oral indirect $P2Y_{12}$ inhibitor, is a prodrug requiring hepatic metabolism by cytochrome P450. It has been associated with a high variability in platelet inhibition response (high on-treatment platelet reactivity), especially during sepsis in both clinical and murine studies [24,30,31]. Conversely, ticagrelor, an oral direct-acting $P2Y_{12}$ inhibitor, has demonstrated a more potent and consistent platelet inhibitory effect, in clinical cardiovascular studies and in murine sepsis model [31,32]. Therefore, ticagrelor might be a better pharmacological approach for targeting sepsis-induced platelet activation and inflammatory response. Indeed, clinical and murine studies reported a beneficial effect of ticagrelor during sepsis [33–39].

Among murine models of sepsis, the cecal ligature and punction model (CLP) is often considered as gold standard [40]. However, this model suffers from several limitations including a high variability in inflammatory response and mortality, associated with the number of cecal punctures and the cecal ligation length, and the difficulty in separating infection from surgery-related effects [40,41]. A new model of polymicrobial abdominal sepsis by intraperitoneal injection of cryopreserved cecal content (cecal slurry-CS) was recently described [42,43]. This CS model demonstrated its ability to induce a reproducible severe sepsis phenotype with systemic inflammatory response and organ injuries, including acute lung injury [42,43]. To our knowledge, this model was never applied to the study of $P2Y_{12}$ inhibition.

Our objective was to evaluate the impact of ticagrelor on inflammatory response, platelet leukocyte interaction and organ injuries in a mouse cecal slurry model. Our hypothesis was that ticagrelor might reduce platelet-leukocyte interaction and subsequently reduce organ injuries in severe sepsis.

Methods

Animals

All experiments were performed using 12- to 16-week-old male C57BL/6JRj mice purchased from Janvier Labs (Saint Berthevin, France). All animals were housed under controlled temperature humidity and lighting with free access to drinking water and food. All animal experiments received ethical approval from the French Ministry of Research (registration number 13799- 2017120613101888-V7).

Cecal slurry model of sepsis

Cecal slurry model was performed as previously described [44]. Briefly, stock cecal slurry (CS) was prepared and stored in aliquots at -80°C (Figure 1A). Cecal content from donor mice were suspended in 10% glycerol-PBS with a ratio of 1 mL for 100 mg of wet cecal content weight. The slurry was filtered using sterile mesh screens (860 µm, 380 µm, 190 µm, 74 µm; Bellco Glass, Vineland, USA), and then dispensed into cryovials (2 mL) and stored at -80°C up to 6 months. As previously described [45], bacterial viability and distribution in CS, assessed in fresh CS and at 6 months by bacterial culture, was not altered by prolonged storage (Figure 1B). For sepsis induction, frozen CS aliquots were thawed in a 37°C water. The CS was homogenized and injected to mice intraperitoneally using a 25-gauge needle. Control mice were injected with the same volume of 10% glycerol-PBS (vehicle). No additional therapeutic intervention was performed. We determined the volume of CS injection in a preliminary study (Figure 2). Mice were injected either with 400 µL glycerol-PBS (control), 300 µL CS or 400 µL CS. Survival was monitored up to 96 hours (Figure 2A). Rectal temperature and murine sepsis score (MSS) were monitored at H0, H1, H2 H6 and H24 after injection (Figure 2B and 2C) [46]. We then chose 300 µL CS for the main study with a final time point at H18 after injection, as it allowed to produce a severe sepsis phenotype with 60% of survival at H24 but without significant mortality at H18, and long enough to induce organ injuries. For the main study, survival, MSS and temperature were monitored at H0, H1, H6 and up to the final timepoint (18 hours after injection). MSS was evaluated blindly by one researcher, after validating inter-operator agreement. Mice were euthanized if MSS exceeded 21 at any timepoint.

Ticagrelor treatment

Ticagrelor (100 mg/kg in 10mM HCl; Brilique® 90 mg, AstraZeneca, Mölndal, Sweden) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Effective inhibition of $P2Y_{12}$ in treated mice was confirmed by measuring platelet reactivity index using VASP assay (CY-QUANT VASP/$P2Y_{12}$, Biocytex, Stago, Asnières sur Seine, France)

Blood and lung collection

Mice were anesthetized 18 hours after CS (or vehicle) injection by intraperitoneal injection of ketamine (80 mg/kg; Clorketam® 1000, Vetoquinol, Lure, France) plus xylazine (10 mg/ kg; Rompun® 2%, Bayer, La GarenneColombe, France). Blood was collected by cardiac puncture into ACD-C solution (1:9 ratio; 13 mM citric acid, 12.6 mM sodium citrate, 11 mM D-glucose). Platelet and white blood cell (WBC) counts were determined in whole blood using an automatic cell counter (MS9, MELET SCHLOESING Laboratoires, Osny, France). Plasma samples were obtained by immediately centrifuging blood at 4°C which were then stored at -80°C until use. After euthanasia, lungs were harvested and fixed in 4% paraformaldehyde for 24h at 4°C for further histological analysis.

Assessment of inflammatory markers and organ injury

Levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and soluble CD62P (P-selectin) in plasma were assessed using Luminex assay (R&D Systems, Minneapolis, USA). Liver function was evaluated by plasma levels of AST (aspartate transaminase) and ALT (alanine transaminase) using Abbott Architect® C-16000 (Abbott Laboratories, USA). Bloodstream infection was assessed by blood culture (BD BACTEC™ Peds Plus™, (Becton, Dickinson, Sparks, USA). Fixed lungs were embedded in paraffin for histological analysis of acute lung injury (ALI). Lungs sections (5 µm) were stained with hematoxylin and eosin and analyzed at 400X total magnification using a light microscope. Acute lung injury was assessed by blinded visual scoring of four random fields per mouse, using the lung injury scoring system [47].

Platelet-neutrophil and platelet-monocyte aggregates quantification

Because lymphocytes are the dominant leukocyte subpopulation in mice [48], and considering the preferential binding of platelets to monocytes and neutrophils [49,50], we decided to quantify platelet-neutrophil and platelet-monocyte aggregates in whole blood by flow cytometry rather than total platelet-leukocyte aggregates.

Briefly, 25 µL of whole blood were labeled using 5 µL of anti-CD41-FITC (Miltenyi Biotec), and either 1 µL of anti-Ly-6G-APC antibodies for neutrophils (Miltenyi Biotec, human) or 1 µL of anti- Ly-6C-APC antibodies for monocytes (Miltenyi Biotec) for 15 minutes at room temperature. Samples were then fixed and lysed (300 µL; BD FACS Lysing, BD Biosciences). Events were acquired on BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, USA). Neutrophils were defined as Ly-6G-positive events and monocytes as Ly-6C-positive events. Aggregates were identified in Ly-6G-gated or Ly-6C-gated dot plots with the co-expression of Ly-6G (or Ly-6C) and CD41, and expressed as percentage of total Ly-6G-positive or Ly-6C-positive events. As a positive control, whole blood samples were stimulated before immunolabelling using PAR4-activating peptide (PAR4-ap,1µM final concentration Bachem, Bubendorf, Switzerland).

Statistical Analyses

Being an exploratory study, no a priori statistical power calculation was conducted. Post hoc statistical analyses were conducted using Prism 9 (GraphPad Software, USA). Mixed models or ANOVA with correction for multiple comparisons were used for comparisons between experimental groups. Mann-Whitney test was used for ALI score comparison between ticagrelor and placebo. Values inferior to the limit of quantification were substituted by zero for cytokines measurement. Data are reported as median with interquartile range or mean+SD. Statistical significance was achieved for $P < 0.05$.

Results

Cecal slurry model induces a severe sepsis phenotype

Sepsis induction by 300 µL of cecal slurry (CS) without ticagrelor administration, induced a severe sepsis phenotype at 18 hours, as compared to control (vehicle-treated, without ticagrelor). CS-treated mice exhibited deep hypothermia (30.0 (27.7-36.8) °C vs 38.2 (37.3-38.5) °C, p<0.0001; Figure 3A) and high murine sepsis score (13 (11-16) vs 0 (0-0), p<0.0001; Figure 3A). Bloodstream infection (bacteremia) was demonstrated in all CS-treated mice (n=6). CS-sepsis was associated with thrombocytopenia (501 (398-621) $10^3/mm^3$ vs 788 (693-812) $10^3/mm^3$, p<0.0001; Figure 4A) and leukopenia (2.1 (1.7-3.0) $10^3/mm^3$ vs 4.6 (3.2-5.8) $10^3/mm^3$, p<0.0001; Figure 4B). Systemic inflammatory response included elevated levels of TNF-α (11.7 (3.1-25.9) pg/mL vs 0 (0-0) pg/mL, p=0.037; Figure 5A), IL-6 (14.4 (5.9-20.1) ng/mL vs 0 (0-0.1) ng/mL, p<0.001; Figure 5B) and soluble P-Selectin (80.9 (61.3-131.5) ng/mL vs 27.0 (24.9-32.2) ng/mL, p=0.009; Figure 5C). CS-sepsis was associated with an increase in platelet-leukocyte interactions, as demonstrated by a trend for higher platelet-neutrophil levels (52.5 (46.6-71.4) % vs 47.4 (45.0-53.5), p=0.201; Figure 6A) and a significant increase in platelet-monocyte aggregates (56.7 (51.6-63.4) % vs 44.5 (38.2-48.5) %, p=0.034; Figure 6B) as compared to non-septic mice. However, this sepsis-induced increase in platelet-leukocyte interactions was not maximal as platelet activation using PAR-4-AP further increased platelet-neutrophil aggregates (76.0 (60.1-93.4) %, p=0.027; Figure 6A) and non-significantly increased platelet-monocyte aggregates (67.4 (56.0-77.0) %, p=0.400; Figure 6B). Finally, CS-treated mice exhibited organ injuries, with acute lung injury (ALI) demonstrated by histological analysis with high ALI scores (Figure 7) and acute liver injury revealed by increased levels of AST (190 (172-288) U/L vs 45 (44-54) U/L, p<0.001; Figure 8A) and ALT (56 (50-71) U/L vs 20 (19-20) U/L, p<0.0001; Figure 8A).

Ticagrelor treatment does not modify blood cell counts or inflammatory markers in healthy mice

First, effective inhibition of $P2Y_{12}$ in ticagrelor-treated mice was confirmed by demonstrating reduced platelet reactivity index using VASP assay as compared to control mice (9±12 % vs 42±6 %; p=0.016; n=4-5). Then we evaluated the impact of ticagrelor treatment in healthy control mice. Ticagrelor administration to control mice (vehicle-treated), as compared to placebo, was not associated with significant modifications of temperature (Figure 3A), blood cells count (Figure 4), inflammatory markers (Figure 5) or platelet-leukocyte aggregates (Figure 6).

Ticagrelor treatment reduces functional impact of sepsis

Ticagrelor treatment before sepsis induction with CS improved sepsis-induced hypothermia as compared to placebo (p=0.006; Figure 3A) but did not completely correct it as compared to non-septic mice (36.0 (32.8-37.3) °C, p=0.018; Figure 3A). Similarly, clinical murine sepsis score (MSS) was improved in ticagrelor-treated septic mice (p<0.0001; Figure 3B) but remained elevated in comparison with non-septic mice (8 (4-10), p<0.0001; Figure 3B).

Ticagrelor treatment improves sepsis-induced thrombocytopenia and leukopenia

Evaluation of blood cells counts demonstrated an improvement of both sepsis-induced thrombocytopenia (p=0.007; Figure 4A) and leukopenia (p=0.028; Figure 4B) in ticagrelor-treated mice, though not completely corrected, either for platelets (637 (534-771) $10^3/mm^3$, p=0.046; Figure 4A) or leukocytes (3.1 (2.3-4.6) $10^3/mm^3$, p=0.004; Figure 4B) as compared to non-septic mice.

Ticagrelor treatment reduces sepsis-induced inflammatory cytokines release but not soluble P-Selectin levels

Ticagrelor administration in CS-treated mice was associated with a reduction of both TNF-α (4.6 (1.7-7.8) pg/mL; Figure 5A) and IL-6 levels (3.2 (0.2-10.7) ng/mL; Figure 5B). However, soluble P-Selectin significantly increased in ticagrelor-treated mice (152.1 (108.8-162.5), p=0.005; Figure 5C). This increase was still significant when adjusting for platelet counts (281 (227-325) ng/10^9 platelets vs 156 (116-252) ng/10^9 platelets, P<0.001).

Sepsis-induced platelet-leukocyte aggregation is not modified by ticagrelor treatment

Cytometric analysis of platelet-leukocyte interactions only revealed a non-significant trend for decreased platelet-leukocyte aggregates, either for neutrophils (48.1 (39.2-52.3) %, p=0.249; Figure 6A) or for monocytes (54.2 (40.6-61.0) %, p=0.960; Figure 6B) as compared to placebo-treated septic mice.

Ticagrelor reduces sepsis-induced acute lung injury

Ticagrelor administration in CS-treated mice was associated with an improvement of acute lung injury score assessed by histological analysis (0.46 (0.35-0.58) vs 0.66 (0.56-0.73), p=0.026; Figure 7D). Liver function however, was not improved in ticagrelor-treated septic mice, either regarding AST (186 (120-213) U/L, p=0.334; Figure 8A) or ALT (58 (49-70) U/L, p=0.974; Figure 8B).

Discussion

This study demonstrates that ticagrelor treatment before the induction of a polymicrobial abdominal sepsis using a cecal slurry mouse model was associated with a significant improvement of murine sepsis score and a reduction of sepsis-induced cytopenia, inflammatory cytokine release and acute lung injury. These results, reported for the first time in a murine cecal slurry model, support previously published clinical and pre-clinical studies using CLP model, demonstrating a beneficial effect of ticagrelor in sepsis [33–39]. Further, the validity of our study is ensured by the fact that 1) our sepsis model reproduced published features of severe sepsis in both mouse and human, including hypothermia, cytopenia, inflammatory cytokine release and organ injuries [40,51]; 2) ticagrelor was associated with a homogeneous improvement of these features.

However, our study does not allow conclusions to be drawn on the mechanisms underlying the effect of ticagrelor in sepsis. On the basis of available literature and our results, four non-mutually exclusive mechanisms can be considered: 1) platelet P2Y12 receptor inhibition; 2) non-platelet P2Y12 receptor inhibition; 3) adenosine-mediated effect following ticagrelor-induced ENT-1 inhibition; 4) direct antibacterial activity of ticagrelor.

The involvement of platelet P2Y12 inhibition was first supported by rodent models of LPS-induced inflammation or CLP-sepsis, using clopidogrel or P2Y12 receptor-deficient mice [25,52]. However, these studies were either limited by the use of LPS-induced sterile-inflammation or by possible variability of the CLP severity [25]. In addition, a recent well-conducted study using mice CLP model reported opposite results without any beneficial effect of clopidogrel treatment or platelet specific deletion of the P2Y12 receptor [28]. Yet, the effect of ticagrelor was reported only for blood cells counts and inflammatory mediators, but not for organ injuries. At first, the differential effect of whole-body versus platelet-specific P2Y12 knockout suggested a lack of involvement of platelet P2Y12 receptor and a potential contribution of non-platelet P2Y12 receptors, especially in leucocytes [24].

However, observational clinical studies further supported clopidogrel lack of efficiency [29,53]. In addition, as described above, clinical and murine studies demonstrated a beneficial effect of ticagrelor on both inflammatory response and organ injuries [33–39]. Two hypotheses can be considered. First, as a prodrug requiring hepatic metabolism, clopidogrel might not be able to effectively inhibit platelet activation during sepsis, as suggested by clinical and murine studies [30,31]. Second, the beneficial effects reported might be mediated by non-P2Y12 effects of ticagrelor.

Indeed, our results are in line with this hypothesis, as we have not been able to demonstrate a significant reduction of eitherplatelet-leukocyte interaction or soluble CD62P levels in association with the improvement of sepsis-induced inflammatory response and acute lung injury. Further, soluble CD62P level was even increased in ticagrelor-treated septic mice, which might be explained by the global effect on sepsis severity and a potential decrease in soluble CD62P during late pre-mortem phase of sepsis.

P2Y12-independent effects of ticagrelor on sepsis can be explained by two mechanisms. First, ticagrelor has been shown to inhibit ENT-1 (equilibrative nucleoside transporter 1) which is a transport protein, notably responsible for adenine uptake by erythrocytes and platelets [24]. In vitro, ticagrelor can increase plasma levels and biological effects of endogenous adenosine. Since high plasma levels of adenosine can induce platelet inhibition and also reduce inflammatory response, ENT-1 inhibition may account for ticagrelor's effects on sepsis [24,54]. Second, a recent study demonstrated that ticagrelor had a bactericidal activity against gram-positive bacteria in vitro and inhibited dissemination of bacteria on a mouse model of implanted Staphylococcus aureus–preinfected disks [55].

Our study has some limitations. First, the use of an animal model does not allow conclusions to be drawn for human pathology, especially sepsis. Second, tough in line with previously published studies, we used a high ticagrelor dose that was above antithrombotic dose for human studies. Third, we did not evaluate the effect of ticagrelor administered after the induction of sepsis. And finally, as discussed earlier, our experimental design did not allow conclusions to be drawn on the mechanisms underlying the effect of ticagrelor in sepsis.

Overall, our study demonstrates for the first time in a cecal slurry mice model that ticagrelor treatment before the induction sepsis was associated with a reduction in both inflammatory response and acute lung injury. Together with previously published studies, our results support the development of prospective clinical studies to evaluate the impact of ticagrelor during sepsis. Further studies will be necessary to explore the mechanisms underlying this effect, especially ticagrelor direct bactericidal activity and adenosine-mediated effects.

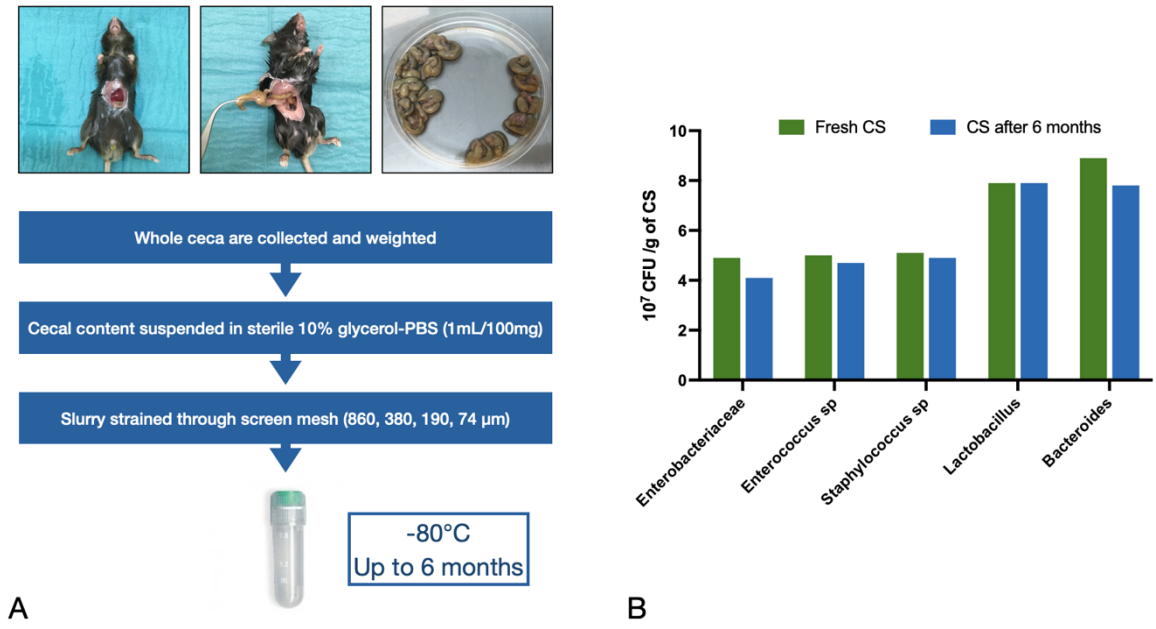


Figure 1. Preparation of cecal slurry and bacteriological characterization. (A) Cecal content from donor mice were suspended in sterile 10% glycerol-PBS with a ratio of 1 mL for 100 mg of wet cecal content weight. The slurry was filtered using sterile mesh screens and then dispensed into cryovials (2 mL) under continuous stirring using a magnetic stir bar and stored at -80°C up to 6 months. (B) Bacterial viability in fresh CS and after 6 months at -80°C was assessed by bacterial culture on specific culture media and expressed as the number of colony-forming unit (CFU) per gram of CS, according to bacterial species.

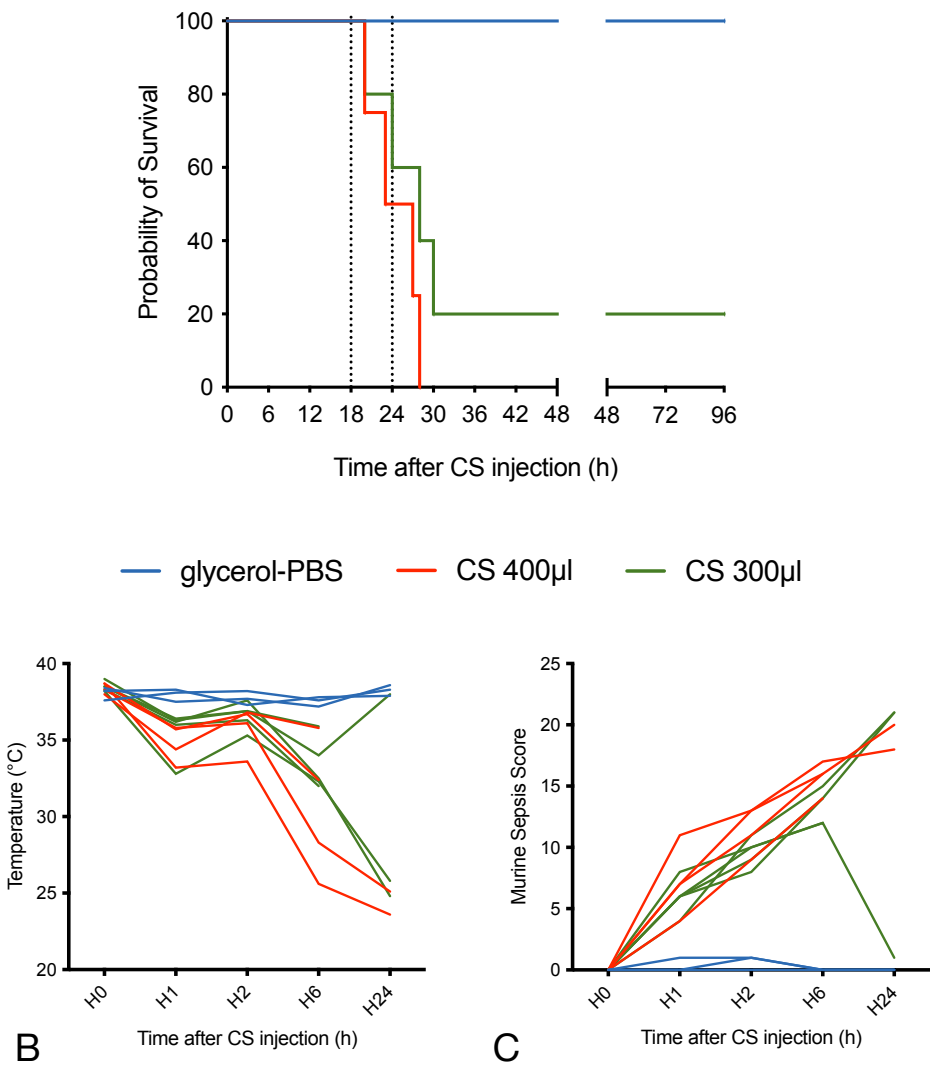


Figure 2. Sepsis severity after injection of cecal slurry (CS). Healthy mice were injected intraperitoneally either with 400 µL glycerol-PBS (vehicle, n=4), 300 µL CS (n=5) or 400 µL CS (n=4). (A) Survival was monitored up to 96 hours. (B) Rectal temperature (°C) was monitored before CS (or glycerol-PBS) injection (H0) and at 1, 2,6 and 24 hours after injection. Murine sepsis score (MSS) was monitored before CS (or glycerol-PBS) injection (H0) and at 1, 2,6 and 24 hours after injection. CS: cecal slurry.

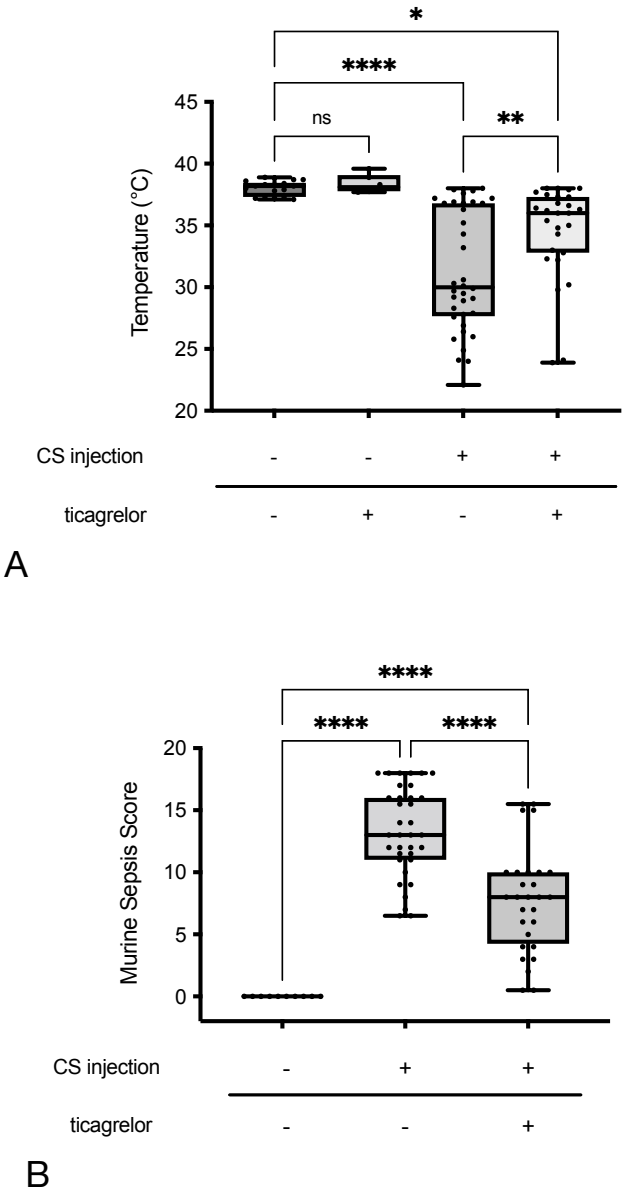


Figure 3. Effect of CS-sepsis and ticagrelor treatment on temperature and murine sepsis score. (A) Mice were injected intraperitoneally either with 300 µL glycerol-PBS (vehicle, n=6-18) or 300 µL CS (n=27-36). Ticagrelor (100 mg/kg in 10mM HCl) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Rectal temperature was measured 18 hours after CS (or vehicle) injection. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant P≥0.05. (B) Mice were injected intraperitoneally either with 300 µL glycerol-PBS (vehicle, n=10) or 300 µL CS (n=28-35). Ticagrelor (100 mg/kg in 10mM HCl) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Murine sepsis score was evaluated 18 hours after CS (or vehicle) injection. * P<0.05, ** P<0.01, **** P<0.0001. CS: cecal slurry.

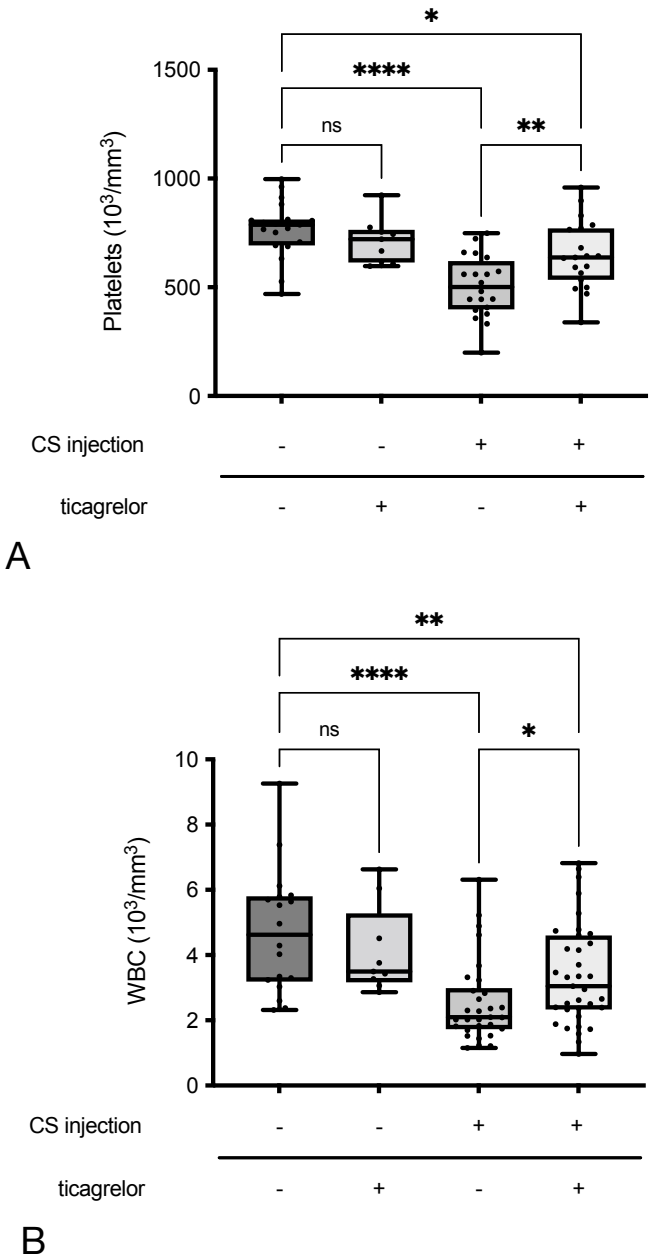


Figure 4. Effect of CS-sepsis and ticagrelor treatment on blood cells counts. (A) Mice were injected intraperitoneally either with 300 µL glycerol-PBS (vehicle, n=9-19) or 300 µL CS (n=19-20). Ticagrelor (100 mg/kg in 10mM HCl) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Platelet count was measured 18 hours after CS (or vehicle) injection. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant P≥0.05. (B) Mice were injected intraperitoneally either with 300 µL glycerol-PBS (vehicle, n=8-18) or 300 µL CS (n=30-35). Ticagrelor (100 mg/kg in 10mM HCl) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Leukocyte count (white blood cell WBC) was evaluated 18 hours after CS (or vehicle) injection. * P<0.05, ** P<0.01, **** P<0.0001. ns: non-significant P≥0.05. CS: cecal slurry, WBC: white blood cell.

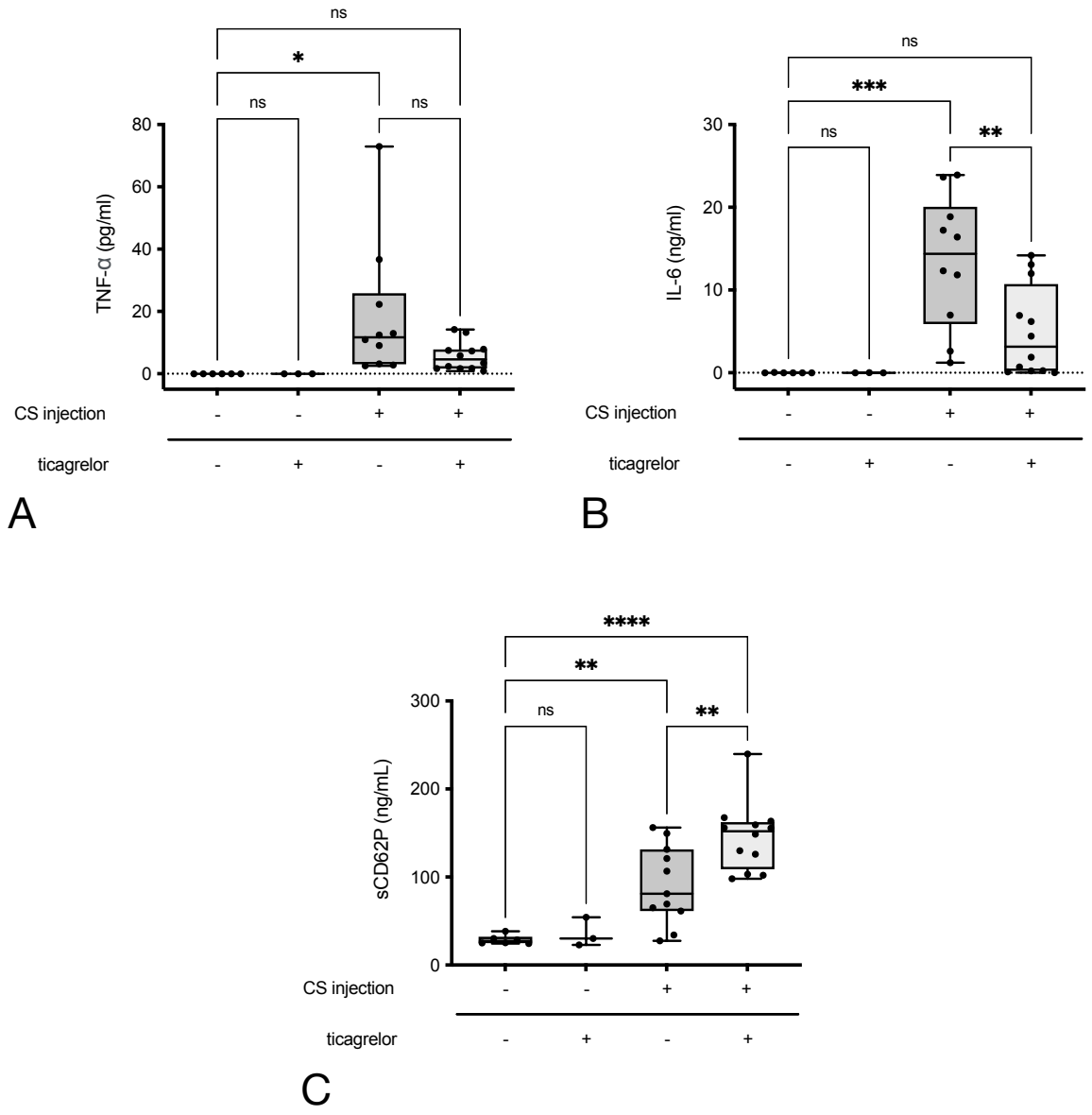
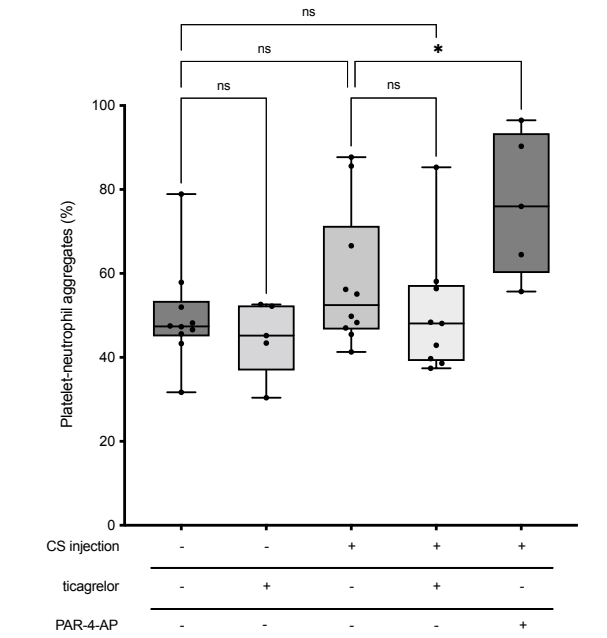


Figure 5. Effect of CS-sepsis and ticagrelor treatment on plasma inflammatory markers. Mice were injected intraperitoneally either with 300 µL glycerol-PBS (vehicle, n=3-6) or 300 µL CS (n=10-12). Ticagrelor (100 mg/kg in 10mM HCl) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Plasma levels of (A) TNF-α, (B) IL-6 and (C) soluble P-Selectin (sCD62P) were measured 18 hours after CS (or vehicle) injection. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant P≥0.05. CS: cecal slurry; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; sCD62P: soluble P-Selectin.



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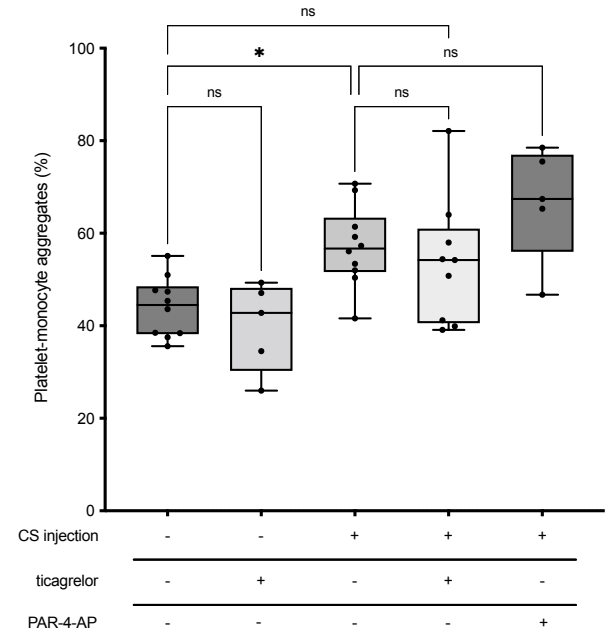

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Figure 6. Effect of CS-sepsis and ticagrelor treatment on platelet-leukocyte interactions. Mice were injected intraperitoneally either with 300 µL glycerol-PBS (vehicle, n=5-10) or 300 µL CS (n=9-10). Ticagrelor (100 mg/kg in 10mM HCl) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Platelet-neutrophil and platelet-monocytes aggregates were quantified in whole blood samples using flow cytometry 18 hours after CS (or vehicle) injection. Blood samples were labeled using anti-CD41-FITC and either anti-Ly-6G-APC antibodies for neutrophils (A) or anti- Ly-6C-APC antibodies for monocytes (B). Aggregates were identified in Ly-6G-gated or Ly-6C-gated dot plots with the co-expression of Ly-6G (or Ly-6C) and CD41, and expressed as percentage of total Ly-6G-positive or Ly-6C-positive events. As a positive control of platelet activation, whole blood samples were stimulated before immunolabelling using PAR4-activating peptide (PAR-4-AP, 1µM final concentration; n=5). * $P<0.05$. ns: non-significant $P\geq0.05$.CS: cecal slurry.

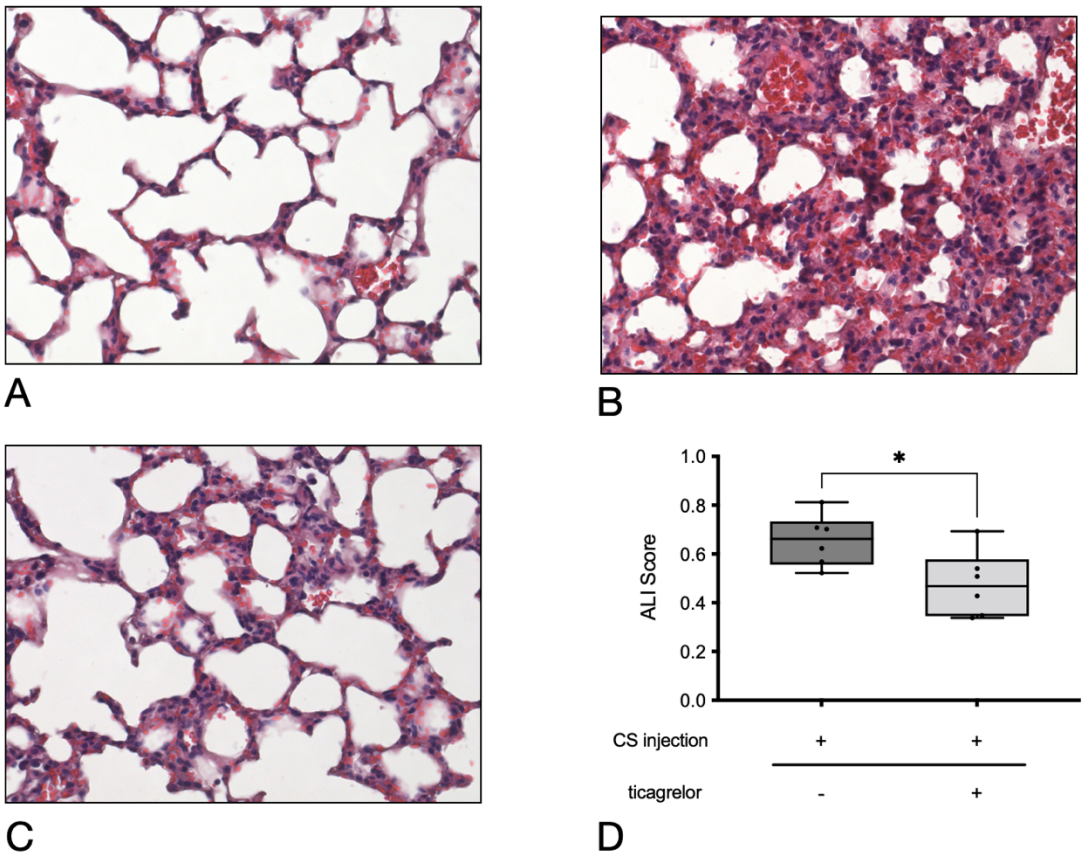

Figure 7. Effect of ticagrelor treatment on sepsis-induced acute lung injury. Mice were injected intraperitoneally either with 300 µL CS (cecal slurry). Ticagrelor (100 mg/kg in 10mM HCl; n=6) or placebo (10mM HCl; n=6) was administered to mice by oral gavage 1 hour before CS injection. Lungs were harvested and fixed 18 hours after CS injection. Lungs sections (5 µm) were stained with hematoxylin and eosin and analyzed at 400X total magnification using a light microscope. (A) Representative stained section of a non-septic control mice. (B) Representative stained section of a placebo-treated septic mice. (C) Representative stained section of a ticagrelor-treated septic mice. (D) Acute lung injury was assessed by blinded visual scoring of four random fields per mouse, using the lung injury scoring system. Lung injury was assessed on a scale of 0–2 for each of the following criteria: A: neutrophils in the alveolar space, B: neutrophils in the interstitial space, C: hyaline membranes, D: proteinaceous debris filling the airspaces, and E: alveolar septal thickening. The final injury score was derived from the following calculation: Score = [20*(A) + 14*(B) + 7*(C) + 7*(D) + 2*(E)] / (number of fields *100). * $P<0.05$. CS: cecal slurry

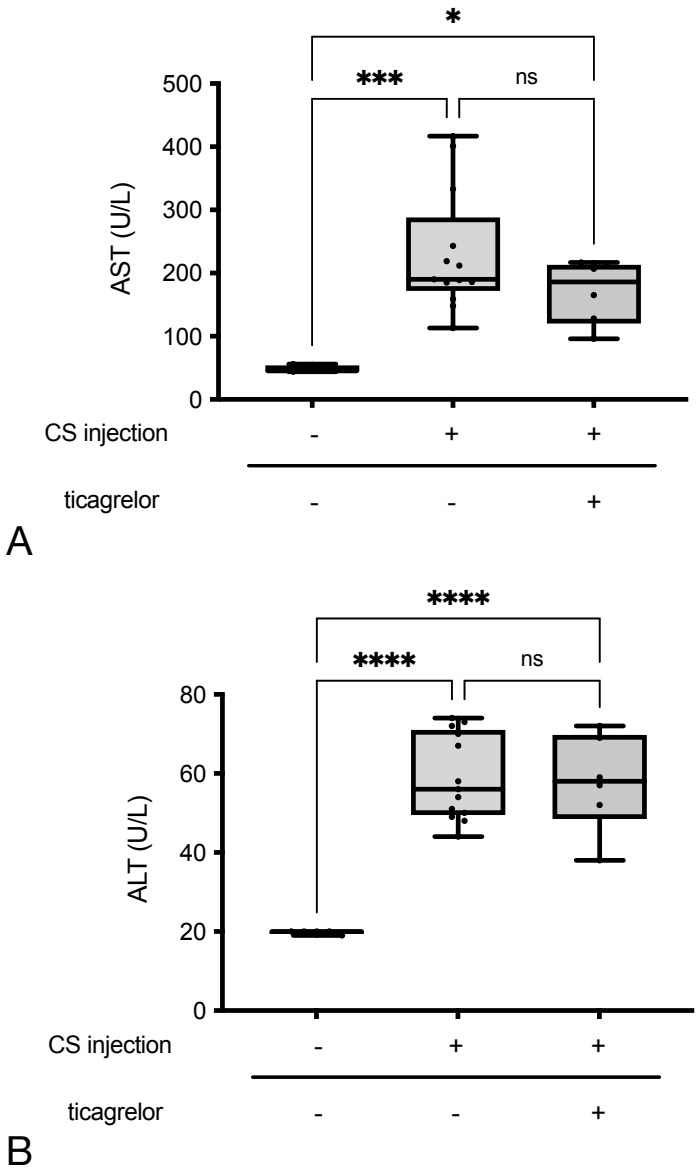

Figure 8. Effect of CS-sepsis and ticagrelor treatment on liver injury. Mice were injected intraperitoneally either with 300 µL glycerol-PBS (vehicle, n=5) or 300 µL CS (n=6-13). Ticagrelor (100 mg/kg in 10mM HCl) or placebo (10mM HCl) was administered to mice by oral gavage 1 hour before CS (or vehicle) injection. Plasma levels of (A) AST and (B) ALT were measured 18 hours after CS (or vehicle) injection. * $P<0.05$, *** $P<0.001$, **** $P<0.0001$. ns: non-significant $P\geq0.05$. CS: cecal slurry; AST: aspartate transaminase; ALT: alanine transaminase.

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Mise au point d'un modèle de stimulation de sang total humain par LPS adapté à l'étude des interactions leuco-plaquettaires et application à la modulation de $P2Y_{12}$

Les limites des modèles animaux, notamment en termes de coût, de reproductibilité et de rapidité de mise en œuvre, justifient la mise au point de modèles in vitro. Dans notre double objectif d'améliorer la compréhension des mécanismes d'interaction plaquettes-leucocytes et d'évaluer l'impact d'inhibiteurs plaquettaires d'utilisation clinique, nous avons souhaité développer un modèle répondant au cahier des charges suivant :

1) Modèle en sang total humain
2) Mise en œuvre rapide
3) Reproductible
4) Induisant une interaction plaquettes-leucocytes et la production de cytokines et de marqueurs d'activation plaquettaire à des niveaux comparables avec la physiologie humaine
5) Utilisable sur des prélèvements sanguins de routine chez le patient de réanimation
6) Permettant d'évaluer un ou plusieurs agents modulant la fonction plaquettaire

Des modèles de stimulation de sang total humain par le LPS ont été développés, y compris au sein de notre équipe, afin d'évaluer la réponse inflammatoire cytokinique, principalement chez les patients septiques[112,113]. Ces modèles, bien caractérisés en termes de production de TNF-α et d'IL-6, n'ont été que très peu utilisés pour évaluer l'interaction plaquettes-leucocytes, a fortiori dans un objectif de recherche translationnelle[114,115].

Nous présenterons ici sous la forme d'un article court la mise au point du modèle, réalisée à Rennes au sein de l'unité INSERM UMR_S 1085 IRSET. Ces travaux feront secondairement l'objet d'une publication complète associant mise au point et effet de l'inhibition de $P2Y_{12}$ (premier auteur Alexandre Mansour).

Development of an in vitro model of LPS-stimulated whole blood to study the pharmacological modulation of platelet-leukocyte interactions

Introduction

Sepsis is a life-threatening condition that arises when the body's response to infection causes injury to its own tissues and organs, by means of dysregulated systemic inflammatory and immune response[1,2]. In 2017, there were an estimated 48.9 million cases and 11 million sepsis-related deaths worldwide, which corresponds to 19,7% of all global deaths[3]. During sepsis, organ failure results from a complex interplay between innate immunity, coagulation but also platelet and endothelium activation[4–7]. Beyond their central role in hemostasis, blood platelets are also key players in the innate immune response and inflammation[8–11]. Upon activation, platelets synthesize and release numbers of pro-inflammatory mediators such as soluble CD40L, PF4 and thromboxane A2 which play a role in paracrine and systemic activation of platelets and leukocytes[12–14]. Above all, during acute inflammation, such as sepsis, platelets can directly interact with leukocytes forming platelet-leukocyte aggregates (PLAs)[9,15–17]. This interaction is a critical step in platelet-mediated inflammation, inducing leukocyte activation, release of inflammatory mediators and formation of Neutrophils Extracellular Traps (NETs) by neutrophils[8,18,19].

Numbers of pharmacological inhibitors of platelet activation and aggregation are available in clinical practice and represent a cornerstone of therapy for patients at risk of major adverse cardiovascular events [20]. Observational clinical studies and rodent preclinical models have suggested a potential beneficial effect of $P2Y_{12}$ inhibitors during sepsis[16]. Besides animal models, there is a need for simple and reproducible *in vitro* models using human whole blood to study pharmacological modulation of platelet-leukocyte interactions and platelet-mediated inflammation. Lipopolysaccharide (LPS) stimulation of human whole blood might be a great candidate for such a model.

LPS originates from the outer membrane of Gram-negative bacteria[21]. Its recognition by TLR-4, a member of the Toll-like receptors family, is able to induce inflammation by activating both

leukocytes and platelets[21–23]. Whole blood stimulation using LPS has been studied as a preclinical model for evaluating cytokine release in sepsis[24,25]. However, this model has rarely been used to study platelet-leukocyte interactions, especially as a translational approach to pharmacological modulation[26,27].

Our objective was to develop an *in vitro* model of LPS-stimulated human whole blood able to induce both platelet-leukocyte interactions and platelet-mediated inflammation. Our hypothesis was that optimal LPS concentration and incubation time could be defined for the evaluation of the main steps of platelet-mediated inflammation. To this end, we chose to measure plasma concentrations of pro-inflammatory cytokines and platelet activation soluble markers and to quantify the platelet-leukocyte interactions by measuring platelet-leukocytes aggregates (PLAs).

Materials and Methods

Human whole blood stimulation

Peripheral venous blood samples were obtained from healthy donors, not taking any anti-platelet medication, after obtaining written informed consent (French Blood Bank Institute, Rennes, France, convention and ethical approval). Blood was drawn by clean venipuncture of a large antecubital vein and was collected into vacuum tubes containing 3.2% buffered sodium citrate (BD Biosciences Vacutainer). Blood samples were stored less than 4 hours at room temperature before processing. Whole blood was diluted 1:1 in RPMI (Gibco, RPMI Medium 1640 (1X) + GlutaMAX^{TM}-I) and stimulated with LPS (E.coli 055:B5, Sigma-Aldrich, St-Quentin Fallavier, France) at different concentrations (0.1, 1, 10 and 100 ng/mL) in 48-well plates. Stimulated whole blood samples were then incubated (37°C, 5% CO_2, Panasonic MCO-18AC-PE) for 1 to 24 hours.

Cytokines and platelet activation markers measurement

Cytokines were measured 4 and 24 hours after LPS stimulation. Blood samples were centrifuged at 2000G for 15 min at 20°C (HETTICH®, Universal 320R) and the supernatants were stored (-20°C). Plasma soluble CD62P (P-selectin), soluble CD40L, TNF-α, IL-6 and IL-8 levels from plasma were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (DuoSet, R&D systems, Minneapolis, MN). A standard concentration-response curve was run on each microplate to allow determination of cytokine levels in samples. Cytokine concentrations were determined with spectrophotometry by measuring the optical density at 450 nm (SpectroStar fluo, BMG Labtech, Thermo Fisher Scientific).

Platelet-leukocyte aggregates measurement

Platelet-leukocytes aggregates (PLAs) were analyzed by flow cytometry at 1 and 24 hours after LPS stimulation. As previously described[28], 20 µL of stimulated whole blood samples were labeled using 2 µL of anti-CD45-APC (Miltenyi Biotec), 2µL anti-CD41-PE antibodies (Miltenyi Biotec) or corresponding istotypes (Miltenyi Biotec) for 20 minutes at room temperature. Samples were then fixed and lysed (BD FACS Lysing, BD Biosciences) in 400

µL. Events were acquired on LSRFortessa (BD Biosciences) one hour after fixation and lysis, and analyzed using FlowLogicTM software (Inivai Technologies). Leukocytes (CD45) were defined as CD45-positive events (Figure 1A) and PLAs were identified in CD45-gated dot plots with the co-expression of CD45 and CD41, and expressed as percentage of total CD45-positive events (Figure 1B). Leukocyte populations (lymphocytes, monocytes and neutrophils) were distinguished by CD45 expression and side scatter characteristics. Subsets of PLAs were then expressed as percentage of CD45-positive events within each leukocyte population.

Statistical Analysis

Being an exploratory study, no a priori statistical power calculation was conducted. Post hoc statistical analyses were conducted using Prism 8 (GraphPad Software, USA). Mixed models with Tukey's correction were used for multiple comparisons between experimental groups for cytokines, platelet activation markers and PLAs analysis. Outliers were not excluded from the analyses. Values inferior to the limit of quantification were substituted by zero for cytokines measurement. Statistical significance was achieved for $P < 0.05$. Data are presented as median with interquartile range.

Results

Cytokines measurement

TNF-α levels are reported in Figure 2. Control studies without LPS did not evidence any spontaneous production of TNF-α. TNF-α levels were raised in a concentration-dependent manner when whole blood was stimulated with increasing concentrations of LPS. A significant increase was observed at 4 hours for LPS 0.1 ng/mL and LPS 1 ng/mL as compared to control (1206 (1104-1292) pg/mL vs 0 (0-16), P<0.001, for LPS 1 ng/mL). The levels of TNF-α seemed to reach a plateau at LPS concentrations of 10 and 100 ng/mL, with values of 1773 (1304-2470) pg/mL and 1487 (1267-2591) pg/mL respectively. Similarly, after 24 hours of incubation, an LPS concentration-dependent increase in TNF-α levels was also observed and reached a plateau at LPS 10 ng/mL. TNF-α levels at 24 hours were lower as compared to levels at 4h with 973 (685-1153) pg/mL for LPS 10 ng/mL.

Control samples without LPS demonstrated a very low production of IL-6 at 4 hours and 24 hours, as described in Figure 3. IL-6 levels were raised in a concentration-dependent manner when whole blood was stimulated with increasing concentrations of LPS. Thus, a significant increase in IL-6 concentration was measured at 4 hours for LPS 1 ng/mL (5284 (3086-6995) pg/mL, p<0.001), as compared to control, and seemed to reach a plateau at LPS 1 ng/mL. After 24 hours of incubation, a LPS concentration-dependent increase in IL-6 levels was also observed despite a higher variability. This increase was sustained up to LPS 100 ng/mL (16684 (14429-20907) pg/mL).

IL-8 levels are reported in Figure 4. Control studies without LPS did not evidence any spontaneous production of IL-8 at 4 hours in contrast to 24 hours, with 151 (27-44) pg/mL in unstimulated blood. IL-8 concentrations were raised in a concentration-dependent manner when whole blood was stimulated with increasing concentrations of LPS after both 4 hours and 24 hours of stimulation. After 4 hours of incubation, maximal response was obtained for LPS 1ng/mL with 517 (486-753) pg/mL. At 24 hours, a significant increase was found for LPS concentrations up to 100 pg/mL, with 1252 (804-2125) pg/mL.

Platelet activation markers

Unstimulated control samples without incubation and after 4 hours of incubation demonstrated similar levels of soluble CD62P (P-selectin) respectively with 18111 (16572-21720) pg/mL and 18281 (13964-27426) pg/mL (Figure 5). Soluble CD62P levels were raised from LPS 1 ng/mL after both 4 hours and 24 hours of incubation, respectively with 27205 (20041-33130) pg/mL and 20848 (17181-28093) pg/mL, and then reached a plateau.

Conversely, soluble CD40L levels were not significantly increased by LPS stimulation, either after 4 or 24 hours of incubation (Figure 6). Compared to unstimulated samples, a non-significant trend for increase was observed after 4 hours at LPS 1ng/mL (71 (28-240) pg/mL vs 29 (22-196) pg/mL, p=0.42).

Platelet-leukocyte aggregates quantification

Platelet-leukocyte aggregates (PLAs) measurement in unstimulated blood samples demonstrated a basal level of PLA formation, with 11.6 (6.2-15.3) % and 14.9 (10.7-16.3) % respectively for 1 hour and 24 hours of incubation (Figure 7). PLAs levels were significantly raised in a concentration-dependent manner with increasing concentrations of LPS after 1 hour of incubation, and reached a plateau at LPS 1ng/mL with 18.6 (7.3-25.4) %. Conversely, this increase was not demonstrated for longer incubation time of 24 hours. Further, compared to unstimulated sample, a non-significant trend for decreased levels was observed at LPS 1 ng/ml (11.5 (6.3-15.7) % vs 14.9 (10.7-16.3) %, p=0.09).

Flow cytometric analysis of platelet-leukocyte aggregate subsets by leukocyte subtypes after 1 hour of incubation are reported in Figure 8. Lymphocytes were slightly aggregated with platelets in unstimulated samples with 4.3 (4.1-5.2) %. LPS stimulation was not associated with a significant increase in platelet-lymphocyte aggregates. While monocytes displayed a higher baseline aggregation with platelets (53.1 (29.4-67.2) % in unstimulated samples), no significant increase was reported with LPS stimulation up to 100 ng/mL (53.9 (17.8-60.4), p=0.12). Conversely, platelet-neutrophil aggregates were significantly raised in a concentration-dependent manner with increasing concentrations of LPS. Compared to unstimulated samples

(8.7 (5.5-11.6) %), this increase was reported from LPS 0.1 ng/mL (16.9 (7.1-20.7) %; $p < 0.01$) and up to LPS 100 n/mL (22.2 (8.5-29.1) %; $p<0.05$ compared to LPS 0.1 ng/mL).

Discussion

This study demonstrates the feasibility of a simple in vitro model of human whole blood stimulation using LPS to study platelet-leucocyte interactions and associated inflammatory response. Indeed, LPS induced an increase in plasma inflammatory cytokines levels (TNF-α, IL-6 and IL-8) and soluble platelet-derived CD62P, in a concentration-dependent manner. This production was associated with an increase in platelet-leucocyte aggregates, mainly driven by neutrophils. A LPS concentration of 1 ng/mL seemed to produce optimal conditions to study the release of leukocytes and platelet mediators after 4 hours of incubation and the formation of platelet-leukocyte aggregates after 1 hour of incubation.

TNF-α concentrations reported in our study were consistent with previous studies[24,29]. TNF-α production was higher after 4 hours of incubation compared to 24 hours. This result was in line with previously published data reporting that leukocytes have a peak of TNF-α release 3 hours after stimulation followed by a decrease in the rate of TNF-α production[30]. The decrease in TNF-α production might be caused by the production of negative feedback regulators such as anti-inflammatory mediator PGE2[31]. In contrast, IL-6 levels increased between 4 and 24 hours, in line with previously published data[29].

Interleukin-8 (IL-8) is a pro-inflammatory cytokine and chemokine, produced by a wide variety of cell types, including leukocytes, endothelial cells and platelets[32,33]. IL-8 is a key activator of neutrophils and is deeply involved in the pathophysiology of inflammation and sepsis, including NETosis[32–34]. Our study reported a LPS-dependent increase in IL-8 levels which was seldom described in the literature in association with platelet-leukocyte aggregates. In addition, the effect of LPS on platelet-neutrophil aggregates in our study raises the question of the impact of IL-8 on neutrophil-platelet interactions.

Unlike soluble CD62P levels reported in our study which were in line with published data[35–37], soluble CD40L levels were low and not increased by LPS stimulation[38–41]. Since the use of whole blood in our experimental design should provide enough CD14 (soluble or membrane bound) to allow LPS/TLR-4 signaling, our results might be explained by low LPS concentration used compared to published data[23].

Baseline levels of PLAs in unstimulated blood samples were consistent with data from previously published studies[28]. Analysis of platelet-leukocyte interactions with long incubation time (24 hours) demonstrated a trend for decrease PLAs levels, despite fixation. This phenomenon might be explained by a dissociation of PLAs, as previously described[42,43].

Despite its novelty, this study is mainly limited by the fact that PLAs, platelet activation markers and cytokines might not be sufficient to completely describe the complex interplay between platelet and inflammation. In this sense, the quantification of NETosis and flow cytometric evaluation of platelet activation might be necessary. In addition, more studies will be needed to evaluate to causality and timing between platelet-leucocyte interaction and the release of inflammatory markers.

In conclusion, LPS-stimulation of human whole blood appears as a simple and reproducible model that might be used for studying pharmacological modulation of platelet-leukocyte interactions and platelet-mediated inflammation.

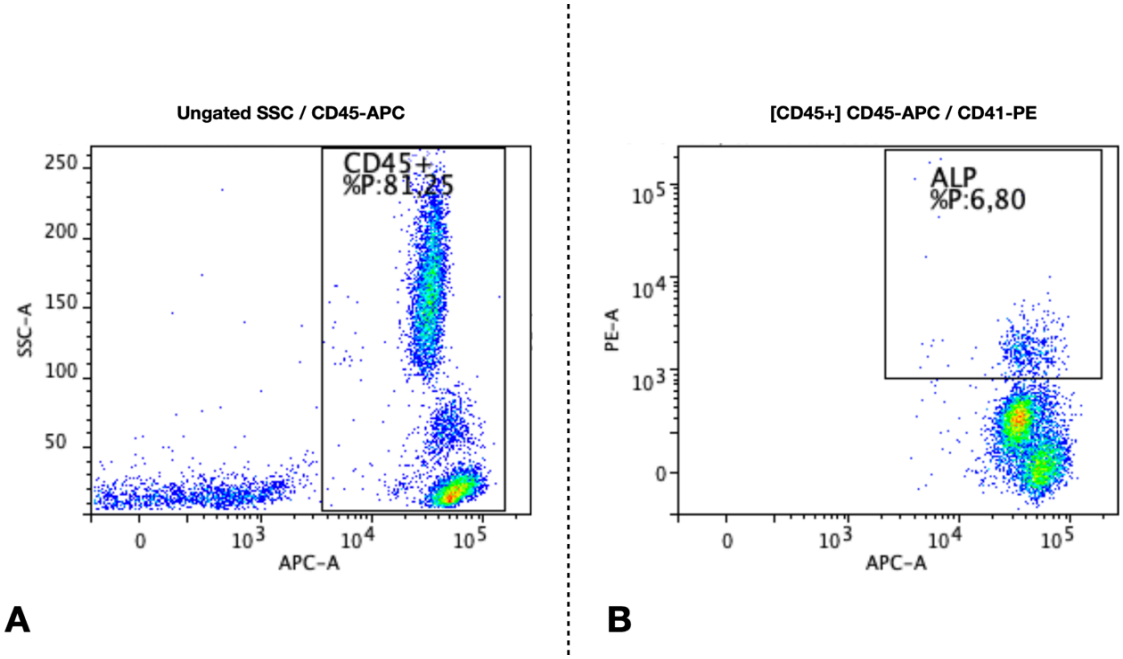

Figure 1. Example of flow cytometric analysis of Platelet-leukocyte aggregates (PLAs). Whole blood samples were labeled using anti-CD45-APC (Miltenyi Biotec, human), anti-CD41-PE antibodies (Miltenyi Biotec, human). Samples were then fixed and lysed (BD FACS Lysing, BD Biosciences). Events were acquired on LSRFortessa (BD Biosciences) and analysed using FlowLogicTM software (Inivai Technologies). Leukocytes (CD45+) were defined as CD45-positive events (Figure 3A). Then PLAs were identified in CD45-gated dot plots with the co-expression of CD45 and CD41, and expressed as percentage of total CD45-positive events (Figure 3B).

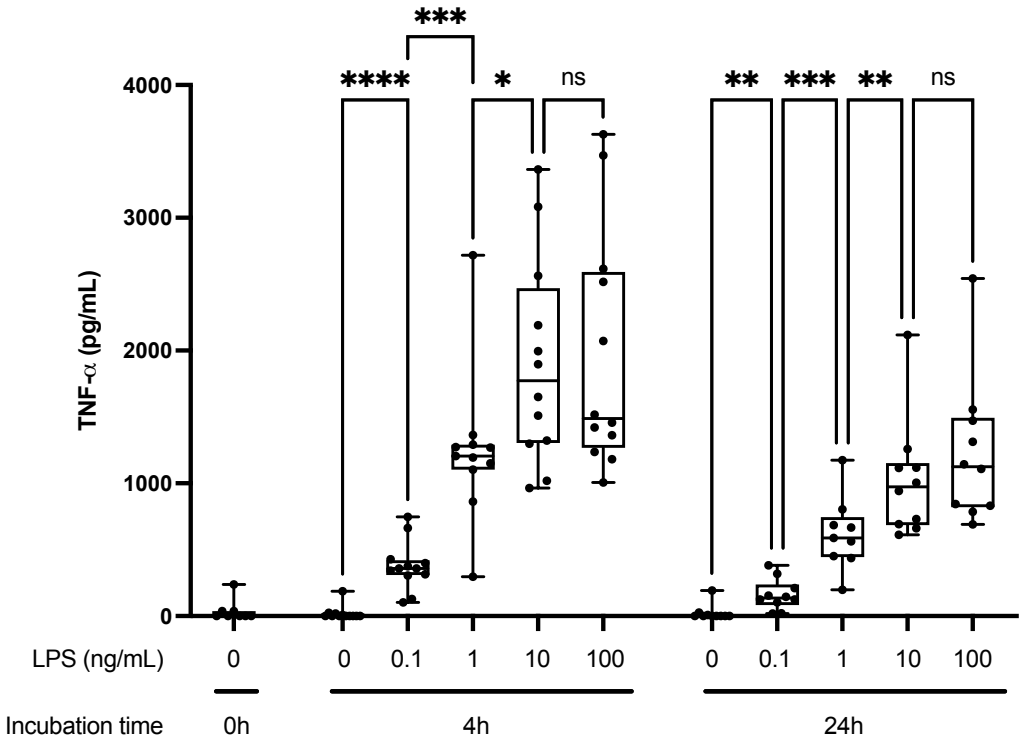

Figure 2. TNF-α at 4 or 24 hours in the supernatant after stimulation of whole blood (WB) with increasing concentrations of LPS. WB from healthy volunteers was stimulated with LPS (0 to 100 ng/mL) from Escherichia coli 055:B5 for 4 or 24h. Unstimulated samples without incubation were used as control. After incubation, the supernatants were collected, and the cytokine levels were measured by ELISA. Data are reported as median, interquartile range and min-max. n=8 (unincubated samples), n=10-12 (incubation time 4h and 24h). Statistical significance is indicated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant; TNF-α :tumor necrosis factor alpha.

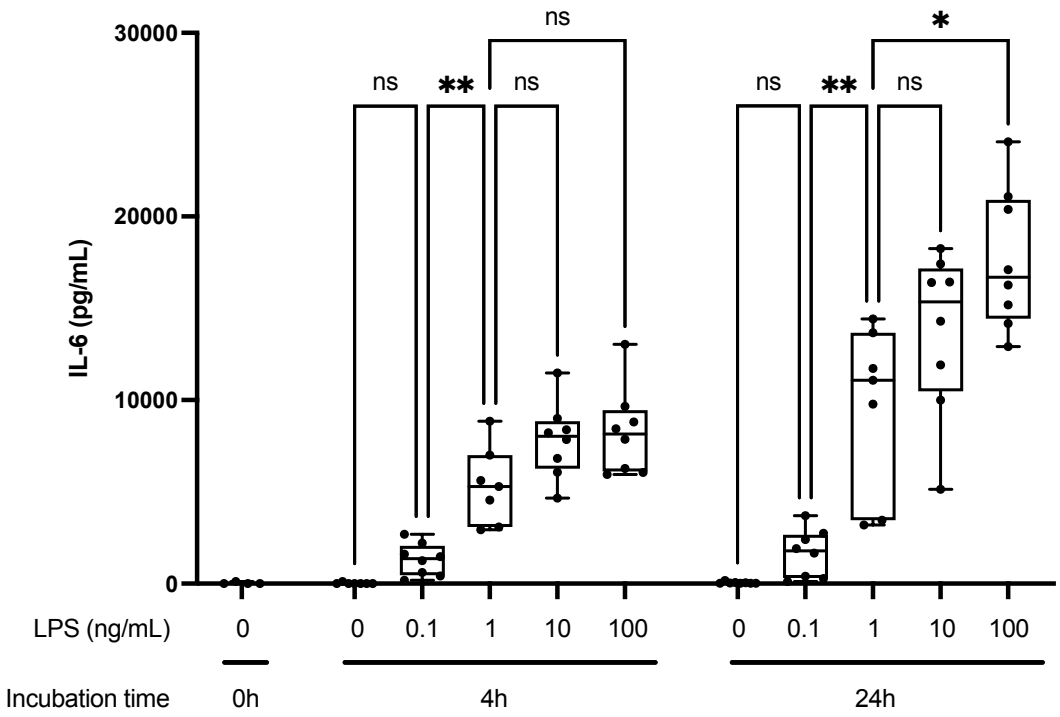

Figure 3. IL-6 at 4 or 24 hours in the supernatant after stimulation of whole blood (WB) with increasing concentrations of LPS. WB from healthy volunteers was stimulated with LPS (0 to 100 ng/mL) from Escherichia coli 055:B5 for 4 or 24h. Unstimulated samples without incubation were used as control. After the incubation, the supernatants were collected, and the cytokine levels were measured by ELISA. Data are reported as median, interquartile range and min-max. n=4 (unincubated samples), n=7-8 (incubation time 4h and 24h). Statistical significance is indicated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant; IL-6: Interleukin 6.

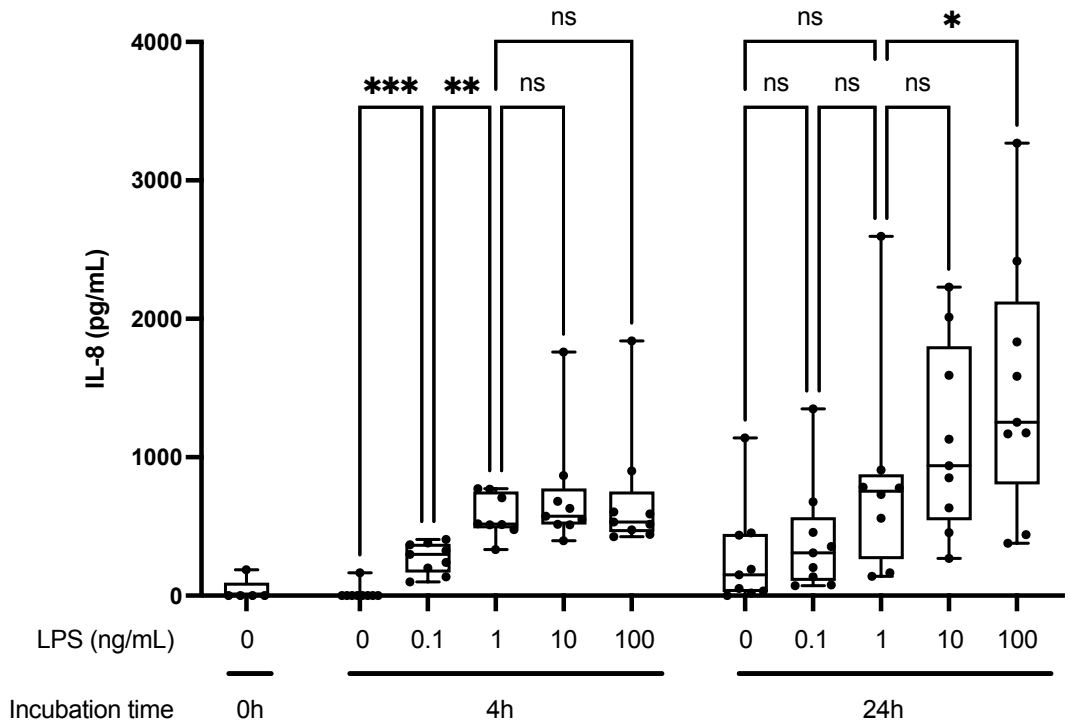

Figure 4. IL-8 at 4 or 24 hours in the supernatant after stimulation of whole blood (WB) with increasing concentrations of LPS. WB from healthy volunteers was stimulated with LPS (0 to 100 ng/mL) from Escherichia coli 055:B5 for 4 or 24h. Unstimulated samples without incubation were used as control. After the incubation, the supernatants were collected, and the cytokine levels were measured by ELISA. Data are reported as median, interquartile range and min-max. n=5 (unincubated samples), n=8-9 (incubation time 4h and 24h). Statistical significance is indicated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant; IL-8: Interleukin 8.

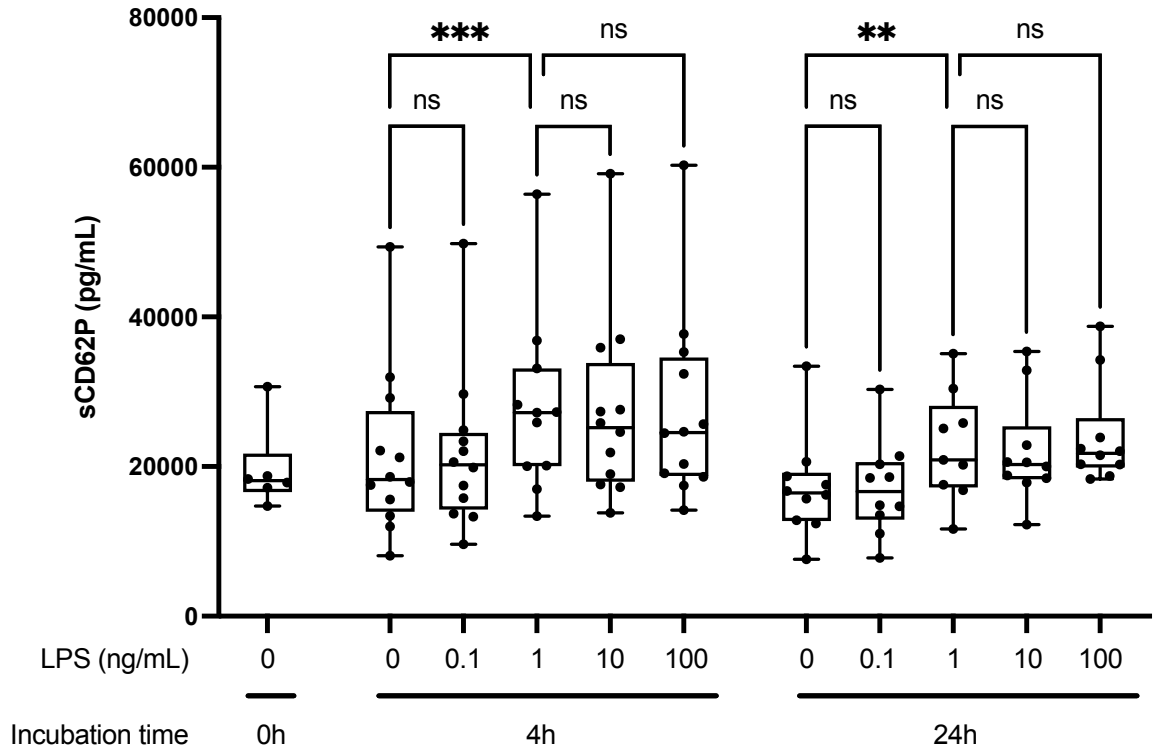

Figure 5. Soluble CD62P (sCD62P) at 4 or 24 hours in the supernatant after stimulation of whole blood (WB) with increasing concentrations of LPS. WB from healthy volunteers was stimulated with LPS (0 to 100 ng/mL) from Escherichia coli 055:B5 for 4 or 24h. Unstimulated samples without incubation were used as control. After the incubation, the supernatants were collected, and CD62P levels were measured by ELISA. Data are reported as median, interquartile range and min-max. n=6 (unincubated samples), n=11-12 (incubation time 4h and 24h). Statistical significance is indicated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant.

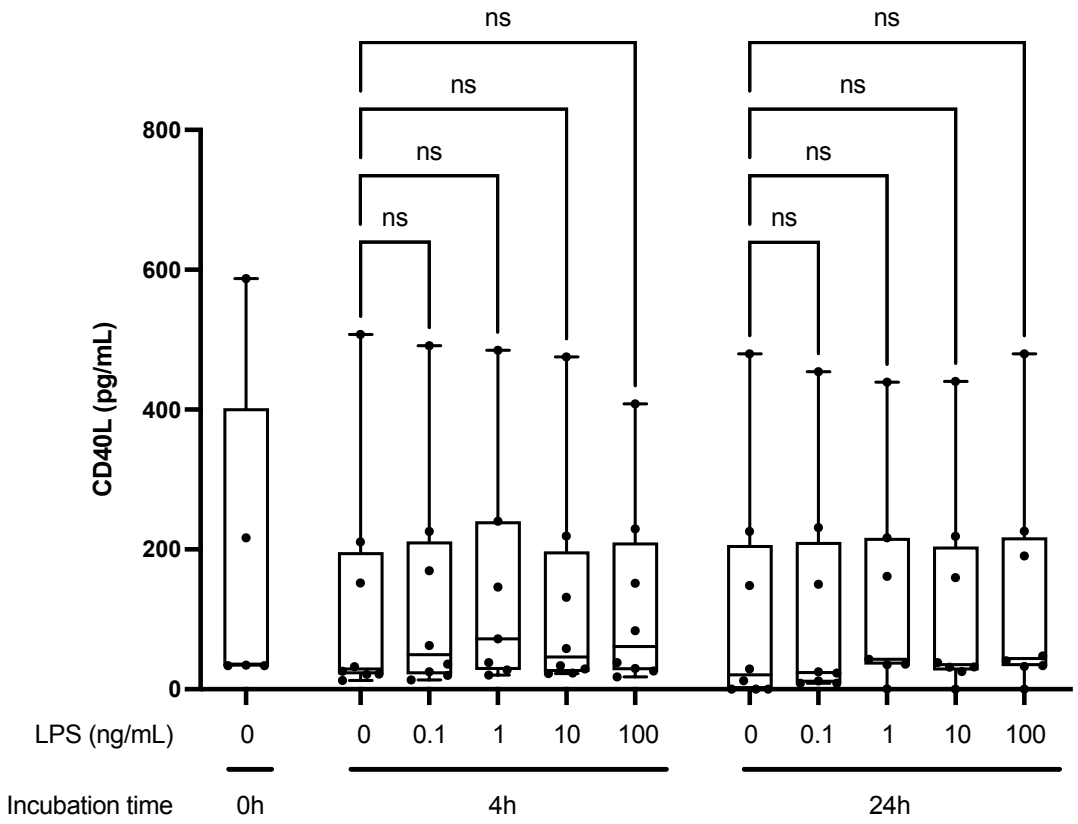

Figure 6. Soluble CD40L at 4 or 24 hours in the supernatant after stimulation of whole blood (WB) with increasing concentrations of LPS. WB from healthy volunteers was stimulated with LPS (0 to 100 ng/mL) from Escherichia coli 055:B5 for 4 or 24h. Unstimulated samples without incubation were used as control. After the incubation, the supernatants were collected, and CD62P levels were measured by ELISA. Data are reported as median, interquartile range and min-max. n=5 (unincubated samples), n=7-8 (incubation time 4h and 24h). Statistical significance is indicated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant;

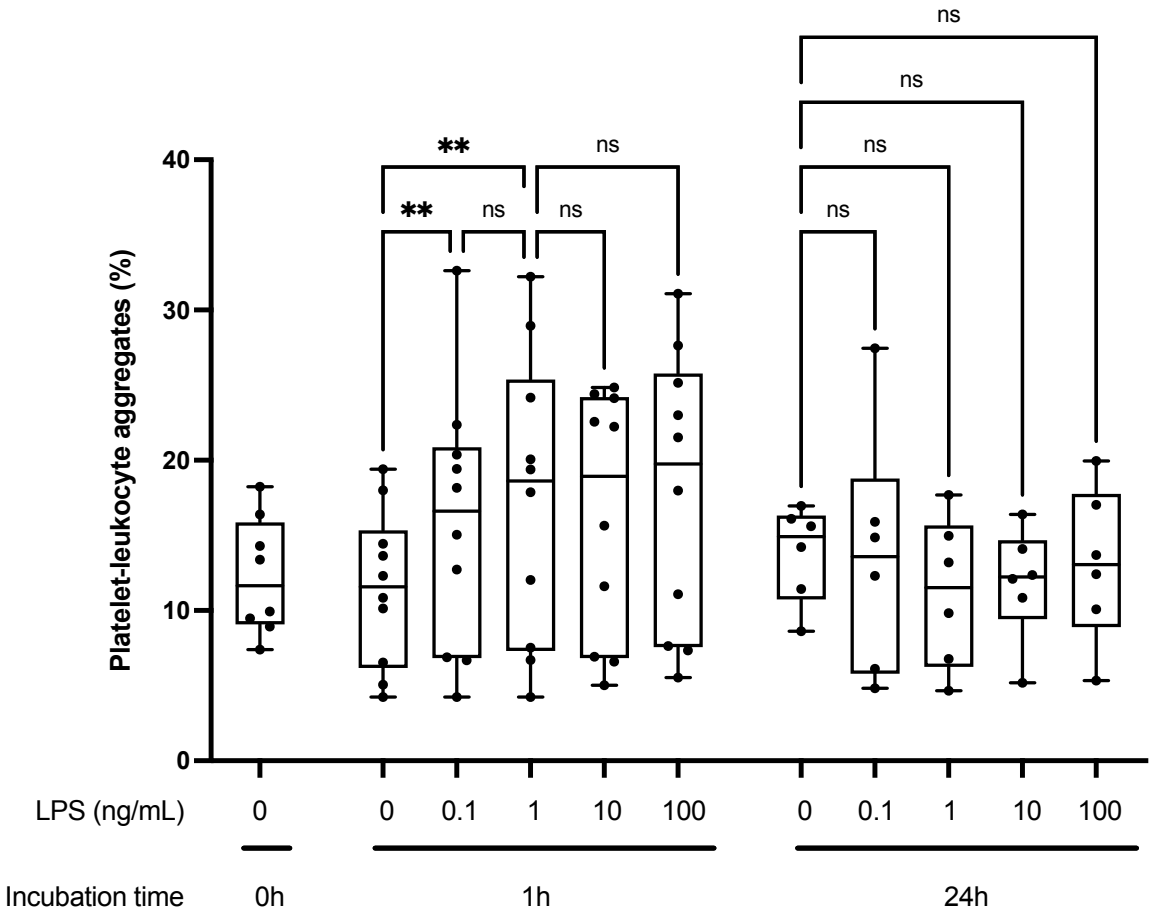

Figure 7. Analysis of platelet-leukocyte aggregates (PLAs) using flow cytometry after 1 or 24 hours of incubation with an increasing concentration of LPS (0 to 100 ng/mL). Unstimulated samples without incubation were used as control. PLAs were identified by flow cytometry with the co-expression of CD45 and CD41, and expressed as percentage of total CD45-positive events. n=8 (unincubated samples), n=6-10 (incubation time 1h and 24h). Statistical significance is indicated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant;

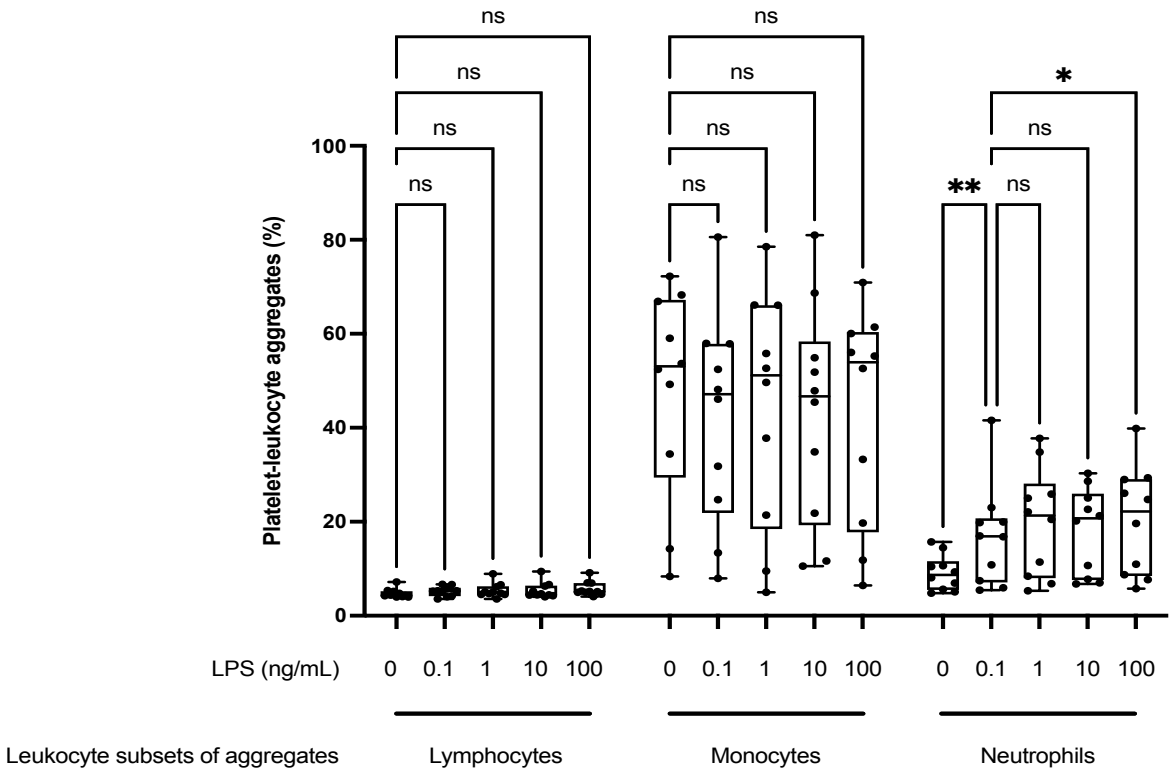

Figure 8. Analysis of platelet-leukocyte aggregates (PLAs) by leukocytes subsets using flow cytometry after 1 or 24 hours of incubation with an increasing concentration of LPS (0 to 100 ng/mL). Unstimulated samples without incubation were used as control. PLAs were identified by flow cytometry with the co-expression of CD45 and CD41. Leukocyte populations (lymphocytes, monocytes and neutrophils) were distinguished by CD45 expression and side scatter characteristics. Subsets of PLAs were then expressed as percentage of CD45-positive events within each leukocyte population. n=8 (unincubated samples), n=6-10 (incubation time 1h and 24h). Statistical significance is indicated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. ns: non-significant;

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Application à la modulation de $P2Y_{12}$

Le modèle in vitro présenté précédemment est actuellement utilisé à Rennes au sein du laboratoire IRSET (co-encadrement de Master 2), et appliqué à l'étude de la modulation des interactions plaquettes-leucocytes par les inhibiteurs du récepteur $P2Y_{12}$. Suite aux résultats du modèle murin présenté au chapitre précédent, les travaux en cours évaluent l'effet du ticagrelor sur l'interaction plaquette-leucocyte et la réponse inflammatoire plasmatique.

Les applications futures de ce modèle seront abordées dans la Discussion Générale de ce manuscrit.

Partie 2 : Étude de l'activation plaquettaire et de son impact dans les situations cliniques inflammatoires septiques ou associées aux circulations extracorporelles

Nous avons rapporté précédemment l'implication potentielle de l'activation plaquettaire dans la réponse inflammatoire et les défaillances d'organes associées au sepsis et aux situations inflammatoires non septiques liées aux dispositifs de circulation extracorporelles, et en particulier les assistances circulatoires (ECMO et CEC de chirurgie cardiaque).

En parallèle des études précliniques présentées en Partie 1, nous avons mené des travaux translationnels et cliniques visant à évaluer l'activation plaquettaire dans des situations inflammatoires septiques ou associées aux circulations extracorporelles.

L'objectif de cette partie clinique était d'améliorer les connaissances physiopathologiques sur le rôle des plaquettes dans des situations de circulation extracorporelle, par une approche transversale combinant des techniques biologiques d'évaluation des fonctions plaquettaires, des outils d'évaluation clinique (base de données) et statistique du devenir de nos patients. Ceci nous permettra dans second temps (voir Discussion Générale) d'établir des bases physiopathologiques, de développer des stratégies pronostiques, et ainsi d'évaluer prospectivement des stratégies thérapeutiques ciblées et basées sur l'inhibition de l'activation plaquettaire et de l'interaction plaquette-leucocyte en anesthésie et en réanimation.

Trois travaux seront ainsi présentés successivement.

Évaluation de l'impact d'une circulation extracorporelle sur l'activation plaquettaire : application au dispositif de récupération sanguine peropératoire i-SEP

Les dispositifs de récupération sanguine peropératoire (RSPO) actuellement utilisés en pratique clinique permettent de récupérer le sang épanché au niveau du site opératoire puis d'en extraire les globules rouges par centrifugation qui sont lavés avant d'être retransfusés au patient. Ces dispositifs ont démontré un bénéfice en chirurgie à haut risque hémorragique en diminuant les besoins transfusionnels en concentrés érythrocytaires et sont par conséquent utilisés couramment en anesthésie, notamment en chirurgie cardiaque sous CEC. Ces dispositifs de RSPO basés sur la centrifugation éliminent les plaquettes sanguines du sang récupéré.

Nous participons depuis plusieurs années au développement et à la validation préclinique et clinique en chirurgie cardiaque sous CEC d'un nouveau dispositif de RSPO (i-SEP, France) basé sur un système de filtration tangentielle. Cette technologie permet de conserver, de laver puis de retransfuser au patient les globules rouges mais également les plaquettes et les leucocytes.

L'utilisation de cette technologie en chirurgie cardiaque pourrait apporter plusieurs bénéfices potentiels en comparaison avec les dispositifs actuels :

1) Une amélioration de l'hémostase périopératoire et des besoins transfusionnels allogéniques
2) Une diminution des complications infectieuses periopératoires consécutive à la diminution des transfusions allogéniques et de l'anémie et thrombopénie post-opératoires

Cependant, l'aspiration chirurgicale, le contact sang-tubulures et la filtration pourraient induire une activation leuco-plaquettaire importante. Connaissant le rôle majeur de l'activation plaquettaire et de l'interaction plaquette-leucocyte dans l'inflammation et l'activation de la coagulation, l'hypothèse d'une majoration du risque inflammatoire, thrombotique et de l'augmentation des défaillances d'organes secondaires doit être envisagée.

Nous avons par conséquent élaboré une stratégie d'évaluation préclinique puis clinique du dispositif.

Dans un premier temps, nous avons évalué les performances du dispositif et son impact sur l'activation plaquettaire et leucocytaire dans une étude in vitro sur sang total humain. Le principe même de la RSPO consiste à concentrer les cellules récupérées au sein d'une solution cristalloïde déplasmatisée. Nous avons ainsi défini en collaboration avec l'HEGP (Professeur Pascale Gaussem), les conditions d'études, dans ce milieu, de l'activation plaquettaire et leucocytaire par techniques de cytométrie en flux. Les résultats de cette étude ont été publiés sous la forme d'un article dans la revue Anesthesiology (Mansour et al. Anesthesiology. 2021 Aug 1;135(2):246-257. doi: 10.1097/ALN.0000000000003820) et présentés ici. Cette étude démontre une bonne performance du dispositif et une faible activation plaquettaire mesurée par l'expression membranaire de CD62P, GP1b et GPIIbIIIa.

Parallèlement nous avons participé à l'évaluation du dispositif dans un modèle de chirurgie hémorragique chez le cochon, publiée sous la forme d'un article dans la revue Plos One (Schreiber et al. PLoS One. 2022 Mar 24;17(3):e0260855. doi: 10.1371/journal.pone.0260855).

Enfin nous menons en collaboration avec le CHU de Bordeaux (Professeur Alexandre Ouattara) les études cliniques d'évaluation du dispositif en chirurgie cardiaque (étude i-TRANSEP NCT04588350, études interventionnelles en cours de développement). Ces études cliniques évaluent entre autres l'impact de la retransfusion sur les complications hémorragiques, thrombotiques et infectieuses post-opératoires, ainsi que sur la réponse inflammatoire biologique et l'immunomodulation.

ANESTHESIOLOGY

Combined Platelet and Erythrocyte Salvage: Evaluation of a New Filtration-based Autotransfusion Device

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Autotransfusion is frequently used intraoperatively for patient blood management, with devices selectively able to salvage and wash red blood cells but not platelets.

What This Article Tells Us That Is New

- A novel filtration-based autotransfusion device salvaged both red blood cells and platelets, without significantly impacting cell integrity and function, with the recovery of 88.1% and 36.8%, respectively. The filtration and washing prevented reinfusion of high concentrations of heparin and did not activate leukocytes.

ABSTRACT

Background: The SAME device (i-SEP, France) is an innovative filtration-based autotransfusion device able to salvage and wash both red blood cells and platelets. This study evaluated the device performances using human whole blood with the hypothesis that the device will be able to salvage platelets while achieving a erythrocyte yield of 80% and removal ratios of 90% for heparin and 80% for major plasma proteins without inducing signification activation of salvaged cells.

Methods: Thirty healthy human whole blood units (median volume, 478 ml) were diluted, heparinized, and processed by the device in two consecutive treatment cycles. Samples from the collection reservoir and the concentrated blood were analyzed. Complete blood count was performed to measure blood cell recovery rates. Flow cytometry evaluated the activation state and function of platelets and leukocytes. Heparin and plasma proteins were measured to assess washing performance.

Results: The global erythrocyte yield was 88.1% (84.1 to 91.1%; median [25th to 75th]) with posttreatment hematocrits of 48.9% (44.8 to 51.4%) and 51.4% (48.4 to 53.2%) for the first and second cycles, respectively. Ektacytometry did not show evidence of erythrocyte alteration. Platelet recovery was 36.8% (26.3 to 43.4%), with posttreatment counts of 88 × 10^9/l (73 to 101 × 10^9/l) and 115 × 10^9/l (95 to 135 × 10^9/l) for the first and second cycles, respectively. Recovered platelets showed a low basal P-selectin expression at 10.8% (8.1 to 15.2%) and a strong response to thrombin-activating peptide. Leukocyte yield was 93.0% (90.1 to 95.7%) with no activation or cell death. Global removal ratios were 98.3% (97.8 to 98.9%), 98.2% (96.9 to 98.8%), and 88.3% (86.6 to 90.7%) for heparin, albumin, and fibrinogen, respectively. The processing times were 4.4 min (4.2 to 4.6 min) and 4.4 min (4.2 to 4.7 min) for the first and second cycles, respectively.

Conclusions: This study demonstrated the performance of the SAME device. Platelets and red blood cells were salvaged without significant impact on cell integrity and function. In the meantime, leukocytes were not activated, and the washing quality of the device prevented reinfusion of high concentrations of heparin and plasma proteins.

(ANESTHESIOLOGY 2021; 135:246–57)

Autotransfusion or cell salvage devices allow processing of blood shed from the surgical field and transfusion of red blood cells back to the patient.[1,2] They play an important role in patient blood management and are recommended by international guidelines.[1,3] Cell salvage has proven a reduction in the need for perioperative allogeneic blood transfusion in high hemorrhagic risk surgery, such as cardiac, orthopedic, gynecologic, and abdominal surgery, and might

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also improve clinical postoperative outcomes in cardiac surgery, including postoperative infections.[1,2,4–6] Finally, the cost-effectiveness of cell salvage has been demonstrated.[5,6] In addition to erythrocyte salvage, current devices can also effectively wash erythrocytes to remove cell breakdown products and activated plasma proteins, to reduce the risk of induced coagulopathy and inflammation.[2,6,7]

However, centrifugation-based autotransfusion devices can only salvage red blood cells, whereas blood platelets are removed during the process.[6] Hence, it has been shown that large amounts of intraoperative cell salvage blood transfusion can be associated with thrombocytopenia and increased use of allogeneic platelet transfusion.[8] Further, thrombocytopenia and platelet function disorders are known conditions associated with perioperative bleeding.[9–11] Although platelet transfusion is commonly used to treat thrombocytopenia-induced bleeding, it is also associated with an increase in postoperative complications including infections and increased length of stay.[3,12–14]

The SAME device (Smart Autotransfusion for Me; i-SEP, France) was designed as an innovative filtration-based autotransfusion device able to salvage and wash both red blood cells and platelets. This new autotransfusion system integrates a hollow fiber filtration technology, comparable to the filters used for plasmapheresis or for ultrafiltration during cardiopulmonary bypass. Using a combination of washing and filtration of salvage blood, the device allows the concentration of red blood cells and platelets within the concentrated blood product, as well as the removal of heparin, free hemoglobin, coagulation factors, and inflammatory mediators such as complement proteins.

We evaluated the i-SEP new autotransfusion device using human whole blood. The objectives of the study were to determine the performance of the device in terms of platelets and erythrocyte yield and function recovery, removal of heparin and of major plasma proteins, and potential impact on blood cell activation. We hypothesized that the device will be able to salvage platelets while achieving a erythrocyte minimal yield of 80% and a minimal removal ratio of 90% for heparin and 80% for major plasma proteins, without significant blood cell activation, with a fast treatment time of less than 5 min.

Materials and Methods

Autotransfusion Device

The SAME device is a medical device consisting of reusable equipment and disposable consumables (Supplemental Digital Content 1, http://links.lww.com/ALN/C621). The device innovative technology and process are described in the following patents: PCT/FR2018/053500 published as WO 2019/129973 on July 4, 2019 (corresponding to U.S. application no. 16/958,473); PCT/FR2018/053501 published as WO 2019/129974 on July 4, 2019 (corresponding to U.S. application no. 16/958,458); and PCT/FR2020/051115 published as WO 2020/260836 on December 30, 2020. Consumables include a dual-lumen suction line (allowing both collection and anticoagulation of shed blood), a blood collection reservoir (including a 40-μm filter), and a treatment set. The treatment set includes tubing, a polyethersulfone hollow fiber cartridge that separates the blood cells from the plasma, a compliant blood treatment bag that ensures the blood washing, a waste bag that receives the plasma and contaminants, and a reinfusion bag that stores the filtered, washed, and concentrated blood cells (Supplemental Digital Content 2, http://links.lww.com/ALN/C622). The reusable equipment is an electromedical medical device composed by several systems required for blood circulation and continuous measurements, including a continuous in-line hematocrit monitor. The i-SEP device is associated with a specific software to drive the different steps of the device installation and blood treatment.

Blood Processing by the i-SEP Device

During clinical use, the first stage of cell salvage with the i-SEP device is the collection of shed blood from the surgical field by the dual-lumen suction line, allowing the anticoagulation of shed blood by a heparinized saline drip. The shed blood is collected in the collection reservoir in which it undergoes a first filtration by the included 40-μm filter, allowing the removal of bone debris and microaggregates before blood treatment by the device. In the current experimental study, the suction line is used without heparinized saline drip as the whole blood is already heparinized (see below, under "Blood Preparation").

Then the treatment set is filled with anticoagulated salvaged blood transferred from the blood collection reservoir when a sufficient volume is collected. During the treatment phase, the blood is processed by the i-SEP device, with simultaneous filtration and washing (Supplemental Digital Content 2, http://links.lww.com/ALN/C622). The volume of the treatment set (300 to 1,000 ml thanks to the compliant treatment bag) limits the amount of collected blood that can be processed in one time, hence defining a treatment cycle. Thus, for research purpose, the volume of a cycle can be programed in the software between 300 and 1,000 ml.

Several simultaneous steps then constitute the innovative i-SEP process: wash solution (normal saline) is pumped into the treatment set; diluted salvaged blood circulates within the treatment set between the treatment bag and the polyethersulfone hollow fiber to allow microfiltration to occur; and fluid is continuously discarded from the treatment circuit into the waste bag through the effluent line. Once the continuously monitored hematocrit reaches the prespecified target, the device automatically transfers the processed blood from the treatment set into the reinfusion bag.

Blood Preparation

Thirty whole human blood units were obtained from the French Blood Bank Institute (Etablissement Français du Sang, Rennes, France, convention and ethical approval

reference No. 79/2019-2022) after obtaining donor written informed consent. Whole blood was collected in citrate–phosphate–dextrose anticoagulant and stored less than 24 h at room temperature before processing. Whole blood unit volume was 478 ml (461 to 511 ml) with a hematocrit of 38.6% (36.2 to 39.9%). Blood preparation is described in figure 1. Blood units were diluted in normal saline (0.9% NaCl; Macopharma, France) up to 1,200 ml (including 200 ml of collection reservoir priming) to obtain clinically relevant hematocrits of 14.4% (13.1 to 15.3%), corresponding to initial hematocrits measured in blood collection reservoir during cardiac and orthopedic surgeries (ranging between 10 and 20%) while preserving between-subject heterogeneity.[7,15–17] A high concentration of unfractionated heparin (Choay heparin; Sanofi-Aventis, France) was added to whole blood before dilution in saline (fig. 1); the final concentration of heparin in the collection reservoir was 12 IU/ml, to evaluate heparin washout in worst-case clinical conditions.

Experimental Procedure for *In Vitro* Study

All experiments were conducted in the Department of Hematology of the University Hospital of Rennes (France). After collection reservoir priming (200 ml of 0.9% NaCl; Macopharma), the blood was collected into the blood collection reservoir under controlled depression level using the suction line, with a vacuum level of −250 mbar (fig. 1). Then the experimental procedure consisted of two consecutive treatment cycles using the i-SEP device standard program. The choice of a two-treatment cycle procedure allows the evaluation of the impact of two consecutive cycles on the same filtering membrane and surface pacification. The first cycle was programed to treat 700 ml, and the second cycle treated 500 ml (total volume, 1,200 ml), using 600 ml of washing volume (0.9% NaCl; Macopharma) for each cycle. This allowed evaluation of the impact of blood volume to washing volume ratio on cell yield and washing performance. A different reinfusion bag was used for each cycle to facilitate the posttreatment sampling. The processing time of each cycle was recorded to be used as a device performance endpoint.

Blood Sample Collection during Processing

Four sample series were realized for each blood unit: (1) blood in the reservoir after collection and before first cycle (first cycle pretreatment), (2) blood cell concentrate in the reinfusion bag at the end of first cycle (first cycle posttreatment), (3) blood in the reservoir before second cycle (second cycle pretreatment), and (4) blood cell concentrate in the reinfusion bag at the end of second cycle (second cycle posttreatment).

The sampling procedure was identical for all tests. Blood was gently homogenized in both the collection reservoir and the transfusion bag before taking samples to ensure homogeneous sampling. Pretreatment samples were taken from the line between the collection reservoir and the treatment set during the transfer of diluted blood into the treatment set, for each cycle. Tubes were cautiously filled in up to the volume indicator. When a centrifugation was needed, the tubes were handled a maximum of 1 h after their sampling. The samples were distinctly stored at room temperature, 4°C, or −80°C or sent, when necessary, at room temperature, in ice, or in dry ice following recommendations.

Laboratory Analyses

Laboratory analyses were performed on all blood units for each sample series (30 blood units, 60 cycles), except for ektacytometry (9 blood units, 18 cycles) and CD64 cytometric analysis (14 blood units, 28 cycles). Complete blood count (including erythrocyte count, leukocyte count, platelet count, hematocrit, and total hemoglobin level) was performed on an EDTA tube using UniCel DxH 800 (Beckman Coulter, USA). Albumin, lactate deshydrogenase, and immunoglobulins were measured in a lithium heparin tube using a Cobas 8000 modular analyzer (Roche Diagnostics, Switzerland). Free hemoglobin was assessed using spectrophotometry (SAFAS, Monaco). Two citrate (109 mM) tubes were taken for each cycle and centrifuged at 2,000*g* to obtain plasma. All plasma samples were frozen (–80°C) before performing the following assays: unfractionated heparin anti-Xa activity using STA-Liquid-anti-Xa (Stago, France), factors II, V, and X coagulant activity using factors II-, V- and X-deficient plasma (Stago) after 1/10 dilution and Neoplastin CI+ (Stago), fibrinogen von Clauss activity (Stago) on a STA R Max coagulometer (Stago), and complement factor 3 assays (Siemens BN nephelometer). Anti-Xa activity was measured in samples diluted to 1:20 or 1:2 in normal pool plasma to allow measurement of high heparin concentration. Blood cell yield was calculated using the following formula: cell yield = [(posttreatment blood volume × posttreatment cell concentration)/(pretreatment blood volume × pretreatment cell concentration)] × 100. Hemolysis was calculated as follows: (100 − hematocrit) × free hemoglobin/total hemoglobin. Removal ratios of major blood proteins were measured as follows: (initial quantity of protein − final quantity of protein)/initial quantity of protein.

Ektacytometry

Osmotic gradient ektacytometry allows measurement of red cell deformability in response to alterations in medium osmolality.[18] It is a useful technique for the diagnosis of inherited red cell membrane disorders and characterization of blood storage lesions.[18–21] We used a LORCA ektacytometer (Centre Hospitalier Universitaire Bicêtre, Le Kremlin Bicêtre, France) to assess the impact of the i-SEP device process on red cell membrane integrity. Samples were run at 37°C. Three parameters were used: osmolality

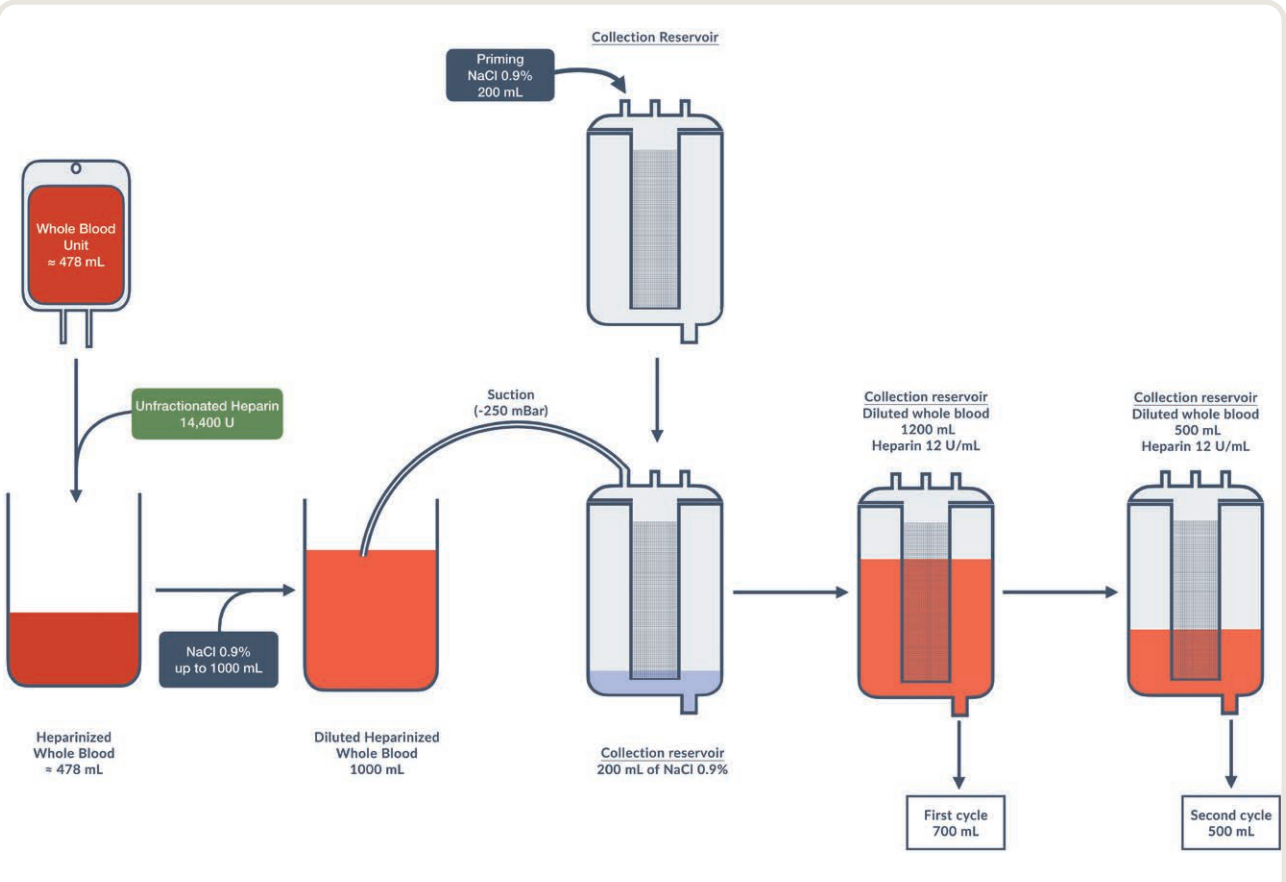

Fig. 1. Blood preparation and experimental procedure for *in vitro* study. Whole human blood units were obtained from the French Blood Bank Institute (Rennes, France) after obtaining donor written informed consent. Whole blood unit volume was 478 ml (461 to 511 ml). Citrate–phosphate–dextrose anticoagulated blood units were diluted in normal saline (0.9% NaCl; Macopharma, France) up to 1,200 ml (including 200 ml of collection reservoir priming). Unfractionated heparin (Choay heparin; Sanofi-Aventis, France) at high concentration was added to whole blood before dilution in saline. The final heparin concentration in the collection reservoir was 12 IU/ml. After collection reservoir priming (200 ml of 0.9% NaCl; Macopharma), the blood was collected into the blood collection reservoir under controlled depression level using the suction line, with a vacuum level of −250 mbar. Then the experimental procedure consisted of two consecutive treatment cycles using the i-SEP device. The first cycle was programed to treat 700 ml and the second cycle treated 500 ml (total volume, 1,200 ml).

corresponding to minimum elongation index, which reflects osmotic fragility; maximum elongation index, which assesses membrane flexibility; and osmololality corresponding to half-maximal elongation in the hypertonic arm of the osmotic gradient, which gives information on intracellular viscosity of the red cell.

Flow Cytometric Evaluation of Platelet Activation

Platelet activation was evaluated by measuring surface expression of three main physiologic platelet glycoproteins: P-selectin, GPIb, and GPIIb. Unlike P-selectin, GPIb and GPIIb are constitutively expressed at the surface of quiescent platelets. Upon activation, surface expression of P-selectin and GPIIb is increased, whereas GPIb surface exposure is reduced. Quantitation of GPIb, GPIIb, and P-selectin on platelet surface was measured using flow cytometry (DxFLEX; Beckman Coulter) and the PLT GP/receptors kit from Stago.[22,23] GPIb and GPIIb expression was reported as the number of receptors per platelet. P-selectin expression was reported as the percentage of P-selectin–positive platelets. Glycoprotein expression at rest, in pretreated and posttreated blood, was used to measure platelet activation as a potential side effect induced by the i-SEP device. Then stimulation by thrombin receptor PAR1-activating peptide 6 (TRAP6) was used as a surrogate to determine whether posttreatment platelets can be fully activated after being recovered by the i-SEP device.

Flow Cytometric Analysis of Leukocyte Viability and Activation State

Leukocyte subset viability and activation states were measured using flow cytometry (DxFLEX; Beckman Coulter). DuraClone IM phenotyping panel (Beckman Coulter) was used to identify leukocyte subpopulations in whole blood

samples. Leukocyte viability was evaluated using 7-amino-actinomycin D antibodies.[24] Alive leukocytes were defined as CD45+/7-amino-actinomycin D and leukocyte viability was reported as a percentage of alive white blood cells among CD45-positive cells. Neutrophils and monocytes were respectively defined as high side scatter size and CD16+/CD14− and CD16−/CD14+ cells. An increase in CD64 surface expression was used as a marker of neutrophil and monocyte activation and was reported as mean fluorescence intensity.[25] The T-cell activation state was evaluated using HLA-DR antibodies.[26] Activated CD4-positive and CD8-positive T cells were defined as CD4+/HLA-DR+ or CD8+/HLA-DR+ double-positive cells and expressed as percentages of HLA-DR–positive cells, respectively, among CD4- or CD8-positive cells, respectively. The data were analyzed with Kaluza software (Beckman Coulter).

Definition of Endpoints

The main goal of this study was to evaluate performance of blood cell salvage by the i-SEP device. Given the lack of consensual guidelines on preclinical evaluation of autotransfusion systems and the low level of evidence in the current literature, we determined prespecified performance criteria based on *in vitro* and clinical assessment of commercially available devices and international guidelines on evaluation of blood products.[27,28] Hence, the erythrocyte minimal recovery rate and hematocrit were respectively set at 80 and 40%, corresponding to 1 SD less than the mean values published for centrifugation-based devices. Washout quality was defined as a minimal removal ratio of 90 and 80%, respectively, for heparin and major plasma proteins. Maximal hemolysis was set at 0.8% in accordance with European guidelines on packed red cell evaluation.[27] Being a major innovative characteristic of the device, no minimal platelet yield criteria was defined. The activation state and function of platelets and leukocytes were considered as exploratory secondary endpoints. The protocol and the choice of endpoints were approved by the French National Agency for Medicines and Health Products Safety (Agence Nationale de Sécurité du Médicament et des Produits de Santé, Saint-Denis, France).

Statistics

Sample size selection was based on the French National Agency for Medicines and Health Products Safety guidelines for therapeutic blood product quality evaluation and was set at 30 replicates (60 cycles).[28] Therefore, no *a priori* statistical power calculation was conducted. *Post hoc* statistical analyses were conducted using Prism 8 (GraphPad Software, USA). The data were tested for normality using the D'Agostino and Pearson normality test. All measured parameters did not show normal distribution. Kruskal–Wallis and Friedman's test with *post hoc* Dunn's correction test were used for multiple comparisons between treatment phases, cycles, and TRAP6 stimulation in the flow cytometric analysis of platelet glycoproteins. The Mann–Whitney test was used for single comparisons between: (1) the first and second cycles for erythrocyte yield, platelet yield, and leukocyte yield and (2) pre- and posttreated blood for erythrocyte lysis markers, ektacytometric parameters, leukocytes viability, and leukocytes activation, with independent analysis of the first and second cycles. All tests used two-tailed hypothesis. Statistical significance was achieved for $P < 0.05$. Statistical analyses used limit-of-quantification values as substitute for values inferior to the limit of quantification. The data are presented as medians with interquartile ranges. The differences between two conditions are reported as actual differences between medians with Hodges–Lehman computed 95% CI. Outliers were not excluded from the analyses. There were no missing data.

Results

Erythrocyte Yield and Impact on Cell Integrity

Whole blood processing using the i-SEP autotransfusion device produced a high recovery rate of red blood cells with a global yield of 88.1% (84.1 to 91.1%). The second cycle steadily achieved significantly higher RBC yield compared to the first cycle (16.3% [12.3 to 18.9%]; $P < 0.001$; fig. 2). The erythrocyte counts in the pretreated blood were 1.5 × 10^{12}/l (1.3 to 1.6) for the first cycle and 1.4 × 10^{12}/l (1.3 to 1.6) for the second cycle. Erythrocyte counts in the posttreated blood were 4.8 × 10^{12}/l (4.4 to 5.1 × 10^{12}/l) for the first cycle and 5.1 × 10^{12}/l (4.8 to 5.3 × 10^{12}/l) for the second cycle. The final posttreatment concentrate volume was 186 ml (152 to 217 ml) for the first cycle and 105 ml (87 to 112 ml) for the second cycle. Likewise, posttreatment hematocrit was consistently above 40%, with 48.9% (44.8 to 51.4%) for the first cycle and 51.4% (48.4 to 53.2%) for the second cycle (fig. 3). Assessment of erythrocyte lysis markers demonstrated a significant increase in global LDH (107 UI/ml [98 to 124 UI/ml]; $P < 0.001$), free hemoglobin (54 mg/dl [46 to 56 mg/dl]; $P < 0.001$), and hemolysis (0.12% [0.11 to 0.14%]; $P < 0.001$) in the posttreated compared to the pretreated blood (table 1).

Ektacytometric analysis of red blood cells demonstrated that blood processing through the i-SEP device did not induce alterations in erythrocyte integrity and deformability, neither between pre- and posttreated blood nor between the first and second cycles for the three parameters analyzed (table 1). Hence, no statistical differences were measured in global osmolality at minimum elongation index (−5 mOsm/kg [−9 to 1 mOsm/kg]; $P = 0.089$), maximum elongation index (0.00 [−0.01 to 0.01]; $P = 0.879$) and osmolality at half-maximal elongation in the hypertonic arm (−1 mOsm/kg [−17 to 13 mOsm/kg]; $P = 0.683$) in the posttreated compared to the pretreated blood (table 1).

Platelet Recovery and Platelet Function Analysis

The device achieved a global platelet recovery rate of 36.8% (26.3 to 43.4%) with significantly higher platelet yield during

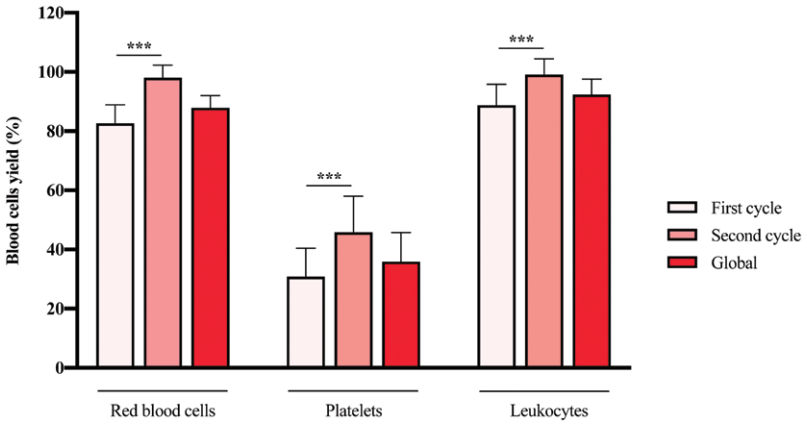

Fig. 2. Blood cell yield, including red blood cells, platelets, and white blood cells, calculated for the first cycle (n = 30) and the second cycle (n = 30). The global yield includes all the results (cycles 1 and 2; n = 30): it represents the global yield of all red blood cells obtained in the concentrated blood compared to all red blood cells from diluted blood. ***$P < 0.001$.

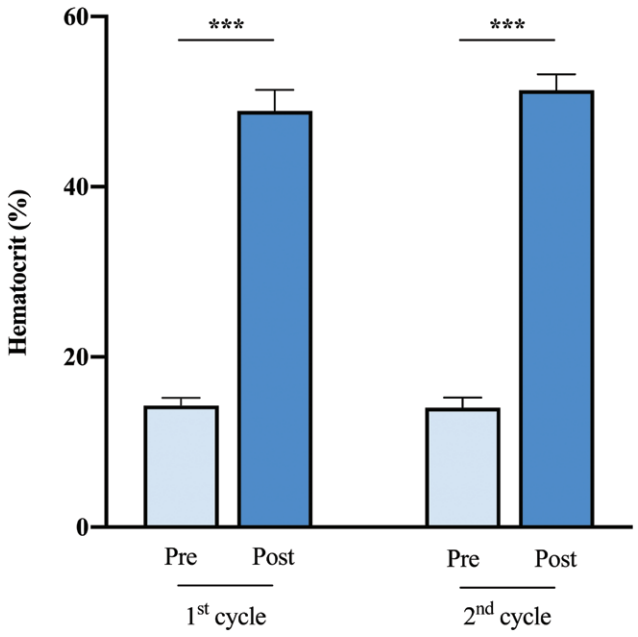

Fig. 3. Hematocrit measurement between pretreatment (Pre) and posttreatment (Post; concentrated) blood for each cycle (n = 30 for cycle 1 and n = 30 for cycle 2). ***$P < 0.001$.

the second cycle compared to the first cycle (14.3% [9.3 to 21.0%]; $P < 0.001$; fig. 2). Platelet counts in the pretreated blood were 74 × 10^9/l (59 to 89 × 10^9/l) and 72 × 10^9/l (60 to 82 × 10^9/l) for the first and second cycles, respectively. Platelet counts in the posttreated blood were 88 × 10^9/l (73 to 101 × 10^9/l) and 115 × 10^9/l (95 to 135 × 10^9/l) for the first and second cycles, respectively. The global number of salvaged platelets was 28.7 × 10^9 platelets (24.2 to 35.4 × 10^9).

Flow cytometric analysis of glycoproteins revealed a limited platelet activation induced by the device, as demonstrated by a significant increase in the percentage of P-selectin–positive platelets in posttreated compared to pretreated blood for both cycles. The percentages of P-selectin–positive platelets were 2.3% (1.5 to 3.4%) and 10.8% (7.2 to 12.6%; $P < 0.001$; fig. 4A) during the first cycle in the pretreated and posttreated blood, respectively, and 3.5% (2.5 to 4.2%) and 10.8% (8.1 to

Table 1. Impact of Blood Processing on Erythrocyte Integrity

	First Cycle		Second Cycle		Global	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Hemolysis, %	0.03 (0.02 to 0.05)	0.18 (0.14 to 0.21)*	0.04 (0.02 to 0.05)	0.15 (0.13 to 0.19)*	0.03 (0.02 to 0.05)	0.16 (0.15 to 0.21)*
Hemoglobin, g/dl	4.8 (4.4 to 5.2)	15.9 (14.7 to 16.5)*	4.7 (4.3 to 5.1)	16.5 (16.0 to 17.0)*	4.8 (4.4 to 5.4)	16.2 (15.6 to 16.7)*
Free hemoglobin, mg/dl	2 (1 to 3)	58 (47 to 65)*	2 (1 to 3)	53 (44 to 65)*	2 (1 to 3)	56 (44 to 64)*
Lactate dehydrogenase, U/l	43 (37 to 57)	145 (123 to 163)*	45 (37 to 50)	169 (150 to 206)*	44 (39 to 58)	151 (140 to 182)*
Ektacytometry						
Osmolality at minimal elongation index, mOsm/kg	154 (151 to 159)	151 (150 to 159)	156 (131 to 158)	148 (147 to 155)	155 (153 to 159)	150 (148 to 155)
Maximal elongation index	0.61 (0.61 to 0.62)	0.62 (0.61 to 0.62)	0.61(0.61 to 0.62)	0.62 (0.61 to 0.62)	0.61(0.61 to 0.62)	0.62 (0.61 to 0.62)
Osmolality at half-maximal elongation in the hypertonic arm, mOsm/kg	464 (449 to 478)	461 (449 to 474)	465 (450 to 477)	461 (445 to 472)	463 (449 to 477)	462 (447 to 473)

The data are expressed as medians and interquartile ranges 25 to 75%. N = 30, except for ektacytometric parameters, for which N = 9. The global results were obtained by pooling the observations of the two cycles for each repetition.

*$P < 0.001$ *versus* pretreatment.

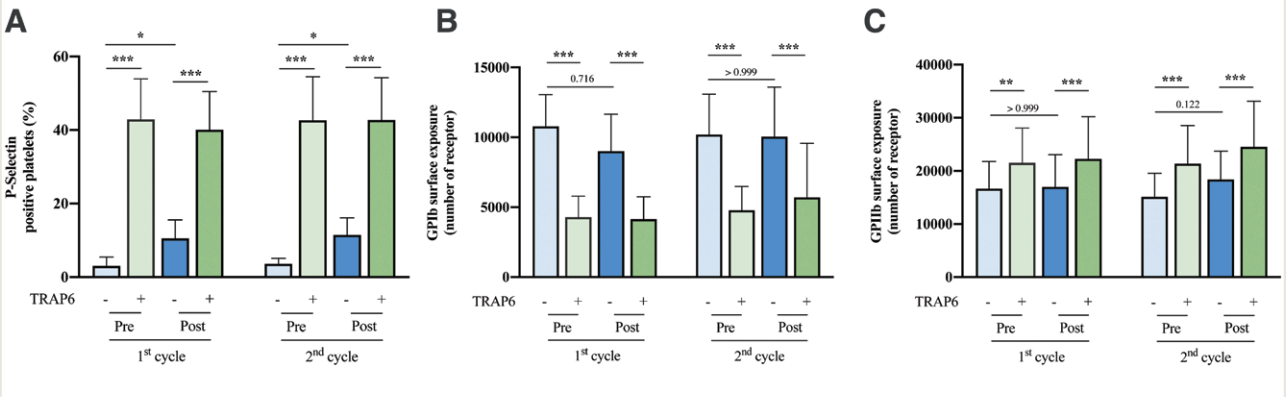

Fig. 4. Effect of i-SEP device processing on P-selectin, GPIb, and GPIIb platelet expression and evaluation of platelet activation potential by thrombin receptor pathway stimulation using thrombin receptor-activating peptide 6 (TRAP6). All flow cytometry experiments were performed on DxFLEX (Beckman Coulter, USA), using the PLT Gp/receptors kit from Stago (Biocytex, France). (A) P-selectin expression reported as the percentage of P-selectin–positive platelets. P-selectin expression was measured between pretreatment (Pre) and posttreatment (Post; concentrated) blood for each cycle (n = 30 for cycle 1 and n = 30 for cycle 2). *$P < 0.05$; ***$P < 0.001$, measurements were performed before (–) or after TRAP6-induced platelet activation (+). (B) GPIb expression reported as the number of receptors per platelet. GPIb expression was measured between pretreatment and posttreatment (concentrated) blood for each cycle (n = 30 for cycle 1 and n = 30 for cycle 2). ***$P < 0.001$. +, platelet stimulation by TRAP6; –, absence of platelet stimulation by TRAP6. (C) GPIIb expression reported as number of receptors per platelet. GPIIb expression was measured between pretreatment and posttreatment (concentrated) blood for each cycle (n = 30 for cycle 1 and n = 30 for cycle 2). **$P < 0.01$; ***$P < 0.001$. +, platelet stimulation by TRAP6; –, absence of platelet stimulation by TRAP6. GpIb and GpIIb expressions were performed before (–) or after TRAP6-induced platelet activation (+).

15.2%; $P < 0.001$; fig. 4A) during the second cycle in the pretreated and posttreated blood, respectively. However, no significant change between pretreated and posttreated blood was found during both cycles regarding GPIb surface expression (fig. 4B) and GPIIb surface expression (fig. 4C), in line with a limited platelet activation.

Compared to unstimulated posttreated blood, TRAP6 stimulation of posttreated blood induced a significant change in platelet glycoprotein surface expression, with increased percentage of P-selectin–positive platelets, decreased GPIb surface expression, and increased GPIIb surface expression. These results clearly demonstrate that recovered platelets retain a high potential of activation (fig. 4, A, B, and C). The percentage of P-selectin–positive platelets was 10.8% (7.2 to 12.6%) and 43.0% (32.6 to 46.7%) during the first cycle in the unstimulated and TRAP6-stimulated posttreated

blood ($P < 0.001$; fig. 4A), respectively, and 10.8% (8.1 to 15.2%) and 47.6% (38.2 to 50.5%) during the second cycle in the unstimulated and TRAP6-stimulated posttreated blood ($P < 0.001$; fig. 4A), respectively. GPIb surface expression was 8,632 (7,611 to 10,849) and 3,809 (3,043 to 5,337) during the first cycle in the unstimulated and TRAP6-stimulated posttreated blood ($P < 0.001$; fig. 4B), respectively, and 9,956 (7,996 to 13,100) and 5,189 (4,067 to 6,152) during the second cycle in the unstimulated and TRAP6-stimulated posttreated blood ($P < 0.001$; fig. 4B), respectively. GPIIb surface expression was 16,297 (13,547 to 20,273) and 23,616 (17,790 to 27,429) during the first cycle in the unstimulated and TRAP6-stimulated posttreated blood ($P < 0.001$; fig. 4C), respectively, and 18,863 (15,047 to 21,424) and 24,892 (19,421 to 30,579) during the second cycle in the unstimulated and TRAP6-stimulated posttreated blood ($P < 0.001$; fig. 4C), respectively.

Leukocyte Yield and Activation State

The device produced a global leukocyte recovery rate of 93.0% (90.1 to 95.7%) with significantly higher leukocyte yield during the second cycle compared to the first cycle (12.4% [7.0 to 13.6%]; $P < 0.001$; fig. 2). Leukocyte counts in the pretreated blood were 2.3×10^9/l (1.9 to 2.7×10^9/l) and 2.3×10^9/l (1.9 to 2.6×10^9/l) for the first and second cycles, respectively. Leukocyte counts in the posttreated blood were 8.3×10^9/l (6.6 to 9.4×10^9/l) and 8.2×10^9/l (6.5 to 9.3×10^9/l) for the first and second cycles, respectively.

Leukocyte viability in pretreated blood was 97.6% (97.0 to 98.5%) for the first cycle and 97.7% (97.3 to 98.5%) for the second cycle. Regarding basal activation of leukocytes in the pretreated blood, respectively, for the first and second cycles: the percentages of HLA-DR positive/CD4-positive cells were 4.4% (3.1 to 6.2%) and 4.4% (3.2 to 6.0%); the percentages of HLA-DR positive/CD8-positive cells were 13.2% (5.5 to 16.3%) and 12.7% (5.2 to 17.5%); CD64 surface expression levels on neutrophils were 1,420 (1,257 to 1,600) and 1,463 (1,297 to 1,721); and CD64 surface expression levels on monocytes were 11,122 (8,900 to 13,157) and 12,219 (9,408 to 14,536).

Blood processing through the device was not associated with leukocyte cell death, as demonstrated by flow cytometric measurement of leukocyte viability in posttreated compared to pretreated blood (0.5% [−0.1 to 1.0%], $P = 0.096$ for the first cycle; 0.4% [−0.1 to 0.6%], $P = 0.281$ for the second cycle). Likewise, cell recovery was not associated with significant leukocyte activation, either regarding CD4-positive cells (−0.1% [−1.2 to 0.7%], $P = 0.535$ for the first cycle; 0.0% [−1.2 to 0.7%], $P = 0.620$ for the second cycle) or CD8-positive cells (−3.2% [−4.6 to 2.2%]; $P = 0.443$ for the first cycle; −2.0% [−4.6 to 1.8%], $P = 0.406$ for the second cycle). Last, blood treatment did not induce any significant increase in CD64 surface expression in posttreated compared to pretreated blood for neutrophils (8 [−119 to 203], $P = 0.701$ for the first cycle; −30 [−238 to 166], $P = 0.635$ for the second cycle) or monocytes (1,905 [−441 to 3,522], $P = 0.125$ for the first cycle; 794 [−1,443 to 3,829], $P = 0.427$ for the second cycle).

Washout Quality

The i-SEP device exhibited a high heparin washing capacity, demonstrated by a global heparin removal ratio of 98.3% (97.8 to 98.9%), despite very high median heparin concentration in the pretreated blood of 11.7 U/ml (11.0 to 13.3 U/ml) for the first cycle and 12.2 U/ml (11.2 to 12.8 U/ml) for the second cycle (table 2). Still, the second cycle achieved a better removal of heparin with a final median concentration of 0.2 U/ml (0.1 to 0.4 U/ml) in the treated-blood and a removal ratio of 99.7% (99.6 to 99.9%), compared to the first cycle with a final median concentration of 1.8 U/ml (1.4 to 2.17 U/ml) and a removal ratio of 97.8% (96.8 to 98.5%).

Likewise, a high washing quality of major plasma proteins was obtained, including albumin, immunoglobulins G, complement factor 3, fibrinogen, and coagulation factors II and VII, demonstrated by global removal ratios > 88% as reported in table 2.

Processing Time

The i-SEP device achieved regular and short median processing times of 4.4 min (4.2 to 4.6 min) for the first cycle and 4.4 min (4.2 to 4.7 min) for the second cycle.

Discussion

This study demonstrates the ability of a filtration-based autotransfusion device to recover and wash both red blood cells and platelets from diluted whole human blood with a fast processing time of less than 5 min. Recovery rates and final hematocrit demonstrated a high quality of erythrocyte salvage by the i-SEP device, comparable to commercially available centrifugation-based devices.[7,16,29–31] Because high suction forces greater than −200 mbar during cell salvage are associated with erythrocyte hemolysis, current centrifugation-based devices use standard vacuum levels of approximately −150 mmHg (−200 mbar). However, those levels can be increased up to −300 mmHg (−400 mbar), in manual mode. Although the i-SEP device is intended to be clinically used with standard vacuum levels of −150 mbar, we decided to increase it to −250 mbar to measure hemolysis in a worst-case clinical scenario. Hemolysis was limited and remained far below acceptable levels for packed red blood cells, according to European and U.S. guidelines, of 0.8 and 1%, respectively.[27,32] Measured hemolysis (0.16%) was comparable to fresh packed red blood cells (less than 7 days of storage).[32] Unlike blood storage, the i-SEP device had no impact on erythrocyte deformability and membrane integrity, as demonstrated by ektacytometry and is therefore comparable to current centrifugation-based devices.[19,33,34]

Table 2. Removal Ratios for Heparin and Major Plasma Proteins

	First Cycle			Second Cycle			Global
	Pretreatment	Posttreatment	Removal Ratio	Pretreatment	Posttreatment	Removal Ratio	Removal Ratio
Heparin, U/ml	11.7 (11.0 to 13.3)	1.8 (1.4 to 2.17)*	97.8 (96.8 to 98.5)	12.2 (11.2 to 12.8)	0.2 (< 0.1 to 0.4)*	99.7 (99.6 to 99.9)	98.3 (97.8 to 98.9)
Albumin, g/l	9.0 (7.9 to 10)	1.5 (0.7 to 1.9)*	97.9 (96.6 to 98.8)	9.3 (8.0 to 10.7)	0.7 (0.2 to 1.1)*	98.8 (97.9 to 99.7)	98.2 (96.9 to 98.8)
Immunoglobulin G, g/l	2.2 (1.8 to 2.9)	0.4 (0.3 to 0.5)*	97.6 (96.7 to 98.7)	2.2 (1.9 to 2.7)	0.1 (0.1 to 0.3)*	99.2 (98.2 to 99.4)	98.1 (97.0 to 98.8)
Complement component 3, g/l	0.25 (0.19 to 0.29)	All values < 0.18*	> 88.4 (86.2 to 90.6)	0.23 (0.19 to 0.29)	All values < 0.18*	> 87.3 (85.4 to 89.6)	> 87.6 (87.0 to 89.4)
Fibrinogen, g/l	0.60 (< 0.4 to 0.69)	All values < 0.4*	> 88.7 (87.3 to 91.2)	0.59 (< 0.4 to 0.64)	All values < 0.4*	> 88.5 (84.8 to 90.7)	> 88.3 (86.6 to 90.7)
Factor II, U/ml	19.5 (16.0 to 24.0)	All values < 10*	> 92.7 (90.9 to 93.4)	20.0 (17.0 to 23.3)	All values < 10*	> 92.3 (91.4 to 93.1)	> 92.4 (91.6 to 93.0)
Factor VII, U/ml	26.5 (23.0 to 30.3)	All values < 10*	> 94.3 (92.7 to 95.7)	27.0 (22.8 to 32.5)	All values < 10*	> 94.4 (92.7 to 95.6)	> 94.3 (92.5 to 95.3)

Blood parameters are expressed as medians and interquartile ranges 25 to 75%. Removal ratios (%) are expressed as medians and interquartile ranges 25 to 75%. Statistical analyses use limit-of-quantification values as substitute for values inferior to the limit of quantification (N = 30).

*$P < 0.001$ *versus* pretreatment.

Platelet recovery is a major innovative feature of the i-SEP autotransfusion device. Overall, the processing of a median 478 ml of whole blood by the device allowed the salvage of 28.7×10^9 platelets, which exceeds minimum platelet content requirement for 1 unit equivalent of platelet concentrate (single-donor whole blood-derived platelet or one sixth of single donor apheresis platelet concentrate). Additionally, recovered platelet function was not altered by the device, as demonstrated by limited platelet activation and strong response to thrombin pathway stimulation. Still, the number of P-selectin positive platelets in the treated blood remained greatly inferior to that of blood bank platelet concentrates, including 1-day storage concentrates (5 to 25% of P-selectin–positive platelets).[35,36] It should be noted that mean platelet GpIb and GpIIb surface expression was inferior to reference values in adult.[37] Blood processing by the i-Sep device cannot be accounted for with this phenomenon, because it was already observed in the pretreated blood. It could rather be explained by platelet activation and glycoprotein shedding during the initial steps of the study, including blood storage, blood dilution with normal saline, and blood suction–induced shear stress, as previously described.[38–40] During blood processing by the i-SEP device, the loss of platelets is probably multifactorial and might involve platelet activation induced by inflammation or shear stress, mechanical destruction during suction and processing, and platelet adhesion to tubing and filtering membranes. We can hypothesize that some mechanisms may be saturable, thanks to tubing and membrane pacification, and will allow for improvement of platelet yield in future device developments. Although centrifugation-based devices theoretically remove platelets from salvaged blood, studies demonstrated that small amounts of platelets remained in the treated blood; however, these platelets were not evaluated in terms of function and activation state.[15] Compared to these centrifugation-based devices, the i-SEP device demonstrated a 6- to 7-fold higher platelet yield. Overall, these results allow further clinical evaluation of the potential benefits of platelet recovery, because we can now hypothesize (1) that the device might decrease perioperative bleeding in the setting of nonmassive surgical bleeding for which platelet transfusion is unlikely and (2) that the processing of undiluted shed blood by the i-SEP device might be sufficient to decrease or overcome the need for platelet transfusion in the setting of massive surgical bleeding.

Although centrifugation-based devices theoretically remove leukocytes from salvaged blood, several studies demonstrated that a significant amount of white blood cells remained in the treated blood, with recovery rates between 27 and 81%.[15,16,29,41] This represents a major concern because leukocyte damage and activation can occur during centrifugation and washing and might induce a systemic inflammatory response.[41–43] We therefore evaluated the impact of i-SEP processing on leukocytes and demonstrated that filtration-based cell salvage and washing did not induce significant leukocyte cell death or activation.

Compared to the first cycle, the second cycle of treatment was steadily associated with a significantly higher erythrocyte, platelet, and leukocyte yield, with second cycle recovery rates sometimes above 100%. This phenomenon can be entirely explained by the fact that a substantial amount of treated blood from first cycle is staying in the i-SEP device circuit and is only released at the end of the second cycle.

Regarding washing quality, the i-SEP device achieved high removal ratios of heparin and major plasma proteins (including albumin, immunoglobulins, complement, and coagulation factors), in the same manner as current centrifugation-based devices.[7,15,16,44,45] This considerably reduces the risk of induced coagulopathy and inflammation. The second cycle steadily exhibited better washing quality as compared to the first cycle, essentially as a result of a smaller treated blood volume (700 ml for the first cycle and 500 ml for the second cycle) for the same processing time and washing volume.

Despite a high heparin removal ratio (98.3%), a substantial heparin concentration remained in the treated blood after the first cycle, with a heparin level greater than 0.5 U/ml.

This phenomenon is explained by the choice of the addition of a high final unfractionated heparin concentration of 12 U/ml, considered to be a worst-case operative condition, whereas studies of centrifugation-based devices commonly used lower heparin concentrations of 5 U/ml.[7,15,16] Indeed, during cardiopulmonary bypass in patients, the heparin concentration in circulating blood frequently exceeds 5 U/ml.[46,47] Also, accidental overheparinization of salvaged blood can occur if the heparinized saline drip is unintentionally increased in the dual-lumen suction tip. In these settings, heparin removal by cell savers might be insufficient to prevent significant heparin reinfusion by commercially available devices in the clinical operative setting, despite high heparin removal ratios.[45] The recently developed cell salvage system HemoSep (Brightwake, United Kingdom) greatly differs from i-SEP device by producing blood cell filtration, without washing, using long processing time (more than 15 min), and was therefore not included in this discussion.[48,49]

A few points have to be considered to evaluate the clinical relevance of our results. First, this study was conducted using diluted whole human blood units, and the results might therefore not be generalized to the clinical setting. Hence, the study was not designed to evaluate clinical efficacy and safety of the device. Multiple factors might indeed interfere with the filtration process during perioperative use, including preexistent coagulopathy, drug-induced platelet dysfunction, cardiopulmonary bypass, suction-induced hemolysis, or systemic inflammatory response. Second, given the nature of posttreated blood cell concentrate, composed only of blood cells and traces of proteins suspended in normal saline, functional analysis of platelets using aggregometry, although considered as the reference test, was impossible. We therefore used thrombin receptor stimulation in combination with flow cytometric analysis as a surrogate for the platelet function test.

This study reports the performance evaluation of a filtration-based autotransfusion device, able to simultaneously recover and wash human platelets and red blood cells. It also provides a detailed cytometric analysis of salvaged platelet and leukocyte viability and activation state. With a fast processing time of less than 5 min, the device was able to recover 88% of red blood cells with minimal hemolysis and without inducing alteration in membrane integrity and deformability. The device achieved 37% of platelet recovery with minimal platelet activation while maintaining platelet ability to be activated by thrombin receptor–activating peptide. The washing process allowed high heparin and plasma protein removal ratios. Together, these results demonstrate the *in vitro* performance of i-SEP new autotransfusion technology. Future trials will be necessary to assess the clinical efficacy and safety of the device.

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Competing Interests

Dr. Decouture is currently employed as project manager by i-SEP (Nantes, France). Dr. Skreko is currently employed as research and development engineer by i-SEP. Dr. Ouattara received expertise fees from i-SEP. Dr. Bachelot-Loza received expertise fees from i-SEP. Dr. Gaussem received expertise fees from Aspen France (Rueil Malmaison, France) and i-SEP. The other authors declare no competing interests.

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Impact de la transfusion allogénique de plaquettes sur les infections nosocomiales après une chirurgie cardiaque

La chirurgie cardiaque sous circulation extracorporelle est associée à un haut risque hémorragique se traduisant par une forte exposition périopératoire à la transfusion allogénique. Du fait du rôle central des plaquettes dans l'hémostase, la transfusion allogénique de plaquettes est recommandée dans la prise en charge périopératoire des saignements actifs associés à une thrombopénie <100G/L ou à une thrombopathie, notamment médicamenteuse, et concerne ainsi 8 à 39% des patients opérés d'une chirurgie cardiaque[116].

La transfusion allogénique de plaquettes est cependant associée à des risques d'alloimmunisation et de contamination bactérienne, rare, mais également à des complications inflammatoires et dysimmunitaires incluant en particulier les réactions fébriles non-hémolytiques et surtout l'œdème pulmonaire lésionnel post-transfusionnel ou transfusion-related acute lung injury (TRALI)[117]. Ces complications, dont la fréquence a été réduite par la déleucocytation des concentrés plaquettaires et les techniques d'inactivation des agents pathogènes, persistent cependant et peuvent impacter le devenir des patients en réanimation.

Le prélèvement et le stockage des concentrés plaquettaires est en effet associé à des modifications structurelles et phénotypiques des plaquettes associées à leur activation, mais également à l'accumulation de médiateurs inflammatoires solubles, en particulier sCD40L, PF4, RANTES, IL-6, HMGB1, et de vésicules extracellulaires plaquettaires[118,119]. Ces médiateurs, sont capables comme on l'a vu précédemment, d'induire des modifications de la réponse inflammatoire et immunitaire. Ainsi, l'implication de la voie de signalisation (s)CD40L/CD40 dans la physiopathologie du TRALI et des événements indésirables graves post-transfusionnels a été récemment démontrée [120,121].

L'effet de ces médiateurs semble particulièrement important dans des situations de pré-activation inflammatoire (two-hit model). C'est le cas, comme nous l'avons décrit précédemment des situations periopératoires et de soins critiques associées aux circulations extracorporelles (chirurgie cardiaque, assistances circulatoires) et au sepsis.

L'activation plaquettaire et les médiateurs associés au stockage des concentrés plaquettaires pourraient également induire une immunomodulation (transfusion-related immunomodulation ou TRIM) et participer à la survenue des complications infectieuses post-opératoires ou en soins critiques. Les données de la littérature en chirurgie cardiaque et en réanimation, uniquement observationnelle, sont discordantes quant à l'impact de la transfusion plaquettaire sur l'augmentation du risque d'infections associées aux soins[122]. Cette discordance, possiblement liée au caractère déleucocyté ou non des concentrés plaquettaires étudiés, pourrait également être expliquée par les limitations méthodologiques de ces études. En effet, les situations cliniques associées à la transfusion plaquettaire en soins critiques sont complexes et nécessitent des approches méthodologiques et statistiques avancées afin de prendre en compte au maximum les biais de confusion.

Nous avons formulé l'hypothèse que la transfusion de concentrés plaquettaires déleucocytés en per-opératoire de chirurgie cardiaque pourrait être associée à la survenue des infections post-opératoires liées aux soins. Pour répondre à cette question, nous avons associé à notre base de données de chirurgie cardiaque, les données issues du laboratoire de bactériologie. Ensuite, nous avons employé des méthodes d'inférence causale et d'ajustement statistique afin de réduire au maximum les biais de confusion. En particulier, l'utilisation de l'overlap-weighting nous a permis de contrôler l'impact des scores de propension extrêmes sur les associations évaluées[123]. Ces résultats sont présentés ici sous la forme d'un article qui sera soumis à courte échéance. Ils démontrent une association, jusqu'ici inédite, entre la transfusion plaquettaire per-opératoire et la survenue de bactériémies en post-opératoire de chirurgie cardiaque sous CEC.

Ces résultats renforcent l'hypothèse d'un modèle à double impact (two-hit model), associant inflammation liée à la chirurgie et à la CEC et modulation inflammatoire et immune liée à la transfusion de concentrés plaquettaires. Ces résultats ouvrent des perspectives de recherche clinique et translationnelle qui seront abordées dans la Discussion Générale de ce travail.

Impact of intraoperative allogeneic platelet transfusion on healthcare-associated infections in cardiac surgery: insights from a large single-center cohort study

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Impact of platelet transfusion on infection after cardiac surgery

Summary

Despite significant improvement in patient blood management-cardiac surgery remains a high hemorrhagic risk procedure. Platelet transfusion is commonly used to treat thrombocytopenia-induced perioperative bleeding. Allogeneic platelet transfusion may induce transfusion-related immunomodulation. However, its association with postoperative healthcare-associated infections is still a matter of debate. Our objective was to evaluate the impact of allogeneic platelet transfusion during cardiac surgery on postoperative HAI incidence. We conducted a retrospective cohort study in a tertiary referral center including all patients undergoing cardiac surgery from 2012 to 2018. Intraoperative platelet transfusion was defined as exposure in a causal model. The primary outcome was the incidence of healthcare-associated infections-comprised of bloodstream infection-hospital-acquired pneumonia and surgical-site infection. Among 7662 included patients, 528 (6.8%) were exposed to intraoperative platelet transfusion and 329 (4.3%) developed 454 postoperative infections. Bloodstream infection affected 106 (1.4%) patients, hospital-acquired pneumonia 174 (2.3%) and surgical-site infection 148 (1.9%). Intraoperative platelet transfusion was associated with an increased risk of bloodstream infection after adjustment by multivariable logistic regression (OR 2.85; 95%CI (1.40-5.8); p=0.004; n=7662), propensity score matching (OR 3.95; 95%CI (1.57-12.0), p=0.007; n=766) and propensity score overlap weighting (OR 3.04; 95%CI (1.51-6.1), p=0.002; n=7762). Surgical-site infection and hospital-acquired pneumonia were not significantly increased by platelet transfusion. Our results suggest that intraoperative allogeneic platelet transfusion is a risk factor for bloodstream infection after cardiac surgery. These results support the development of patient blood management strategies aiming at minimizing perioperative platelet transfusion in cardiac surgery.

Introduction

Patients undergoing cardiac surgery are at high risk of excessive bleeding, related to the invasiveness of the procedures, the need for high-dose anticoagulation and the exposure to cardiopulmonary bypass (CPB)[1]. Thrombocytopenia and platelet function disorders are known conditions associated with perioperative bleeding in cardiac surgery[2–4]. The mechanisms involved include dilution, platelet activation or consumption, as well as frequent use of antiplatelet agents in cardiac surgery patients[5]. Since platelets are a critical component of coagulation and fibrin clot formation, platelet transfusion is commonly used to treat thrombocytopenia or platelet dysfunction-related bleeding[1,6,7]. However, platelet transfusion has been found associated with an increase in healthcare-associated infections (HAI) after cardiac surgery or in critically-ill patients[8–12], for which transfusion-induced immunomodulation is thought to be a major pathophysiological mechanism. Indeed, platelets are now recognized as a critical player in both innate and acquired immune response[13–15]. Further, allogeneic platelet storage has been associated with platelet activation and accumulation of platelet extracellular vesicles and soluble factors, such as soluble P-selectin, soluble CD40-Ligand (sCD40L) and platelet factor 4 (PF4), which may alter the immune response after transfusion[15,16]. Indeed, sCD40L seems to play a critical role in the pathogenesis of platelet transfusion-induced TRALI (transfusion-related acute lung injury)[17,18]. In addition to allogeneic platelet-mediated effects, leukocyte content of non-leukoreduced platelet concentrates may contribute to the platelet transfusion-induced immunomodulation[16]. Leukocyte reduction, which is now required for platelet concentrates preparation in France, might attenuate platelet transfusion-induced immunomodulation [16]. Indeed, a recent meta-analysis of six studies mainly reporting transfusion of leukoreduced platelet concentrates did not find any association between platelet transfusion and postoperative infections after cardiac surgery [19]. At the opposite in medical-surgical critically ill patients (including cardiovascular patients) an association with HAI and platelet transfusion has been observed despite the use of leukoreduced blood products[8]. Unfortunately, these studies were not exclusively focused on postoperative infections

and suffered from several limitations, including heterogeneous definitions of postoperative infection, lack of subgroup analysis by type of infection and statistical methodology issues [9,20–24].

Therefore, the main objective of our study was to determine whether intraoperative platelet transfusion with leukoreduced concentrates was associated with postoperative healthcare-associated infections in a large cohort of cardiac surgery patients. We hypothesized that immunomodulation properties of platelets themselves could alter the immune response promoting healthcare-associated infections.

Materials and Methods

Study design

This study was performed using data prospectively collected for all consecutive patients undergoing cardiac surgery in our center as part of an ongoing quality improvement program. Data were collected by research assistants and followed from the day of surgery to death or discharge from hospital and up to 1-year for survival status. Data included patient characteristics and comorbidities, surgery and anaesthesia management, post-operative ICU management, complications and outcomes. For the present study, we analyzed all consecutive adult patients from January, 1st 2012 up to December, 31th 2018, with a minimum hospital length of stay of two days and for whom data regarding healthcare-associated infections were available. This study has been approved by Rennes University Hospital ethics committee. According to the French legislation, written consent was waived because of the observational design of the study. The analysis followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (Supplementary Table 1).

Patient management

Patients were managed in the cardiothoracic department of a 1500-bed tertiary university hospital, comprising both cardiothoracic surgery and intensive-care units. Hospital hygiene standard protocols followed the recommendations of the French Society for Hospital Hygiene regarding the prevention and treatment of HAI. Further, all patients were decolonized using intranasal mupirocin five days before surgery and received parenteral 2nd generation cephalosporin during surgery as antimicrobial prophylaxis.

Preoperative antiplatelet therapy using P2Y12-inhibitors was discontinued prior to elective and semi-urgent surgeries (5 days for clopidogrel and ticagrelor, 7 days for prasugrel) while preoperative aspirin was continued throughout the perioperative period. Intraoperative allogeneic platelet transfusion was indicated in case of significant bleeding associated with thrombocytopenia or known platelet function

disorder. No point-of-care platelet function testing was available. All platelet concentrates were prepared using pooled whole blood derived buffy-coat or apheresis, with leucocyte reduction.

Outcomes and variables

Our primary outcome was the incidence of HAI. Based on previous publications reporting that the majority of HAI were due to hospital-acquired bloodstream infections (BSI), hospital-acquired pneumonia (HAP) and surgical-site infection (SSI), and because other HAI (e.g., urinary tract infections) have not been associated with an increased mortality, only BSI, HAP, and SSI were included in this study. Secondary outcomes were the incidence of BSI, HAP and SSI and microorganisms' distribution associated with HAI. According to Centers for Disease Control and Prevention (CDC) guidelines, HAI were defined as postoperative infections diagnosed more than 48 hours after admission and who were not incubating on admission. BSI definition required at least one positive blood culture, except for common skin contaminants (coagulase-negative Staphylococcus species, Corynebacterium species, Propionibacterium acnes, and Micrococcusspecies) for whom two distinct positive blood cultures were needed. HAP definition included both ventilated and nonventilated patients and was based on clinical signs (fever), radiographic findings (new infiltrate on chest x-ray or computed tomography scan), leukocytosis and a positive microbiological culture of an endotracheal aspirate ($\geq 10^6$ colony-forming units/mL) or a bronchoalveolar lavage ($\geq 10^4$ colony-forming units/mL). Finally, SSI included only thoracic wound infections and were defined by fever, clinical or radiological evidence of wound infection and a positive microbiological culture of surgically retrieved deep soft tissues. Diagnosis of HAI was made by the treating physician and independently confirmed by two ICU physicians who reviewed relevant imaging, microbiologic data, and medical records.

In addition, the following variables were included in the present study: patient characteristics and comorbidities (age, sex, body mass index, diabetes, stroke, chronic kidney failure, chronic respiratory failure, cirrhosis), pre-surgery characteristics (EuroSCORE predicted mortality, hemoglobin level, antiplatelet agent use, shock, circulatory support), surgery and anaesthesia management variables

(year of surgery, type of surgery, endocarditis, emergency surgery, circulatory arrest, length of cardiopulmonary bypass, red blood cells, platelets and fresh frozen plasma transfusion), post-operative ICU management complications and outcomes (post-operative circulatory support, dobutamine and norepinephrine use, reoperation for bleeding, ventilation duration > 48 hours, post-operative stroke, HAI, BSI, HAP, SSI, in-hospital, 90 days and 1 year survival).

Statistical Analysis

Patient characteristics are expressed as number (proportion) for categorical variables and median (IQR [range]) for continuous variables. For comparison between platelet-transfused and non-platelet-transfused patients, a $\chi 2$ test or a Fisher's exact test were used for categorical variables and a Wilcoxon's rank sum test for continuous variables. A statistical analysis plan was made prior to accessing the data. No a priori statistical power calculation was conducted. Only pre-surgery variables, anesthesia-related and surgery-related variables were included in the following multivariable analyses to prevent competing risk bias. All analyses were conducted on complete cases.

In addition to univariable logistic regression between platelet transfusion and healthcare-associated infections, we used a multivariable logistic regression model as main statistical analysis. A directed acyclic graph was used to describe our model of causal associations between intra-operative platelet transfusion (exposure variable), patient-related confounders, anesthesia and surgery-related confounders, and healthcare-associated infections, using DAGitty software (Supplementary Figure 1) [25]. No variables were analyzed as effect modifiers. The set of potential confounders sufficient for adjustment was: age, sex, body mass index, diabetes, cirrhosis, chronic kidney failure, preoperative hemoglobin, preoperative antiplatelet agent use, preoperative shock, preoperative circulatory support, year of surgery, type of surgery, emergency surgery, circulatory arrest, length of cardiopulmonary bypass, intraoperative red blood cells transfusion, intraoperative platelet concentrate transfusion and intraoperative fresh frozen plasma transfusion. A multivariable logistic regression model was then used to estimate odd ratios between intra-operative platelet transfusion (exposure variable) and HAI, BSI,

HAP and SSI. Absence of multicollinearity and linearity of continuous independent variables and log-odds was checked. Confounders entered in the model were defined a priori using the directed acyclic graph. Treatment effect was expressed as odd ratio (OR) with corresponding 95% confidence interval.

Two additional propensity-based models were developed as sensitivity analyses, using matching and overlap weighting. Propensity score was estimated using a non-parsimonious multivariable logistic regression including all available pre-surgery, anesthesia-related and surgery-related variables. Matching was performed without replacement using 1:1 nearest neighbor matching. Overlap weighting was performed by using the average treatment effect for the overlap population (ATO) as target estimand. Standardized mean difference (SMD) was used to assess covariate balance quality after both matching and weighting and was reported using Love plots. Residual imbalance was defined as a SMD value higher than 0.10. Treatment effect after matching was estimated using univariable logistic regression. Treatment effect after overlap weighting was estimated using univariable weighted logistic regression with robust variance estimator. All tests used two-tailed hypothesis. Statistical significance was achieved for $P < 0.05$. Statistical analyses were performed using R 4.1.1 statistical software, including packages WeightIt and MatchIt.

Results

Study population

Among the 8969 patients undergoing cardiac surgery between January, 1st 2012 up and December, 31th 2018, 48 were younger than 18 years of age, 40 had missing data on post-operative healthcare-associated infections and 76 had hospital length of stay less than two days, leaving 8805 patients included in the present study (Figure 1). Complete case analyses were performed on 7662 patients. Characteristics and distribution of missing data in the 8805 included patients are reported in Supplementary Table 2.

Among the complete case population, median age was 69 (61-77 [18-90]) years, 73% were males and had a median body mass index of 26.3 (23.7-29.3 [14.4-49.1]) $kg.m^{-2}$ (Table 1). Thirty-eight percent of patients were exposed to antiplatelet agent before surgery. Predicted mortality according to EuroSCORE was 1.2 (0.7-2.7 [0.5-61.3]) %. Surgeries were mainly elective (87%) with a median cardiopulmonary bypass time of 77 (58-108 [20-468]) minutes. Twenty percent of patients received intraoperative red blood cells transfusion while 9.6% received intraoperative fresh frozen plasma transfusion. Survival was 98% at hospital discharge and 96% at one-year.

Intraoperative platelet transfusion

Intraoperative platelet concentrate transfusion was performed in 528 patients (6.8%). In unadjusted comparison, patient comorbidities and surgery and anesthesia management significantly differed according to intraoperative platelet transfusion status (Table 1). Patients receiving intraoperative platelet transfusion were more likely to undergo emergency surgery (32% vs 12%, p<0.001) and complex surgery with higher EuroSCORE predicted mortality (1.8% vs 1.1%, p<0.001), preoperative circulatory support (1.9% vs 0.3%, p<0.001) and endocarditis (5.5% vs 3.3%, p=0.009), and more frequently required deep hypothermic circulatory arrest (27% vs 2.8%, p<0.001) and longer cardiopulmonary bypass time (151 min vs 74 min, p<0.001). They also more frequently received concurrent intraoperative RBC (55% vs 17%, p<0.001) and FFP (79% vs 4.4%, p<0.001) transfusions.

Likewise, in unadjusted comparison post-operative outcomes significantly differed according to intraoperative platelet transfusion status (Table 2). Platelet-transfused patients were more likely to receive postoperative circulatory support (25% vs 4%, p<0.001), dobutamine (41% vs 18%, p<0.001) and norepinephrine (45% vs 34%, p<0.001) and to suffer worse outcomes, including stroke (3.2% vs 1%, p<0.001), reoperation for bleeding (6.2% vs 1.5%, p<0.001), prolonged mechanical ventilation (6.2% vs 1.5%, p<0.001), HAI (12% vs 3.7%, p<0.001) and one-year survival (87% vs 97%, p<0.001).

Healthcare-associated infections

Of 7662 patients included in the analysis, 329 (4.3%) developed 454 healthcare-associated infections (HAI): 106 (1.4%) had at least one BSI (122 events), 174 (2.3%) at least one HAP (184 events) and 148 (1.9%) had SSI (148 events) (Table 2). Among confounders included in multivariable logistic regression, factors independently associated with the occurrence of overall HAI were body mass index (by each $kg.m^{-2}$, OR(95%CI) 1.05(1.02-1.07), p=0.001), diabetes (1.40(1.03-1.88), p=0.028), chronic kidney failure (1.87(1.22-2.80), p=0.003), preoperative circulatory support (12.3(5.2-30.8), p<0.001), type of surgery (coronary artery bypass grafting (1.59(1.15-2.20), p=0.005); combined surgery (1.65(1.14-2.36), p=0.007); other surgery (2.52(1.70-3.70), p<0.001)), emergency surgery (1.67(1.23-2.26), p=0.001) and length of cardiopulmonary bypass (by each min, 1.01(1.00-1.01), p<0.001). Year 2017 (0.48(0.295-0.77), p=0.003) and 2018 (0.50(0.308-0.80), p=0.004) were independently associated with a decrease in overall HAI incidence (Supplementary Table 3).

Impact of intraoperative platelet transfusion on healthcare-associated infections

On univariable analysis, platelet transfusion was associated with higher incidence of HAI (12% vs 3.7%, p<0.001), BSI (5.5% vs 1.1%, p<0.001) and HAP (9.1% vs 1.8%, p<0.001), but not SSI (2.5% vs 1.9%, p=0.359) (Table 2). Intraoperative platelet transfusion was not independently associated with the incidence of overall HAI, in main multivariable logistic regression (1.36(0.87-2.13), p=0.176) and in propensity-based sensitivity analyses, either using matching (1.30(0.81-2.10), p=0.280) or overlap

weighting (1.37(0.90-2.08), p=0.139) (Table 3).

In contrast to HAP and SSI, intraoperative platelet transfusion was consistently found independently associated with the occurrence of BSI, in main multivariable logistic regression (2.85(1.40-5.8), p=0.004) and in propensity-based sensitivity analyses, either using matching (3.95(1.57-12.0), p=0.007) or overlap weighting (3.04(1.51-6.1), p=0.002) (Table 3). Complete logistic regression models for HAI, BSI, SSI and HAP are reported in Supplementary Materials (Supplementary Tables 3 to 6). Covariate balance and treatment effect for propensity-based analyses are reported in Supplementary Materials (Supplementary Tables 7 and 8, and Supplementary Figures 2 and 3).

Median delay between surgery and BSI occurrence did not differ when associated with platelet transfusion (6 (4-20 [1-94]) days vs 6 (5-14 [0-58]) days, p=0.691) (Table 4). Likewise, microorganisms' distribution remained unaffected by platelet transfusion, largely driven by staphylococci (coagulase-negative and aureus), enterobacteriacae and pseudomonas aeruginosa (Table 4).

Discussion

We report the impact of intraoperative platelet transfusion on postoperative healthcare-associated infections in a large cohort of cardiac surgery patients, using a robust statistical approach. The main results of our study are the following: 1) The proportion of intraoperative platelet transfusion was 6.8%; 2) Intraoperative platelet transfusion was not found independently associated with postoperative HAP or SSI; 3) Intraoperative platelet transfusion was independently associated with postoperative BSI.

Despite existing guidelines, allogeneic platelet transfusion practices vary considerably in cardiac surgery [12,26]. Indeed, in this setting the incidence of total perioperative platelet transfusion ranges between 8% and 39%, with intraoperative platelet concentrate use between 7.6% and 13.3% [9,10,12,20–24,26–28]. In our study, intraoperative platelet transfusion was performed in 6.8% of patients, which was lower than most published data. Likewise, incidence of intraoperative RBC and FFP transfusion were in line with previously published studies [27–29]. Healthcare-associated infections, especially bloodstream infections, pneumonia and surgical site infection, are associated with both morbidity and mortality after cardiac surgery [30–33]. In our study, both incidence and distribution of healthcare-associated infections were in line with previous studies in cardiac surgery[30,31,33].

While the impact of red blood cell transfusion on healthcare-associated infections has been consistently demonstrated, including in cardiac surgery [34,35], conflicting results have been reported regarding the impact of allogeneic platelet transfusion on postoperative HAI after cardiac surgery[9,10,12,20–24]. However, as clearly highlighted in a recent meta-analysis, these studies suffer from serious limitations [19]. Indeed, many studies reported the association with all HAI, without specific analysis for separate infection types, including BSI, HAP and SSI. Further, most of them did not exclusively focus on postoperative HAI, but rather on overall postoperative outcomes. This frequently resulted in heterogeneous definition and thus inaccurate identification of HAI and might also have led to sub-optimal statistical modelling. Indeed, the decision to transfuse platelet concentrates in cardiac surgery, based on clinical judgment, is affected by patient's comorbidities and treatments, surgical

complexity, center practices and adherence to patient blood management[12,19,26]. Platelet transfusion is also associated with the decision to transfuse RBC and FFP [26]. As a result, large differences can be observed between transfused and non-transfused patients in terms of comorbidities, surgical risk, anesthesia and transfusion management, that were also demonstrated in our cohort[19,21]. Hence, platelet transfusion shares common risk factors with healthcare-associated infections in cardiac surgery[31]. In this setting, there is high risk of bias due to uncontrolled confounding, that might partly explain the discrepancy between previously published studies investigating the impact of platelet transfusion on HAI in cardiac surgery. A robust statistical methodology is therefore required to minimize confounding bias[36–40]. To that end, we performed a statistical analysis using 1) a multivariable logistic regression using a causality-based variable selection, 2) a non-parsimonious propensity score matching and 3) a propensity score overlap weighting to help controlling the impact of extreme propensity scores.

Our study demonstrated that, unlike overall HAI, SSI and HAP, intraoperative platelet transfusion was consistently found independently associated with the occurrence of BSI, in multivariable logistic regression and in propensity-based analyses, either using matching or overlap weighting. To our knowledge, this is the largest available study investigating the specific association between allogeneic platelet transfusion and BSI after cardiac surgery. The results are in line with recently published data in medical-surgical critically ill patients [8]. Direct bacterial contamination of platelet concentrates appears very unlikely responsible for these results, given its low reported incidence and the median delay between surgery and BSI exceeding 24 hours[41–43]. Instead, platelet transfusion may be responsible for transfusion-induced immunomodulation, promoting the occurrence of BSI. First, platelet transfusion might elicit TRIM through their MHC antigen expression[44]. Second, the preparation and storage of platelet concentrates has been associated with the accumulation of biological response modifiers, including cytokines, platelet receptors, growth factors and microparticles[15,16]. Similarly, washing of platelet concentrates might be associated with reduced adverse reactions to platelet transfusion[45,46]. In particular, platelet concentrate storage has been associated with elevated levels of soluble CD40L

(sCD40L) which might play a role in transfusion-related acute lung injury pathogenesis[17,18,47]. The CD40L/CD40 interaction is associated with numbers of proinflammatory pathways involving, platelets, leukocytes and endothelium[48]. Further, it has been associated with endothelial dysfunction and increased vascular permeability [49]. Together, these mechanisms might participate in the occurrence of bloodstream infection after cardiac surgery in a two hit-model of inflammation initiated by surgery and cardiopulmonary bypass[50].

Compared with previously published observational data, our study has the following strengths. First, the use of a prospectively maintained database associated with microbiological data, clear HAI definitions and independent confirmation of suspected HAI helped reducing potential information bias. Indeed, both incidence and distribution of HAI were in line with previous studies in cardiac surgery. Second, both allogeneic transfusion practices and clinical outcomes were in line with current data and guidelines in perioperative management of cardiac surgery patient, thus ensuring generalizability of our findings. Third, the use of both causality-based multivariable regression and propensity score matching and overlap weighting in this large cohort ensured a better control of potential confounders. Similarly, the consistent results with these different statistical models strengthens the confidence in our results.

This study has several limitations. First, the observational design of the study precludes any definitive conclusion regarding causality. Second, despite the use of a robust statistical approach, residual confounding by unmeasured factors cannot be excluded. In particular, our database lacks information regarding preoperative platelet count and at the time of platelet transfusion decision, and postoperative blood product transfusion. Third, as an observational study relying on patients' medical records, this study might be subject to information bias. Fourth, although HAI diagnosis was independently confirmed by two ICU physicians, the primary diagnosis was made by the treating physician. This might constitute a limitation, especially for the diagnosis of HAP, which was made using

a set of clinical criteria rather than for SSI and BSI for which diagnosis relied on documented culture data. Finally, as all platelet concentrates transfused were leukoreduced, our results cannot be generalized to non-leukoreduced platelet transfusion.

Conclusion

In a large single-center observational study, intraoperative allogeneic platelet transfusion was associated with a significant increase in postoperative bloodstream infection after cardiac surgery. Surgical-site infection and hospital-acquired pneumonia were not significantly increased by platelet transfusion. These results support the development of patient blood management strategies aiming at minimizing perioperative platelet transfusion in cardiac surgery. Future translational research should aim at understanding and regulate the impact of platelet activation on perioperative inflammatory response.

Competing Interests

No external funding

AM - received payments made to his institution from i-SEP for consulting fees-and LFB for lecture fees.

TS - no competing interests declared.

NM - no competing interests declared.

IGT - no competing interests declared.

AA - no competing interests declared.

AP - no competing interests declared.

CP - no competing interests declared.

EF - no competing interests declared.

NN - no competing interests declared.

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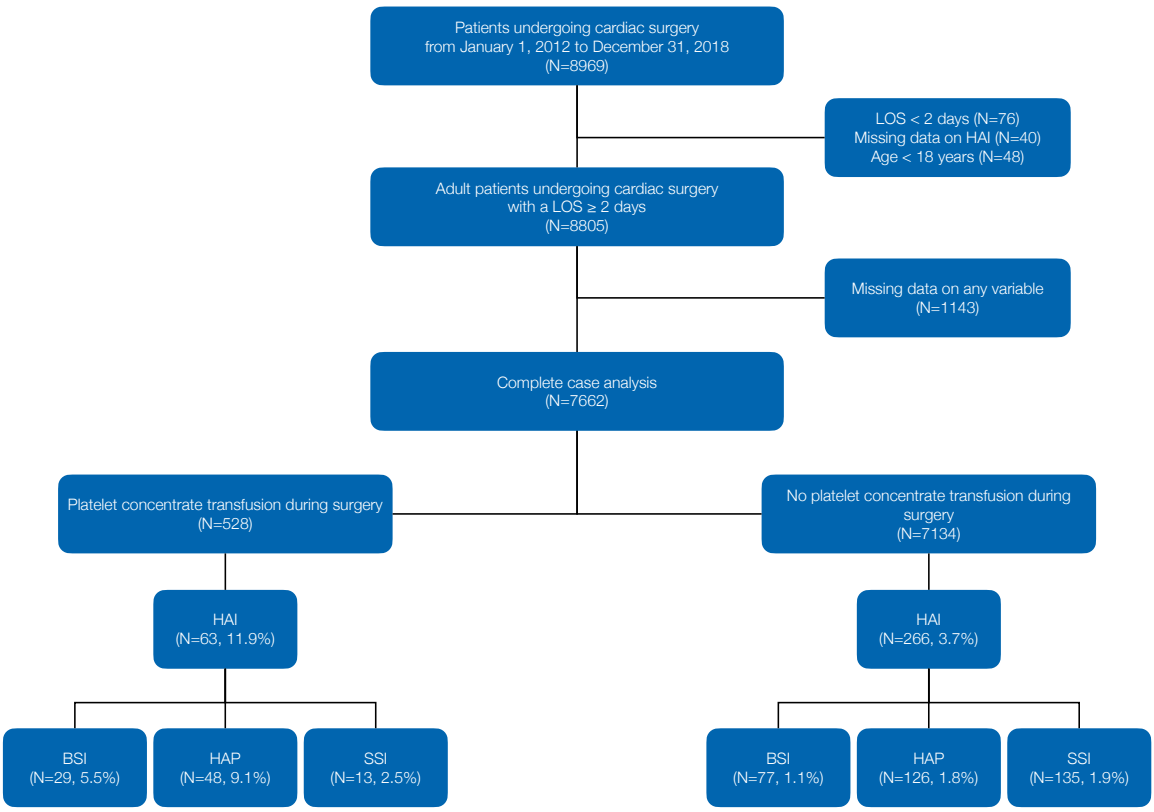
Figure 1 : Flowchart



LOS : Length of Stay; HAI Healthcare-Associated Infections; BSI Bloodstream infection; HAP Hospital-acquired pneumonia; SSI Surgical site infection

Table 1: Patient comorbidities, surgery and anesthesia management

Characteristic	All patients (N = 7662)	No platelet transfusion (N = 7134)	Platelet transfusion (N = 528)	p-value
Age — years	69 (61-77 [18-90])	70 (62-77 [18-90])	66 (57-72 [20-86])	<0.001
Male	5620 (73%)	5226 (73%)	394 (75%)	0.493
BMI — $kg.m^{-2}$	26.3 (23.7-29.3 [14.4-49.1])	26.3 (23.7-29.4 [14.4-49.1])	25.7 (22.8-28.7 [15.4-41.2])	<0.001
Comorbidities				
Diabetes	1194 (16%)	1143 (16%)	51 (9.7%)	<0.001
Stroke	168 (2.2%)	157 (2.2%)	11 (2.1%)	0.859
Chronic kidney failure	339 (4.4%)	302 (4.2%)	37 (7.0%)	0.003
Chronic respiratory failure	549 (7.2%)	518 (7.3%)	31 (5.9%)	0.232
Cirrhosis	44 (0.6%)	34 (0.5%)	10 (1.9%)	<0.001
Pre-surgery				
EuroSCORE predicted mortality— %	1.2 (0.7-2.7 [0.5-61.3])	1.1 (0.7-2.5 [0.5-61.3])	1.8 (0.9-4.2 [0.5-36.0])	<0.001
Hemoglobin level — g/dL	13.7 (12.7-14.7 [6.9-19.9])	13.7 (12.7-14.7 [6.9-19.9])	13.4 (11.8-14.4 [7.4-19.1])	<0.001
Antiplatelet agent	2887 (38%)	2741 (38%)	146 (28%)	<0.001
Shock	25 (0.3%)	17 (0.2%)	8 (1.5%)	<0.001
Circulatory support	29 (0.4%)	19 (0.3%)	10 (1.9%)	<0.001
Surgery				
Year of surgery				<0.001
2012	746 (9.7%)	709 (9.9%)	37 (7.0%)	
2013	1163 (15%)	1074 (15%)	89 (17%)	
2014	1184 (15%)	1068 (15%)	116 (22%)	
2015	1178 (15%)	1098 (15%)	80 (15%)	
2016	1153 (15%)	1067 (15%)	86 (16%)	
2017	1142 (15%)	1061 (15%)	81 (15%)	
2018	1096 (14%)	1057 (15%)	39 (7.4%)	
Type of Surgery				<0.001
Isolated valve surgery	4210 (55%)	3947 (55%)	263 (50%)	
CABG	2028 (26%)	1982 (28%)	46 (8.7%)	
Combined surgery	859 (11%)	780 (11%)	79 (15%)	
Other	565 (7.4%)	425 (6.0%)	140 (27%)	
Surgery for endocarditis	266 (3.5%)	237 (3.3%)	29 (5.5%)	0.009
Emergency surgery	1023 (13%)	853 (12%)	170 (32%)	<0.001
Deep hypothermic circulatory arrest	342 (4.5%)	197 (2.8%)	145 (27%)	<0.001
Length of CPB — min	77 (58-108 [20-468])	74 (57-101 [20-410])	151 (118-187 [45-468])	<0.001
RBC transfusion	1540 (20%)	1248 (17%)	292 (55%)	<0.001
FFP transfusion	732 (9.6%)	313 (4.4%)	419 (79%)	<0.001

Data are expressed as number (proportion) or median (IQR [range]).
BMI: body mass index; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma

Table 2: ICU management and postoperative outcomes

Characteristic	All patients (N = 7662)	No platelet transfusion (N = 7134)	Platelet transfusion (N = 528)	p-value
Circulatory support	420 (5.5%)	287 (4.0%)	133 (25%)	<0.001
Dobutamine use	1522 (20%)	1304 (18%)	218 (41%)	<0.001
Norepinephrine use	2642 (34%)	2402 (34%)	240 (45%)	<0.001
Reoperation for bleeding	377 (4.9%)	324 (4.5%)	53 (10%)	<0.001
Ventilation duration >48h	142 (1.9%)	109 (1.5%)	33 (6.2%)	<0.001
Stroke	85 (1.1%)	68 (1.0%)	17 (3.2%)	<0.001
HAI	329 (4.3%)	266 (3.7%)	63 (12%)	<0.001
BSI	106 (1.4%)	77 (1.1%)	29 (5.5%)	<0.001
HAP	174 (2.3%)	126 (1.8%)	48 (9.1%)	<0.001
SSI	148 (1.9%)	135 (1.9%)	13 (2.5%)	0.359
In-hospital survival	7484 (98%)	7002 (98%)	482 (91%)	<0.001
Survival at 90 days	7431 (97%)	6957 (98%)	474 (90%)	<0.001
Survival at 1 year	7384 (96%)	6925 (97%)	459 (87%)	<0.001

Data are expressed as number (proportion)
HAI: Healthcare-Associated Infections; BSI: Bloodstream infection; HAP: Hospital-acquired pneumonia; SSI: Surgical site infection

Table 3: Impact of intraoperative platelet transfusion on postoperative healthcare-associated infections

Outcomes	Unadjusted logistic regression N=7662			Multivariable logistic regression N=7662			PS matching N=766			PSOW analysis N=7662		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
HAI	**3.50**	**2.60-4.6**	**<0.001**	1.36	0.87-2.13	0.176	1.30	0.81-2.10	0.280	1.37	0.90-2.08	0.139
BSI	**5.3**	**3.33-8.1**	**<0.001**	**2.85**	**1.40-5.8**	**0.004**	**3.95**	**1.57-12.0**	**0.007**	**3.04**	**1.51-6.1**	**0.002**
HAP	**5.4**	**3.69-7.9**	**<0.001**	1.24	0.73-2.10	0.426	1.15	0.68-1.95	0.596	1.37	0.85-2.21	0.199
SSI	1.31	0.70-2.24	0.360	0.72	0.319-1.56	0.416	0.66	0.256-1.61	0.368	0.68	0.317-1.47	0.328

Data are expressed odd ratio (OR) with 95% confidence interval (95% CI).
PSOW: propensity score overlap weighting; HAI: Healthcare-Associated Infections; BSI: Bloodstream infection; HAP: Hospital-acquired pneumonia; SSI: Surgical site infection

Table 4: Incidence and comparison of microorganisms distribution during BSI.

	All BSI events (122 events)	No platelet transfusion (88 events)	Platelet transfusion (34 events)	p-value
Delay between surgery and BSI - days	6 (5-14 [0-94])	6 (5-14 [0-58])	6 (4-20 [1-94])	0.691
Microorganisms				0.918
Coagulase-negative *Staphylococci*	34 (25%)	26 (26%)	8 (22%)	
Staphylococcus aureus	34 (25%)	25 (25%)	9 (24%)	
Enterobacteriacae	24 (18%)	18 (18%)	6 (16%)	
Pseudomonas aeruginosa	20 (15%)	13 (13%)	7 (19%)	
Enterococcus sp.	9 (6.6%)	6 (6.1%)	3 (8.1%)	
Streptococcus sp.	7 (5.1%)	4 (4.0%)	3 (8.1%)	
Anaerobes	6 (4.4%)	5 (5.1%)	1 (2.7%)	
Candida sp.	2 (1.5%)	2 (2.0%)	0 (0%)	

Data are expressed as number (proportion) or median (IQR [range]).
BSI: Bloodstream infection

Supplementary Table 1: Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines

	Recommendation	Page
Title and abstract	(a) Indicate the study's design with a commonly used term in the title or the abstract	
	(b) Provide in the abstract an informative and balanced summary of what was done and what was found	
Background/rationale	Explain the scientific background and rationale for the investigation being reported	
Objectives	State specific objectives, including any prespecified hypotheses	
Study design	Present key elements of study design early in the paper	
Setting	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	
Participants	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	
	(b) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources measurement	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	
Bias	Describe any efforts to address potential sources of bias	
Study size	Explain how the study size was arrived at	
Quantitative variables	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	(a) Describe all statistical methods, including those used to control for confounding	
	(b) Describe any methods used to examine subgroups and interactions	
	(c) Explain how missing data were addressed	
	(d) If applicable, explain how loss to follow-up was addressed	
	(e) Describe any sensitivity analyses	
Participants	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
	(b) Give reasons for non-participation at each stage	
	(c) Consider use of a flow diagram	
Descriptive data	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
	(b) Indicate number of participants with missing data for each variable of interest	
	(c) Summarise follow-up time (eg, average and total amount)	
Outcome data	Report numbers of outcome events or summary measures over time	
Main results	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95 confidence interval). Make clear which confounders were adjusted for and why they were included	
	(b) Report category boundaries when continuous variables were categorized	
	(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Key results	Summarise key results with reference to study objectives	
Limitations	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	
Interpretation	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	Discuss the generalisability (external validity) of the study results	
Funding	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	

Supplementary Table 2: Patient characteristics and distribution of missing data in the 8805 included patients

Characteristic	N	All patients (N = 8805)	No platelet transfusion (N = 8260)	Platelet transfusion (N = 545)	p-value
Age — years	8805	70 (62-77 [18-94])	70 (62-77 [18-94])	65 (56-72 [18-86])	<0.001
Male	8805	6466 (73%)	6064 (73%)	402 (74%)	0.859
BMI — kg/m^2	8797	26.3 (23.7-29.4 [14.4-51.9])	26.3 (23.8-29.4 [14.4-51.9])	25.7 (22.9-28.7 [15.4-41.2])	<0.001
Comorbidities					
Diabetes	8805	1411 (16%)	1357 (16%)	54 (9.9%)	<0.001
Stroke	8805	188 (2.1%)	175 (2.1%)	13 (2.4%)	0.677
Chronic kidney failure	8805	428 (4.9%)	389 (4.7%)	39 (7.2%)	0.010
Chronic respiratory failure	8805	663 (7.5%)	630 (7.6%)	33 (6.1%)	0.178
Cirrhosis	8805	53 (0.6%)	43 (0.5%)	10 (1.8%)	0.001
Presurgery					
EuroSCORE mortality — %	8805	0.012 (0.007-0.028 [0.005-0.613])	0.011 (0.007-0.025 [0.005-0.613])	0.019 (0.009-0.042 [0.005-0.360])	<0.001
Hemoglobin level — g/dL	8250	13.70 (12.70-14.70 [6.90-19.90])	13.80 (12.70-14.70 [6.90-19.90])	13.40 (11.80-14.40 [7.40-19.10])	<0.001
Antiplatelet agent	8805	3280 (37%)	3132 (38%)	148 (27%)	<0.001
Shock	8805	26 (0.3%)	18 (0.2%)	8 (1.5%)	<0.001
Circulatory support	8805	30 (0.3%)	20 (0.2%)	10 (1.8%)	<0.001
Surgery					
Year of surgery	8805				<0.001
2012		1233 (14%)	1193 (14%)	40 (7.3%)	
2013		1277 (15%)	1186 (14%)	91 (17%)	
2014		1280 (15%)	1162 (14%)	118 (22%)	
2015		1271 (14%)	1190 (14%)	81 (15%)	
2016		1254 (14%)	1167 (14%)	87 (16%)	
2017		1264 (14%)	1177 (14%)	87 (16%)	
2018		1226 (14%)	1185 (14%)	41 (7.5%)	
Type of Surgery	8802				<0.001
Isolated valve surgery		4501 (51%)	4230 (51%)	271 (50%)	
CABG		2555 (29%)	2509 (30%)	46 (8.5%)	
Combined surgery		910 (10%)	830 (10%)	80 (15%)	
Other		836 (9.5%)	691 (8.4%)	145 (27%)	
Endocarditis	8805	282 (3.2%)	253 (3.1%)	29 (5.3%)	0.004
Emergency surgery	8805	1129 (13%)	950 (12%)	179 (33%)	<0.001
Circulatory arrest	8805	369 (4.2%)	215 (2.6%)	154 (28%)	<0.001
Lenght of CPB — min	8134	77 (58-109 [20-468])	74 (57-102 [20-410])	152 (119-187 [45-468])	<0.001
RBC transfusion	8805	1581 (18%)	1278 (15%)	303 (56%)	<0.001
FFP tranfusion	8805	756 (8.6%)	322 (3.9%)	434 (80%)	<0.001

Data are expressed as number (proportion) or median (IQR [range]).
BMI: body mass index; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma

Supplementary Table 3: Impact of pre and per-operative variables on the incidence of overall HAI by multivariable logistic regression.

Characteristic	OR	95% CI	p-value
Age — years	1.01	1.00-1.02	0.203
Sex (male)	1.17	0.87-1.58	0.301
BMI — kg.m^{-2}	**1.05**	**1.02-1.07**	**0.001**
Diabetes	**1.40**	**1.03-1.88**	**0.028**
Chronic kidney failure	**1.87**	**1.22-2.80**	**0.003**
Cirrhosis	0.314	0.017-1.52	0.260
Preoperative hemoglobin — g/dL	0.93	0.86-1.00	0.059
Preoperative antiplatelet agent	1.08	0.83-1.40	0.561
Preoperative shock	2.05	0.56-5.9	0.222
Preoperative circulatory support	**12.3**	**5.2-30.8**	**<0.001**
Year of surgery			
2012	—	—	
2013	0.76	0.49-1.18	0.212
2014	0.68	0.44-1.06	0.087
2015	0.77	0.50-1.19	0.240
2016	0.76	0.49-1.17	0.207
2017	**0.48**	**0.295-0.77**	**0.003**
2018	**0.50**	**0.308-0.80**	**0.004**
Type of surgery			
Isolated valve surgery	—	—	
CABG	**1.59**	**1.15-2.20**	**0.005**
Combined surgery	**1.65**	**1.14-2.36**	**0.007**
Other	**2.52**	**1.70-3.70**	**<0.001**
Emergency surgery	**1.67**	**1.23-2.26**	**0.001**
Circulatory arrest	0.73	0.44-1.19	0.221
Lenght of CPB — min	**1.01**	**1.00-1.01**	**<0.001**
Intraoperative RBC transfusion	0.98	0.70-1.36	0.899
Intraoperative FFP transfusion	1.26	0.80-1.95	0.304
Intraoperative PC transfusion	1.36	0.87-2.13	0.176

Data are expressed odd ratio (OR) with 95% confidence interval (95% CI).
BMI: body mass index; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma; PC: Platelet concentrates.

Supplementary Table 4: Impact of pre and per-operative variables on the incidence of BSI by multivariable logistic regression.

Characteristic	OR	95% CI	p-value
Age — years	1.00	0.98-1.02	0.697
Sex (male)	1.23	0.76-2.07	0.408
BMI — kg.m-²	**1.06**	**1.01-1.10**	**0.015**
Diabetes	1.59	0.95-2.59	0.070
Chronic kidney failure	2.17	1.10-3.96	0.017
Cirrhosis	0.90	0.050-4.5	0.921
Preoperative hemoglobin — g/dL	**0.88**	**0.78-1.00**	**0.050**
Preoperative antiplatelet agent	1.02	0.64-1.60	0.936
Preoperative shock	**5.0**	**1.00-16.4**	**0.017**
Preoperative circulatory support	**6.4**	**2.20-17.5**	**<0.001**
Year of surgery			
2012	—	—	
2013	0.78	0.375-1.64	0.494
2014	**0.368**	**0.160-0.83**	**0.016**
2015	0.69	0.329-1.46	0.317
2016	0.78	0.383-1.64	0.504
2017	0.42	0.180-0.97	0.042
2018	0.63	0.290-1.37	0.235
Type of surgery			
Isolated valve surgery	—	—	
CABG	0.99	0.54-1.77	0.97
Combined surgery	1.41	0.73-2.60	0.282
Other	**2.06**	**1.08-3.82**	**0.025**
Emergency surgery	1.59	0.93-2.67	0.084
Circulatory arrest	0.62	0.248-1.37	0.264
Lenght of CPB — min	1.00	1.00-1.01	0.240
Intraoperative RBC transfusion	1.02	0.58-1.79	0.935
Intraoperative FFP transfusion	1.15	0.53-2.39	0.715
Intraoperative PC transfusion	**2.85**	**1.40-5.8**	**0.004**

Data are expressed odd ratio (OR) with 95% confidence interval (95% CI).
BMI: body mass index; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma; PC: Platelet concentrates.

Supplementary Table 5: Impact of pre and per-operative variables on the incidence of HAP by multivariable logistic regression.

Characteristic	OR	95% CI	p-value
Age — years	**1.02**	**1.01-1.04**	**0.003**
Sex (male)	1.26	0.85-1.90	0.258
BMI — $kg.m^{-2}$	1.01	0.97-1.04	0.791
Diabetes	1.33	0.85-2.04	0.202
Chronic kidney failure	1.39	0.73-2.45	0.279
Cirrhosis	0.58	0.032-2.93	0.603
Preoperative hemoglobin — g/dL	1.02	0.92-1.13	0.725
Preoperative antiplatelet agent	1.03	0.71-1.48	0.871
Preoperative shock	1.24	0.178-5.1	0.795
Preoperative circulatory support	**3.40**	**1.25-8.8**	**0.013**
Year of surgery			
2012	—	—	
2013	0.81	0.47-1.42	0.458
2014	0.62	0.352-1.10	0.098
2015	0.76	0.44-1.33	0.329
2016	**0.289**	**0.142-0.57**	**<0.001**
2017	0.52	0.277-0.95	0.035
2018	**0.325**	**0.160-0.64**	**0.001**
Type of surgery			
Isolated valve surgery	—	—	
CABG	0.79	0.46-1.33	0.390
Combined surgery	1.47	0.90-2.36	0.117
Other	**3.75**	**2.35-6.0**	**<0.001**
Emergency surgery	**2.54**	**1.71-3.76**	**<0.001**
Circulatory arrest	**0.45**	**0.234-0.81**	**0.010**
Lenght of CPB — min	**1.01**	**1.00-1.01**	**<0.001**
Intraoperative RBC transfusion	1.09	0.70-1.66	0.712
Intraoperative FFP transfusion	**2.03**	**1.19-3.38**	**0.008**
Intraoperative PC transfusion	1.24	0.73-2.10	0.426

Data are expressed odd ratio (OR) with 95% confidence interval (95% CI).
BMI: body mass index; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma; PC: Platelet concentrates.

Supplementary Table 6: Impact of pre and per-operative variables on the incidence of SSI by multivariable logistic regression.

Characteristic	OR	95% CI	p-value
Age — years	1.01	0.99-1.03	0.350
Sex (male)	1.20	0.77-1.92	0.432
BMI — kg.m^{-2}	**1.08**	**1.04-1.12**	**<0.001**
Diabetes	**1.71**	**1.15-2.50**	**0.007**
Chronic kidney failure	**1.96**	**1.06-3.39**	**0.022**
Cirrhosis	0.000	0.000-7.48	0.97
Preoperative hemoglobin — g/dL	0.97	0.87-1.10	0.662
Preoperative antiplatelet agent	1.10	0.76-1.58	0.622
Preoperative shock	1.51	0.081-8.1	0.697
Preoperative circulatory support	**9.6**	**2.90-29.4**	**<0.001**
Year of surgery			
2012	—	—	
2013	1.35	0.64-3.03	0.444
2014	1.43	0.70-3.16	0.352
2015	1.15	0.54-2.60	0.719
2016	**2.10**	**1.06-4.5**	**0.043**
2017	0.90	0.41-2.09	0.807
2018	1.18	0.56-2.67	0.668
Type of surgery			
Isolated valve surgery	—	—	
CABG	**2.52**	**1.62-3.94**	**<0.001**
Combined surgery	**2.27**	**1.34-3.79**	**0.002**
Other	1.71	0.83-3.37	0.130
Emergency surgery	1.43	0.89-2.23	0.124
Circulatory arrest	1.47	0.64-3.14	0.337
Lenght of CPB — min	**1.01**	**1.00-1.01**	**0.020**
Intraoperative RBC transfusion	1.08	0.65-1.76	0.771
Intraoperative FFP transfusion	1.05	0.50-2.10	0.887
Intraoperative PC transfusion	0.72	0.319-1.56	0.416

Data are expressed odd ratio (OR) with 95% confidence interval (95% CI).
BMI: body mass index; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma; PC: Platelet concentrates.

Supplementary Table 7: Covariates balance after propensity score matching

Characteristic	Overall-N = 766	0-N = 383	1-N = 383	SMD	P-value
Age — years	66 (57-74)	66 (56-75)	66 (57-73)	-0.004	0.830
Sex (male)	570 (74%)	289 (75%)	281 (73%)	-0.021	0.508
BMI — kg/m²	25.7 (22.8-28.7 [14.9-44.4])	25.8 (23.0-28.7 [14.9-44.4])	25.7 (22.8-28.7 [16.8-41.2])	0.016	0.961
Diabetes	84 (11%)	42 (11%)	42 (11%)	<0.001	>0.999
Stroke	23 (3.0%)	14 (3.7%)	9 (2.3%)	-0.013	0.290
Chronic kidney failure	54 (7.0%)	29 (7.6%)	25 (6.5%)	-0.010	0.572
Chronic respiratory failure	51 (6.7%)	25 (6.5%)	26 (6.8%)	0.003	0.885
Cirrhosis	11 (1.4%)	4 (1.0%)	7 (1.8%)	0.008	0.362
Preoperative hemoglobin — g/dL	13.40 (11.83-14.40 [6.90-19.70])	13.30 (11.85-14.40 [6.90-19.70])	13.50 (11.85-14.50 [7.40-19.10])	0.035	0.511
Preoperative antiplatelet agent	219 (29%)	108 (28%)	111 (29%)	0.008	0.810
Preoperative shock	9 (1.2%)	4 (1.0%)	5 (1.3%)	0.003	>0.999
Preoperative circulatory support	10 (1.3%)	5 (1.3%)	5 (1.3%)	<0.001	>0.999
Year of surgery					0.865
2012	59 (7.7%)	34 (8.9%)	25 (6.5%)	-0.024	
2013	138 (18%)	69 (18%)	69 (18%)	<0.001	
2014	142 (19%)	71 (19%)	71 (19%)	<0.001	
2015	124 (16%)	65 (17%)	59 (15%)	-0.016	
2016	125 (16%)	59 (15%)	66 (17%)	0.018	
2017	115 (15%)	56 (15%)	59 (15%)	0.008	
2018	63 (8.2%)	29 (7.6%)	34 (8.9%)	0.013	
Type of surgery					0.819
Isolated valve surgery	396 (52%)	204 (53%)	192 (50%)	-0.031	
CABG	80 (10%)	40 (10%)	40 (10%)	<0.001	
Combined surgery	117 (15%)	55 (14%)	62 (16%)	0.018	
Other	173 (23%)	84 (22%)	89 (23%)	0.013	
Endocarditis	51 (6.7%)	28 (7.3%)	23 (6.0%)	-0.013	0.469
Emergency surgery	228 (30%)	115 (30%)	113 (30%)	-0.005	0.874
Circulatory arrest	136 (18%)	64 (17%)	72 (19%)	0.021	0.449
Lenght of CPB — min	139 (103-169 [25-410])	139 (100-170 [25-410])	139 (105-168 [45-362])	0.006	0.894
Intraoperative RBC transfusion	372 (49%)	185 (48%)	187 (49%)	0.005	0.885
Intraoperative FFP transfusion	544 (71%)	270 (70%)	274 (72%)	0.010	0.750

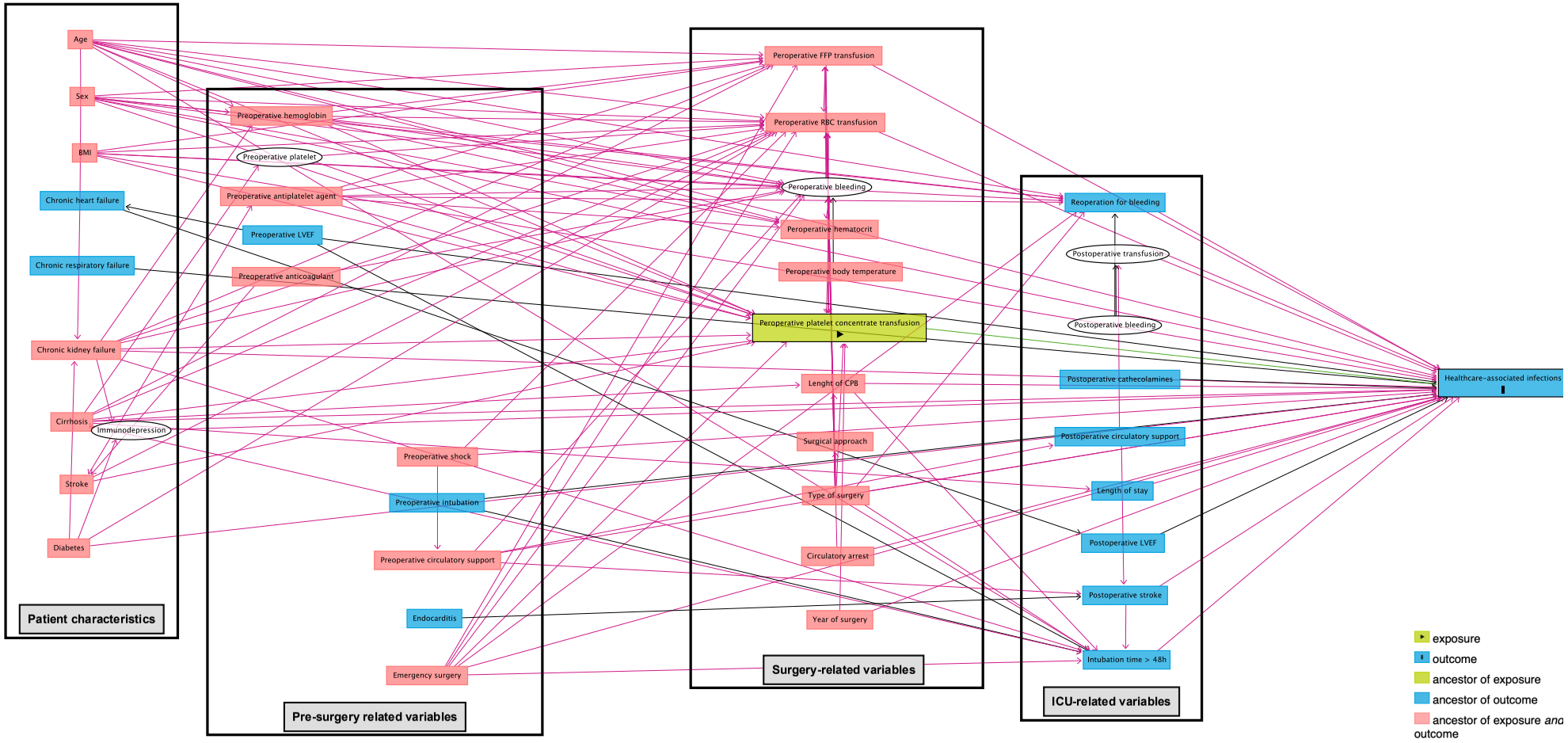
Data are expressed as number (proportion) or median (IQR [range]).
BMI: body mass index; CABG: coronary artery bypass grafting; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma

Supplementary Table 8 : Incidence of HAI according to platelet transfusion status after propensity score matching

Characteristic	Overall N = 766	No platelet transfusion N = 383	Platelet transfusion N = 383	P-value
HAI	77 (10%)	34 (8.9%)	43 (11%)	0.280
BSI	**24 (3.1%)**	**5 (1.3%)**	**19 (5.0%)**	**0.004**
HAP	62 (8.1%)	29 (7.6%)	33 (8.6%)	0.596
SSI	20 (2.6%)	12 (3.1%)	8 (2.1%)	0.365

Data are expressed as number (proportion)HAI Healthcare-Associated Infections; BSI Bloodstream infection; HAP Hospital-acquired pneumonia; SSI Surgical site infection

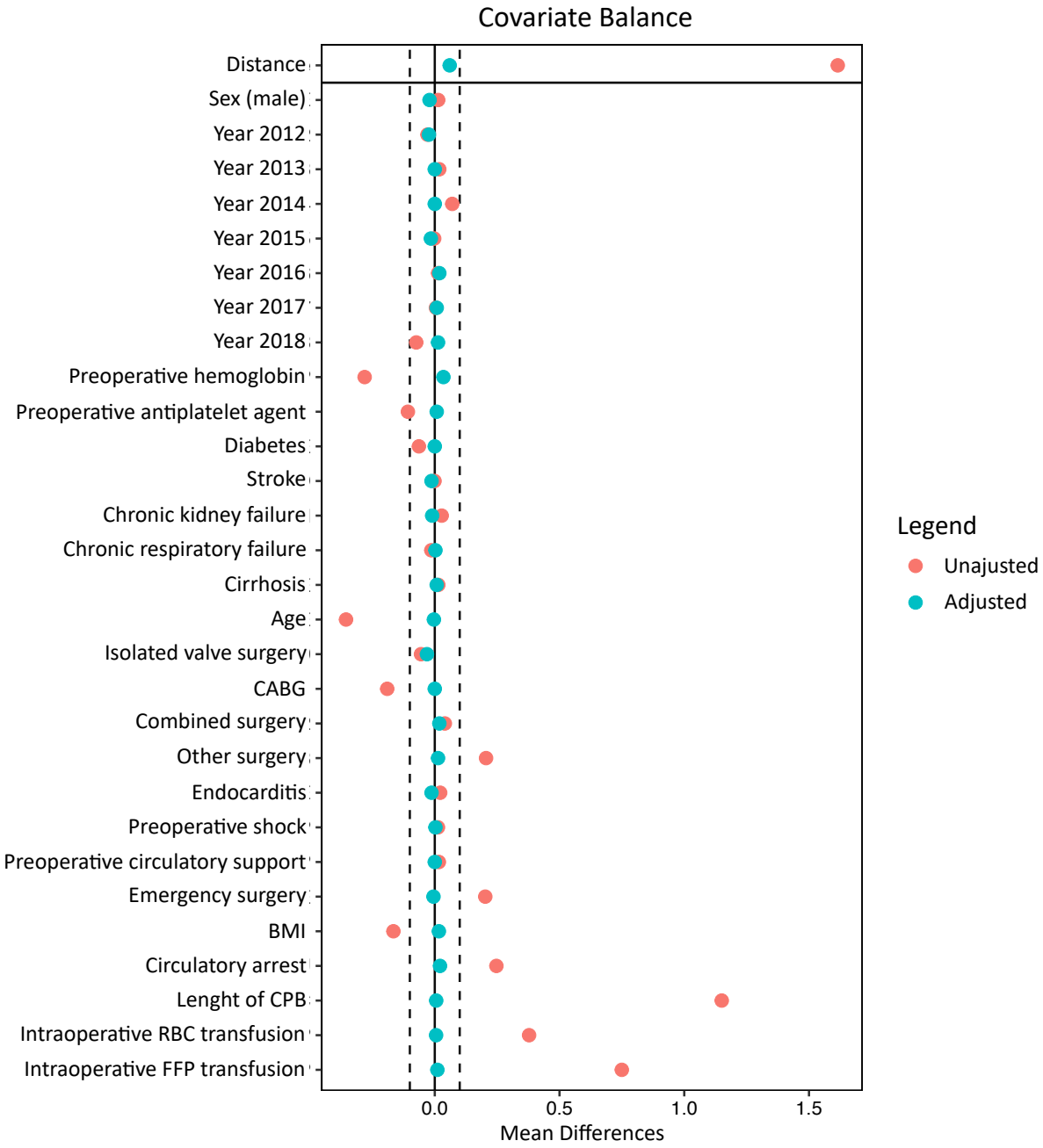
Supplementary Figure 1 : Directed acyclic graph describing associations between platelet transfusion (exposure) and healthcare-associated infections (outcome).



Directed acyclic graph describing our model of causal associations between peroperative platelet concentrate transfusion (exposure), patient-related confounders, pre-surgical-related confounders, per-surgical-related confounders, ICU-related confounders and healthcare-associated infections (outcome), using DAGitty software. Exposure variables are color-coded in green with a right pointing arrow. Outcome is color-coded in blue with a bar. Ancestors of exposure are color-coded in green; ancestors of outcome are color-coded

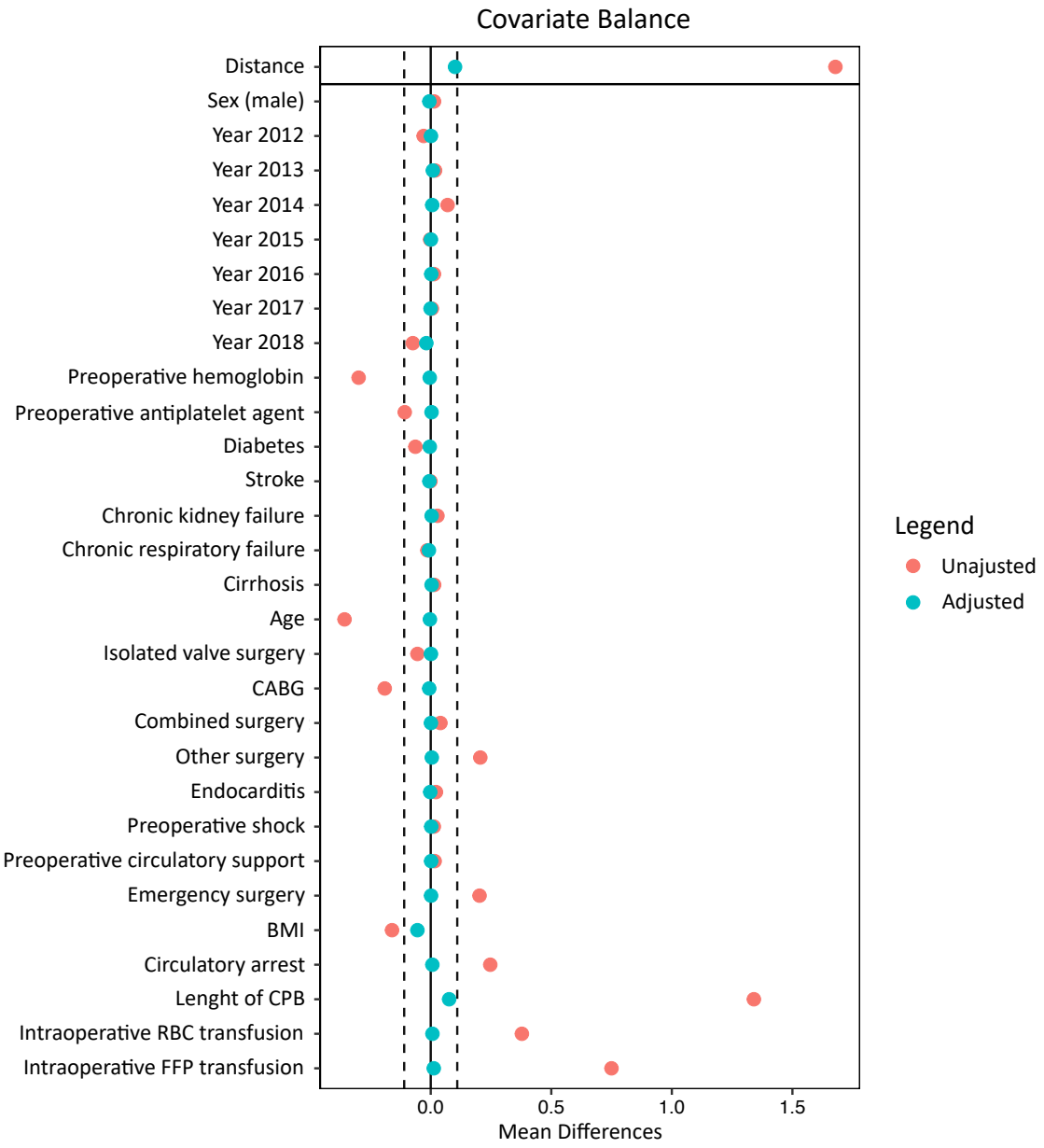
in blue, and ancestors of both exposure and outcome are color-coded in pink. The set of potential confounders sufficient for adjustment was: age, sex, body mass index, diabetes, cirrhosis, chronic kidney failure, preoperative hemoglobin, preoperative antiplatelet agent use, preoperative shock, preoperative circulatory support, year of surgery, type of surgery, emergency surgery, circulatory arrest, length of cardiopulmonary bypass, intraoperative red blood cells transfusion, intraoperative platelet concentrate transfusion and intraoperative fresh frozen plasma transfusion.

Supplementary Figure 2: Covariate balance before and after propensity score matching



Data are expressed as standardized mean differences
CABG: coronary artery bypass grafting; BMI: body mass index; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma.

Supplementary Figure 3: Covariate balance before and after propensity score overlap weighting (PSOW)



Data are expressed as standardized mean differences
CABG: coronary artery bypass grafting; BMI: body mass index; CPB: cardiopulmonary bypass; RBC: red blood cells; FFP: fresh frozen plasma.

Évaluation des marqueurs plasmatiques d'activation plaquettaire à la phase aiguë des infections sévères à SARS-CoV-2

La survenue récente de la pandémie de SARS-CoV-2, virus responsable de la COVID-19 (Coronavirus Disease 2019), a été l'occasion de mettre en lumière le rôle central des interactions plaquette-leucocyte dans la réaction inflammatoire et la survenue de défaillances d'organe.

En effet, la COVID-19 dans ses formes modérées (hospitalisation conventionnelle) et sévères (hospitalisation en réanimation – nécessité de ventilation invasive) est associée à l'ensemble des mécanismes thrombo-inflammatoires vu précédemment, incluant activation plaquettaire, endothéliale et leucocytaire, activation de la coagulation et NETose[124,125]. En plus de constituer des marqueurs de sévérité, ces processus semblent participer activement à l'initiation et à la progression de l'atteinte pulmonaire.

Dès la première vague de COVID19 (mars 2020) et en collaboration avec le laboratoire INSERM SAINBIOSE et l'EFS de Saint-Etienne (Dr Hind Hamzeh-Cognasse, Dr Fabrice Cognasse), nous avons cherché à caractériser la cinétique des marqueurs solubles d'activation plaquettaire sCD62 et sCD40L au cours de la progression du COVID-19 chez les patients hospitalisés en unité de surveillance continue ou en réanimation. Ce travail a fait l'objet d'un Brief Report publié et présenté ici.

Nous rapportons ainsi des taux de sCD62P et sCD40L élevés comparativement à une cohorte de patients COVID-19 convalescents, ainsi qu'une différence de cinétique de ces deux marqueurs. Il est important de noter qu'au cours de la première vague épidémique, les critères d'entrée en USC et en réanimation étaient moins stricts qu'au cours des vagues suivantes, de même que les indications de ventilation invasive. Ainsi, les patients inclus dans ce travail présentaient en majorité des formes classées aujourd'hui comme modérées (mortalité hospitalière 3.4%, ventilation invasive 44.8%, durée de séjour hospitalier 11j). La cinétique décroissante du sCD40L semble être ainsi corrélée à l'amélioration clinique des patients.

Ces résultats seront discutés et mis en perspective avec la littérature récente dans la partie Discussion Générale.

Virology Journal

SHORT REPORT

Open Access

Platelet-derived sCD40L: specific inflammatory marker for early-stage severe acute respiratory syndrome coronavirus 2 infection

Hind Hamzeh-Cognasse[1†], Alexandre Mansour[2,3†], Florian Reizine[4], Patrick Mismetti[1,5,6], Isabelle Gouin-Thibault[3,7] and Fabrice Cognasse[1,8*]

Abstract

Background: The SARS-CoV-2 virus is the causing agent of the Coronavirus disease 2019 (COVID-19) characterized by a huge pro-inflammatory response and coagulation disorders that may lead to for its severe forms, in organ failure or even death. As major players of thrombo-inflammation, platelets release large amounts of immunomodulatory molecules and regulate leukocyte and endothelial activity, which are both altered in COVID-19. Altogether, this makes platelets a very likely actor of the thrombo-inflammatory complications of COVID-19. Thus, we propose to identify a platelet inflammatory signature of severe COVID-19 specifically modulated throughout the course of the disease.

Methods: Luminex technology and enzyme-linked immunosorbent assay were used to assess plasma levels of platelet inflammatory markers in patients with severe acute respiratory syndrome coronavirus 2 infection on admission and for 14 days afterwards.

Results: In accordance with the observations of other teams, we evidence that the plasma levels of the platelet soluble (s)CD40L is significantly elevated in the early stages of the disease. Interestingly we observe that the plasma level of sCD40L decreases overtime while that of sCD62P increases significantly.

Conclusions: Our data suggest that there is a platelet signature of inflammatory response to SARS-COv-2 infection which varies overtime and could serve as monitoring biomarkers of patient inflammatory state.

Clinical trial registration number: 2020-A01100-39; title: Human Ab Response & immunoMONItoring of COVID-19 Patients, registration date: 05/25/2020; URL of the registry: https://clinicaltrials.gov/ct2/history/NCT04373200?V_5=View.

Keywords: Platelets, Innate immunity, CD40L, Inflammation, SARS-CoV2

Background

Beyond their contribution to hemostatic response, platelets behave as immune cells owing to their innate immunity receptors, including toll-like receptors, enabling them to sense danger signals, as neutrophils, macrophages, or dendritic cells do [1]. Upon activation, platelets release immune mediators and chemokines such as soluble (s)CD40L, sCD62P, or CXCL4 from their

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granules or membranes [1]. sCD40L and sC62P from platelets mediate thrombotic and inflammatory processes, contributing to inflammation associated to viral infection and increased cardiovascular disease risk [2]. In patients with coronavirus disease (COVID-19), platelet hyperreactivity [3] and upregulated release of soluble immunomodulatory factors [4] have been described, which suggests a platelet involvement in COVID-19 thromboinflammation. Early detection and monitoring of COVID-19 increase the survival rate.

Thus, a continuous search for new biomarkers of severe acute respiratory syndrome coronavirus 2 infection is necessary for early diagnosis and stratification of COVID-19 severity to improve patient management. We hypothesized that circulating sCD40L and sCD62P levels are significantly modulated throughout the disease course of COVID-19 and significantly different from those in COVID-19 convalescent patients in the PLASMACOV cohort. We included 29 patients who attended the Pontchaillou University Hospital of Rennes, France, between March 2020 and July 2020. The patients with severe acute respiratory syndrome coronavirus 2 infection (with polymerase chain reaction positive tests) were hospitalized for severe COVID-19 in a continuing care or intensive care unit and received oxygen therapy (Table 1). Blood sampling was performed on intensive care unit admission (day 1) and days 3–5, 7, and 14. Plasma samples were obtained by centrifugation of citrated whole blood samples and stored at − 80 °C until assay. For the PLASMACOV cohort, in accordance with the recommendations of the European Blood Alliance, convalescent patients were considered eligible for plasma donation at least 14 days after symptom resolution and underwent the standard plasma apheresis procedure for healthy volunteers. Apheresis plasma samples from 26 convalescent patients were processed as usual with pathogen inactivation treatment and cryopreserved until clinical use. The sCD40L and sCD62P levels were measured using the Luminex technology or enzyme-linked immunosorbent assay (RnD Systems), respectively. Owing to some missing values due to the absence of blood samples for certain patients at some time points, a mixed model was used to evidence the significance of the changes of the sCD62P and sCD40L levels over time. The Tukey multiple-comparisons test was used to compare data from patients with COVID-19 with those from convalescent patients. Statistical differences were considered significant at $p < 0.05$.

Median age was 57 (54–68) years, 73% of the patients were men and the median BMI was 27.9 (24.4–31.5) (Table 1). The changes in the sCD40L and sCD62P concentrations are plotted in Fig. 1. The sCD62P level significantly increased over time from 24,251 to 33,784 pg/ml, whereas the sCD40L level decreased from 2396 to 1497 pg/ml ($p < 0.0001$). Moreover, the mixed model analysis revealed a significant interaction between the platelet inflammatory molecule and the time variables ($p < 0.0001$), indicating that the kinetics of the sCD40L plasma level significantly differed from that of sCD62P. Finally, the plasma levels of sCD62P and sCD40L of COVID-19 patients were significantly higher than those observed in the plasma samples of convalescent patients (22,886 and 290 pg/ml, for sCD62P and sCD40L respectively), regardless of sampling time, except for the sCD62P level assessed in the first time point, which did not differ significantly from those in convalescent patients. The strength of our study is the 14-day sequential sampling. Our data suggest that these factors are well associated with acute-phase COVID-19.

However, this study has several limitations. Indeed, the size of the population remains modest, even though we were able to perform several sequential samples on the same patients. Our observations need to be confirmed in a larger cohort of patients that would also allow to stratify COVID-19 patients according to

Table 1 Patient characteristics and outcomes

	All patients (N = 29)	Convalescent patients (N = 26)
Age—years	57 (54–68)	37.6 (22–57)
Sex		
Female	8 (27.6%)	7 (26.9%)
Male	21 (72.4%)	19 (73.1%)
BMI—kg/m^2	27.9 (24.4–31.5)	N/C
Days from illness onset to first blood sampling—days	11 (9–13)	N/A
SAPS II score on day 1	27 (16–35)	N/A
SOFA score on day 1	2 (1–8)	N/A
Platelet count on day 1–10^9/L	219 (153–260)	N/A
Lymphocyte count on day 1–10^9/L	0.8 (0.6–1.1)	N/A
Invasive ventilation	13 (44.8%)	N/A
ARDS	14 (48.3%)	N/A
Most pathological PaO2/FiO2	156 (89–238)	N/A
Acute renal failure	8 (27.6%)	N/A
Septic shock	2 (6.9%)	N/A
Thromboembolic event	4 (13.8%)	N/A
Hospital length of stay—days	11 (7–19)	N/A
ICU length of stay—days	4 (2–13)	N/A
Hospital death	1 (3.4%)	N/A

Results are presented as n (%) or median (IQR)

BMI, body mass index; SAPS II, Simplified Acute Physiology Score II; PaO2, arterial oxygen tension; FiO2, Fraction of Inspired Oxygen

N/C: Data not collected

N/A: Not applicable

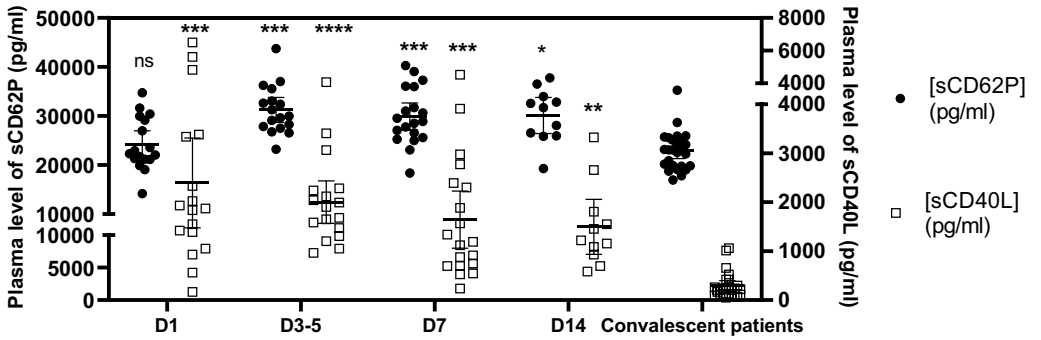

Fig. 1 Plasma levels of sCD40L and sCD62P biomarkers in patients with severe COVID-19 throughout the disease course. Individual values and mean ± 95% confidence intervals are plotted for each sampling day (D1, D3–5, D7, and D14). A mixed model was used to evidence the significant changes in sCD62P and sCD40L levels in patients with COVID-19. The Tukey multiple-comparisons test was used to compare sCD62P and sCD40L values between COVID-19 and convalescent patients (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$; ****$p < 0.0001$)

clinical criteria, such as the most pathological PaO2/FiO2 or thromboembolic events.

On the other hand, it would be interesting to complete the kinetic analysis of the platelet inflammatory signature in COVID-19 patients by assessing other platelet factors. It would also be particularly relevant to combine the platelet inflammatory signature with other biological parameters, such as the percentage of circulating platelet-leukocyte complexes, in order to have a panel of biomarkers allowing to precisely characterize the thrombo-inflammatory state of COVID-19 patients.

Conclusions

The management of COVID-19 infection remains complex partly because of the lack of reliable severity markers. Furthermore, our data highlight the difference in the temporal dynamics of these factors and the importance of monitoring relevant factors, which should include platelet factors, in the early stages of COVID-19 infection. Thus, the follow-up of platelet inflammatory parameters during the course of COVID-19 could be of particular interest for clinicians. Indeed, the assessment of the platelet signature of the thrombo-inflammation associated with severe COVID-19, particularly at early stages of the disease, will help patient monitoring, evaluation of the efficiency of therapeutic strategies on thrombo-inflammation and evidence the need for treatment adaptation, if levels of inflammatory factors are sustained.

Finally, our study suggest that platelets could be relevant therapeutic targets allowing clinicians to intervene early and simultaneously on two major systems, haemostasis and inflammation, that are profoundly deregulated during COVID-19.

Abbreviations

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus-2; COVID-19: Coronavirus disease; (s)CD40L: Soluble (s)CD40L; (s)C62P: Soluble (s)CD62P.

Acknowledgements

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Authors' contributions

HHC, AM, IGT, and FC formulated the study hypothesis, analyzed the results, and wrote the manuscript. FR enrolled the patients and provided blood samples. PM analyzed the results and wrote the manuscript. All authors reviewed the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon individual specific and reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the local ethics committee (2020-A01100-39) with respect to the Declaration of Helsinki. Written informed consent was obtained from all the patients or their trusted persons. Data collection from the PLASMACOV cohort was approved by the French national ethics committee (2020-A00728-31).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Principaux résultats et acquis à l'issue de la thèse

Comme nous l'avons décrit en préambule de ce manuscrit, c'est au lit du malade que ce travail de thèse prend sa source car l'inflammation majeure associée au sepsis et aux circulations extracorporelles est une préoccupation quotidienne des médecins anesthésistes et réanimateurs. L'hypothèse que les cellules plaquettaires puissent jouer un rôle important dans ce processus, nous a conduit à formuler les présupposés suivants (Figure 13) :

1) L'activation plaquettaire pourrait jouer un rôle dans la réponse inflammatoire aiguë
2) L'inhibition de l'activation plaquettaire par la voie de l'AMP cyclique et en particulier par l'inhibition de $P2Y_{12}$ pourrait permettre de moduler cette réponse inflammatoire et ainsi le devenir des patients.

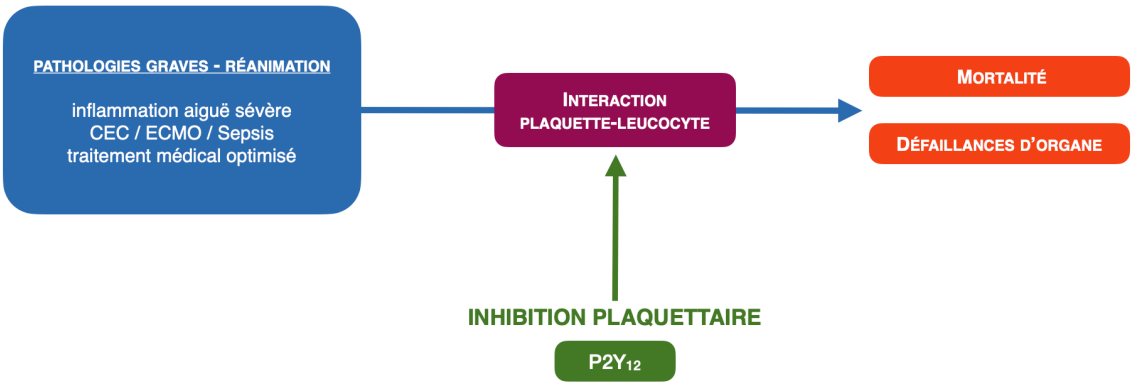

Figure 13. Hypothèses initiales

Ce travail a été structuré autour de la mise en place de modèles précliniques d'une part (modèle murin de sepsis cecal slurry et modèle in-vitro de stimulation de sang total), et de travaux translationnels et cliniques d'autre part.

Plusieurs conclusions scientifiques peuvent être tirées de ces résultats (Figure 14). En premier lieu, nous avons démontré que le traitement par ticagrelor était associé à une diminution de la réponse inflammatoire et des lésions d'agression pulmonaire aiguë dans un modèle murin de sepsis polymicrobien novateur (cecal slurry). Cet effet nous permet d'envisager plusieurs hypothèses mécanistiques (effet de $P2Y_{12}$ plaquettaire, effet de $P2Y_{12}$ non plaquettaire, effet de

l'inhibition de la recapture de l'adénosine et effet direct antibactérien). En second lieu, nous avons mis en évidence une association indépendante entre la transfusion peropératoire de concentrés plaquettaires déleucocytés et la survenue de bactériémies en post-opératoire de chirurgie cardiaque sous CEC. Ce résultat permet d'envisager un rôle direct des plaquettes allogéniques et des médiateurs inflammatoires issus du stockage des concentrés plaquettaires, et en particulier de l'axe CD40L/CD40. Enfin, nous avons pu montrer que le CD40L soluble, principalement d'origine plaquettaire, était précocement associé aux formes sévères de COVID-19 et pourrait constituer un biomarqueur d'intérêt dans ce contexte.

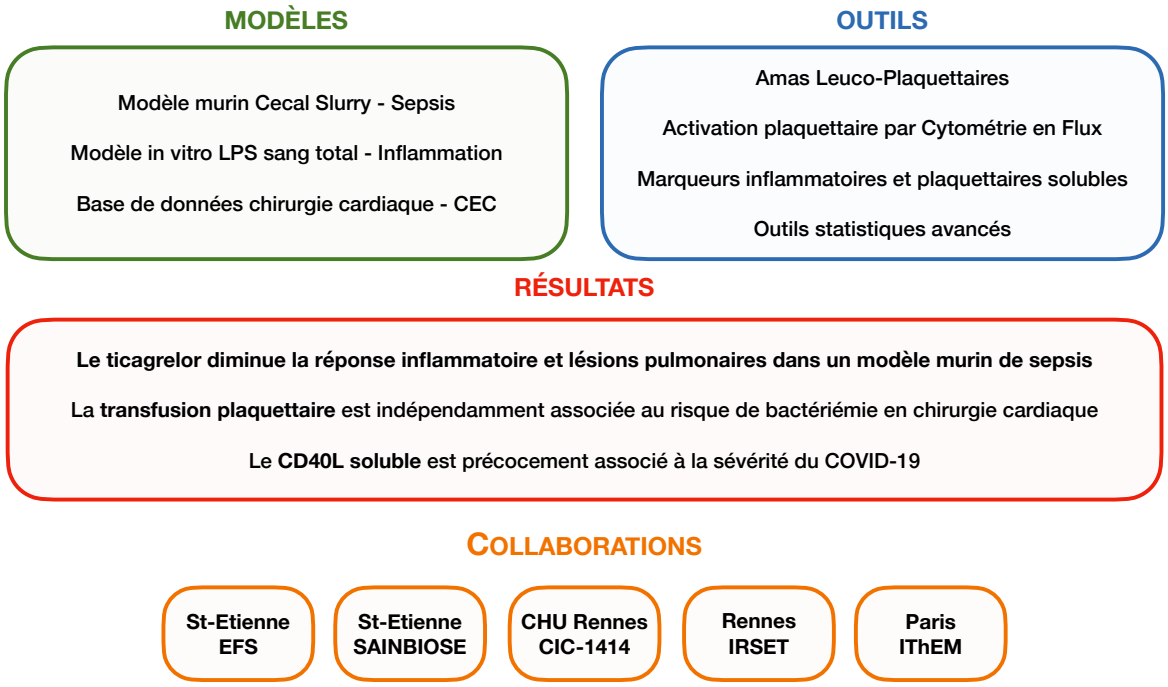

Figure 14. Principaux résultats et acquis à l'issue de la thèse

Ces travaux nous ont permis d'élaborer et de mettre au point des modèles d'études précliniques et des bases de données cliniques et biologiques en chirurgie cardiaque mais également des outils qui viennent enrichir les capacités techniques de notre équipe à Rennes. C'est le cas en particulier des techniques de mesure des amas leuco-plaquettaires par cytométrie en flux mais également de l'apprentissage de méthodes statistiques avancées permettant d'exploiter au mieux l'ensemble des données générées par l'activité clinique.

Enfin, ce travail nous a également permis de renforcer les collaborations entre les équipes cliniques et de recherche à Rennes, mais également de développer de nouvelles collaborations pérennes (Paris-HEGP-IThEM, Saint-Etienne-EFS-SAINBIOSE, UCSF-Matthay lab).

Mise en perspective : expérience de la COVID-19

Les résultats de nos travaux et le travail bibliographique qui les accompagne, s'est rapidement confronté en cours de thèse, à l'émergence de la pandémie de SARS-CoV-2. Comme discuté précédemment (Partie 2), l'extrême engouement pour la recherche translationnelle sur la COVID-19 a mis en lumière le rôle central des interactions plaquette-leucocyte dans la réaction inflammatoire et la survenue de défaillances d'organe.

En effet, les premières constatations cliniques, auxquelles nous avons participé (Tacquard C, Mansour A, Godon A, et al Chest. 2021 Jun;159(6):2417-2427), ont mis en évidence un phénotype pro-thrombotique et inflammatoire associé aux formes sévères de COVID-19. De très nombreux travaux ont par la suite suggéré un rôle direct de l'immunothrombose dans la physiopathologie de l'atteinte respiratoire des formes graves de COVID-19 [124,125].

Ce rationnel a fort logiquement conduit à mettre en place des études randomisées contrôlées évaluant l'effet de thérapeutiques antiplaquettaires sur la mortalité ou les défaillances d'organes. A ce jour, trois études ont évalué l'effet de l'aspirine et/ou des inhibiteurs de $P2Y_{12}$ en association avec une anticoagulation héparinique préventive dans des populations de patients atteints de COVID-19 modérée à sévère[126–128]. Aucune de ces trois études n'est parvenue à mettre en évidence un effet bénéfique de ces antiplaquettaires. Deux études évaluant un anticorps inhibiteur de la P-Sélectine (crizanlizumab, CRITICAL, NCT04435184) et un anticorps inhibiteur de la GPVI (glenzocimab, GARDEN, NCT04659109) sont toujours en cours.

Alors que cette piste thérapeutique semble logiquement s'éteindre pour la COVID-19, notamment à la faveur du bénéfice préventif de la vaccination, ces résultats viennent paradoxalement soutenir le changement de paradigme que nous opérons au sein de l'équipe, à la fois dans notre conception des mécanismes inflammatoires et des possibilités de modulation pharmacologiques, mais également dans le choix de critères de jugement cliniques d'évaluation de ces thérapeutiques (Figure 15).

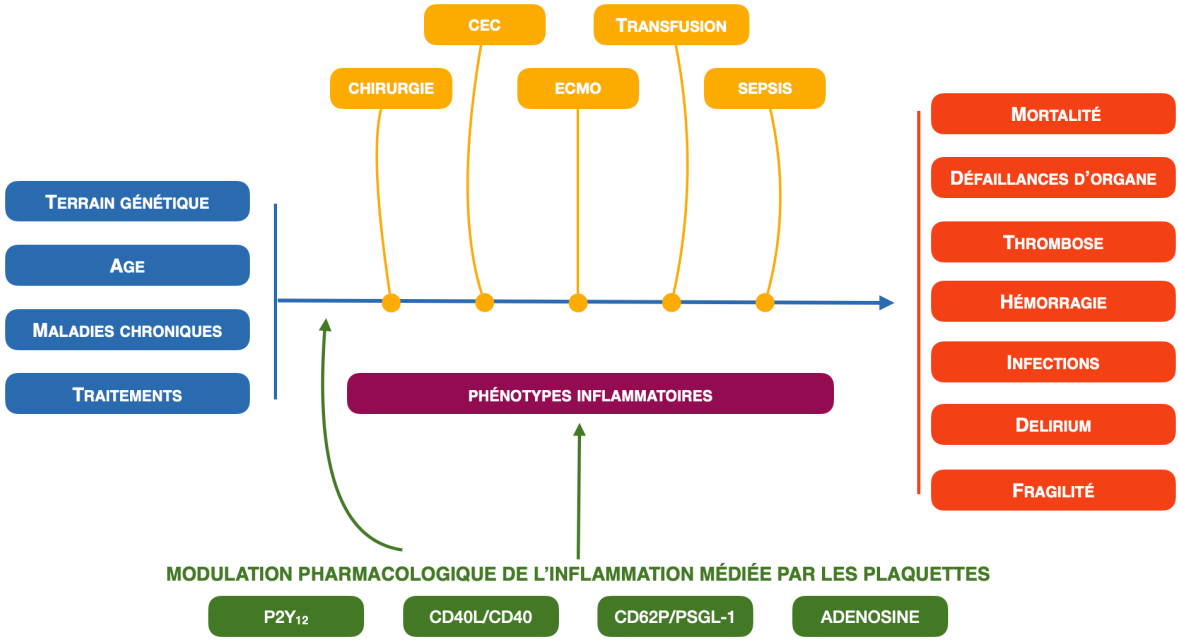

Figure 15. Actualisation des hypothèses de recherche à l'issue de la thèse

Cette nouvelle conception diffère de nos hypothèses initiales sur plusieurs points. Premièrement, le terrain génétique et acquis du patient et son exposition à des traitements médicamenteux pourrait jouer un rôle dans sa susceptibilité à développer des phénotypes inflammatoires sévères et devrait être pris en compte dans les évaluations cliniques. Deuxièmement, l'intensité de la réponse inflammatoire et son potentiel pathologique pourraient être dépendants de l'association synergique de plusieurs phénomènes pro-inflammatoires successifs (multi-hit model). Le patient de réanimation cardiothoracique en est un parfait exemple en associant parfois une pathologie inflammatoire chronique, l'athérosclérose, une chirurgie sous CEC, une exposition à la transfusion allogénique et des complications circulatoires ou infectieuses. Troisièmement, il nous semble nécessaire, à la lumière de nos résultats et de la littérature, d'élargir l'évaluation thérapeutique à d'autres cibles pharmacologiques (ou association de cibles pharmacologiques) dans l'objectif d'accroitre l'effet anti-inflammatoire sans toutefois majorer le risque hémorragique notamment décrit chez les patients COVID-19. Enfin, nous pensons que la mesure de l'efficacité clinique de ces stratégies devrait être étudiée non plus seulement sur la mortalité et les défaillances multiviscérales, mais également sur la survenue, par exemple, d'infections associées aux soins ou de phénomènes d'immunomodulation à court ou long terme. Cette nouvelle conception s'inscrit de fait dans une démarche de personnalisation des soins en réanimation.

Perspectives de recherche à l'issue de la thèse

En parallèle de ma carrière hospitalière et universitaire en Anesthésie-Réanimation cardiothoracique, nous poursuivons les travaux de recherche translationnelle initiés en thèse au sein de l'UMR_S 1085 IRSET (Pr Olivier Fardel – Dr Isabelle Gouin-Thibault) avec encadrement de Master. L'étude de la modulation de l'inflammation par les antiplaquettaires est également évaluée dans un modèle de sclérodermie systémique où elle fait l'objet d'une thèse au sein de cette unité (Dr Adeline Pontis – Dr Isabelle Gouin-Thibault). Plusieurs axes de recherche sont actuellement développés ou envisagés dans les années à venir (Figure 16).

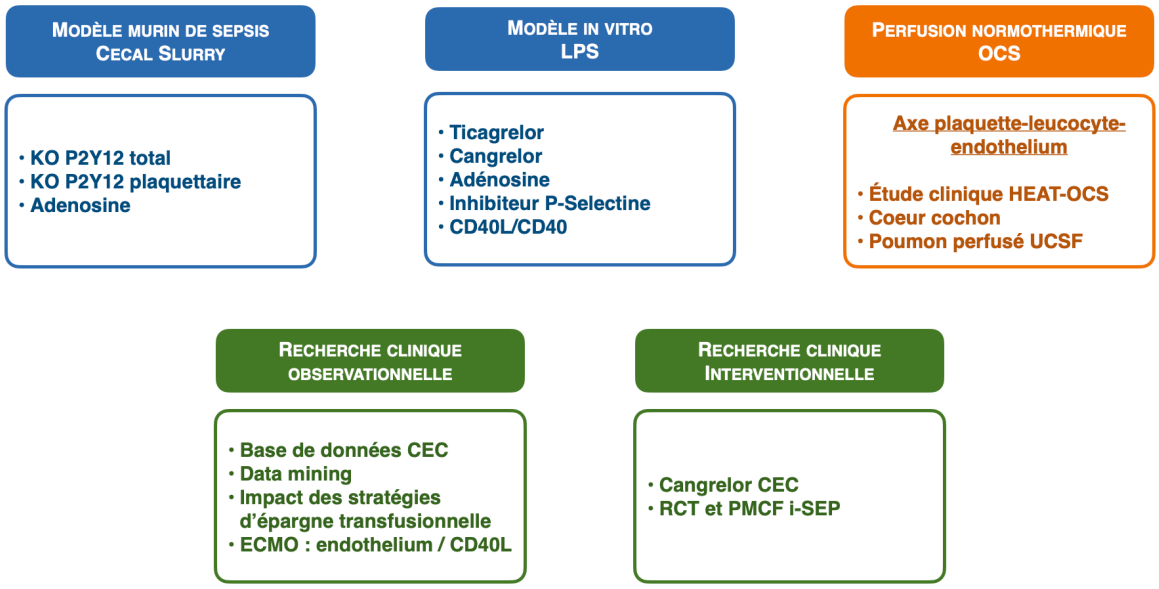

Figure 16. Perspectives de recherche à l'issue de la thèse

Modèles précliniques

Le modèle in vitro présenté précédemment est actuellement utilisé à Rennes et appliqué à l'étude de l'effet du ticagrelor. Nous avons prévu de poursuivre ce travail par l'évaluation d'autres inhibiteurs du $P2Y_{12}$ (cangrelor) mais également d'autres voies d'activation plaquettaire et d'interaction plaquette-leucocyte (adénosine, P-Sélectine, CD40L/CD40). Une approche plus mécanistique est également envisagée, notamment par l'inhibition de la formation des ALP.

Le modèle cecal slurry n'a pas encore été transféré à Rennes, mais sa mise en place est envisagée. Il pourrait permettre l'évaluation précise des mécanismes expliquant l'effet bénéfique du ticagrelor mis en évidence dans notre étude.

Perfusion ex-vivo normothermique d'organes

La perfusion ex-vivo normothermique de cœur et de poumon à l'aide de circulations extracorporelles et de sang total est un modèle applicable à l'étude des interactions plaquettes-leucocytes-endothélium, dans un objectif de recherche fondamentale et translationnelle.

Trois projets utilisant ce modèle sont en cours dans notre équipe. Premièrement l'étude HEAT-OCS évalue les marqueurs inflammatoires et d'activation plaquettaire et endothéliale au cours de la perfusion de greffons cardiaques humains avant transplantation. Deuxièmement, ce projet sera complété l'année prochaine par l'encadrement d'un travail de master 2 similaire utilisant des cœurs de cochon perfusés. Enfin, nous poursuivons la collaboration avec le Pr Matthay (UCSF) en analysant la NETose dans des prélèvements issus de perfusions ex-vivo de poumons humains (Leligdowicz A, Ross JT, Nesseler N, Matthay MA. Intensive Care Med Exp. 2020 Sep 21;8(1):56.)

Recherche clinique

Pour terminer, nous poursuivons et développons des travaux de recherche clinique observationnelle et interventionnelle en lien avec la thématique thrombo-inflammatoire. Ainsi, nous souhaitons poursuivre l'analyse des bases de données de chirurgie cardiaque afin de mettre en évidence les déterminants des réponses inflammatoires dérégulées ainsi que des travaux évaluant la thrombo-inflammation au cours de l'ECMO. Les données issues des études interventionnelles du dispositif i-SEP seront également analysées dans l'objectif d'évaluer l'impact sur l'immunomodulation. Enfin, nous prévoyons d'évaluer l'impact de l'utilisation d'inhibiteurs de $P2Y_{12}$ au cours des chirurgies cardiaques sous CEC.

Conclusion

L'étude du rôle de l'activation plaquettaire médiée par la voie du récepteur $P2Y_{12}$ dans l'inflammation aiguë nous a conduit à développer une approche transversale, basée sur l'utilisation de modèles précliniques murin et *in vitro,* associés à l'analyse de données cliniques. Ce travail a ainsi permis de mettre en évidence un effet bénéfique du ticagrelor dans un modèle murin de sepsis. Ces résultats permettent d'envisager plusieurs hypothèses mécanistiques, associant l'inhibition plaquettaire à des mécanismes extra-plaquettaires, qui devront être évaluées dans des études complémentaires. Nos résultats apportent également des données sur le rôle de l'activation plaquettaire dans des situations inflammatoires aiguës en réanimation, comme le sepsis ou l'utilisation des circulations extracorporelles. Ces données constituent un rationnel solide pour bâtir de futurs travaux de recherche clinique et translationnelle évaluant l'utilisation d'approches pharmacologiques d'inhibition plaquettaire dans l'objectif d'améliorer le devenir des patients en soins critiques sous assistance extracorporelle.

Annexes

En parallèle de ces travaux translationnels et cliniques présentés précédemment, j'ai également participé à des travaux publiés de l'unité UMR_S1140, portant sur la régulation de l'AMP cyclique plaquettaire par MRP4 (Annexe 1) ainsi que sur l'évaluation comparative des tests utilisés pour mesurer l'effet de l'aspirine chez la souris (Annexe 2).

Enfin, en collaboration avec le CHU de Lille (Professeur André Vincentelli), nous avons mis en place un registre prospectif national incluant les patients COVID-19 bénéficiant d'une assistance par ECMO. Ce registre à fait l'objet d'une première publication dans la revue Anesthesiology (Nesseler et al. Anesthesiology 2022; 136:732–748). J'ai par la suite mené une analyse de ce registre afin d'évaluer l'incidence et l'impact des complications thrombotiques et hémorragiques dans le contexte très pro-inflammatoire de l'association COVID-19 et circulation extracorporelle. Ce travail a fait l'objet d'un article en cours de révision (Annexe 3).

Annexe 1. Role of membrane lipid rafts in MRP4 (ABCC4)-dependent regulation of the cAMP pathway in blood platelets

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Role of membrane lipid rafts in MRP4 (ABCC4)-dependent regulation of the cAMP pathway in blood platelets

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Abstract:
Background. Platelet cytosolic cAMP levels are balanced by synthesis, degradation, and efflux. Efflux can occur via MRP4 (multidrug resistant protein-4, ABCC4) present on dense granule and/or plasma membranes. As lipid rafts have been shown to interfere on cAMP homeostasis, we evaluated the relationships between the distribution and activity of MRP4 in lipid rafts and cAMP efflux.
Methods. Platelet activation and cAMP homeostasis were analyzed in human and wild-type or MRP4-deleted mouse platelets in the presence of methyl-β-cyclodextrin (MβCD) to disrupt lipid rafts, and of activators of the cAMP signalling pathways. Human platelet MRP4 and effector proteins of the cAMP pathway were analyzed by immunoblots in lipid rafts isolated by differential centrifugation.
Results. MβCD dose-dependently inhibited human and mouse platelet aggregation without affecting per se cAMP levels. An additive inhibitory effect existed between the adenylate cyclase (AC) activator forskolin and MβCD that was accompanied by an overincrease of cAMP, and which was significantly enhanced upon MRP4 deletion. Finally, an efflux of cAMP out of resting platelets incubated with PGE1 was observed that was partly dependent on MRP4. Lipid rafts contained a small fraction (≈ 15%) of MRP4 and most of the inhibitory G-protein Gi, whereas Gs protein, AC3, and phosphodiesterases PDE2 and PDE3A were all present as only trace amounts.
Conclusion. Our results are in favor of part of MRP4 present at the platelet surface, including in lipid rafts. Lipid raft integrity is necessary for cAMP signalling regulation, although MRP4 and most players of cAMP homeostasis are essentially located outside rafts.

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Role of membrane lipid rafts in MRP4 (ABCC4)-dependent regulation of the cAMP pathway in blood platelets

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Abstract

Background. Platelet cytosolic cAMP levels are balanced by synthesis, degradation, and efflux. Efflux can occur via MRP4 (multidrug resistant protein-4, ABCC4) present on dense granule and/or plasma membranes. As lipid rafts have been shown to interfere on cAMP homeostasis, we evaluated the relationships between the distribution and activity of MRP4 in lipid rafts and cAMP efflux.

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and effector proteins of the cAMP pathway were analyzed by immunoblots in lipid rafts isolated by differential centrifugation.

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Conclusion. Our results are in favor of part of MRP4 present at the platelet surface, including in lipid rafts. Lipid raft integrity is necessary for cAMP signalling regulation, although MRP4 and most players of cAMP homeostasis are essentially located outside rafts.

Key-words: platelets, MRP4 (ABCC4), cAMP, lipid rafts, microdomains

Category: original article, cellular haemostasis and platelets

Introduction

Platelets play a central role in hemostasis and thrombosis. In a physiological situation, circulating blood platelets are maintained functionnally inactive to avoid any inappropriate stimulation and aggregate formation. This regulated resting state is mostly under the control of cyclic nucleotide homeostasis[1]. Endothelial cells (EC) are the main source of two major platelet inhibitory molecules, namely prostacyclin (PGI_2, prostaglandin I_2) and adenosine, the latter being produced from extracellular ATP catabolized by ecto-ATPases such as CD39 and CD73 expressed on EC[2]. PGI_2 and adenosine bind to specific platelet receptors, PGI_2 through IP receptor and adenosine through A_{2A} and A_{2B} receptors, through which they activate adenylate cyclases (AC) that generate cyclic adenosine monophosphate (cAMP)[3]. To date, only AC3 and AC5/6 isoforms of AC have been identified in platelets[4]. EC also release nitric oxide (NO), a free radical messenger that activates guanylate cyclase (GC) to produce cyclic guanosine monophosphate (cGMP)[5].

Cyclic nucleotides stimulate cAMP- or cGMP-dependent protein kinases (PKA and PKG, respectively) to phosphorylate a broad panel of substrate proteins that maintain

platelets in a resting state[6]. PKA activation inhibits cytoskeleton reorganisation, integrin αIIbβ3 activation, granule secretion and Ca^{2+} mobilization, all processes implicated in platelet activation. To limit the extent to which cAMP/cGMP-dependent pathways repress platelet activation, phosphodiesterases (PDE) can rapidly hydrolyse cyclic nucleotides and thus stop their downstream signalling pathways[7]. In platelets, several PDE isoforms, PDE2, PDE3A and PDE5A[4], are activated by various mechanisms, and in particular through PKC and PKA activities, leading to a negative control of cAMP homeostasis[8]. Similarly, cGMP induces a negative feedback on its own synthesis by activating PDE2A[9] and PDE5A[10].

Platelets are activated following vascular injury, as they first adhere to denuded sub-endothelium components, such as collagen or von Willebrand factor (VWF), or to an activated inflammatory endothelium. Then platelets undergo a shape change and a relocation of their granule content before secretion. Among secreted molecules is ADP that acts as a platelet agonist mostly by reducing the level of cytosolic cyclic nucleotides[11]. Indeed, ADP binding to the G protein-coupled receptor (GPCR) $P2Y_{12}$, a Gi-coupled ADP receptor, inhibits AC *via* the $G\alpha_i$ subunit, thus rapidly lowering intraplatelet levels of cAMP and subsequently PKA activation. The balance between platelet activation and inhibition is thus largely under the control of cAMP, as witnessed by the efficacy of pharmacological molecules targeting this pathway, such as anti-$P2Y_{12}$ antiplatelet drugs, PDE inhibitors, and PGI_2 analogues[12,13].

Although the major role of ADP, cAMP and cGMP in platelet activation and its regulation has been demonstrated for years[1], the underlying molecular mechanisms are only beginning to emerge. Besides synthesis and degradation, the platelet cytosolic cAMP levels are also balanced by transport out of the cytosol either to dense granules or to the extracellular space[14,15]. The ATP-binding cassette (ABC) transporters have been reported to regulate cAMP homeostasis in several cell types, and notably the multidrug resistance protein-4 (MRP4, ABCC4) that can indeed transport cyclic nucleotides and nucleotide analogues[14,16,17]. More recently, its implication has been established in platelet activation since several groups, including ours, have shown that MRP4 regulates platelet activation and clot formation by modulating the cAMP-PKA signalling pathway[18–20]. Indeed, *in vivo* and *in vitro* platelet aggregation and thrombus formation are defective in transgenic MRP4-deficient mice due to an impaired MRP4-dependent efflux of cAMP and consequently, its accumulation in the cytosol[15,19]. Different studies have localized MRP4 either on dense

granule membranes, on the plasma membrane, or both[15,21,22]. Although precise MRP4 subcellular localization(s) in resting as well as in activated platelets remains a matter of debate, its diversified localization underlines the emerging concept of cAMP compartmentation in platelets. The so-called “cAMP-dependent microdomains” represent a major regulatory mechanism for the biological activities of this cyclic nucleotide in other cell types[23–25]. In these specific microdomains, A-kinase anchoring proteins (AKAP) play a major role by assembling and colocalizing enzymes such as PDE, phosphatases, AC, PKA and receptors that together provide spatial regulation of cAMP generation and signalling. As a consequence, PKA activity can be focused on specific substrates in different subcellular compartments[26]. In platelets, little is known about this structural and functional entity but it is likely that similar structures exist[27].

Lipid rafts are membrane microdomains identified as dynamic assemblies of sphingolipids and cholesterol that are present in all cell types including platelets[28]. They play a critical role in maintaining multiprotein complexes at the cytoplasmic side of the plasma membrane[29]. In platelets, the role of lipid rafts has been described in critical signalling mechanisms, such as activation induced by G-protein-coupled receptors[28]. So far, one group has linked lipid rafts to the cAMP pathway in platelets, showing that lipid rafts are important in the regulation of platelet-VWF interactions[27] and in the inhibitory effect on platelet activation of PGI_2 or forskolin[30]. Thus, putative platelet cAMP microdomains, whether or not associated with lipid rafts, could regulate the availability and/or distribution of cAMP in platelets, controlling the resting state of platelets by assembling the players of the cAMP pathway both for synthesis, degradation and efflux. However, nothing is known on the presence of MRP4 in lipid rafts, and/or in cAMP microdomains.

The main objective of the present work was to clarify the relationship between the role of MRP4, lipid rafts and the regulation of cAMP homeostasis in platelets with the aim to further analyze whether the cAMP microdomains, belong to, or are related to lipid rafts, particularly for what is concerned with the compartmentation of cAMP.

Results

MRP4 deletion enhances the combined effect of cAMP activation and of lipid raft disruption on inhibition of platelet aggregation

Given the reported respective roles of MRP4 and lipid rafts on cAMP homeostasis as detailed in the indroduction, we investigated the links between these factors in the regulation of platelet activation. For this purpose, transgenic MRP4-deficient mouse platelets were compared to those of WT animals for PAR4-ap-induced aggregation (i) in the presence of MβCD that, by depleting membranes from their cholesterol, is able to disturb lipid rafts, and (ii) upon the addition of an activator of the cAMP pathway. We previously showed that MRP4-deficient platelets exhibit a diminished aggregation response when activated by low PAR4-ap concentrations, due to increased basal levels of cytosolic cAMP[19]. In the present study, to overcome this defect of activation, platelet aggregation was induced by high PAR4-ap concentration, i.e. 100 µM. In this condition, maximal aggregations did not differ between genotypes with values of 69% [54-74] and 62% [53-69] for WT and MRP4-deficient platelets, respectively.

When platelets were exposed to increasing amounts of MβCD prior to PAR4-ap activation, a concentration-dependent inhibition of aggregation was observed. Low MβCD concentration (0.25 mM) did not significantly inhibit platelet aggregation, with 17% inhibition [1-38] for WT and 24% [0-38] for MRP4-deficient platelets, respectively ($p > 0.05$ when compared to control) (Figure 1, A-left panel and B: upper graphs for representative aggregation tracings). At 0.5 mM MβCD, a significant inhibition of platelet aggregation was evidenced for both genotypes, with 27% inhibition [20-61] for WT and 46% [12-68] for MRP4-deficient platelets, respectively. This effect increased with MβCD concentration to reach more than 90% inhibition at 1 mM MβCD. No significant differences were noted between genotypes whatever the MβCD concentration, even if a trend to a higher inhibition existed for MRP4 deficient platelets at MβCD concentrations above 0.75 mM. These results suggest that under such conditions of strong platelet activation, aggregation depending on the maintenance of lipid rafts does not or barely implicates MRP4 activity.

Similar experiments were conducted in parallel in the presence of forskolin that activates AC and thus induces cAMP generation. When lipid rafts were first disturbed with MβCD, the inhibition of WT platelet activation induced by forskolin was enhanced when compared to that in the absence of MβCD (Figure 1, A-right panel and B: lower graphs). These results suggest that cAMP signalling resulting from AC activation does not need the integrity of lipid

rafts to develop its inhibitory activity, but even more that forskolin inhibitory activity is increased as lipid rafts are progressively disrupted. Of note is that similar results were obtained with washed human platelets (Supplemental Figure 1).

In these conditions of strong platelet activation, the inhibition produced by 1 µM forskolin in the absence of MβCD did not differ between genotypes, with 34% [25-39] inhibition for MRP4-deficient platelets *vs* 24% [19-28] for WT platelets (p>0.05) (Figure 1). However, with the pre-exposure of platelets to MβCD 0.5 or 0.75 mM, the inhibition of MRP4-deficient platelet aggregation was significantly higher compared to that of WT platelets (79 % [72-93] vs 62% [51-70] (p=0.01) and 94 % [92-97] vs 70 % [46-75] (p<0.05 and p<0.001), respectively). Thus, whereas no interaction was observed between the inhibitory effect of MβCD and that due to the absence of MRP4 in the absence of forskolin as described above (p>0.05, Figure 1 left panel), a significant interaction occurred when forskolin was present (p=0.026) (Figure 1 right panel). These results suggest that the absence of MRP4 increases the inhibitory effect of cAMP on platelet activation, as seen consecutively to the destruction of lipid rafts by MβCD and when AC is activated by forskolin.

Quantification of cAMP according to lipid raft integrity in combination with MRP4 deletion

We next quantified the effect of MβCD on cAMP level in mouse platelets at rest. Under basal conditions, in the absence of forskolin or PGE_1, total cAMP level was very low, whether or not WT platelets were treated with MβCD (9.2 [7.2-19.6] and 8.6 [6.1-18.3] pmol/10^9 platelets, with and without MβCD (0.5 mM), respectively; p>0.05, n=10). Therefore, all experiments comparing WT and MRP4-deficient platelets for cAMP levels were performed after activation of the cAMP pathway. For this purpose, platelets were thus pre-incubated with PGE_1 to activate the Gs protein-coupled receptor IP, leading to AC activation with the aim to take into account the entire signalling pathway. As previously reported[19], deletion of MRP4 did not impact total cAMP level in platelets following PGE_1 addition, with concentrations of 58.7 [32.6-113.7] and 55.5 [18.8-104.7] pmol/10^9platelets for WT and MRP4-deficient platelets, respectively (p=0.92) (Figure 2). When platelets were pretreated with 0.5 mM MβCD, then exposed to PGE_1, total cAMP levels significantly increased to 140.4 [80.5-236.8] and 121.4 [39.3-192.3] pmol/10^9platelets for WT and MRP4-deficient platelets, respectively (p<0.001 and p=0.0016 compared to WT and MRP4-deficient platelets respective control values without MβCD) (Figure 2), with no difference between genotypes

(p=0.76). The 0.5 mM MβCD concentration was chosen as it exerted an additive effect of inhibition of platelet aggregation in the presence of forskolin in MRP4-deficient platelets as compared to WT platelets (Figure 1). These results thus suggest that, if maintenance of membrane lipid rafts appears to play a major role in the regulation of total cAMP levels in platelets exposed to PGE_1, it occurs independently of MRP4, notwithstanding cAMP intracellular distribution in MRP4-deleted platelets as compared to WT platelets. Similar results were obtained using forskolin instead of PGE_1 (data not shown).

MRP4 is involved in an efflux of cAMP out of resting platelets incubated with PGE1

MRP4 present on the platelet dense granule membrane has been reported to efflux cAMP from cytosol to granules[14,18,20]. However, the presence of MRP4 on the platelet plasma membrane remains discussed. Interestingly, when quantifying cAMP levels in the extracellular space of mouse platelet suspensions at rest in the presence of PGE_1, we measured a basal efflux of this cyclic nucleotide from WT platelets, amounting 12 [8.5-16.3] pmol/10^9platelets (Figure 3, white boxes), which represented 17.3% [12.2-23.5] of the total cAMP of the platelet suspension (compare the corresponding boxes in Figures 2 and 3). The cAMP spontaneously released by MRP4-deficient platelets (7.3 [2.3-10.2] pmol/10^9platelets) was significantly lower compared to WT platelets (p=0.006) (Figure 3, hatched boxes), corresponding to 10.4% of the total cAMP [3.3-14.5] (p<0.001 compared to WT platelets), thus suggesting that this basal efflux of cAMP was partially dependent on the presence of MRP4 on the surface of platelets exposed to PGE_1. Of note is that platelets were prepared in the presence of inhibitors, PGE_1 and apyrase (see Methods section) to avoid activation during isolation, further ascertained by the absence of CD62P exposure on their plasma membrane, a marker of alpha granule exocytosis during platelet activation (data not shown). Interestingly, addition of 0.5 mM MβCD that significantly increased total platelet cAMP in combination with PGE_1 (see Figure 2), did not significantly enhance the basal cAMP efflux in WT platelets (13.7 [7.9-20.2] in the absence of MβCD vs 12 [8.5-16.3 pmol/10^9platelets in its presence, p=0.09) nor in MRP4-deficient platelets (9 [5.9-11.7] vs 7.3 [2.3-10.2] pmol/10^9platelets, p=0.17) (Figure 3), suggesting a saturable transport of cAMP outside the cells. As a consequence, when expressed as a percentage of total cAMP, efflux was found significantly lower in the presence of MβCD, as compared to that seen for platelets with intact lipid rafts, with values of 9.4% [5.5-13.8] (p<0.001) for WT platelets and 5.1 % [3.3-6.7]

(p=0.0087) for MRP4-deficient platelets. Interestingly, this difference in basal efflux observed between genotypes in the absence of MβCD was maintained after preexposure to MβCD (p=0.004 for absolute values and p=0.017 for percentages), suggesting that lipid rafts integrity does not play a major role in the part of basal cAMP efflux that is mediated by MRP4, and represents about half of the efflux.

Evaluation of the presence of MRP4 and of proteins involved in the platelet cAMP pathway in membrane lipid rafts

The fact that a significant interaction existed , in the presence of forskolin, between the inhibitory effect on aggregation of 1/ MβCD and 2/ the absence of MRP4; independently of total cAMP concentration, prompted us to characterize the potential relationships between lipid rafts, MRP4, and their role in the maintenance of the intracellular cAMP homeostasis. We thus investigated whether or not MRP4 could be localized in lipid rafts, together with various effectors that can operate in the cAMP signalling pathway.

Membrane lipid rafts were isolated from human washed platelets at rest or after activation with PAR1-ap, using ultracentrifugation on a sucrose density gradient as detailed in the Method section. Lipid rafts are characterized by their enrichment with specific proteins, in particular the transmembrane protein LAT[31,32]. In platelets at rest, LAT was mainly found in fractions of intermediate density in sucrose gradients (Figure 4, top panel), similar to those previously described as containing lipid rafts insoluble in 0.5 % Triton X-100[33]. Approximately two thirds of LAT protein was associated with lipid rafts, whereas the residual amount was recovered in the high-density sucrose fractions. When platelets were preexposed to MβCD for depletion of membrane cholesterol, the consequent disruption of lipid rafts was ascertained by the disappearance of LAT from the intermediate density sucrose fractions and its shift to high-density fractions (Figure 4, middle panel). Upon platelet activation, the distribution of LAT protein in sucrose gradients was similar to that seen for resting platelets, suggesting that activation does not drastically alter lipid rafts (Figure 4, bottom panel).

We first investigated if the proteins responsible for the synthesis of cAMP (Gs and AC3), for the inhibition of its synthesis or for its degradation (Gi, PDE2 and PDE3A), as well as its principal effector (PKA) were detectable in the lipid rafts. The most important proteins found in the lipid raft fractions were the Gi protein and the regulatory subunit I of PKA, accounting for ≈ 70% and ≈ 50% of their total amount, respectively. The presence of the scaffold AKAP

family member moesin was evaluated in these fractions. Interestingly, the bulk (≥ 95% of the total protein) of moesin was found outside the lipid rafts, suggesting that moesin was not the major AKAP for localization of PKA into lipid rafts. After platelet activation, a similar part of the PKA-RI and of the Gi proteins remained associated with rafts (Figure 4). On the contrary, Gs as well as PDE2 and 3A isoforms were found as traces (≤ 5% of the total proteins) in the lipid raft fractions in both resting and activated platelets.

Concerning MRP4, a minor fraction (≈ 15%) of the total platelet was present in lipid rafts in both resting and activated platelets (Figure 4). Of note, exposing platelets to MβCD shifted the entire MRP4 as well as PKA to high density fractions exactly as seen for LAT, thus confirming that these proteins are partially associated with lipid rafts (data not shown).

Discussion

The aim of our study was to characterize the potential relationships between membrane lipid rafts and the MRP4 transporter in the maintenance of the intracellular cAMP homeostasis. Indeed, in other cell types, the cAMP pathway is regulated at the molecular level through involvement of many actors, including MRP4, which are found to be structurally organized into submembrane microdomains associated or not with the lipid raft domains[24,34]. However, if the interdependence between these processes has been addressed previously for what is concerned with blood platelets[30], the potential location of MRP4 within rafts and/or cAMP microdomains and its interactions with the regulation of the cAMP pathway remain to be characterized.

Given the complex distribution of the different players of the cAMP pathway, in and/or outside lipid rafts, we sought to characterize more precisely the potential interaction between rafts and MRP4 in cAMP homeostasis. To that aim, we used platelets from WT or MRP4-deficient mice pre-exposed to MβCD, a compound known to deplete biological membranes from their cholesterol and thus to disrupt lipid rafts[35]. Our results showed a concentration-dependent inhibitory effect of MβCD on PAR4-ap-induced mouse platelet aggregation, confirming that lipid raft disorganisation alters platelet reactivity to agonists[36]. The same features were observed for PAR1-ap-induced human platelet activation upon exposure to similar concentration of MβCD. In these conditions, namely a strong pro-

aggregatory activation occurring in the absence of a specific activation of the cAMP pathway in parallel, the extent of the inhibitory effect of MβCD did not differ between mouse platelets expressing MRP4 or those deleted for the transporter. These results indicate that, in the absence of specific activation of the cAMP pathway, regulation of aggregation depending on the integrity of lipid rafts does not depend on MRP4. To further evaluate the relationships between lipid rafts, MRP4 and the cAMP pathway, platelets previously treated with MβCD were exposed to forskolin in order to stimulate cAMP synthesis. In such conditions, the extent of inhibition of platelet aggregation significantly exceeded that produced by MβCD or forskolin used separately for both WT and MRP4-deficient platelets (compare figure 1 left and right panels). Whereas no interaction was observed between the inhibitory effect of MβCD and that due to the absence of MRP4 in the absence of forskolin, a significant interaction existed between these two variables in the presence of forskolin. Overall, these results suggest that lipid rafts participate to the control of cAMP homeostasis and that the absence of MRP4 increases the inhibitory effect of cAMP on platelet activation once the cAMP synthesis pathway is specifically stimulated.

In the aim to verify if the effect described above is due to a modification of cAMP metabolism, platelet cAMP levels were compared in MRP4-deficient *versus* WT platelets in the presence of MβCD. Destruction of lipid rafts induced a three-fold increase in total cAMP in platelet suspensions exposed to PGE_1, which is in agreement with the inhibition of PAR4-ap-induced aggregation in both WT and MRP4-deficient platelets exposed to forskolin described above. However, MRP4 deficiency did not modify total cAMP levels, whatever the presence or the absence of lipid raft integrity, which is in favor of the absence of a role for MRP4 in the regulation of cAMP metabolism (synthesis and/or degradation).

Next step was to search for the presence of players of cAMP pathway in lipid rafts in the aim to evaluate if potential cAMP microdomains, as described above[23-26], are located in lipid rafts and if the destruction of these structures might explain the increased level of cAMP induced by MβCD in combination with forskolin or PGE_1. Considering that it was already shown that a minor fraction of the platelet AC5/6 isoforms of adenylate cyclase are present in the lipid rafts[30], we focused on the AC3 isoform since it has been formerly identified in human platelets[37]. It appears from our results that AC3, but also PDE2A and PDE3A could be detected only as trace amounts in lipid rafts of resting as well as activated platelets. By

contrast, and in agreement with previous reports[38], we identified the major fraction of the platelet Gi protein in lipid rafts, where it should be theoretically able to inactivate the small fraction of AC isoforms present in rafts. On the opposite, the Gs protein was found outside lipid rafts. This is in line with previous data showing Gs as well as the PGI_2 receptor IP to be entirely located outside of the rafts[30]. When analysing the location of MRP4 within lipid rafts in human platelets, we found that most part of the MRP4 protein was detected outside lipid rafts. However, approximately 15 % of MRP4 was found associated with these membrane structures. Our results are therefore in favor of players in the synthesis and degradation of cAMP being essentially outside the membrane lipid rafts, while MRP4 shows a minor fraction in lipid rafts. The signalling activity of cAMP being dependent of its effector PKA, the presence of the latter in the lipid rafts was also evaluated. We found PKA-RI to be located both inside and outside lipid rafts, in line with previous data[27].

Thus, our present results together with previously published data[39] are in favor of cAMP microdomains mostly including Gs, PDE, moesin and PKA[23,24], to be spatially distinct from lipid rafts in platelets. It remains to clearly demonstrate the existence of such structures in platelets. Lipid rafts would in contrast play a downregulatory role on cAMP-signalling pathway, probably by separating various players, as previously suggested[30]. Indeed, the major players of cAMP synthesis were found outside the rafts, and the bulk of PDE2 and PDE3 that is localized outside rafts could represent a “barrier” regulating the cAMP available in the cytosol as previously proposed in nuclear cells such as cardiomyocytes or smooth muscle cells, as part of a cAMP microdomain[40,41].

Besides, the role of rafts in the metabolism of cAMP, as shown by cAMP increase when rafts are disrupted, suggest that, by trapping part of the PKA-RI platelet pool, lipid rafts would keep it non available for activation by cAMP. Therefore, the potentiation of the inhibitory effect of forskolin after lipid raft disruption, both in human platelets and in WT or MRP4-deficient mouse platelets, could be explained by a release of PKA-RI from the rafts that would reinforce the in situ activity of the PKA-RI present outside rafts. PKA-RI location near the cytosolic leaflet of the plasma membrane by scaffold AKAP has been proposed to be essential to its function[27], and moesin may be one of the AKAP responsible for this process in platelet lipid rafts[42]. In a model involving platelet activation via the von Willebrand factor/platelet GPIb receptor axis, the use of a peptide blocking the binding of PKA to AKAP

resulted in a decreased activity of PGI_2 as an antagonist of platelet activation, thus underlining the importance of the binding of PKA by AKAP for a full biological activity of cAMP, at least in this model[27]. However, our results establish that moesin is barely detectable in lipid rafts of resting platelets, and thus should not be the major AKAP that allows localization of PKA-RI at least in rafts. Therefore, further identification, characterization and distribution of platelet AKAPs remain important goals to get further insights about the spatial regulation of cAMP pathway.

Finally, and as expected, MRP4 played a role in platelet activability. To explain the significant interaction between MRP4 deficiency and lipid raft destruction on platelet inhibition (Figure 1), we searched for a potential impact of lipid destruction on cAMP efflux in particular the efflux dependent on MRP4, which may rely on the fraction found in lipid rafts. To this purpose, we used WT platelets incubated with PGE_1 and evidenced an efflux of cAMP corresponding to about one fifth of the total platelet cAMP. We provide evidence that this cAMP efflux, which is independent of platelet activation and granule secretion, is partly dependent on MRP4, since it was markedly diminished in MRP4-deficient platelets (figure 3) despite the fact that these platelets have similar total cAMP level than WT platelets. Whereas we and others[15,19] have identified the major role for MRP4 in the regulation of cytosolic cAMP level, its presence on plasma membrane and/or on the dense granule membrane remains a matter of debate. However, the present results bring new arguments in favour of the presence of MRP4, at least partially, on the platelet plasma membrane at rest where it may participate to cAMP efflux. Since MRP4-deficient platelets were still able to efflux part of the cAMP accumulated through synthesis activated by PGE_1, it is likely that another transporter is also involved in this efflux. Noteworthy, results obtained using MβCD to disrupt lipid rafts bring further information. Indeed, the fact that the increase in total platelet cAMP observed in such conditions, which is similar in normal and in MRP4-deficient resting platelets (figure 2), did not result in corresponding increased efflux would suggest a saturated transport for this efflux. Other explanation would be that the MRP4-independent fraction of the efflux is partially dependent on the integrity of the rafts. Furthermore, the fact that the difference in cAMP efflux between WT and MRP4-deficient platelets, incubated with PGE_1, was still observed after exposure to MβCD suggests the MRP4-dependent basal efflux of cAMP is independent of lipid raft integrity despite the presence of a fraction of

MRP4 in lipid rafts. Therefore, the synergistic effect between increased cAMP level due to lipid raft destruction and the absence of MRP4 is most likely due to the result of the increase of cAMP level and the absence of MRP4-dependent efflux, responsible for greater in situ inhibitory activity of the cAMP signalling. Thus, differential spatial distribution of all players and lipid raft integrity are both necessary for maintaining cAMP homeostasis in platelets.

In conclusion, our results confirm a role for lipid rafts on cAMP signalling homeostasis in platelets. However, MRP4 that has the capacity to efflux cAMP from cytosol to dense granules and outside the cells and that is partly associated to lipid rafts, was not or only slightly involved in this particular mechanism. We propose the existence of cAMP microdomains containing AKAP and PDE outside lipid rafts in the membrane of resting platelets. However, independently of rafts, the transporter MRP4 plays a positive role in platelet activability, by decreasing cytosolic cAMP[19], not only by its capacity to allow storage of cAMP in dense granules[21], but also, as shown here and previously by others, by participating to a basal cAMP efflux from resting platelets[15]. All these data point to MRP4 as a potential target for an antithrombotic agent with vascular properties[14], beside molecules targeting directly the platelet cAMP pathway at the production level, such as anti-$P2Y_{12}$ and PDE inhibitors[12,13].

What is known on this topic?

- MRP4 is a transporter for cyclic nucleotides
- MRP4 localization on platelet dense granules and/or plasma membrane is still a matter of debate
- Nothing is known on the presence of MRP4 in lipid rafts

What this paper adds?

- MRP4 is present, at least partly, on platelet plasma membrane
- MRP4 is present at 14% in lipid rafts
- Most of players of cAMP pathway in platelets are located outside lipid rafts

Authors' Contributions

TBR conceived the study, designed and performed research, analysed data and wrote the manuscript.

AL participated in the research, analysed data, and participate in writing the initial and revised versions of the manuscript.

AM, BD, FM, SC, MR, HG, BD, CBL performed research and, together with DP, revised the manuscript and gave final approval.

CBL and PG conceived the study, designed research, analysed data and wrote the manuscript.

Conflicts of interest

Authors declare no conflict of interest.

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Figure 1: Plasma membrane lipid rafts counteract the inhibition of platelet activation resulting from engagement of the cAMP signalling pathway in a manner partially dependent on MRP4

Washed platelets of WT (A: empty boxes; B: left panels) and MRP4-deficient mice (A: plain boxes; B: right panels) were treated with increasing MβCD concentrations for 30 min at 37°C and thereafter incubated with vehicle (A: left panel; B: upper graphs) or 1 µM forskolin (A:

right panel; B: lower graphs) for 15 sec. Platelet activation was then induced by 100 µM PAR4-ap. A: Results are expressed as the percentage of inhibition of platelet aggregation at 120 sec relative to aggregation obtained in the presence PAR4-ap alone (n≥5) ; B: representative tracings of aggregations measured under stirring in a microplate reader set at 37°C as described in supplemental data.

Figure 2. Lipid rafts negatively control cAMP levels in platelets, independently of MRP4.

WT (empty boxes) or MRP4-deficient (hatched boxes) washed platelets were treated with 0.5 mM MβCD or vehicle for 30 min at 37°C. At the end of the incubation, 1 µM PGE_1 was added for 90 sec. cAMP was quantified on total non activated-platelet lysates obtained by addition of lysis buffer containing 4 mM IBMX and 20 mM EGTA. cAMP from total lysates was quantified using cAMP Dynamic 2 kit (Cisbio Bioassays). Results are expressed as pmol/10^9 platelets (n≥7).

Figure 3. An efflux of cAMP from resting platelets incubated with PGE_1 exists and is partly dependent on MRP4, irrespective of the lipid raft integrity.

WT (empty boxes) or MRP4-deficient (hatched boxes) washed platelets were treated with 0.5 mM MβCD or vehicle for 30 min at 37°C. At the end of the incubation, 1 µM PGE_1 was added for 90 sec. cAMP was quantified on extracellular fractions obtained by centrifugation (30 sec at 16000 g) of the platelet suspension supplemented with 20 mM EGTA and then collection of supernatants and addition of lysis buffer with IBMX. cAMP from supernatants was quantified using cAMP Dynamic 2 kit (Cisbio Bioassays). Results are expressed as pmol/10^9 platelets (n≥7).

Figure 4: Players of the cAMP signalling pathway, including MRP4, are mostly located outside membrane lipid rafts.

Resting (upper panel) or PAR1-ap-activated (20 µM, 4 min, 37°C; lower panel) human washed platelets (500 µl at 10^9/mL) were lysed in the presence of 0.5% Triton X- 100 and submitted to insoluble lipid raft isolation through ultracentrifugation on sucrose density gradients as described in Materials and Methods. In some experiments, a sample of ~~resting~~ non activated washed platelets was preincubated 30 min with MβCD to disrupt lipid raft

domains (middle panel). Twelve fractions were harvested starting from the top of the sucrose density gradient. Proteins from each fraction of the gradient were precipitated by trichloroacetic acid (TCA) and acetone, submitted to PAGE and immunoblotted with specific antibodies directed against LAT (36 kDa), Gi (41 kDa), PKA (48 kDa), MRP4 (150 kDa), AC3 (130 kDa), PDE3A (125 kDa), PDE2A (100 kDa), moesin (75 kDa) and Gs (46 kDa). Visualization was obtained with Dylight-800 or -680 goat anti-rabbit or anti-mouse antibodies and then the bands were quantified using the software ImageJ and expressed as the percentage in or outside the lipid raft fractions (right tables; mean ± SEM). Blots are representative of 10 independent experiments using platelets from 10 donors.

Annexe 2. Evaluation of commonly used tests to measure the effect of single-dose aspirin on mouse hemostasis.

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Original research article

Evaluation of commonly used tests to measure the effect of single-dose aspirin on mouse hemostasis

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ABSTRACT

Discrepancies in preclinical studies of aspirin (ASA) antiplatelet activity in mouse models of bleeding and arterial thrombosis led us to evaluate commonly reported methods in order to propose a procedure for reliably measuring the effects of single dose ASA on mouse hemostasis.

FVB and C57Bl6 mice received 100 mg/kg of ASA or vehicle orally 30 min or 3 h prior to investigate either hemostasis using the tail bleeding assay or carotid thrombosis induced by $FeCl_3$, or to blood sampling for isolated platelet aggregation and TXB_2 generation.

Expected inhibition of COX1 by ASA was ascertained by a strong decrease in TXB_2 production, and its effect on platelet function and hemostasis, by decreased collagen-induced aggregation and increased bleeding time, respectively. Strikingly, we determined that anti-hemostatic effects of ASA were more predictable 30 min after administration than 3 h later. Conversely, ASA did not alter time to arterial occlusion of the carotid upon $FeCl_3$-induced thrombosis, suggesting ASA not to be used as reference inhibitor drug in this model of arterial thrombosis.

1. Background

Acetyl salicylic acid (ASA or aspirin) at low dose is broadly used for its antiplatelet effect in primary and secondary prevention of cardiovascular diseases [1]. ASA irreversibly acetylates the Ser529 residue of COX1, thereby leading to a steric hindrance of the COX channel that prevents access of the substrate to the catalytic site of the enzyme [2]. COX1 is responsible for the conversion of arachidonic acid into prostaglandins, that are precursors of thromboxane A_2 (TXA_2) in platelets [3]. By binding the thromboxane and prostanoid receptor (TP receptor), platelet-secreted TXA_2 enhances platelet activation initially triggered by agonists such as collagen. By limiting one major amplification pathway of platelet activation [4], ASA is therefore a relevant comparator for preclinical studies of antiplatelet agents under development.

Interestingly, whereas ASA pharmacology is well known in humans, its antiplatelet activity is inconsistently described in mouse models. The discrepancies between studies, such as inconsistent effects on mouse arterial thrombosis or bleeding time, could be the consequence of differences regarding the animal strain, the route of administration and ASA doses, as well as the hemostatic parameters evaluated *in vivo* and *ex vivo*. Doses most frequently found in the literature may vary from 1 to 100 mg/kg, given as single or repeated doses [5–10]. Various routes are also reported: intravenous, oral, intraperitoneal or subcutaneous. Whereas the most used bleeding model is the tail-tip transection, arterial thrombosis models in use are more diverse. Indeed, arterial thrombosis of carotid, cremaster or mesenteric artery may be induced by $FeCl_3$, laser or mechanical injury [11]. Finally, the delay of testing the effect of ASA after administration may vary from 10 min to 24 h after administration [7,9,12].

2. Objectives

Starting from commonly used experimental conditions concerning delivery of ASA and testing its antiplatelet effects in mice, our aim was

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to determine a procedure for reliably measuring the effects of single dose ASA on mouse hemostasis.

3. Methods

3.1. Animals

FVB and C57Bl6 (C57BL/6NRj) mice (males, 8–12 weeks old; weight 27.6 gr ± 2.3, mean ± SD) were from Janvier Labs (Le Genest-Saint-Isle, France). Anesthesia was induced by intraperitoneal injection of 80 mg/kg ketamine (Clorketam® 1000, Vetoquinol, Lure, France) and 10 mg/kg xylasine (Rompun® 2%, Bayer, La Garenne-Colombe, France), or with 2.5% inhaled isofluorane (Vetflurane®, Virbac, Carros, France) when performing tail bleeding time experiments. ASA (DL-lysine acetylsalicytate, Aspegic®, Sanofi-Aventis, Gentilly, France) dissolved in distilled water was administered orally by force-feeding or by intraperitoneal (i.p.) injection (100 mg/kg, 10 µl/gr). In *vivo* experiments were done by an observer blinded to the treatment group. All animal studies were approved by the Ethics Committee on Animal Resources of Paris Descartes University (registration number: CEEA34.CBL.131.12).

3.2. Platelet preparation

Blood was collected by cardiac puncture into ACD-C solution (13 mM citric acid, 12.6 mM sodium citrate, 11 mM D-glucose) and diluted with wash buffer (36 mM citric acid, 5 mM D-glucose, 5 mM potassium chloride, 2 mM calcium chloride, 1 mM magnesium chloride, 103 mM sodium chloride, pH 6.5) containing apyrase (0.1 U/ml; Sigma-Aldrich, St. Louis, MO, USA) and prostaglandin E_1 (PGE_1, 1 µM; Sigma-Aldrich). Diluted platelet-rich plasma (PRP) was obtained by 7 min centrifugation at 170*g*. Platelets were washed twice with wash buffer containing apyrase and PGE_1, and then centrifuged for 10 min at 750*g*. The pelleted platelets were resuspended in assay buffer (10 mM HEPES, 140 mM sodium chloride, 3 mM potassium chloride, 5 mM sodium bicarbonate, 0.5 mM magnesium chloride, 10 mM D-glucose, pH 7.35) to a concentration of 3.5×10^8 /ml. Calcium chloride 2 mM was then added.

3.3. Platelet aggregation studies

Platelet aggregation was measured on a Discovery HT-R microplate reader (MWG Biotech AG, Ebersberg, Germany) coupled to the KC4 software for analysis of the data. FVB mouse washed platelets (3.5×10^8/ml) were incubated for 2 min at 37 °C under stirring in wells of a 96-well microplate (Greiner Bio-one, Frickenhausen, Germany) in a volume of 90 µl, then aggregation was induced by adding 5 µg/ml fibrillar type-I collagen from equine Achilles tendon (Horm, Nycomed, Linz, Austria) or 1 µM U46619, a TP synthetic agonist (Calbiochem, Merck, Darmstadt, Germany). Aggregation was monitored for 5 min and expressed as the percentage change in absorbance at 405 nm as previously described [13].

3.4. Bleeding assay

Tails of anaesthetized mice were pre-incubated in a 37 °C saline solution during 5 min to homogenize vessel dilatation between animals. Then, bleeding time was measured following a 3-mm tail-tip transection, and immediate immersion of the tail in 10 ml of isotonic saline at 37 °C. Bleeding time was set at cessation of blood leakage for at least 1 min. Blood loss was estimated by measuring the hemoglobin concentration in the saline, using the Drabkin method.

3.5. Thrombosis assay

Mice were anaesthetized and maintained at 37 °C on a heating plate. The left carotid artery was exposed and dissected away from the vagus nerve and surrounding tissues. Carotid artery blood flow was monitored with a Doppler flow meter equipped with a Transonic flow probe (Model MA0.5PSB, Transonic System Inc, Ithaca, NY). Arterial thrombosis was induced by placing a 15% $FeCl_3$-saturated filter paper on the artery, 5 mm upstream the flow probe, for 2 or 4 min. Monitoring of blood flow was maintained for 5 min after the cessation of flow, and the time required for occlusion was recorded.

3.6. Thromboxane assay

Thromboxane B_2 (TXB_2) level was measured with the thromboxane assay kit from R&D system (Abingdon, UK). Assays were performed on washed platelet supernatant obtained after collagen-induced platelet aggregation. Ten min after the addition of collagen, 20 mM EDTA was added and the sample were centrifuged 2 min at 12,000 g. The supernatant was kept frozen at −20 °C until tested.

3.7. Data analysis

Data were expressed as medians [95% confidence interval (CI)] for non-normally distributed variables. Statistical analysis was performed with the Prism software package (GraphPad Software, Inc., San Diego, CA, USA). The Mann-Whitney test was used to compare each parameter. Differences were considered significant when $P < .05$.

4. Results

The impact of ASA administration on mouse hemostasis was assessed by varying the time elapsed between ASA administration and testing (30 min *versus* 3 h), as well as the mouse strain (FVB *versus* C57Bl6).

4.1. Bleeding experiments

FVB mice. The impact of the period of time between oral ASA administration and bleeding time measurement was first tested in FVB mice. When ASA was given 30 min before measuring bleeding time, this hemostatic parameter was strongly increased compared to vehicle (88 s [95% CI, 44–95] *vs*343 s [95% CI, 250–600] for vehicle and ASA respectively; $P < .0001$; Fig. 1A). Conversely, when administered 3 h before the bleeding assay, ASA inconsistently modified the bleeding time compared to vehicle (161 s [95% CI, 45–600] *vs*213 s [95% CI, 99–600] for vehicle and ASA respectively; $P > .05$; Fig. 1A), mostly because a high variability in values. However, no significant difference in bleeding time between the ASA groups was observed (30 min *vs* 3 h, $p = .16$).

Compared to bleeding time, measuring blood loss to evaluate the anti-hemostatic effect of ASA was not informative since it did not significantly differ from controls neither 30 min (14 µl [95% CI, 6–29] *vs* 21 µl [95% CI, 11–97] for control and ASA respectively; $P > .05$; Fig. 1B) nor 3 h after ASA administration (15 µl [95% CI, 6–23] *vs* 36 µl [95% CI, 10–73] for control and ASA respectively; $P > .05$; Fig. 1B).

C57Bl6 mice. To evaluate the potential relevance of the animal genetic background on responsiveness to ASA, we tested whether the widely used C57Bl6 (C57BL/6NRj) strain could give comparable results than the FVB strain. When ASA was given to C57Bl6 mice 30 min before the assay, the bleeding time was also found strongly increased (44 s [95% CI, 5–83] *vs*320 s [95% CI, 205–600] for control and ASA respectively; $P < .01$; Fig. 1C). On another hand, blood loss was significantly, although slightly, increased by ASA (7.5 µl [95% CI, 5.8–9.7] *vs* 10.5 µl [95% CI, 9–18] for control and ASA respectively; $P < .05$; Fig. 1D). However, such as for FVB mice, ASA administered 3 h before the tail cutting did not modify the bleeding time (53 s [95% CI, 44–64] *vs*128 s [95% CI, 5–600] for control and ASA respectively; $P > .05$; Fig. 1C) nor blood loss values (5.5 µl [95% CI, 3.6–13.3] *vs* 10.4 µl [95%

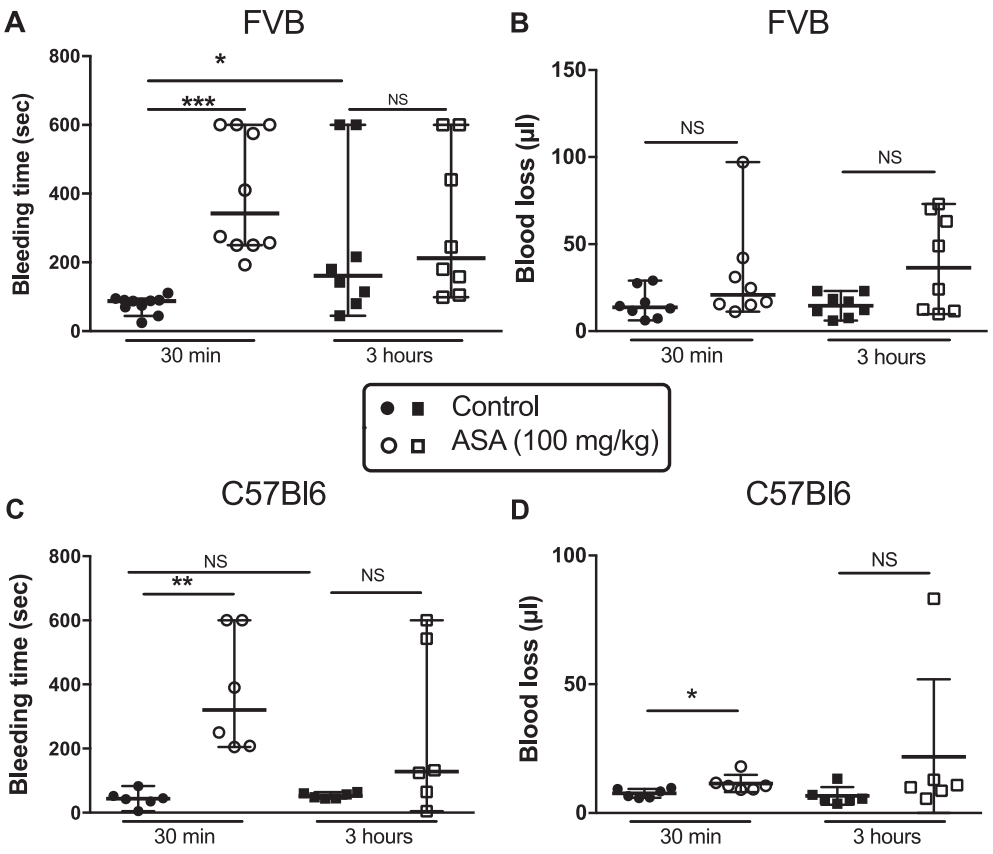

Fig. 1. *In vivo* ASA effect on bleeding time and blood loss. Bleeding time was measured 30 min or 3 h after vehicle (closed circles) or ASA at 100 mg/kg oral administration (open circles) for FVB (A; $n \geq 8$) or C57Bl6 (C; $n \geq 6$) mice. Hemoglobin concentration in the chamber effluent (containing 10 ml NaCl 0.9%) following bleeding time was measured for FVB (B; $n \geq 8$) and C57Bl6 (D; $n \geq 6$) mice.

CI, 5.5–83.2] for control and ASA respectively; $P > .05$; Fig. 1D). No significant difference in bleeding time between the ASA groups was observed (30 min *vs* 3 h, $p = .18$, Fig. 1C).

4.2. Carotid artery thrombosis

We next tested the impact of ASA on the most commonly used *in vivo* artery thrombosis model: the $FeCl_3$-induced injury of the carotid artery [11]. Surprisingly, ASA administered by oral route 30 min or 3 h before the experiment did not modify the time to the total occlusion whatever the mouse strain, FVB or C57Bl6 (Table 1).

Then, we tested a repeated administration of ASA for 4 days in C57Bl6 mice. In order to check if the absence of effect of aspirin on the thrombosis model was not due to a lack of sensitivity of our method, the $FeCl_3$ patch was left during only 2 min. Again, no difference in the time to occlusion was evidenced between aspirin and placebo groups (17 min [95% CI, 13.8–19.5] *vs* 16.3 min [95% CI, 15.1–17.3], respectively) (Supplemental Fig. 1A).

Moreover, in order to verify that the route of administration was not responsible for the absence of anti-thrombotic activity of ASA in this model, we reproduced experiments using the i.p. route, which is largely used in preclinical pharmacology in mice, including ASA studies [6,9,14]. Similarly to the results obtained with the oral route, and whatever the time to assay after ASA administration (30 min or 3 h), we did not observe any effect of i.p.-administered ASA on carotid artery thrombosis, neither for FVB nor for C57Bl6 mice (Table 1).

4.3. Platelet aggregation and thromboxane generation

To ensure that the absence of anti-thrombotic effect was not due to a pharmacological inefficacy of a single oral ASA administration for COX-1 inhibition, platelet response to ASA exposure was also evaluated by testing platelet functions *ex vivo*. Aggregation assays were performed on washed platelets isolated from FVB mice, in response to 5 µg/ml collagen or 1 µM U46619, a specific agonist of the TP receptor.

When platelet isolation was done 30 min after ASA had been administered by the oral route, and in line with the results obtained for bleeding time measurement, collagen-induced platelet aggregation was significantly reduced compared to control animals receiving vehicle alone (76% [95% CI, 62–88] *vs* 34% [95% CI, 20–83] for control and

Table 1
In vivo ASA effect on artery thrombosis.

	30 min			3 h		
	Control	ASA (100 mg/Kg)		Control	ASA (100 mg/Kg)	
Oral						
FVB	14.7 [10.7–21.2]	17.4 [10.3–25]	NS	12.8 [9.6–16.5]	14.0 [10.5–25]	NS
C57Bl6	11.7 [8.0–21.1]	14.2 [12.3–25]	NS	11.1 [9.4–25]	14.4 [10.5–16.1]	NS
I.P.						
FVB	11.9 [8.9–15.7]	12.5 [10.1–17]	NS	10.6 [10–11]	11.8 [9.6–14.9]	NS
C57Bl6	11.4 [7.3–14.6]	12.7 [7.9–16.2]	NS	10.4 [8.2–13.4]	12.5 [10.4–15]	NS

Vehicle or ASA were administered to FVB or C57Bl6 mice by the oral or intraperitoneal route (I.P.). Thirty min or 3 h after ASA administration, carotid artery thrombosis was induced and the time to occlusion was recorded. Results are in minutes and expressed as median [95% CI].

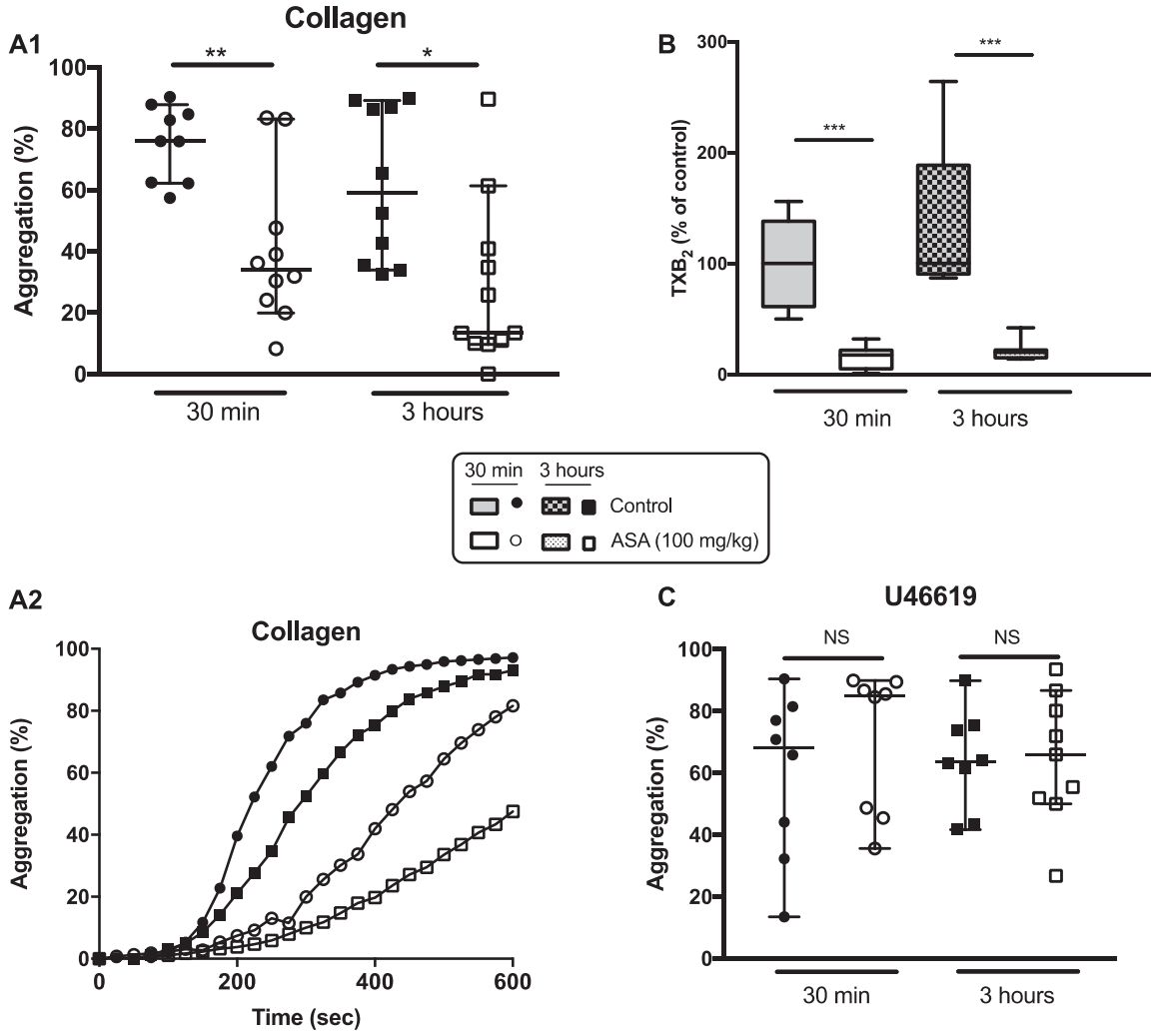

Fig. 2. *Ex vivo* ASA effect on platelet aggregation and TXB_2 synthesis. Blood from FVB mice was taken 30 min or 3 h after vehicle (closed symbols) or ASA at 100 mg/kg oral administration (open symbols). Washed platelet aggregation was measured by monitoring OD (405 nm) variations of a platelet suspension at a concentration of 3.5×10^8 platelets per milliliter under stirring conditions in response to 5 µg/ml collagen (A1; $n \geq 9$; A2: typical aggregation curves) or 1 µM U46619 (C; $n \geq 8$). Results are expressed as the percentage of aggregation (seen as a decrease in OD) 300 s after agonist addition. After collagen-induced aggregation and a centrifugation step, TXB_2 was quantified in the supernatant (B; $n \geq 5$), and results are normalized relative to the vehicle condition (*Control, grey bars*) for each time point.

ASA respectively; $P < .01$; Fig. 2A). This inhibition of platelet aggregation was associated with a high and significant reduction of about 85% TXB_2 generation by platelets of the ASA group (563 pg/10^8 platelets [95% CI, 356–1430] *vs* 81 pg/10^8 platelets [95% CI, 12–297] for control and ASA respectively; $P < .001$; Fig. 2B).

When ASA was administered 3 h before platelet isolation, collagen-induced platelet aggregation was still significantly reduced compared to control platelets without ASA (59% [95% CI, 34–89] *vs* 13% [95% CI, 10–61] for control and ASA respectively; $P < .05$; Fig. 2A). Of note, and whereas TXB_2 generation in platelet supernatant was also significantly decreased at 3 h (1058 pg/10^8 platelets [95% CI, 545–1532] *vs* 210 pg/10^8 platelets [95% CI, 82–270] for control and ASA respectively; $P < .05$; Fig. 2B), the effect of ASA on platelet response to collagen was less significant compared to the 30 min time point (Fig. 2A). Notably, there was no significant difference in collagen-induced aggregation between the ASA groups (30 min *vs* 3 h; $p = .25$).

The efficacy of aspirin after 4 days administration was also checked by a significant inhibition of collagen-induced washed platelet aggregation (82% ± SEM 12% inhibition) and TXB_2 generation in the supernatant (97% ± SEM 0.4% inhibition) (Supplemental Fig. 1B-C). Overall, platelet inhibition measured *ex vivo* was more pronounced after ASA repeated administration compared to the single dose.

As a control for platelet responsiveness, aggregation induced by 1 µM U46619, which directly activates platelets *via* the TP receptor independently of TXA_2 synthesis, did not differ whatever the time of blood sampling after ASA delivery to animals (68% [95% CI, 30–88] *vs* 85% [95% CI, 49–92] and 64% [95% CI, 50–78] *vs* 66% [95% CI, 46–22] at 30 min and 3 h respectively, for control and ASA, respectively; $P > .05$; Fig. 2C).

5. Discussion

Since aspirin remains the gold standard of antiplatelet treatment and an unavoidable reference for other antiplatelet molecules under development, this work was designed to settle appropriate in-house conditions to evidence its effects under a single dose regimen on mouse hemostasis. Indeed, there are major discrepancies found in the literature concerning *in vivo* effects of aspirin in mice. Our present results actually support other studies that failed to demonstrate the antiplatelet effect of ASA in various experimental setups. The two main parameters we chose to evaluate and to settle were (*i*) the period elapsed between ASA administration and bleeding or thrombosis assays, and (*ii*) the *in*

vivo test to evaluate drug efficacy.

Regarding the ASA doses to be administered *per os* in such an evaluation, those that can be found in the literature are more frequently between 5 and 100 mg/kg [6,7,9,14–17]. We show here that a 100 mg/kg ASA dose increased bleeding time as well as efficiently inhibited platelet activation *in vitro*, as evaluated by two recognized assays [18]: washed platelet aggregation and TXB_2 production. We also used a tenfold lower single dose of ASA (10 mg/kg) in some experiments, which turned to result in a high and unacceptable variability in read out data values (data not shown).

Importantly, we analyzed the influence of the period elapsed between ASA administration and assays, and how it can affect evaluation of the ASA effect. In some previously reported studies, assays for ASA efficacy were performed between 16 and 24 h after drug administration [12,19]. However Evangelista et al. have shown that a significant amount of newly released platelets with a fully active COX were present in the circulation 24 h after ASA administration [20]. Therefore, and as they are frequently used in published procedures, we focused on two short periods of time, 30 min and 3 h after ASA was administered orally. These time-points are in agreement with the rapid ASA effect reported for humans after an oral single dose [21]. Whatever the *in vivo* or *ex vivo* endpoint hemostatic test (bleeding time or platelet aggregation), our results show that the 30 min time point ensures more reproducible results as compared to 3 h. In these conditions, we also show that bleeding time is a better parameter than the blood loss to evidence an effect of ASA on hemostasis.

Considering the demonstration by Schiviz et al. of a variability for hemostatic parameters in mice in the absence of drug exposure, depending on the genetic background even between different strains of C57Bl6 [22], we compared the basal and ASA-modulated bleeding parameters in FVB and in C57Bl6 (C57BL/6NRj) mice, all males aged 8–12 weeks. We used these genetic backgrounds that correspond to the KO models currently used in our and many other labs [13,23]. Interestingly, in the absence of ASA treatment, bleeding time was significantly about twice shorter for control C57Bl6 as compared to FVB mice at 30 min, and thrice shorter at 3 h (Fig. 1A *vs* 1C, black dots). In line, blood loss was also always higher in FVB as compared to C57Bl6 mice at either 30 min or 3 h (Fig. 1B *vs* 1D, black dots). In the whole, bleeding experiments appeared to be more predictable using the C57Bl6 (C57BL/6NRj) strain within the limits of the conditions tested.

Finally, we investigated the impact of ASA on arterial thrombus formation with a commonly used model, the carotid artery thrombosis induced by $FeCl_3$ [11]. Although we used a 15% $FeCl_3$ concentration for 4 min, we obtained similar results (see Table 1) than Li et al. who found a time to occlusion of 11.3 ± 3.16 min when using 7.5% $FeCl_3$ on C57Bl6 [24]. Whatever the delay after ASA administration and the mouse strain used, no anti-thrombotic effect of the drug was observed. Therefore, we wondered if this negative result could be due to the administration route. Huang et al. have shown that doses of ASA up to 150 mg/kg given intravenously were not sufficient to increase the time for occlusion of the mesenteric venule exposed to fluorescein sodium, and that 250 mg/kg was needed to observe an effect of ASA [15]. In the same study, however, the 150 mg/kg dose was, nevertheless, found to efficiently increase the tail bleeding time [15]. The same study showed, however, an effect of 40 mg/kg oral ASA on occlusion time. To note, the study used male ICR mice, a strain we did not use in the present work. Intraperitoneal administration being a commonly used route in pharmacological studies in murine models and ASA treatment [6,9,12,14,16,19,25], we also evaluated this type of administration. However, we did not either observe any effect of ASA on thrombus formation under this particular condition. On the whole, and given that an effective inhibitory activity of ASA on platelet functions was observed *ex vivo* at 30 min and at 3 h (see Fig. 2A and B), we can conclude that the carotid artery thrombosis induced by $FeCl_3$ is not a suitable model for the evaluation of ASA anti-platelet effects, at least within the frame of our experimental conditions. Using the $FeCl_3$ injury model, some authors also failed to show any effect or a very moderate effect of ASA on thrombosis, if any [6,9,25,26]. In a model of femoral artery thrombosis, Kondo et al. showed that ASA can increase the time to occlusion of the artery when thrombosis is induced photochemically by using rose Bengal [7], while Nonne et al. failed to show any effect of intravenous ASA in laser-injured mesentery thrombosis in C57Bl6 strain [17]. More recently, Adili et al. showed that ASA induced a decreased platelet recruitment into the arterial wall thrombus in a model of laser-induced cremaster artery thrombosis, without affecting the increase in platelet surface P-selectin-expression within thrombi [27]. Thus, taken together, the already published and our current data show a wide variability in response to ASA treatment in arterial thrombosis models in mice. Data currently converge to a lesser contribution of platelets in the $FeCl_3$ injury model compared to mechanical injury; indeed, $FeCl_3$ injury induces significant damage of subendothelial proteins and attachment of platelets to bodies containing ferric ions and exposing large amounts of tissue factor [28].

Limitations of our study are (*i*) that models using laser to induce carotid injury were not considered, and (*ii*) that thrombus formation was not monitored using real-time intravital microscopy. Major reason for this is that we wanted to use the more commonly $FeCl_3$ injury model. On purpose, testing of a single ASA administration was firstly considered mostly because the objective of the present study was to evaluate this frequently used procedure, and after having verified that a strong TXA2 generation inhibition was reached after a single dose. However, we cannot exclude that a daily administration of ASA could be more effective in limiting thrombosis. Indeed in a model of thrombus formation induced by *in vivo* injection of platelet agonist, Armstrong et al. observed that a chronic ASA dosing (300 mg/kg/day for 7 days) reduced thrombus formation [29]. However, more pathophysiologically relevant studies carried out in models of atherothrombosis did not observe an effect of daily administration of ASA on the lesion [5,10]. To address this controversy, we tested the repeated administration of ASA for 4 days in C57Bl6 mice. Interestingly, whereas global platelet inhibition measured *ex vivo* was more pronounced 4 days after ASA repeated administration compared to the single dose, again no difference in time to occlusion was evidenced between aspirin and placebo groups in the carotid thrombosis model (Supplemental Fig. 1).

Moreover, it must be noted that, upon completion of our study, a recent publication suggested that the time to occlusion might not be the best parameter in order to analyze arterial thrombosis, and authors suggested including reflow events to maximize data interpretation [30].

Third limitation of our study is that the high dosage of aspirin used is not the one used in chronic treatment in patients with high cardiovascular risk.

We have thus demonstrated that specific experimental conditions are required in order to observe and adequately evaluate the effect of ASA on mouse hemostasis. Here, we show that the most relevant endpoint is the tail bleeding time performed with a cut at 3 mm of the tip and at 30 min after oral 100 mg/kg ASA administration to C57Bl6 mice, as our optimal in-house experimental conditions. We do not recommend time to occlusion of $FeCl_3$-induced carotid arterial thrombosis as an index of ASA efficacy on platelets since it is inconsistently altered by ASA. A future consensus debate should define the more relevant method to explore the ASA antithrombotic effect.

CRediT authorship contribution statement

Benoit Decouture: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft. **Alexandre Leuci:** Investigation, Writing - review & editing. **Blandine Dizier:** Data curation. **Tiphaine Belleville-Rolland:** Investigation, Data curation. **Alexandre Mansour:** Investigation, Data curation. **Fanny Martin:** . **Dominique Pidard:** Writing - review & editing. **Pascale Gaussem:** Conceptualization, Supervision, Data curation, Funding acquisition, Validation, Writing - original draft, Writing - review & editing. **Christilla Bachelot-Loza:**

Conceptualization, Supervision, Data curation, Funding acquisition, Validation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2019.08.002.

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Annexe 3. Bleeding and thrombotic events in patients with severe COVID-19 supported with extracorporeal membrane oxygenation: a nationwide cohort study

Title Page

1. Article Title: **Bleeding and thrombotic events in patients with severe COVID-19 supported with extracorporeal membrane oxygenation: a nationwide cohort study**

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Abstract

Purpose: To describe bleeding and thrombotic events and their risk factors in patients receiving ECMO for severe COVID-19 and to evaluate their impact on in-hospital mortality.

Methods: The ECMOSARS registry included COVID-19 patients supported by ECMO in France. We analyzed all patients included up to March 31, 2022 without missing data regarding bleeding and thrombotic events. The association of bleeding and thrombotic events with in-hospital mortality and pre-ECMO variables was assessed using multivariable logistic regression models.

Results: Among 620 patients supported by ECMO, 29% had only bleeding events, 16% only thrombotic events and 20% both bleeding and thrombosis. Cannulation site (18% of patients), ear nose and throat (12%), pulmonary bleeding (9%) and intracranial hemorrhage (8%) were the most frequent bleeding types. Device-related thrombosis and pulmonary embolism/thrombosis accounted for most of thrombotic events. In-hospital mortality was 55.7%. Bleeding events were associated with in-hospital mortality (adjOR=2.91[1.94–4.4]) but not thrombotic events (adjOR=1.02[0.68–1.53]). Intracranial hemorrhage was strongly associated with in-hospital mortality (adjOR=13.5[4.4–41.5]). Ventilation duration before ECMO ≥7 days and length of ECMO support were associated with bleeding. Thrombosis-associated factors were fibrinogen ≥6 g/L and length of ECMO support.

Conclusion: In a nationwide cohort of COVID-19 patients supported by ECMO, bleeding incidence was high and associated with mortality. Intracranial hemorrhage incidence was higher than reported for non-COVID patients and carried the highest risk of death. Thrombotic events were less frequent and not associated with mortality. Length of ECMO support was

associated with a higher risk of both bleeding and thrombosis, supporting the development of strategies to minimize ECMO duration.

Trial registration number: NCT04397588 (May 21, 2020)

Keywords

ECMO; COVID-19; bleeding; thrombosis; anticoagulation

Take Home Message

In COVID-19 patients supported by ECMO, bleeding incidence was high and associated with mortality, with intracranial hemorrhage carrying the highest risk of death. Thrombotic events were less frequent and not associated with mortality. Length of ECMO support was associated with a higher risk of both bleeding and thrombosis.

Statements and Declarations

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Introduction

Veno-venous (VV) and veno-arterial (VA) extracorporeal membrane oxygenation (ECMO) are increasingly used in the management of refractory respiratory and circulatory failure [1–4]. However, ECMO complication rates remain high. Bleeding and thrombosis on ECMO are particularly frequent and carry a high risk of both morbidity and mortality[5–13]. They occur as a result of a complex interplay between the underlying critical illness, blood exposure to shear stress and non-biological surfaces and antithrombotic strategies.

Since the beginning of the SARS-CoV-2 pandemic, ECMO has been widely used for COVID-19 related acute respiratory distress syndrome (ARDS) and, to a lesser extent, for COVID-19 associated circulatory failure[14–18]. Immunothrombosis is thought to be a key mechanism contributing to the pathogenesis of severe COVID-19 and to its high reported thrombotic risk[19–21]. This putative relationship has led to an ongoing research effort to evaluate optimal antithrombotic strategies and, frequently, to an intensification of anticoagulant dosing for COVID-19 patients in the ICU[22–26]. Although the rates and mechanisms of bleeding and thrombosis in COVID-19 patients have been extensively studied, relatively little is known about bleeding and thrombosis risks of COVID-19 patients on ECMO. The existing data are limited to small single-center series and one multicenter study[27–37].

Therefore, the goals of this prospective multicenter cohort study were: 1) to report bleeding and thrombotic events in patients receiving ECMO for severe COVID-19; 2) to evaluate their impact on in-hospital mortality; and 3) to identity factors associated with their occurrence. We hypothesized that bleeding and thrombotic events would be frequent and associated with worse outcomes.

Materials and methods

Data collection

The French national ECMOSARS registry (ClinicalTrials.gov Identifier: NCT04397588) was launched in April 2020 and is still currently recruiting COVID-19 patients supported by ECMO (VV or VA). The registry has been approved by the Rennes University Hospital ethics committee (n° 20.43). According to the French legislation, written consent was waived because of the observational design of the study. The data collection methodology has previously been described in the first report of the registry [17]. Briefly, data were collected by research assistants using an electronic case report form, and consistency tests were performed by data managers. Collected data included patient characteristics and comorbidities, management of COVID-related ARDS before ECMO cannulation, patient characteristics at ECMO cannulation and the day after, therapeutics, complications and patient outcomes on ECMO (see Supplementary Table S1 for the definition of the main variables). Patient and ECMO management, including anticoagulation, screening for bleeding/thrombosis complications and weaning protocol, was at the discretion of each center.

Study design and population

For the present study, we analyzed all consecutive patients included in the registry from the first patient included on February 25, 2020 up to March 31, 2022 without missing data regarding bleeding and thrombotic events. The analysis followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (Supplementary Table S2).

Outcomes and variables

Our primary outcome was the incidence of thrombotic and bleeding events. Secondary outcomes were in-hospital mortality, mortality at day 28, mortality at day 90, ICU length of stay and hospitalization duration.

The ECMOSARS registry captures all clinically relevant bleeding and thrombotic events, irrespective of their assumed severity (see Supplementary Table S1 for the definition of bleeding and thrombotic events). No systematic screening was performed for both bleeding and thrombosis complications. Bleeding events included: intracranial bleeding, upper or lower gastrointestinal hemorrhage, peripheral cannulation site bleeding, retroperitoneal bleeding and pulmonary hemorrhage. Thrombotic events included: ischemic stroke, deep vein thrombosis, pulmonary embolism (or pulmonary thrombosis), acute mesenteric ischemia, acute limb ischemia, macroscopic thrombus of circuit and/or membrane without needing to change the circuit or the oxygenator, oxygenator failure requiring change due to clot formation, acute circuit thrombosis requiring change. In addition, the following variables were included in the present study: pre-ECMO patient-related variables (baseline demographics and comorbidities), pre-ECMO hospitalization related variables (center, Simplified Acute Physiology Score (SAPS) II, non-invasive ventilation, high-flow oxygen therapy, neuromuscular blocking agents, prone position, antiviral therapy, antibiotic therapy), variables at ECMO cannulation (ventilation duration, Sequential Organ Failure Assessment (SOFA) score, ARDS, vasoactive and inotropic drugs, lactatemia, pH, PaO_2/FiO_2 ratio, $PaCO_2$ change within 24 hours after ECMO initiation, renal replacement therapy, anticoagulation, platelet count, prothrombin time (PT) expressed as percentage of standard value, fibrinogen), cannulation-related variables (retrieval and transport, type of ECMO), on-ECMO variables (antiplatelet agent, anticoagulation-related variables, antithrombin monitoring, length of ECMO support, transfusion requirements, vasoactive and inotropic drugs, acute kidney injury, infectious complications).

Statistical Analysis

Patient characteristics are expressed as number (percentage) for categorical variables and median with interquartile range for continuous variables. For comparison between bleeding and thrombotic complications, a $\chi 2$ test or a Fisher's exact test were used for categorical variables and a Kruskal-Wallis test for continuous variables. For comparison between VV and VA-ECMO and between epidemic waves, a $\chi 2$ test or a Fisher's exact test were used.

A statistical analysis plan was made prior to accessing the data. No a priori statistical power calculation was conducted. Only pre-ECMO variables and variables at ECMO cannulation were included in the following multivariable analyses to prevent competing risk bias.

A directed acyclic graph was used to describe our model of causal associations between bleeding and thrombotic events (exposure variables), patient-related confounders, pre-ECMO hospitalization-related confounders, at ECMO cannulation confounders and in-hospital mortality, using DAGitty software (Supplementary Figure S1)[38]. No variables were analyzed as effect modifiers. The set of potential confounders sufficient for adjustment was: age, body mass index, center, PT at cannulation, type of ECMO (VV or VA), renal replacement therapy before ECMO, ventilation duration before ECMO and PaO_2/FiO_2 at cannulation. A multivariable logistic regression model was then used to estimate odd ratios between bleeding and thrombotic events (exposure variables) and in-hospital mortality. Confounders entered in the model were defined a priori using the directed acyclic graph. Centers were included in analysis as stratification factor. Patients who were still hospitalized at the time of database lock were not included in this analysis. Mortality at day 90 was also evaluated for any bleeding, any thrombosis and intracranial hemorrhage using the same model, as a post-hoc secondary analysis.

Multivariable logistic regression models were also used to identify variables independently associated with bleeding and thrombotic events. Variables entered in the models were defined a priori, based on published ECMO and COVID-19 literature [9, 10, 21, 27, 36, 37, 39–46]. No

further variable selection was done. The set of variables entered in the model for bleeding events was: age, body mass index, type of ECMO, ventilation duration before ECMO, anticoagulation before ECMO, PT at cannulation, platelet count < 100 G/L at cannulation, fibrinogen < 1.5 g/L at cannulation, pH ≥ 7.25 at cannulation, PaO2/FiO2 ratio at cannulation, renal replacement therapy at cannulation, $PaCO_2$ change within 24 hours after ECMO initiation and length of ECMO support. The set of variables entered in the model for thrombotic events was: age, body mass index, type of ECMO, ventilation duration before ECMO, anticoagulation before ECMO, PT at cannulation, platelet count ≥ 350 G/L at cannulation, fibrinogen ≥ 6 g/L at cannulation, pH ≥ 7.25 at cannulation, PaO2/FiO2 ratio at cannulation, renal replacement therapy at cannulation, $PaCO_2$ change within 24 hours after ECMO initiation, length of ECMO support and history of venous thromboembolism. A sensitivity analysis was performed by removing from the multivariable models, the variables imputed with more than 30% of missing data (PT and fibrinogen).

Linearity of continuous independent variables and log-odds was checked. If not, those variables were transformed into categorical variables in accordance with previously published works[3, 9, 10, 17, 46].

Multiple imputation was used to account for missing values in variables. We used fully specified chained equations in the SAS MI procedure. For continuous variables, the regression method was used to impute missing values and discriminant function methods were used for binary and categorical variables. Passive imputation was used for the derived variables (BMI), meaning that each variable needed for the calculation was imputed prior to the calculation of the derived variable. Fifty imputed datasets were created and combined using standard between/within-variance techniques.

In order to describe the clinical management and outcomes over the course of the pandemic, a post-hoc analysis was performed by splitting the cohort between the first epidemic wave (up to July 1st, 2020 [47]), and the next waves (from July 1st, 2020 to March 31, 2022). Indeed, substantive changes were made regarding ICU management of COVID-19 patients in France

after the first wave, including improved healthcare organization at a national scale, widespread use of Dexamethasone[48], increased use of non-invasive ventilation[49] and SARS-CoV2 vaccination (starting January 2021).

All tests used two-tailed hypothesis. Statistical significance was achieved for $P < 0.05$. Statistical analyses were performed with SAS version 9.4 software (SAS Institute, Cary, North Carolina, USA).

Results

Study population

Among the 701 patients included in the ECMOSARS registry at the time of database lock, 81 had missing data regarding bleeding and thrombotic events, leaving 620 patients included in the present study (Figure 1). Five hundred sixty-eight patients were supported by VV-ECMO, and 52 by VA-ECMO. Median age was 55 (47–61) years, 22.9% were females, and had a median body mass index of 30 (27–34) kg/m^2 (Table 1). Median SAPS II was 42 (31–57). ICU management before ECMO cannulation included non–invasive ventilation (32.5%), high–flow oxygen therapy (51.6%), neuromuscular blocking agents (94.8%), prone positioning (90.4%), antiviral therapy (49.2%) and antibiotics (90.1%). At the time of ECMO cannulation, 96.0% met Berlin criteria for ARDS with a median PaO_2/FiO_2 ratio of 68 (57–85) mmHg, and 11.9% were on renal replacement therapy.

Coagulation management

At ECMO cannulation, 90.1% (430/477) of patients had received anticoagulation (therapeutic-dose 45.3%, prophylactic-dose 44.9%). Median fibrinogen level was 7.4 (5.6–8.7) g/L, median PT was 73 (64–82) % and median platelet count was 255 (184–345) G/L (Table 2).

During ECMO support, the majority of patients received systemic anticoagulation (95.3%) and the preferred anticoagulant was unfractionated heparin (98.1%; n=468). Unfractionated heparin was monitored using anti-factor Xa activity (91.6%), activated partial thromboplastin time (7.4%) and activated clotting time (ACT) (0.9%; n=431). Median time to achieve anticoagulation target defined by centers for each patient was 9 (4–44) hours. The anti-factor Xa activity target was ≥0.3 IU/mL in 86.2% of patients (n=354; Supplementary Table S3 and S4). Antithrombin (AT) levels were monitored for 27.3% of patients (n=476), for whom the lowest AT level was 62 (50–73) %. Forty patients (8.5%) received AT supplementation

(n=471). Anticoagulation management was not significantly modified over the course of the pandemic (Supplementary Table S4).

Incidence of bleeding and thrombosis

Overall, 406 (65.5%) patients suffered from bleeding or thrombosis during ECMO support (306 with bleeding and 225 with thrombosis), of whom 181 (29%) had only bleeding events, 100 (16%) only thrombotic events and 125 (20%) both bleeding and thrombotic events (Table 3; Figure 1). Of 725 total events, 382 (53%) were bleeding events (Figure 2A). Cannulation site (114 events, 18.4% of patients) and ear nose and throat (76 events, 12.3% of patients) were the most frequent bleeding types. Intracranial hemorrhage accounted for 6.8% of total events (49 events, 8.0% of patients). Ten percent of bleeding events (40 events) were associated with a massive transfusion (>10U PRBCS/24h). Device-related thrombosis accounted for most thrombotic events with 82 circuit changes due to acute thrombosis (13.2% of patients), 59 oxygenator failures (9.5% of patients) and 72 macroscopic thrombi of circuit or membrane without needing to change circuit or oxygenator (11.6% of patients). Pulmonary embolism/thrombosis was diagnosed in 9.4% of patients (58 events). No significant difference was observed between VV and VA-ECMO regarding overall incidence of bleeding and thrombosis (Supplementary Table S5, S10 and S11). VA-ECMO support, however, was associated with a significant increase in gastrointestinal bleedings (15.4% vs 6.5%, p=0.043), leg ischemia (13.5% vs 1.1%, p<0.001) and ischemic stroke (9.6% vs 0.9%, p<0.001). While thrombosis incidence remained stable over the course of the pandemic (32.7% vs 37.6%, p=0.267, Supplementary Table S6), overall bleeding increased after the first epidemic wave (58.2% vs 46.2%, p=0.008, Supplementary Table S6).

Outcomes and bleeding/thrombosis events

In-hospital mortality was 55.7% (336/603) with a median follow-up of 51 (34-78) days for survivors and 17 (8-28) days for deceased patients. Mortality at day 90 was 62.6% (330/527).

Bleeding events were associated with higher in-hospital mortality with 71.8% for bleeding only, 69.4% for bleeding and thrombosis, 42.4% for thrombosis only and 40.3% for no bleeding or thrombosis ($p<0.001$; Table 4). On multivariable analysis, overall bleeding was independently associated with in-hospital mortality (adjOR= 2.91[1.94-4.4]; Figure 2B, Supplementary Table S7), unlike overall thrombosis which was not associated with increased in-hospital mortality (adjOR= 1.02 [0.68-1.53]; Figure 2B, Supplementary Table S8). Likewise, mortality at day 90 was increased in patients with bleeding complications (adjOR=3.21[2.03–5.1]; Supplementary Table S7). Among bleeding types, intracranial hemorrhage was independently associated with in-hospital mortality (adjOR=13.5 [4.4-41.5]; Figure 2B, Supplementary Table S9) and mortality at day 90 (adjOR=23.9 [4.6–124.8]; Supplementary Table S9). Pulmonary bleedings were also independently associated higher in-hospital mortality (adjOR= 2.67 [1.27-5.6]; Figure 2B). Successive bleeding events in a patient were associated with higher mortality rates with adjusted odd-ratios of 1.87 [1.19-2.96] for one event, 3.84 [2.05-7.2] for two events and 3.63 [1.80-7.3] for three or more events. On univariate analysis, bleedings complications were associated with transfusion requirements on ECMO (packed red blood cells, fresh frozen plasmas and platelet concentrates) and acute kidney injury (Table 4).

Factors associated with the occurrence of bleeding and thrombotic events

Factors independently associated with the occurrence of all bleeding events were ventilation duration before ECMO ≥ 7 days (adjOR=1.62 [1.09–2.41]) and length of ECMO support (per 5 days increase, adjOR=1.08 [1.01–1.15]; Supplementary Table S10). Factors independently associated with the occurrence of all thrombosis events were fibrinogen ≥ 6 g/L at cannulation (adjOR=1.94 [1.00–3.75]) and length of ECMO support (per 5 days increase, adjOR=1.17 [1.09–1.26]; Supplementary Table S11). Sensitivity analyses removing PT and fibrinogen, imputed with more than 30% of missing data, from the variable selection did not change these results (Supplementary Tables S10 and S11).

Discussion

Our study reports bleeding and thrombotic events at a nationwide level in a large multicenter cohort of COVID-19 patients supported by ECMO. The main findings were as follows. First, bleeding complications were common, occurring in 49% of patients, and were independently associated with in-hospital mortality and mortality at day 90. Second, thrombotic events, while also common (36%), were associated with a fibrinogen ≥ 6 g/L at cannulation but not with mortality. Third, duration of ECMO support was associated with a higher risk of both bleeding and thrombosis. Fourth, intracranial hemorrhage was frequent (8.0%) and associated with high mortality rates (in-hospital and at day 90). And finally, the vast majority of the patients (95.3%) received a systemic anticoagulation with unfractionated heparin as the drug of choice (98.1%), mainly monitored by anti-factor Xa activity.

The incidence of bleeding complications in our study was high, with almost half of patients experiencing at least one bleeding event, which was higher than previously published studies on both COVID-19 and non-COVID patients supported by ECMO. This might however be explained by the fact that the ECMOSARS registry captures all bleeding events, irrespective of their assumed severity (unlike ELSO registry or ISTH major bleeding criteria) [3, 11–13, 16, 27, 28]. Overall, bleeding was independently associated with in-hospital mortality with a cumulative effect of bleeding recurrence, as already reported [11–13, 27]. Also, our study demonstrates a sustained impact of bleeding on mortality at day 90. Finally, bleeding incidence seemed to increase over the course of the pandemic, which might be compared with the increased mortality previously reported [47, 50–52]. However, these findings will need to be confirmed in larger and more extensive studies.

Intracranial hemorrhage (ICH) incidence was higher than previously reported for both VV and VA-ECMO in non-COVID patients[3, 9, 11–13]. This seems, however, in line with recent data suggesting a higher incidence of ICH for COVID-19 patients supported by VV-ECMO [12–14, 16, 29–31, 34]. Notably, non-severe COVID-19 seems to be associated with a small but significant increase in the incidence of ICH[53, 54]. Unfortunately, many of these studies suffer from bias and heterogeneity in the diagnosis and reporting of intracranial hemorrhage, limiting their interpretation. The cause of the comparatively higher ICH incidence in Covid-19 patients on ECMO compared to other ECMO patients is not clear, but may be explained in part by the SARS-CoV2 neurotropism hypothesis, or possibly by the intensification of anticoagulation in Covid-19 patients on ECMO[22, 26]. However, evidence for the link between anticoagulant dosing and bleeding remains limited for non-COVID patients on ECMO[11, 55], and therapeutic anticoagulation seems to be associated with only a non-significant trend for higher bleeding in COVID-19 patients[23–25, 56]. Finally, ICH independently associated with mortality, in line with earlier studies on both COVID and non-COVID patients[12, 13, 27].

In addition to ICH, cannulation-related bleeding and ear nose and throat (ENT) bleeding accounted for more than a quarter of total events, but did not have a significant impact on in-hospital mortality. Compared to cannulation-related bleeds [12, 13], ENT bleeds, which are not included in the ELSO registry, have been rarely reported and their impact on mortality is largely unknown[11]. Moreover, the impact of the return cannula in a jugular position on the risk of ENT bleeding deserves further evaluation. In line with published data in non-COVID patients, pulmonary bleedings were frequent and independently associated with mortality[12, 13, 57]. Unlike previous reports, gastrointestinal bleeds, despite a trend towards higher mortality, did not reach statistical significance in multivariable analysis[12, 13].

Thrombosis incidence was lower than bleeding in our cohort, which contrasts with the recent ELSO report on VV-ECMO[12], even though our study included deep vein thrombosis and

pulmonary embolism/thrombosis which are not recorded in the ELSO registry. Our results also differ from the high rates of thrombosis in early reports of COVID-19 patients on VV-ECMO[27, 28, 34]. Two factors might have influenced these results. First, as previously highlighted, intensification of anticoagulant dosing during the COVID-19 pandemic may have reduced thrombosis incidence, in particular circuit clotting and oxygenator failure[58]. Second, thrombosis reporting is highly dependent on clinical and radiological screening protocols. Systematic ultrasound or computed-tomography assessment of thrombosis on ECMO is likely accountable for the discrepancy between our results and recently published COVID-19 reports, especially regarding pulmonary embolism/thrombosis[27, 28, 34].

Unlike bleeding, neither overall thrombosis nor any thrombosis subtypes were significantly associated with in-hospital mortality. These results are partially in line with recent findings on COVID-19 ECMO patients[27] but differ from large multicenter studies in non-COVID ECMO patients which reported a significant impact of thrombosis on in-hospital survival, although weaker than bleeding [12, 13]. Two factors might explain these results. First, the use of a causal approach for model building using a DAG may have enabled a better control of confounding variables [38, 59]. Second, the smaller number of patients and events in our report might have underpowered the analysis.

We identified specific variables independently associated with the occurrence of bleeding or thrombosis. As already reported from the ELSO registry, the length of ECMO run was independently associated with both bleeding and thrombosis, supporting strategies aiming at minimizing ECMO duration, including daily assessment of readiness to liberate from ECMO[12, 13]. Longer duration of mechanical ventilation (MV) prior to ECMO (≥ 7 days) was also independently associated with increased risk of bleeding. This may reflect a higher disease severity, nutritional deficiencies, increased inflammation and endothelial activation. This may also help to explain the reported association between survival and duration of MV before

ECMO in both COVID and non-COVID patients [17, 18, 46]. Finally, a high fibrinogen level (≥ 6 g/L) was associated with greater odds of thrombosis. Elevated fibrinogen levels have been associated with thrombosis risk in the general population[60, 61] but the evidence in ECMO patients remains scarce[62]. While fibrinogen, as inflammatory marker, is associated with COVID-19 severity and mortality[63], its ability to predict thrombosis appears low[39]. To date, only one single-center study, though limited in size, reported an association between high fibrinogen levels and thrombosis in COVID-19 patients supported by VV-ECMO[64].

Finally, we reported anticoagulation management practice during ECMO support at a nationwide level. As previously reported in an international survey[65], and in accordance with current international guidelines[66], the vast majority of the patients received a systemic anticoagulation, with UFH being the drug of choice. Heparin was essentially monitored using anti-factor Xa activity, which contrasts with the international practice in adult ECMO[65]. Antithrombin monitoring and supplementation, though significant, was lower than previously published[65], which might reflect the negative results of a recent randomized control trial[67].

Our study has several strengths. First, we report bleeding and thrombosis for the first time in a large multicenter sample of COVID-19 patients, at a nationwide level. The excellent adherence to recommended medical interventions in ARDS patient management during the pre-ECMO period supports the generalizability of our results. Second, our registry captured data regarding ENT bleeding, venous thromboembolism and anticoagulation management practice that are not collected by the ELSO registry. Third, the use of a causal approach for multivariable model building and the sustained effect of bleeding on mortality up to day 90 strengthens confidence in our results.

Although the multicenter nature of this study prevented us from collecting high frequency clinical and biological data on ECMO, we were able to report important data regarding

coagulation management, hitherto unpublished. These data highlight the considerable heterogeneity of practices and underline 1) the need for harmonization of procedures and practices across centers regarding hemostasis management on ECMO and 2) the crucial need for prospective interventional studies of anticoagulation management during both VA and VV-ECMO. To this extent, we believe our study reports valuable data that might help setting up prospective interventional studies.

Limitations

Our study has several limitations. The observational nature of this study prevented us from inferring causality. The timing of bleeding and thrombotic events occurrence was not available and sequential assessment of anticoagulation, ECMO parameters and biological markers on ECMO was not collected, precluding any time-to-event analysis. In addition, D-dimer levels, though described as markers of disease severity and thrombotic risk, were not available. Finally, the absence of systematic screening protocols for both bleeding and thrombosis before and during ECMO might have led to under-reporting of these events.

Conclusion

In a large nationwide cohort of patients supported by ECMO for severe COVID-19, bleeding incidence was high and associated with mortality. Besides, intracranial hemorrhage carried the highest risk of death. Thrombotic events were less frequent and were not associated with mortality. Length of ECMO support was associated with a higher risk of both bleeding and thrombosis, supporting the development and use of strategies to minimize ECMO duration. Our results highlight the need for harmonization of practices across centers regarding hemostasis management on ECMO and for prospective studies to evaluate anticoagulation strategies during ECMO support.

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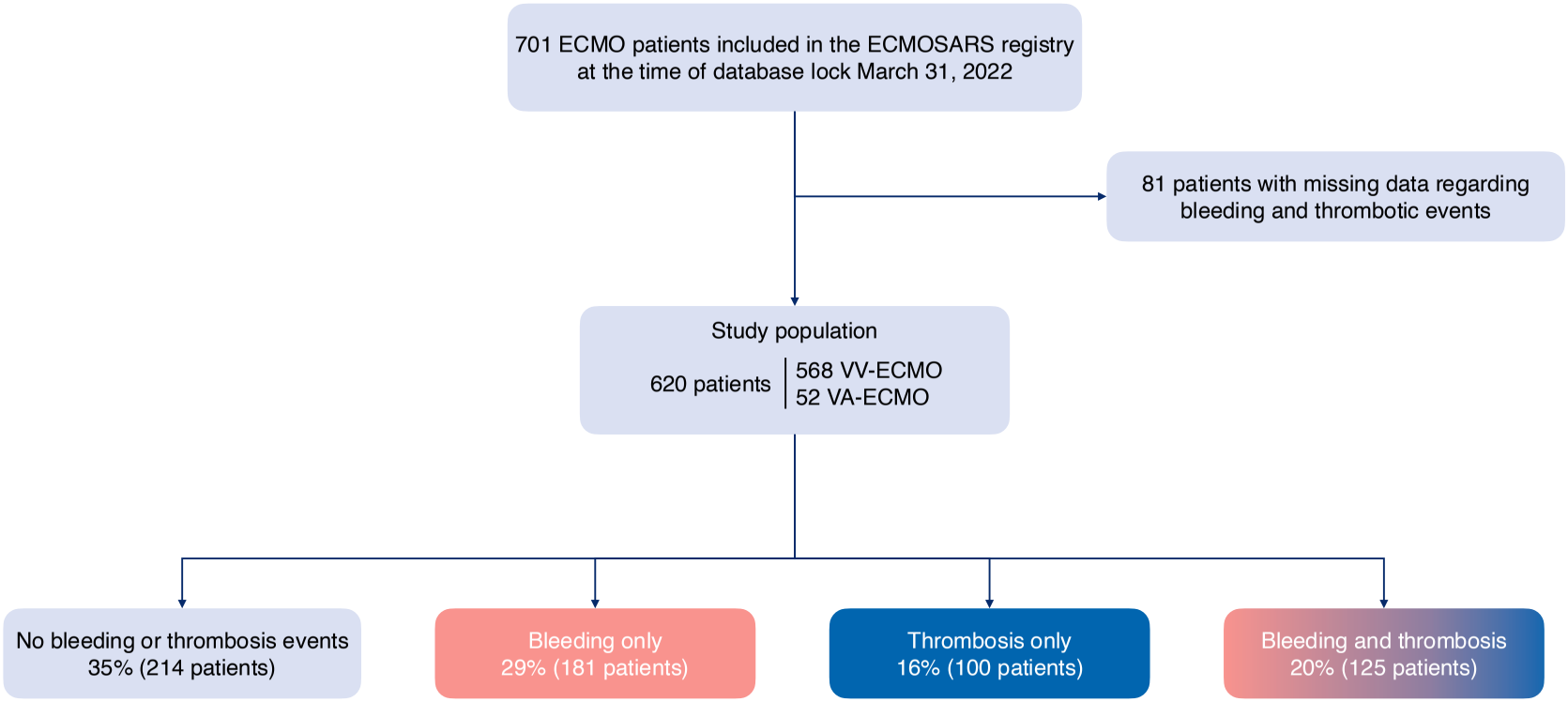
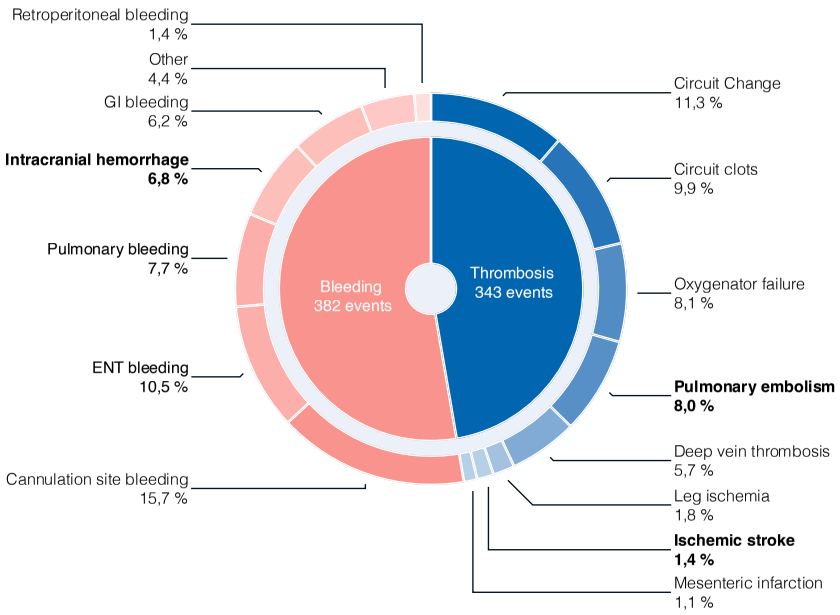
Figure Legends:

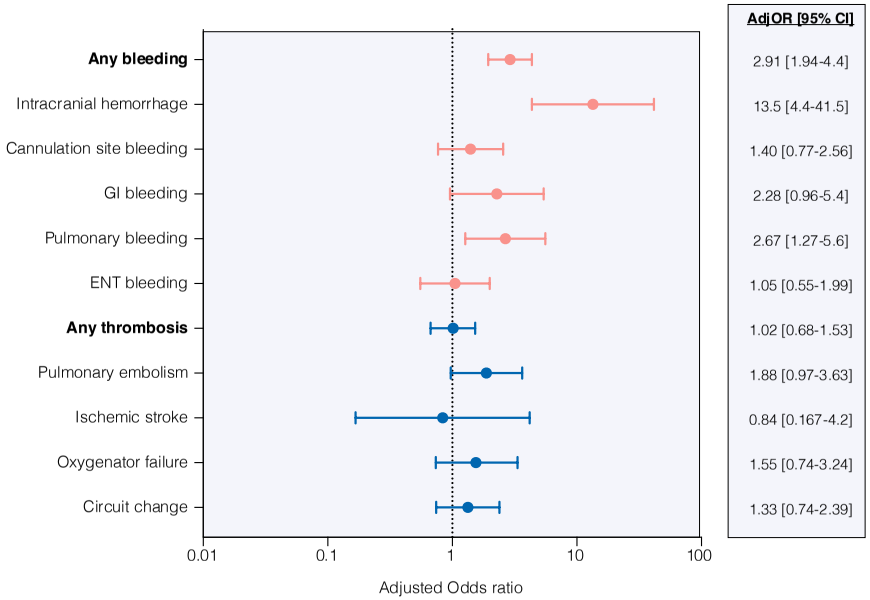
Figure 1: Flow chart of ECMO patients included in the study

Figure 2: Incidence and impact of bleeding and thrombotic events during ECMO support for severe COVID-19. A. Distribution of bleeding and thrombotic events on ECMO, expressed as percentage of total events (n=725). Bleeding events are represented in red, thrombotic events in blue. B. Independent association of main bleeding and thrombotic events with in-hospital mortality. GI, gastrointestinal; ENT, ear nose and throat; AdjOR, adjusted odds ratio; CI, confidence interval.


701 ECMO patients included in the ECMOSARS registry at the time of database lock March 31, 2022
81 patients with missing data regarding bleeding and thrombotic events
Study population
620 patients
568 VV-ECMO
52 VA-ECMO
No bleeding or thrombosis events
35% (214 patients)
Bleeding only
29% (181 patients)
Thrombosis only
16% (100 patients)
Bleeding and thrombosis
20% (125 patients)


Retroperitoneal bleeding
1,4 %
Other
4,4 %
GI bleeding
6,2 %
Intracranial hemorrhage
6,8 %
Pulmonary bleeding
7,7 %
ENT bleeding
10,5 %
Cannulation site bleeding
15,7 %
Bleeding
382 events
Thrombosis
343 events
Circuit Change
11,3 %
Circuit clots
9,9 %
Oxygenator failure
8,1 %
Pulmonary embolism
8,0 %
Deep vein thrombosis
5,7 %
Leg ischemia
1,8 %
Ischemic stroke
1,4 %
Mesenteric infarction
1,1 %

A


Any bleeding
Intracranial hemorrhage
Cannulation site bleeding
GI bleeding
Pulmonary bleeding
ENT bleeding
Any thrombosis
Pulmonary embolism
Ischemic stroke
Oxygenator failure
Circuit change
0.01
0.1
1
10
100
Adjusted Odds ratio
AdjOR [95% CI]
2.91 [1.94-4.4]
13.5 [4.4-41.5]
1.40 [0.77-2.56]
2.28 [0.96-5.4]
2.67 [1.27-5.6]
1.05 [0.55-1.99]
1.02 [0.68-1.53]
1.88 [0.97-3.63]
0.84 [0.167-4.2]
1.55 [0.74-3.24]
1.33 [0.74-2.39]

B

Table 1- Patient characteristics at the time of ECMO cannulation

Characteristics	No.	All patients (n = 620)	Bleeding/thrombosis status				P value
			None (n=214)	Bleeding (n=181)	Thrombosis (n=100)	Both (n=125)	
Age — years	618	55 (46–61)	54 (44–61)	57 (50–62)	53 (46–59)	55 (48–60)	0.040
Female sex	620	142 (22.9)	56 (26.2)	36 (19.9)	26 (26.0)	24 (19.2)	0.291
Body mass index — kg/m^2	597	30 (27–34)	30 (27–35)	29 (26–33)	30 (26–35)	30 (27–34)	0.196
Comorbidities							
Chronic respiratory failure	620	19 (3.1)	10 (4.7)	5 (2.8)	2 (2.0)	2 (1.6)	0.441
Congestive heart failure	498	11 (2.2)	1 (0.7)	4 (2.5)	3 (3.9)	3 (2.6)	0.360
Coronary artery disease	620	34 (5.5)	9 (4.2)	10 (5.5)	8 (8.0)	7 (5.6)	0.593
Chronic kidney disease	499	24 (4.8)	9 (6.0)	9 (5.7)	3 (3.9)	3 (2.6)	0.579
Cancer	496	6 (1.2)	3 (2.0)	1 (0.6)	1 (1.3)	1 (0.9)	0.695
Hematological malignancy	496	5 (1.0)	2 (1.3)	2 (1.3)	0 (0.0)	1 (0.9)	0.935
Active smoker	614	27 (4.4)	10 (4.7)	8 (4.4)	3 (3.0)	6 (4.9)	0.929
History of venous thromboembolism	496	22 (4.4)	5 (3.4)	6 (3.8)	4 (5.2)	7 (6.2)	0.663
Pre–ECMO ICU management							
Simplified acute physiology score II	619	42 (31–57)	41 (29–56)	41 (31–58)	43 (34–55)	40 (29–56)	0.842
Delay from hospitalization to ICU	618	0 (0–0)	0 (0–0)	0 (0–1)	0 (0–1)	0 (0–0)	0.077
Non–invasive ventilation	615	200 (32.5)	60 (28.3)	70 (39.3)	24 (24.0)	46 (36.8)	0.020
High–flow oxygen therapy	496	256 (51.6)	72 (47.7)	85 (54.8)	38 (49.4)	61 (54.0)	0.574
Neuromuscular blocking agents	616	584 (94.8)	196 (92.5)	170 (95.0)	96 (96.0)	122 (97.6)	0.198
Prone position	617	558 (90.4)	187 (88.2)	165 (91.7)	90 (90.0)	116 (92.8)	0.501
Antiviral therapy	494	243 (49.2)	62 (41.9)	74 (47.4)	37 (48.1)	70 (61.9)	0.013
Antibiotic therapy	495	446 (90.1)	135 (91.2)	134 (85.4)	69 (89.6)	108 (95.6)	0.046
Characteristics at ECMO cannulation							
Ventilation time before ECMO— d	615	5 (2–8)	4 (1–7)	6 (3–9)	5 (3–8)	6 (3–8)	< 0.001
SOFA score	552	9 (8–12)	10 (8–12)	10 (8–12)	8 (6–11)	8 (7–12)	0.032
ARDS (Berlin criteria)	607	583 (96.0)	200 (95.7)	168 (94.9)	94 (95.9)	121 (98.4)	0.477
Vasoactive/inotropic drugs							
Norepinephrine	487	290 (59.5)	84 (57.5)	93 (60.8)	41 (54.7)	72 (63.7)	0.593
Epinephrine	494	29 (5.9)	8 (5.4)	11 (7.1)	3 (3.9)	7 (6.2)	0.815
Dobutamine	492	26 (5.3)	10 (6.7)	8 (5.2)	3 (3.9)	5 (4.4)	0.844
Lactatemia — mmol/L	543	1.7 (1.3–2.5)	1.8 (1.3–2.6)	1.7 (1.2–2.6)	1.7 (1.3–2.2)	1.6 (1.2–2.4)	0.362
pH	593	7.33 (7.25–7.40)	7.32 (7.24–7.40)	7.33 (7.24–7.40)	7.35 (7.26–7.42)	7.33 (7.25–7.38)	0.593
PaO2/FiO2 ratio — mmHg	586	68 (57–85)	66 (55–85)	69 (58–85)	72 (59–90)	67 (59–85)	0.233
ΔPCO2	563	-9 (-21 to 0)	-10 (-21 to 1)	-10 (-22 to 1)	-9 (-19 to -1)	-7 (-17 to 2)	0.722
Renal replacement therapy	612	73 (11.9)	27 (12.8)	21 (11.7)	9 (9.1)	16 (13.0)	0.788
ECMO cannulation							
Retrieval and transport	617						0.383
Referral center		385 (62.4)	121 (56.8)	119 (66.1)	63 (63.6)	82 (65.6)	
Mobile ECMO unit, no transfer		45 (7.3)	14 (6.6)	13 (7.2)	8 (8.1)	10 (8.0)	
Mobile ECMO unit, transfer to referral center		187 (30.3)	78 (36.6)	48 (26.7)	28 (28.3)	33 (26.4)	
Type of ECMO	620						0.840
Veno–venous ECMO		575 (92.7)	201 (93.9)	166 (91.7)	93 (93.0)	115 (92.0)	
Veno–arterial ECMO		45 (7.3)	13 (6.1)	15 (8.3)	7 (7.0)	10 (8.0)	

Results are presented as n(%) or median (IQR). SOFA: Sequential Organ Failure Assessment; ARDS: Acute Respiratory Distress Syndrome; PT: prothrombin time; PaO2: partial pressure of oxygen; FiO2: fraction of inspired oxygen; ΔPaCO2: difference between day 1 and cannulation partial pressure of carbon dioxide.

Table 2- Hemostasis laboratory results and coagulation management pre- and during ECMO support

Clinical condition and management	No.	All patients (n = 620)	Bleeding/thrombosis status				p value
			None (n=214)	Bleeding only (n=181)	Thrombosis only (n=100)	Both (n=125)	
At ECMO cannulation							
Anticoagulation	477						0.010
No		47 (9.9)	18 (12.5)	17 (11.0)	11 (15.5)	1 (0.9)	
Therapeutic-dose		216 (45.3)	60 (41.7)	76 (49.0)	25 (35.2)	55 (51.4)	
Prophylactic-dose		214 (44.9)	66 (45.8)	62 (40.0)	35 (49.3)	51 (47.7)	
Platelet count — G/L	477	255 (184–345)	253 (176–325)	250 (187–341)	264 (187–353)	257 (201–367)	0.663
PT —% [a]	411	73 (64–82)	75 (66–83)	73 (60–81)	74 (63–83)	72 (64–82)	0.646
Fibrinogen — g/L	398	7.4 (5.6–8.7)	7.1 (5.3–8.7)	7.0 (4.7–8.5)	7.87 (6.1–9.0)	7.6 (6.4–8.9)	0.032
During ECMO support							
Antiplatelet agents	492	48 (9.8)	11 (7.4)	18 (11.5)	11 (14.5)	8 (7.1)	0.235
Anticoagulation strategy	509						0.464
Without systemic anticoagulation		24 (4.7)	8 (5.4)	10 (6.4)	3 (3.5)	3 (2.5)	
Systemic anticoagulation		485 (95.3)	140 (94.6)	146 (93.6)	82 (96.5)	117 (97.5)	
Therapeutic–target–achieving time — hours	313	9 (4–44)	7 (4–28)	8 (4–28)	12 (2–60)	11 (4–48)	0.364
Type of anticoagulant	468						0.241
Unfractionated heparin		459 (98.1)	131 (96.3)	145 (99.3)	72 (97.3)	111 (99.1)	
Nonheparin Anticoagulants		9 (1.9)	5 (3.7)	1 (0.7)	2 (2.7)	1 (0.9)	
Unfractionated heparin monitoring method	431						0.682
Anti–Factor Xa activity		395 (91.6)	104 (88.1)	132 (93.0)	60 (93.8)	99 (92.5)	
aPTT		32 (7.4)	13 (11.0)	9 (6.3)	3 (4.7)	7 (6.5)	
ACT		4 (0.9)	1 (0.8)	1 (0.7)	1 (1.6)	1 (0.9)	
Antithrombin monitoring	476	130 (27.3)	32 (23.0)	46 (29.7)	21 (29.6)	31 (27.9)	0.587
Lowest antithrombin level — %	129	62 (50–73)	65 (54–77)	59 (50–73)	65 (57–70)	56 (45–74)	0.710
Antithrombin supplementation	471	40 (8.5)	6 (4.4)	15 (9.8)	5 (7.0)	14 (12.6)	0.118

Results are presented as n(%) or median (IQR).
PT: prothrombin time; aPTT: activated partial thromboplastin time; ACT: activated clotting time.
[a] Expressed as percentage of the standard value

Table 3- Bleeding and thrombotic events during ECMO support and associated in-hospital mortality

Characteristics	All patients (n = 620)	In-hospital mortality[a]
Bleeding		
Any bleeding *No of events*	306 (49.4) *382*	71.3%
Cannulation site bleeding	114 (18.4)	67.6%
ENT bleeding	76 (12.3)	69.9%
Intracranial hemorrhage	49 (8.0)	93.9%
Pulmonary bleeding	56 (9.0)	76.8%
GI bleeding	45 (7.3)	80.0%
Retroperitoneal bleeding	10 (1.6)	82.5%
Other bleeding	32 (5.2)	70.0%
Thrombosis		
Any thrombosis *No of events*	225 (36.3) *343*	57.4%
Circuit Change	82 (13.2)	65.9%
Circuit clots	72 (11.6)	50.0%
Oxygenator failure	59 (9.5)	74.6%
Pulmonary embolism / thrombosis	58 (9.4)	60.3%
Deep vein thrombosis	41 (6.6)	30.0%
Leg ischemia	13 (2.1)	75.0%
Ischemic stroke	10 (1.6)	70.0%
Mesenteric infarction	8 (1.3)	100.0%

Results are presented as n (%).
[a]In-hospital mortality was not available for 17 patients who were still hospitalized at the time of database lock (n=603).
ENT: ear, nose and throat; GI: gastrointestinal.

Table 4- Complications during ECMO support and outcomes

Characteristics	No.	All patients (n = 620	Bleeding/thrombosis status				p value
			None (n=214)	Bleeding only (n=181)	Thrombosis only (n=100)	Both (n=125)	
Complications on ECMO							
Length of ECMO support, days	541	12 (7–21)	11 (6–19)	13 (7–21)	13 (8–24)	18 (10–32)	<0.001
Transfusion requirements on ECMO							
Number of PRBC transfused	476	4 (2–8)	2 (0–3)	6 (4–11)	2 (0–4)	9 (5–14)	<0.001
Number of FFP transfused	469	0 (0–0)	0 (0–0)	0 (0–2)	0 (0–0)	0 (0–2)	<0.001
Number of PC transfused	469	0 (0–0)	0 (0–0)	0 (0–1)	0 (0–0)	0 (0–2)	<0.001
Vasoactive/inotropic drugs on ECMO							
Norepinephrine	492	422 (85.8)	123 (83.7)	139 (89.1)	51 (68.0)	109 (95.6)	<0.001
Epinephrine	493	40 (8.1)	11 (7.4)	17 (10.8)	5 (6.6)	7 (6.3)	0.493
Dobutamine	493	40 (8.1)	13 (8.8)	12 (7.7)	4 (5.3)	11 (9.7)	0.717
Acute kidney injury on ECMO	615	292 (47.5)	86 (40.8)	95 (52.8)	39 (39.0)	72 (58.1)	0.003
Renal replacement therapy	288	225 (78.1)	65 (77.4)	77 (81.9)	27 (71.1)	56 (77.8)	0.596
Infectious complications on ECMO	616	307 (49.8)	109 (50.9)	77 (43.0)	55 (55.6)	66 (53.2)	0.151
Outcomes							
ICU length of stay — days	580	28 (15–45)	27 (15–45)	25 (12–41)	30 (15–48)	29 (17–46)	0.086
Hospitalization duration — days	565	34 (17–54)	40 (19–60)	28 (14–46)	39 (18–64)	33 (18–52)	0.003
Mortality at day 28	620	271 (43.7)	72 (33.6)	105 (58.0)	33 (33.0)	61 (48.8)	<0.001
Mortality at day 90	527	330 (62.6)	82 (47.1)	122 (77.2)	42 (49.4)	84 (76.4)	<0.001
In–hospital mortality	603	336 (55.7)	83 (40.3)	125 (71.8)	42 (42.4)	86 (69.4)	<0.001

Results are presented as n (%) or median (IQR)
ECMO: extracorporeal membrane oxygenation; PRBC: packed red blood cells; FFP: fresh frozen plasma; PC: platelet concentrate; ICU: intensive care unit.

Table S1- Definition of variables

Variable	Definition
Chronic respiratory failure	Arterial pO2 on room air less than 60 mmHg for 3 months or more
Chronic kidney disease	Glomerular filtration rate <60 mL/min/ 1.73 m2 for 3 months or more
Cancer	Ongoing carcinologic treatment
ARDS	Berlin Criteria for ARDS. JAMA. 2012;307(23):2526-2533. doi:10.1001/jama.2012.5669
SAPS II	Simplified Acute Physiology Score II according to Le Gall et al. PMID: 8254858 DOI: 10.1001/jama.270.24.2957
SOFA score	Sequential-related Organ Failure Assessment score, according to Vincent et al. PMID: 8844239 DOI: 10.1007/BF01709751
Bleeding complications on ECMO	One or more of the following complications: intracranial bleeding, upper or lower gastro-intestinal hemorrhage, peripheral cannulation site bleeding, retroperitoneal bleeding, pulmonary hemorrhage or massive hemorrhage. Peripheral cannulation site bleeding defined as a bleeding from a peripheral cannulation site requiring PRBC transfusion and/or surgical intervention. Massive transfusion definition required >10U PRBCS/24 hrs.
Thrombotic complications on ECMO	One or more of the following complications: ischemic stroke, deep vein thrombosis, pulmonary embolism or thrombosis, acute mesenteric ischemia, acute coronary syndrome, acute limb ischemia, macroscopic thrombus of circuit/membrane without needing to change the circuit, oxygenator failure requiring change due to clot formation, acute circuit thrombosis requiring change
Acute kidney injury on ECMO	Acute kidney injury according to KDIGO classification kidney International Supplements (2012) 2, 8–12; doi:10.1038/kisup.2012.7
Infections on ECMO	One or more of the following complications: bloodstream infection, ventilator-associated pneumonia and cannula-related infection.

Table S2 – STROBE Statement

	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	7
Objectives	3	State specific objectives, including any prespecified hypotheses	7
Methods			
Study design	4	Present key elements of study design early in the paper	8
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	8
		(b) For matched studies, give matching criteria and number of exposed and unexposed	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	8-9
Data sources measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	8-9
Bias	9	Describe any efforts to address potential sources of bias	10
Study size	10	Explain how the study size was arrived at	10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	9-10
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	9-10
		(b) Describe any methods used to examine subgroups and interactions	9-10
		(c) Explain how missing data were addressed	9-10
		(d) If applicable, explain how loss to follow-up was addressed	NA
		(e) Describe any sensitivity analyses	9-10
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	12
		(b) Give reasons for non-participation at each stage	12
		(c) Consider use of a flow diagram	Fig 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	12
		(b) Indicate number of participants with missing data for each variable of interest	12-15, Tables
		(c) Summarise follow-up time (eg, average and total amount)	13
Outcome data	15*	Report numbers of outcome events or summary measures over time	13
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95 confidence interval). Make clear which confounders were adjusted for and why they were included	13-14, tables
		(b) Report category boundaries when continuous variables were categorized	14
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	14
Discussion			
Key results	18	Summarise key results with reference to study objectives	15
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	18-19
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15-18
Generalisability	21	Discuss the generalisability (external validity) of the study results	18
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	6

Table S3 – Description of anti-factor Xa activity therapeutic targets

Anti-factor Xa activity range (IU/mL)	Frequency (N=354)
0.1-0.2	5 (1,4)
0.1-0.3	1 (0,3)
0.15-0.3	12 (3,4)
0.2-0.3	28 (7,9)
0.2-0.4	3 (0,8)
0.2-0.5	3 (0,8)
0.2-0.6	1 (0,3)
0.3-0.4	11 (3,1)
0.3-0.5	26 (7,3)
0.3-0.6	111 (31,4)
0.3-0.7	125 (35,3)
0.3-0.8	1 (0,3)
0.4-0.5	2 (0,6)
0.4-0.6	10 (2,8)
0.4-0.7	1 (0,3)
0.5-0.6	3 (0,8)
0.5-0.7	10 (2,8)
0.6-0.8	1 (0,3)

Results are presented as number (percentage).

Table S4- Coagulation management pre- and during ECMO support over the course of the COVID-19 pandemic

Clinical condition and management	No.	All patients (n = 620)	First epidemic wave Before July 1st, 2020 (n = 455)	After July 1st: 2020 (n = 165)	p value
At ECMO cannulation					
Anticoagulation	477				0.512
No		47 (9.9)	28 (8.8)	19 (12.0)	
Therapeutic-dose		216 (45.3)	145 (45.5)	71 (44.9)	
Prophylactic-dose		214 (44.9)	146 (45.8)	68 (43.0)	
During ECMO support					
Antiplatelet agents	492	48 (9.8)	26 (7.9)	22 (13.6)	0.045
Anticoagulation strategy	509				0.586
Without systemic anticoagulation		24 (4.7)	15 (4.4)	9 (5.5)	
Systemic anticoagulation		485 (95.3)	329 (95.6)	156 (94.5)	
Therapeutic–target–achieving time — hours	313	9 (4–44)	10 (4–48)	8 (4–39)	0.664
Type of anticoagulant	468				0.283
Unfractionated heparin		459 (98.1)	306 (97.5)	153 (99.4)	
Nonheparin Anticoagulants		9 (1.9)	8 (2.5)	1 (0.6)	
Unfractionated heparin monitoring method	431				0.061
Anti–Factor Xa activity		395 (91.6)	256 (89.5)	139 (95.9)	
aPTT		32 (7.4)	26 (9.1)	6 (4.1)	
ACT		4 (0.9)	4 (1.4)	0 (0.0)	
Anti-factor Xa activity target	354				0.094
Prophylactic (< 0.3 IU/mL)		49 (13.8)	29 (11.7)	20 (18.7)	
Therapeutic (≥ 0.3 IU/mL)		305 (86.2)	218 (88.3)	87 (81.3)	
Antithrombin monitoring	476	130 (27.3)	88 (28.1)	42 (25.8)	0.585
Lowest antithrombin level —	129	62 (50–73)	60 (47–72)	65 (52–75)	0.198
Antithrombin supplementation	471	40 (8.5)	25 (8.1)	15 (9.3)	0.666

Results are presented as n(%) or median (IQR).
aPTT: activated partial thromboplastin time; ACT: activated clotting time.
[a] Expressed as percentage of the standard value

Table S5- Comparison of bleeding and thrombosis between VV and VA-ECMO

Characteristics	All patients (n = 620)	VV-ECMO (n = 568)	VA-ECMO (n = 52)	p value
Bleeding				
Any bleeding	306 (49.4)	275 (48.4)	31 (59.6)	0.122
Any bleeding No of events	382	343	39	
Cannulation site bleeding	114 (18.4)	102 (18.0)	12 (23.1)	0.362
ENT bleeding	76 (12.3)	71 (12.5)	5 (9.6)	0.544
Intracranial hemorrhage	49 (8.0)	46 (8.2)	3 (5.8)	0.788
Pulmonary bleeding	56 (9.0)	54 (9.5)	2 (3.8)	0.213
GI bleeding	45 (7.3)	37 (6.5)	8 (15.4)	0.043
Retroperitoneal bleeding	10 (1.6)	9 (1.6)	1 (1.9)	0.586
Other bleeding	32 (5.2)	24 (4.2)	8 (15.4)	0.003
Thrombosis				
Any thrombosis	225 (36.3)	207 (36.4)	18 (34.6)	0.793
Any thrombosis - No of events	343	315	28	
Circuit Change	82 (13.2)	77 (13.6)	5 (9.6)	0.422
Circuit clots	72 (11.6)	69 (12.1)	3 (5.8)	0.169
Oxygenator failure	59 (9.5)	56 (9.9)	3 (5.8)	0.461
Pulmonary embolism / thrombosis	58 (9.4)	54 (9.5)	4 (7.7)	0.807
Deep vein thrombosis	41 (6.6)	40 (7.0)	1 (1.9)	0.240
Leg ischemia	13 (2.1)	6 (1.1)	7 (13.5)	<0.001
Ischemic stroke	10 (1.6)	5 (0.9)	5 (9.6)	<0.001
Mesenteric infarction	8 (1.3)	8 (1.4)	0 (0.0)	>0.999
Total number of events per patient				0.274
None	231 (37.3)	217 (38.2)	14 (26.9)	
One event	197 (31.8)	179 (31.5)	18 (34.6)	
Two events	96 (15.5)	84 (14.8)	12 (23.1)	
Three events or more	96 (15.5)	88 (15.5)	8 (15.4)	

Results are presented as n (%).
ENT: ear, nose and throat; GI: gastrointestinal.

Table S6 - Comparison of bleeding and thrombosis over the course of the COVID-19 pandemic

Characteristics	All patients (n = 620)	First epidemic wave Before July 1st, 2020 (n = 455)	After July 1st; 2020 (n = 165)	p value
Bleeding				
Any bleeding	306 (49.4)	210 (46.2)	96 (58.2)	0.008
Any bleeding No of events	382	256	126	
Cannulation site bleeding	114 (18.4)	85 (18.7)	29 (17.6)	0.754
ENT bleeding	76 (12.3)	46 (10.1)	30 (18.2)	0.007
Intracranial hemorrhage	49 (8.0)	39 (8.6)	10 (6.1)	0.317
Pulmonary bleeding	56 (9.0)	35 (7.7)	21 (12.7)	0.053
GI bleeding	45 (7.3)	29 (6.4)	16 (9.7)	0.159
Retroperitoneal bleeding	10 (1.6)	5 (1.1)	5 (3.0)	0.141
Other bleeding	32 (5.2)	17 (3.7)	15 (9.1)	0.008
Thrombosis				
Any thrombosis	225 (36.3)	171 (37.6)	54 (32.7)	0.267
Any thrombosis - No of events	343	259	84	
Circuit Change	82 (13.2)	56 (12.3)	26 (15.8)	0.263
Circuit clots	72 (11.6)	67 (14.7)	5 (3.0)	<0.001
Oxygenator failure	59 (9.5)	34 (7.5)	25 (15.2)	0.004
Pulmonary embolism / thrombosis	58 (9.4)	51 (11.2)	7 (4.2)	0.009
Deep vein thrombosis	41 (6.6)	34 (7.5)	7 (4.2)	0.153
Leg ischemia	13 (2.1)	6 (1.3)	7 (4.2)	0.050
Ischemic stroke	10 (1.6)	7 (1.5)	3 (1.8)	0.730
Mesenteric infarction	8 (1.3)	4 (0.9)	4 (2.4)	0.219
Total number of events per patient				0.044
None	231 (37.3)	184 (40.4)	47 (28.5)	
One event	197 (31.8)	135 (29.7)	62 (37.6)	
Two events	96 (15.5)	66 (14.5)	30 (18.2)	
Three events or more	96 (15.5)	70 (15.4)	26 (15.8)	

Results are presented as n (%).
ENT: ear, nose and throat; GI: gastrointestinal.

Table S7 – Multivariable analysis of the impact of any bleeding on in-hospital and day 90 mortality

Variable	adjOR for in-hospital mortality (n=562)	adjOR for mortality at day 90 (n=484)
Any bleeding	2.909 [1.943–4.357]	3.208 [2.025–5.083]
Age (by 10 years)	1.656 [1.367–2.006]	1.671 [1.344–2.079]
Body mass index		
< 25	1	1
[25-30[	0.854 [0.466–1.564]	1.003 [0.507–1.981]
[30-35[	0.752 [0.400–1.415]	0.668 [0.329–1.358]
[35-40[	0.669 [0.310–1.441]	0.637 [0.274–1.479]
≥ 40	0.832 [0.350–1.977]	0.818 [0.319–2.096]
VV-ECMO (vs VA-ECMO)	0.441 [0.187–1.041]	0.658 [0.251–1.726]
Ventilation duration before ECMO ≥ 7 days	1.315 [0.859–2.012]	1.475 [0.910–2.393]
PT (by 5%[a]) at cannulation	0.978 [0.912–1.049]	0.966 [0.890–1.048]
PaO2/FiO2 ratio (by 10) at cannulation	0.992 [0.957–1.027]	0.988 [0.953–1.025]
Renal replacement therapy at cannulation	1.831 [0.983–3.410]	1.540 [0.777–3.053]
Length of ECMO support (by 5 days)	0.987 [0.918–1.061]	0.978 [0.905–1.056]

Results are presented as OR [95CI]
VV-ECMO: venovenous extracorporeal membrane oxygenation; VA-ECMO: venoarterial extracorporeal membrane oxygenation; PT: prothrombin time; PaO2: partial pressure of oxygen; FiO2: fraction of inspired oxygen.
[a]Expressed as percentage of standard value

Table S8 – Multivariable analysis of the impact of any thrombosis on in-hospital and day 90 mortality

Variable	adjOR for in-hospital mortality (n=562)	adjOR for mortality at day 90 (n=484)
Any thrombosis	1.016 [0.677–1.524]	1.187 [0.753–1.873]
Age (by 10 years)	1.675 [1.389–2.021]	1.721 [1.391–2.128]
Body mass index		
< 25	1	1
[25-30[	0.845 [0.470–1.520]	1.017 [0.528–1.957]
[30-35[	0.752 [0.408–1.386]	0.688 [0.349–1.357]
[35-40[	0.589 [0.282–1.231]	0.573 [0.256–1.282]
≥ 40	0.794 [0.344–1.833]	0.812 [0.328–2.010]
VV-ECMO (vs VA-ECMO)	0.431 [0.191–0.975]	0.609 [0.244–1.520]
Ventilation duration before ECMO ≥ 7 days	1.457 [0.965–2.199]	1.601 [1.002–2.559]
PT (by 5%[a]) at cannulation	0.980 [0.916–1.049]	0.968 [0.894–1.048]
PaO2/FiO2 ratio (by 10) at cannulation	0.991 [0.957–1.026]	0.985 [0.950–1.022]
Renal replacement therapy at cannulation	1.847 [1.007–3.387]	1.567 [0.799–3.070]
Length of ECMO support (by 5 days)	1.000 [0.932–1.074]	0.994 [0.921–1.073]

Results are presented as OR [95CI]
VV-ECMO: venovenous extracorporeal membrane oxygenation; VA-ECMO: venoarterial extracorporeal membrane oxygenation; PT: prothrombin time; PaO2: partial pressure of oxygen; FiO2: fraction of inspired oxygen.
[a]Expressed as percentage of standard value

Table S9 – Multivariable analysis of the impact of intracranial hemorrhage on in-hospital and day 90 mortality

Variable	adjOR for in-hospital mortality (n=562)	adjOR for mortality at day 90 (n=484)
Intracranial hemorrhage	13.456 [4.359–41.534]	23.926 [4.588–124.772]
Age (by 10 years)	1.632 [1.370–1.943]	1.582 [1.304–1.920]
Body mass index		
< 25	1	1
[25-30[	0.957 [0.546–1.677]	1.070 [0.581–1.970]
[30-35[	0.793 [0.441–1.427]	0.763 [0.407–1.431]
[35-40[	0.751 [0.375–1.504]	0.646 [0.306–1.364]
≥ 40	0.921 [0.426–1.991]	0.880 [0.387–2.004]
VV-ECMO (vs VA-ECMO)	0.306 [0.146–0.645]	0.395 [0.184–0.852]
Ventilation duration before ECMO ≥ 7 days	1.202 [0.820–1.762]	1.155 [0.753–1.771]
PT (by 5%[a]) at cannulation	0.980 [0.917–1.047]	0.955 [0.886–1.029]
PaO2/FiO2 ratio (by 10) at cannulation	0.991 [0.962–1.020]	0.984 [0.954–1.014]
Renal replacement therapy at cannulation	1.961 [1.108–3.471]	1.893 [1.015–3.532]
Length of ECMO support (by 5 days)	1.022 [0.955–1.094]	1.011 [0.939–1.088]

Results are presented as OR [95CI]
VV-ECMO: venovenous extracorporeal membrane oxygenation; VA-ECMO: venoarterial extracorporeal membrane oxygenation; PT: prothrombin time; PaO2: partial pressure of oxygen; FiO2: fraction of inspired oxygen.
[a]*Expressed as percentage of standard value*

Table S10 – Pre-ECMO variables associated with bleeding

Variables	Model 1	Model 2
Age (by 10 years)	1.132 [0.945–1.357]	1.129 [0.943–1.352]
Sex (Male)	1.190 [0.759–1.865]	1.200 [0.768–1.876]
Body mass index		
< 25	1	1
[25-30[	0.901 [0.508–1.599]	0.892 [0.504–1.577]
[30-35[	0.945 [0.519–1.721]	0.935 [0.515–1.698]
[35-40[	0.615 [0.297–1.273]	0.620 [0.301–1.279]
≥ 40	0.882 [0.386–2.018]	0.866 [0.381–1.969]
VV-ECMO (vs VA-ECMO)	0.666 [0.312–1.421]	0.642 [0.306–1.350]
Ventilation duration before ECMO ≥ 7 days	**1.618 [1.088–2.405]**	**1.619 [1.091–2.403]**
Anticoagulation before ECMO		
No anticoagulation	1	1
Therapeutic anticoagulation	1.745 [0.912–3.337]	1.735 [0.909–3.311]
Prophylactic anticoagulation	1.411 [0.735–2.708]	1.405 [0.734–2.689]
PT (by 5%[a]) at cannulation	1.000 [0.937–1.067]	–
Platelet count < 100 G/L at cannulation	1.047 [0.451–2.430]	1.072 [0.466–2.468]
Fibrinogen < 1.5 g/L at cannulation	1.285 [0.734–2.251]	–
pH ≥ 7.25 at cannulation	0.828 [0.516–1.326]	0.826 [0.518–1.316]
PaO2/FiO2 ratio (by 10) at cannulation	0.991 [0.956–1.028]	0.991 [0.957–1.028]
Renal replacement therapy at cannulation	1.055 [0.590–1.885]	1.049 [0.588–1.870]
ΔPCO2 (by 5 mmHg)	1.031 [0.967–1.100]	1.032 [0.969–1.099]
Length of ECMO support (by 5 days)	**1.077 [1.005–1.153]**	**1.076 [1.005–1.151]**

Results are presented as OR [95CI]
VV-ECMO: venovenous extracorporeal membrane oxygenation; VA-ECMO: venoarterial extracorporeal membrane oxygenation; PT: prothrombin time; PaO2: partial pressure of oxygen; FiO2: fraction of inspired oxygen; ΔPaCO2: difference between day 1 and cannulation partial pressure of carbon dioxide.
[a]Expressed as percentage of standard value

Table S11 – Pre-ECMO variables associated with thrombosis

Variables	Model 1	Model 2
Age (by 10 years)	0.869 [0.718–1.051]	0.874 [0.723–1.055]
Sex (Male)	1.005 [0.626–1.614]	0.994 [0.623–1.584]
Body mass index		
< 25	1	1
[25-30[	1.126 [0.610–2.078]	1.152 [0.629–2.109]
[30-35[	1.012 [0.537–1.906]	1.041 [0.557–1.945]
[35-40[	1.015 [0.477–2.161]	0.996 [0.475–2.091]
≥ 40	1.151 [0.494–2.679]	1.184 [0.513–2.732]
VV-ECMO (vs VA-ECMO)	1.825 [0.797–4.178]	2.028 [0.896–4.593]
Ventilation duration before ECMO ≥ 7 days	0.994 [0.652–1.517]	0.974 [0.642–1.479]
Anticoagulation before ECMO		
No anticoagulation	1	1
Therapeutic anticoagulation	0.929 [0.447–1.932]	0.934 [0.450–1.937]
Prophylactic anticoagulation	1.144 [0.568–2.303]	1.141 [0.569–2.286]
PT (by 5%[a]) at cannulation	1.000 [0.932–1.073]	–
Platelet count ≥ 350 G/L at cannulation	1.058 [0.643–1.742]	1.115 [0.681–1.825]
Fibrinogen ≥ 6 g/L at cannulation	**1.939 [1.003–3.747]**	–
pH ≥ 7.25 at cannulation	0.801 [0.490–1.310]	0.810 [0.502–1.309]
PaO2/FiO2 ratio (by 10) at cannulation	1.034 [0.998–1.070]	1.031 [0.996–1.068]
Renal replacement therapy at cannulation	0.842 [0.454–1.561]	0.858 [0.465–1.582]
ΔPCO2 (by 5 mmHg)	1.019 [0.953–1.089]	1.017 [0.953–1.085]
Length of ECMO support (by 5 days)	**1.170 [1.089–1.257]**	**1.170 [1.090–1.256]**
History of venous thromboembolism	1.659 [0.604–4.557]	1.617 [0.591–4.425]

Results are presented as OR [95CI]
VV-ECMO: venovenous extracorporeal membrane oxygenation; VA-ECMO: venoarterial extracorporeal membrane oxygenation; PT: prothrombin time; PaO2: partial pressure of oxygen; FiO2: fraction of inspired oxygen; ΔPaCO2: difference between day 1 and cannulation partial pressure of carbon dioxide
[a]Expressed as percentage of standard value

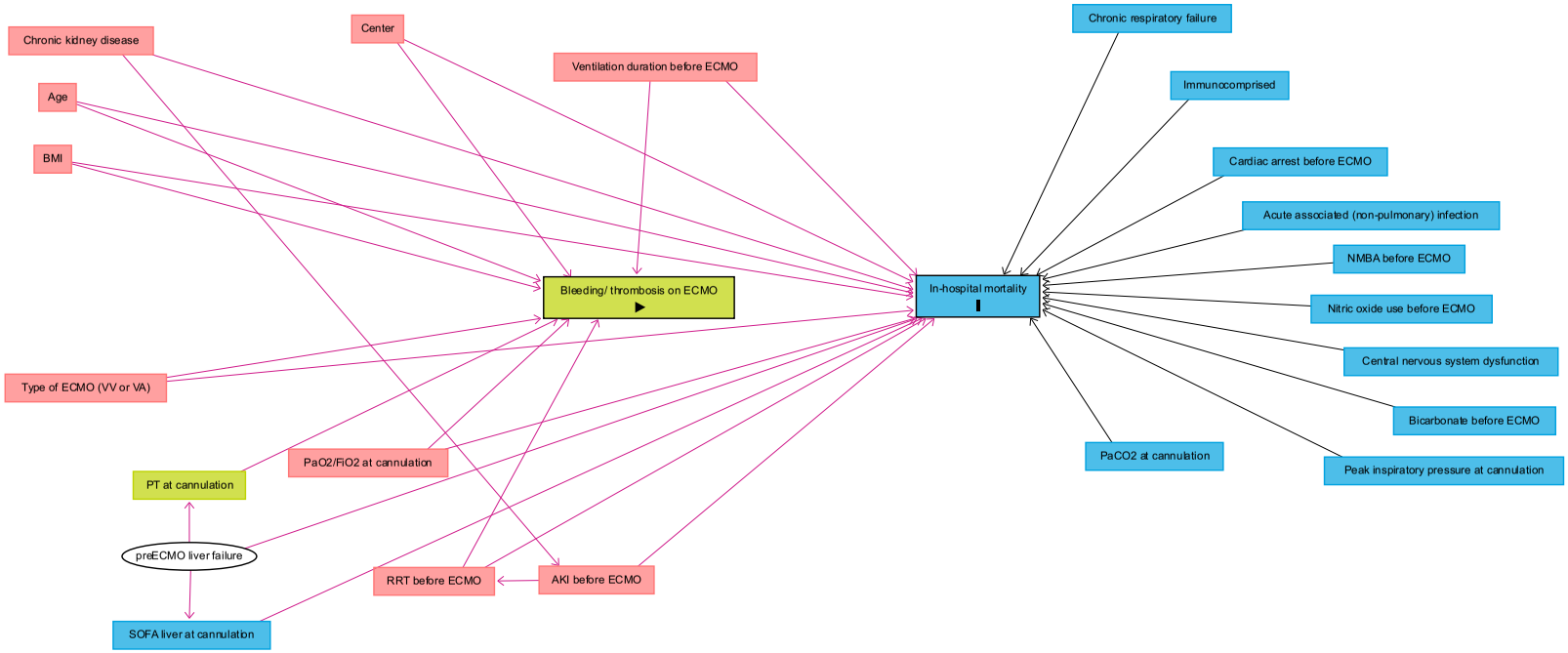


Figure S1: Directed acyclic graph describing associations between bleeding/thrombosis (exposure), pre-ECMO confounders and in-hospital mortality (outcome).

Directed acyclic graph describing our model of causal associations between bleeding/thrombosis (exposure), patient-related confounders, pre-ECMO hospitalization-related confounders and in-hospital mortality, using DAGitty software. Exposure variables are color-coded in green with a right pointing arrow. Outcome is color-coded in blue with a bar. Ancestors of exposure are color-coded in green; ancestors of outcome are color-coded in blue, and ancestors of both exposure and outcome are color-coded in pink. The set of potential confounders sufficient for adjustment was: age, body mass index, center, PT at cannulation, type of ECMO (VV or VA), RRT before ECMO, ventilation duration before ECMO, PaO2/FiO2 at cannulation.

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UNIVERSITÉ DE RENNES 1

Titre : **Implication de la voie de l'AMP cyclique plaquettaire dans la modulation de l'inflammation**
Application à l'étude de l'inhibition du récepteur P2Y12 au cours du sepsis et de l'inflammation liée aux circulations extracorporelles

Mots clés : plaquettes, inflammation, $P2Y_{12}$, ticagrelor, sepsis, circulations extracorporelles

Résumé : En plus de leur rôle central dans l'hémostase, les plaquettes sanguines sont désormais reconnues comme des acteurs importants de la réponse inflammatoire aiguë. Notre hypothèse était que l'inhibition de l'activation plaquettaire par la voie du récepteur $P2Y_{12}$ pourrait permettre de moduler la réponse inflammatoire associée aux états septiques graves et aux circulations extracorporelles. Nous avons développé une approche transversale, basée sur l'utilisation de modèles précliniques (modèle murin de sepsis, modèle in-vitro de stimulation de sang total par LPS, évaluation d'un dispositif de récupération sanguine peropératoire), associés à l'analyse de données cliniques. Ce travail démontre que le traitement par ticagrelor est associé à une diminution de la réponse inflammatoire et des lésions d'agression pulmonaire aigüe dans un modèle murin de sepsis polymicrobien (cecal slurry). Ces résultats permettent d'envisager plusieurs hypothèses mécanistiques, associant l'inhibition plaquettaire à des mécanismes extra-plaquettaires, qui devront être évaluées dans des études complémentaires

Nous avons également mis en évidence une association indépendante entre la transfusion peropératoire de concentrés plaquettaires déleucocytés et la survenue de bactériémies en post-opératoire de chirurgie cardiaque sous circulation extracorporelle. Ce résultat permet d'envisager un rôle direct des plaquettes allogéniques et des médiateurs inflammatoires issus du stockage des concentrés plaquettaires. Enfin, nous avons pu montrer que le CD40L soluble, principalement d'origine plaquettaire, était précocement associé aux formes sévères de COVID-19 et pourrait constituer un biomarqueur d'intérêt dans ce contexte.
Ces données constituent un rationnel solide pour développer de futurs travaux de recherche clinique et translationnelle évaluant l'utilisation d'approches pharmacologiques d'inhibition plaquettaire dans l'objectif d'améliorer le devenir des patients en soins critiques sous assistance extracorporelle.

Title: **Role of platelet cyclic AMP in the modulation of inflammation**
Application to the study of $P2Y_{12}$ inhibition during sepsis and extracorporeal circulation-mediated inflammation

Keywords: platelets, inflammation, $P2Y_{12}$, ticagrelor, sepsis, extracorporeal circulation

Abstract: Besides their central role in hemostasis, platelets are now recognized as key players in acute inflammatory response. Our hypothesis was that inhibition of platelet activation using $P2Y_{12}$ inhibitors might reduce extracorporeal circulation- and sepsis-mediated inflammation. We employed a transversal approach, using both preclinical models (murine sepsis model, LPS-stimulated whole blood model, evaluation of a new filtration-based autotransfusion device) and clinical data. Our study demonstrates that, in a mouse model of polymicrobial sepsis (cecal slurry), ticagrelor treatment was associated with a significant reduction of sepsis-induced cytopenia, inflammatory cytokine release and acute lung injury. Several hypotheses might be considered, including platelet and non-platelet-mediated mechanisms, that will require further studies.

We also reported that intraoperative allogeneic platelet transfusion was associated with a significant increase in postoperative bloodstream infection after cardiac surgery, suggesting a possible role for platelet activation and platelet-derived soluble mediators in platelet concentrates. Finally, we reported that soluble CD40L, a platelet-activation marker, was increased early in the clinical course of severe COVID-19 and might therefore be further evaluated as biomarker.
Together, these results provide a strong basis for developing clinical and translational research using pharmacological modulation of platelet functions to improve clinical outcomes of patients receiving extracorporeal circulation in ICU.