Étude génomique et protéomique des concentrés plaquettaires ayant induit une réaction post-transfusionnelle

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RÉSUMÉ

Malgré la mise en œuvre de la leucoréduction systématique, les transfusions plaquettaires restent génératrices de réactions post-transfusionnelles (encore appelées « Effets Indésirables Receveur, EIR »). Nous savons que les plaquettes sanguines relarguent des molécules proinflammatoires (e.g. CD40 ligand soluble ou sCD40L) durant leur préparation et stockage et leurs taux augmentés sont associées aux EIR. Le travail de cette thèse vise à mieux comprendre les mécanismes de survenue des EIR post transfusion plaquettaire, dans le contexte de l'inflammation.

La première partie de nos travaux n'a pas retrouvé de polymorphismes génétiques du gène *CD40LG* qui pourraient modifier l'affinité du couple Récepteur/Ligand, en cas d'EIR. Dans un second temps, une caractérisation haplotypique de *CD40LG* n'a pas non plus permis de retrouver d'association avec l'apparition d'EIR.

Nous avons ensuite tenté d'identifier des marqueurs génétiques de surexpression de sCD40L (et par conséquence, d'EIR). Pour cela, nous avons étendu l'investigation à douze polymorphismes de *CD40LG* mais aussi des marqueurs génétiques connus comme indépendamment liés à l'expression de sCD40L : au niveau de son récepteur *CD40* et d'*ITGA2*. Ont été retrouvés associés à une modification significative de sécrétion de sCD40L, d'une part le polymorphisme rs126643 (*ITGA2*), un haplotype étendu de CD40LG et aussi un haplotype interchromosomique (*CD40LG–CD40–ITGA2*). Toutefois, ces haplotypes n'ont pas pu être retrouvés associés à l'apparition d'EIR.

Nous avons également montré que lors du stockage de concentrés plaquettaires (CP) non leucoréduits, les « modificateurs de réponse biologique » (BRM) leucocytaires diffèrent d'une part, dominent et influencent d'autre part les BRM d'origine plaquettaire. Nous nous sommes ensuite intéressés à un autre BRM individualisé dans les produits transfusés : l'ADN mitochondrial, classé comme un signal de danger endogène (ou DAMP) et nous avons retrouvé une augmentation significative de ceux-ci dans les CP ayant induit un EIR.

Nous avons alors entrepris une étude protéomique (LC-MS/MS) et transcriptomique (RNA-seq) sur des CP ayant induit ou non un EIR. L'enrichissement des protéines et gènes différentiellement exprimés présente principalement un rôle dans l'activation plaquettaire, la coagulation, l'apoptose et la dégranulation qui représentent des processus en liaison étroite. Le transcriptome plaquettaire a confirmé l'étiologie apoptique par la mise en évidence de la

dérégulation calcique mitochondriale. De plus, l'obtention de l'enrichissement de sa voie intrinsèque permet d'expliquer l'abondance des ADNmt retrouvés précédemment dans les CP associés aux EIR. Par ailleurs, l'état d'activation et la dégranulation des plaquettes expliquent l'abondance des BRM retrouvés en cas d'EIR.

Ainsi, nous avons identifié de nouveaux marqueurs inflammatoires hautement exprimés dans le groupe EIR. Après validation de ces résultats par des études complémentaires, ces marqueurs pourraient être proposés comme cibles de prévention d'EIR en modifiant, par exemple la préparation des CP.

LISTE DES ABRÉVIATIONS

A. fumigatus : Aspergillus fumigatus

ACD : Acide citrique-Citrate trisodique-Dextrose

ADN : acide désoxyribonucléique

ADNmt : ADN mitochondrial

ADP : Adenosine diphosphate

ANSM : agence nationale de sécurité médicale

ARMS : Amplification Refractory Mutation System

ARN : acide ribonucléique

ARNm : ARN messager

ATP : Adenosine triphosphate

BC : *Buffy-Coat*

BRM : Biological Response Modifier

C. albicans : Candida albicans

CCI : Corrected Count Increment

CCL : Chemokine (C-C motif) ligand

CD : cluster de différentiation

CD40L : CD40 ligand

c-Mpl : Thrombopoietin receptor

CNV : Copy Number Variant

COFRADIC : combined fractional diagonal chromatography

CP : concentrés de plaquettes

CPA : Concentrés de Plaquettes d'Aphérèse

CPD : Citrate-Phosphate-Dextrose

CPU : Concentré Plaquettaires Unitaire

CXCL : Chemokine (C-X-C motif) ligand

DEHP : phtalate de 2-diéthyl-hexyle

DGGE : Denaturing Gradient Gel Electrophoresis

dHPLC : denaturing High Performance Liquid Chromatography

DIGE : Differential In-Gel Electrophoresis

EIR : effets indésirables receveur

ELISA : ensyme linked immuno-sorbent assay

Fc : Fragment crystallizable (e.g. FcγRIIa, FceRII)

FGF-4 : Fibroblast growth factor 4

GM-CSF : Granulocyte macrophage colony-stimulating factor

GP : glycoprotéine (e.g. GPIIb-IIIa)

Granules α : granules alpha

GTP : Guanosine triphosphate

H. pylori : Helicobacter pylori

HRM : High Resolution Melt

HTLV-I, HTLVII

ICAM-1 : Intercellular Adhesion Molecule 1

ICAT : isotope-coded affinity tags

Ig : immunoglobulin

IL- : Interleukine

iTRAQ : isotope tags for relative and absolute quantitation

JAM : « Junction Adhesion Molecule »

LAMP-2 : lysosomal associated membrane protein 2

LC/MS-MS chromatographie liquide / spectrométrie de masse en tandem

LD : Linkage Disequilibrium = déséquilibre de liaison

LPS : lipopolysaccharides

LSP : lésion de stockage des plaquettes

Mac-1 : Macrophage-1 antigen

MAF : Minor Allele Frequency)

MCP : mélange de concentrés plaquettaires

MCP-3 : monocyte chemotactic proteins 3

miARN : microARN

MIP-1α : macrophage inflammatory protein 1-alpha

MSE : matrice sous-endothéliale

NET : Neutrophil extracellular traps

NGS : Next Generation Sequencing = séquençage nouvelle génération

NO : monoxyde d'azote

P. falciparum : Plasmodium falciparum

PAS : Platelet Additive Solution

pb : paires de bases

PCR : Réaction de polymérisation en chaîne = Polymerase Chain Reaction

PDGF : Platelet-derived growth factor

PECAM-1 : Platelet endothelial cell adhesion molecule

PF4 : Platelet factor 4

PMN : polymorphonuclear

PRP : Plasma Riche en Plaquette

PRR : pathogen recognition receptor

PSGL-1 : P-selectin glycoprotein ligand-1

PVC : chlorure de polyvinyle

qPCR : PCR quantitative

RANTES : Regulated on Activation, Normal T Cell Expressed and Secreted

RFLP : Restriction Fragment Length Polymorphisms

RFNH : réactions fébriles non-hémolytiques

ROS : reactive oxygen species

RT-PCR : transcription inverse

S. aureus : Staphylococcus aureus

S. epidermidis : Staphylococcus epidermidis

S. gordonii : Streptococcus gordonii

S. pneumoniae : Streptococcus pneumoniae

S. sanguinis : Streptococcus sanguinis

SAGE : Serial Analysis of Gene Expression

sCD40L : CD40L soluble

sCD62P : P-selectine soluble

SDF-1 : Stromal cell-derived factor 1

SDS-PAGE : électrophorèse sur gel de polyacrylamide dodécyl sulfate de sodium

SELDI : surface-enhanced laser desorption/ionization

SMI : système de membrane invaginée

SNP : Single Nucleotide Polymorphisms

SSCP : Single Strand Conformation Polymorphism

STRP Short Tandem Repeat Polymorphisms

TGF-β : Transforming growth factor béta

TLR : Toll-like receptor

TNF : tumor necrosis factor

TPO : Thrombopoïétine

TRALI : syndrome de détresse respiratoire aiguê

TREM-1 = TLT-1 : triggering receptor expressed on myeloid cells like 1

TxA2 : Thromboxane A2

UTP : Uridine triphosphate

UTR : Untranslated région

VCAM-1 : vascular cell adhesion molecule 1

VHC : virus de l'hépatite C

VIH : virus de l'immunodéficience humaine

VNTR Variable Number of Tandem Repeat

VWF : Von Willebrand factor

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I. Généralités sur les plaquettes sanguines

Les plaquettes sanguines sont les plus petits éléments figurés du sang avec un diamètre compris entre 2 et 4 µm et un volume de 6 à 10 femto litre. Ces sont des cellules anucléées qui jouent un rôle de sentinelles parcourant le système vasculaire assurant son intégrité prévenant ainsi les saignements et minimisant les blessures des vaisseaux sanguins. Outre leur rôle dans l'hémostase, les plaquettes sont maintenant confirmées comme étant des cellules de l'immunité innée et adaptative.

Pour assurer ces rôles, 150 à 450 10^9/L plaquettes circulent dans le sang d'un adulte et leur durée de vie moyenne est de 8 à 10 jours. Afin de maintenir une numération normale de plaquettes, 220 10^9/L plaquettes (environ) sont produites quotidiennement par les mégacaryocytes.

I.1. Du mégacaryocyte aux plaquettes

I.1.1. Maturation et développement des mégacaryocytes

Les mégacaryocytes sont des cellules très spécialisées qui assurent la formation et la libération des plaquettes afin de maintenir un taux approprié de plaquettes circulantes. Les mégacaryocytes se développent à partir des cellules souches hématopoïétiques qui se trouvent principalement dans la moelle osseuse, mais sont également présentes dans d'autres organes comme le foie, la rate et les poumons[1,2].

La biologie des mégacaryocytes et des plaquettes a été révolutionnée grâce à la découverte de la thrombopoïétine (TPO), et de son récepteur spécifique, le c-Mpl. La TPO est une cytokine produite de manière constitutive principalement par les hépatocytes qui agit sur les cellules souches hématopoïétiques et les progéniteurs des mégacaryocytes dans la moelle osseuse pour promouvoir la différenciation des mégacaryocytes[3]. Ce processus nécessite également plusieurs signaux moléculaires comprenant le GM-CSF, l'IL-3, l'IL-6, l'IL-11, le SDF-1, le FGF-4 et l'érythropoïétine[4,5]. Les mégacaryocytes utilisent un processus TPO-dépendant par lequel ils deviennent polyploïdes à travers des cycles de réplication de l'ADN sans division cellulaire ; il s'agit de l'endomitose. Au cours de leur cycle de vie, les mégacaryocytes subissent une première étape de prolifération 2n où leur progression à travers le cycle cellulaire est identique à celle des autres cellules hématopoïétiques. Ensuite, ils débutent l'endomitose et accumulent une teneur en ADN de 4n, 8n, 16n, 32n, 64n, et même 128n dans un seul noyau polylobé avant de procéder à leur maturation finale et la formation de proplaquettes ultérieurement[6].

Il est supposé que les mégacaryocytes sont polyploïdes afin de produire de grandes quantités d'ARNm et de protéines qui vont être stockées dans les granules plaquettaires ultérieurement, tout en conservant leur capacité à effectuer plusieurs fonctions, telles que la génération du système membranaire invaginé et la formation de proplaquettes[6,7].

Les nombreux rôles que les plaquettes jouent dans différents processus biologiques et pathologiques sont en grande partie dus à la présence de divers réservoirs contenus dans leurs granules hérités du mégacaryocyte. Dans les mégacaryocytes, les granules sont dérivés du bourgeonnement de petites vésicules qui contiennent un réservoir granulaire dérivant du réseau trans-Golgien[8]. Ces vésicules peuvent être livrées directement dans des corps multivésiculaires où les protéines sont triées et emballées par la suite sous forme de granules[9,10]. Les organelles et les granules sont ensuite transportés individuellement à partir du corps cellulaire mégacaryocytaire, le long de la tige de proplaquettes où elles se déplacent de façon bidirectionnelle jusqu'à ce qu'elles soient capturées à la pointe proplaquettaire[11].

Toutes ces vésicules et granules sont formés grâce au système de membrane invaginée (SMI) connu aussi sous le nom de « système de membrane de démarcation » produit pendant la maturation des mégacaryocytes. Il s'agit de la formation progressive d'un système de membrane complexe qui est continu avec la membrane plasmique et qui imprègne le cytoplasme[12]. Le SMI, dérivé de la membrane plasmique, maintient le contact avec le milieu extracellulaire et fonctionne comme un réservoir à membrane pour la formation de proplaquettes[13,14].

I.1.2. Formation des plaquettes : la thrombopoïèse

I.1.2.1 Formation des proplaquettes

Les mégacaryocytes matures se prolongent en longues ramifications dites proplaquettes dans les vaisseaux sinusoïdaux de la moelle osseuse. Le processus commence au niveau d'un site unique de la membrane plasmique du mégacaryocyte. A ce point, il se forme des pseudopodes qui s'allongent et se rétrécissent en un tube d'un diamètre moyen de 2 à 4 µm, appelé tuyau proplaquettaire[15].

Les proplaquettes fonctionnent comme des chaînes de montage de la production de plaquettes. Elles sont constituées de renflements qui ont la taille des plaquettes dans des réseaux en tandem le long du tuyau proplaquettaire[16]. Une grande partie des organites qui vont ultérieurement constituer les plaquettes matures se forment à partir du cytoplasme

mégacaryocytaire et le SMI. Ces organites migrent du corps cellulaire mégacaryocytaire aux extrémités des proplaquettes[11].

Le noyau polylobé reste dans le corps cellulaire du mégacaryocyte alors que le reste se transforme en proplaquettes. Une fois que la totalité du corps de la cellule mégacaryocytaire est transformée en proplaquettes, le noyau est extrudé et dégradé[14,17].

Ainsi les plaquettes sont entièrement formées à l'extrémité des proplaquettes, leur libération peut alors être achevée.

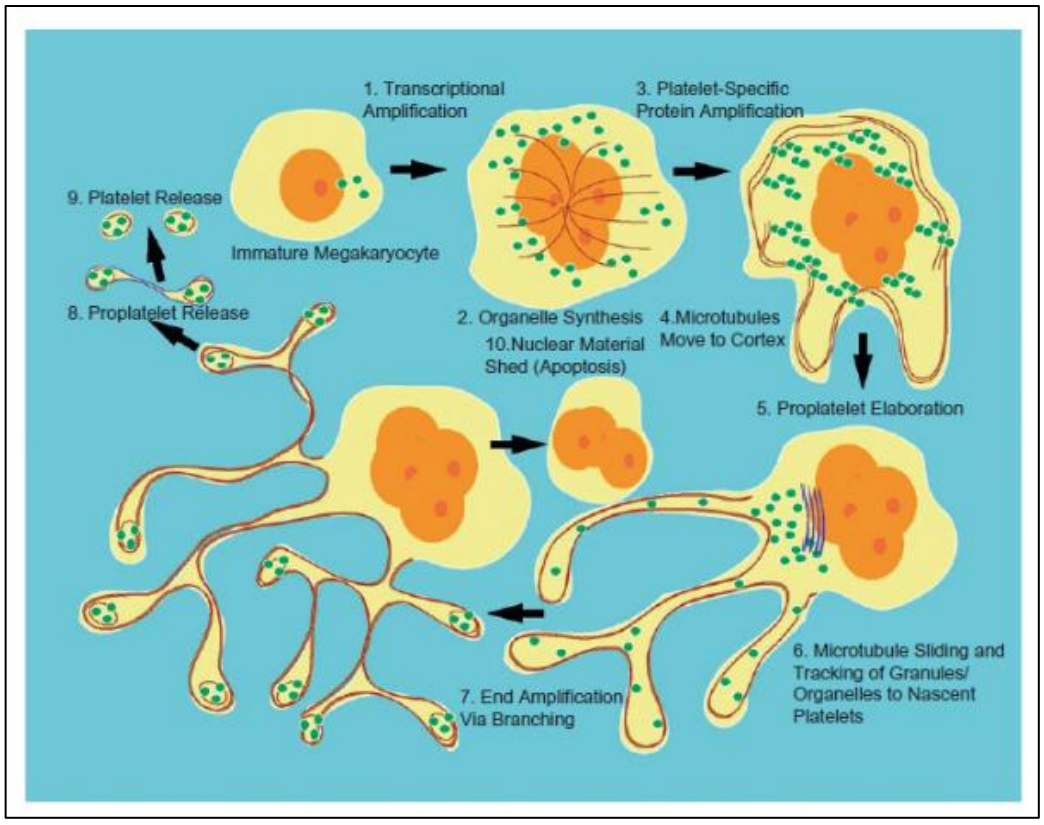

Figure 1: Les différentes étapes de la formation des plaquettes[1].

(1) Après différenciation, les mégacaryocytes subissent l'endomitose et une activation de la transcription. (2) Au cours de l'expansion cytoplasmique et de la maturation, le mégacaryocyte débute la synthèse des organites et (3) l'amplification des protéines spécifiques des plaquettes. Les microtubules émanant d'un centrosome sont clairement établis. (4) Avant le début de la formation des proplaquettes, les centrosomes se désassemblent et les microtubules sont transloquées vers le cortex cellulaire. (5) La production de proplaquettes s'initie par la formation de grandes pseudopodes allongées et forment des procédés de proplaquettes minces avec des extrémités bulbeuses ; ces extrémités contiennent un faisceau périphérique de microtubules qui forme des boucles. (6) Les organites sont déplacés individuellement sur les microtubules dans les extrémités des proplaquettes où les plaquettes naissantes s'assemblent. (7) La croissance et l'extension des processus de proplaquettes sont associées à la flexion répétée et à la bifurcation actine-dépendante, ce qui amplifie les extrémités de proplaquettes libres. Les proplaquettes forment des rétrécissements le long de leur longueur, leur donnant un aspect de perle. (8) L'ensemble du cytoplasme des mégacaryocytes est

converti en proplaquettes qui se libèrent du corps du mégacaryocyte après une rétraction rapide. Un « haltère » de deux particules ayant la taille de plaquettes liées par un petit pont cytoplasmique peut constituer une étape intermédiaire. (9) Les proplaquettes subissent une fragmentation en plaquettes individuelles. (10) Enfin, le noyau nu extrudé restant après l'excrétion presque complète du cytoplasme du mégacaryocyte subit l'apoptose.

I.1.2.2 Libération des plaquettes

A la base, les mégacaryocytes sont localisés dans la moelle osseuse. Après maturation en mégacaryocytes « plaquettogènes », ils migrent depuis la niche ostéoblastique vers la région sous-endothéliale près des sinusoïdes veineuses. L'imagerie in vivo a montré que les plaquettes peuvent être clivées à partir des extensions proplaquettaires au niveau des sinusoïdes médullaires[17]. Les proplaquettes peuvent être aussi libérées dans la circulation sanguine où elles libèrent des plaquettes par fragmentation de leurs extrémités bulbeuses. Cette imagerie *in vivo* a souligné le rôle du flux sanguin qui permet de rompre efficacement l'extrémité des proplaquettes libérant ainsi les plaquettes.

Les mégacaryocytes matures peuvent aussi migrer à travers les cellules endothéliales et sont détectés dans le sang circulant[18]. Plusieurs auteurs ont remarqué que les mégacaryocytes sont présents en grand nombre dans les petits vaisseaux des poumons et se retrouvent **"**piégés**"** dans les capillaires pulmonaires où ils libèrent des plaquettes à partir de l'extrémité des proplaquettes grâce aux forces de cisaillement élevées intravasculaires, en particulier dans la microcirculation pulmonaire[19,20].

En 2010, Thon et al.[21] ont décrit une nouvelle étape dans la formation des plaquettes identifiées dans les mégacaryocytes en culture et dans les frottis de sang périphérique. Il s'agit des préplaquettes, particules discoïdes anucléées ayant 2 à 10 µm de diamètre qui peuvent se convertir de manière réversible en proplaquettes en forme haltère. Deux autres études ont constaté que les préplquettes sont capables de former directement des plaquettes à la fois *in vitro* et après transfusion chez des souris *in vivo*[22,23]. Les préplaquettes peuvent être **"**piégées**"** dans les microcapillaires de la moelle osseuse, des poumons, ou de la rate, où les forces de cisaillement intravasculaires (processus controlé par polymérisation des microtubules[22]) peuvent alors aussi conduire à une trombopoïèse périphérique à partir de préplaquettes mais aussi des plaquettes eux-mêmes qui sont capables de générer d'autres[23].

I.2. Biologie des plaquettes

I.2.1. Morphologie des plaquettes

Les plaquettes sont les plus petits éléments figurés du sang circulant. Anucléées, elles ont en moyenne 2 à 5 µm de diamètre, 0,5 µm d'épaisseur et présente un volume moyen de 6 à 10 femto litres. Leur forme est discoïde à l'état de repos et irrégulière avec des prolongements dendritiques après activation. En microscopie électronique, les plaquettes possèdent 3 principales composantes : le système membranaire, le cytosquelette et les organelles intracellulaires[24].

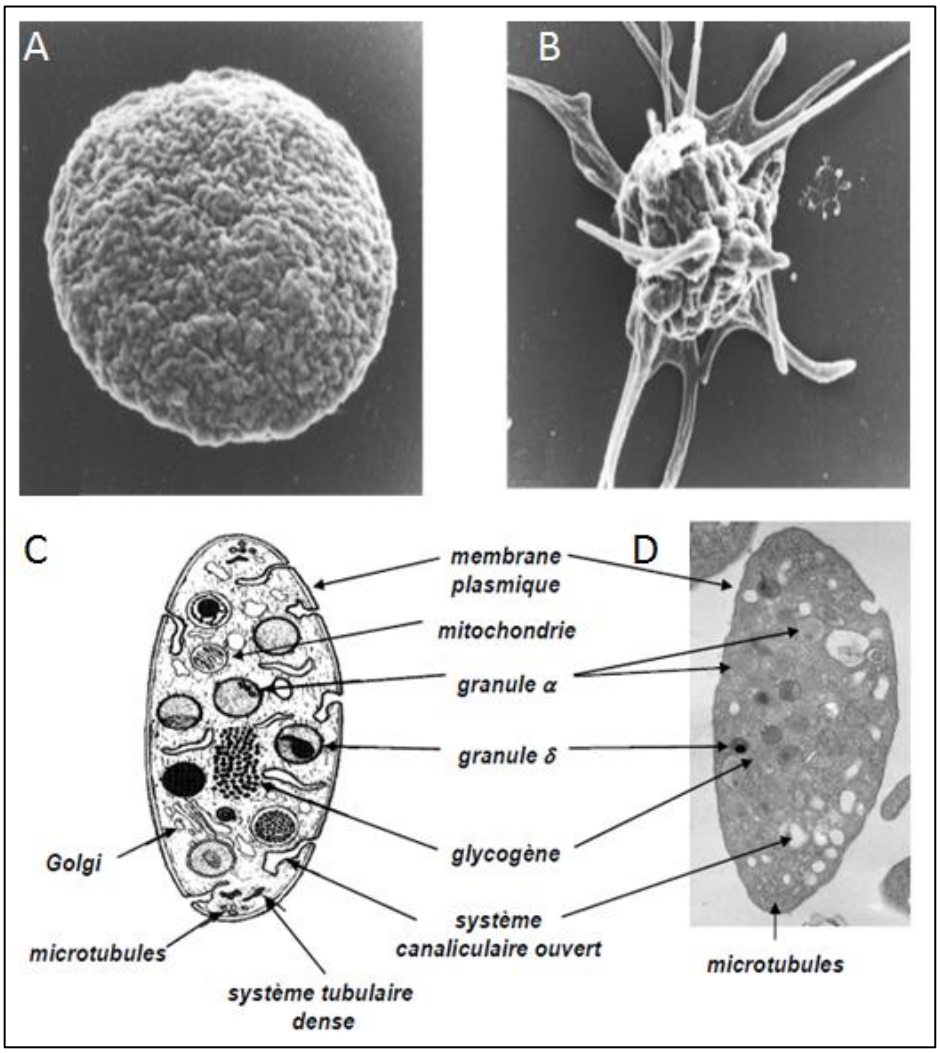

Figure 2 : Morphologie des plaquettes.

A) Plaquette discoïde vue au microscope électronique à balayage ; B) Forme dendritique de plaquette activée vu au microscope électronique à balayage ; C) Schéma des principales caractéristiques ultrastructurales observées sur une coupe longitudinale d'une plaquette discoïde ; D) Une coupe de plaquette discoide au repos examinée en microscopie électronique à transmission[24].

I.2.2. Structure des plaquettes

I.2.2.1 Le système membranaire extérieur

La membrane plasmique plaquettaire est relativement lisse comparée à celle des leucocytes. Elle est constituée d'une bicouche lipidique sur laquelle repose un épais manteau membranaire extérieur, le glycocalyx. Sur la surface intérieure, elle contient un système relativement régulier de minces filaments ressemblant à des filaments d'actine qui relie la membrane plasmique au cytosquelette[25–27].

Le système membranaire extérieur ne sert pas seulement de barrière pour séparer le contenu interne des plaquettes du milieu extérieur. Il constitue, au contraire, une structure très dynamique qui détecte les changements dans le compartiment vasculaire nécessitant une réponse hémostatique des plaquettes aux sites de lésions vasculaires.

Le glycocalyx est couvert de différents types de récepteurs, principalement les glycoprotéines, nécessaires pour i) faciliter l'adhérence des plaquettes à une surface endommagée, ii) déclencher leur activation complète, iii) promouvoir leur agrégation et leur interaction avec d'autres éléments cellulaires, iv) d'accélérer le processus de rétraction du caillot[24].

Le feuillet externe de la membrane plasmique présente de nombreuses invaginations ouvertes sur l'extérieur formant ainsi le système canaliculaire ouvert, un réseau anastomosé de canaux membranaires ou tubules qui parcourent toute la membrane plaquettaire. Ces canaux permettent aux petites molécules de pénétrer dans le cytoplasme d'une manière semi-sélective en limitant l'entrée de grandes protéines telles que certains anticorps. En cas d'activation plaquettaire, le système canaliculaire ouvert sert de point de fusion entre les granules et la membrane plasmique[24].

I.2.2.2 Le cytosquelette

Le cytosquelette, constitué de polymères d'actine et de tubuline et d'autres protéines qui leurs sont associées, sert de système d'entretoises et de poutres moléculaires qui définit la forme discoïde des plaquettes au repos et maintient l'intégrité cellulaire. La forme de plaquettes rencontre des forces de cisaillement élevées du fluide généré par l'écoulement du sang au-dessus de l'endothélium.

Les éléments essentiels de ce système sont, de la membrane plasmique vers l'intérieur, un squelette à base de spectrine adhérant à la face cytoplasmique de la membrane plasmique, une

bobine de microtubules qui court le long du périmètre du disque et un réseau rigide de filaments d'actine réticulés qui remplit l'espace cytoplasmique de la cellule[28–30].

Le changement de forme des plaquettes après activation est aussi contrôlé par le cytosquelette. En effet, pour se propager, la plaquette doit réorganiser son cytosquelette et assembler de nouveaux filaments d'actine. Ce processus complexe dépend de la dynamique cytoplasmique des polymères d'actine et implique plusieurs protéines qui régulent l'architecture et la dynamique de l'actine[31].

I.2.2.3 Les organelles plaquettaires

Etant donné que les plaquettes sont anucléées, elles contiennent différents types de granules qui sont nécessaires à leur fonctionnement (Tableau 1).

Tableau 1 : Les principales caractéristiques des granules plaquettaires[32,33].

	Nombre/ plaquette	Diamètre (nm)	Surface (μm^2)/ plaquette	Marqueurs communs	Fonction principale
Granules α	50 à 80	200 - 500	14	VWF CXCL4 (PF4) CD62P	Hémostase/thrombose Inflammation Angiogenese Défense de l'hôte Mitogénèse
Granules denses	3 à 8	150	<1	CD63 Sérotonine	Hémostase/thrombose Inflammation
Lysosomes	< 3	200 - 250	<1	Acide phosphatase	Digestion endosomale

I.2.2.3.1 Les granules α

Les granules α sont spécifiques aux plaquettes. Ce sont les granules les plus abondants (environ 50 à 80 granules par plaquette, dix fois plus que les granules denses). Ils ont une taille de 200 à 500 nm[34].

Les granules α acquièrent leur contenu moléculaire à la fois de la synthèse des protéines endogènes par le mégacaryocyte et par l'internalisation des protéines plasmatiques par endocytose[35]. La molécule la plus connue pour être endocytée est le fibrinogène qui est internalisé après sa fixation sur l'intégrine αIIbβ3 (CD41/CD61)[36].

Les granules α contiennent à la fois des protéines liées à la membrane qui s'expriment à la surface des plaquettes et des protéines solubles qui sont libérées dans l'espace extracellulaire (Tableau 2). La plupart des protéines liées à la membrane des granules α sont également présentes sur la membrane cytoplasmique des plaquettes au repos[37,38]. L'endocytose de

membrane plasmique peut contribuer à la présence de molécules d'adhérence dans les granules α[37]. Cependant, toutes les protéines associées à la membrane des granules α, ne sont pas présentes sur la membrane plasmique des plaquettes au repos (e.g, CD109, CD62P, CD40L)[38].

En ce qui concerne les molécules solubles, les plaquettes en contiennent plus de 300[39]. Elles sont stockées dans des réservoirs d'une façon sélective et hétérogène[40] et sont secrétées d'une façon très spécifique par différents stimuli (coagulation sanguine, adhérence cellulaire, inflammation, croissance cellulaire et défense de l'hôte).

Après l'activation plaquettaire, les granules α se dirigent vers le système canaliculaire ouvert, leurs deux membranes fusionnent, ce qui permet aux plaquettes d'augmenter leur surface spécifique par deux à trois fois. Ainsi, les protéines membranaires se présentent à la surface des plaquettes et les protéines contenues dans les réservoirs vont être libérées dans le milieu extérieur.

Tableau 2 : Contenu des granules α (liste non exhaustive)[32,33].

Type de protéine	Exemples
Protéines membranaires	αIIbβ3 (CD41/CD61), GPIbα-IX-V, GPVI, TLT-1, CD62P, CD40L
Coagulants, anticoagulants, et protéines fibrinolytiques	Factor V, factor IX, factor XIII, antithrombin, proteinS, tissue factor pathway inhibitor, plasminogen, plasminogen activator inhibitor1, α2-macroglobuline
Protéines d'adhérence	Fibrinogene, facteur von Willebrand, thrombospondine
Chimiokines	CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP-1α), et CCL5 (RANTES)
Facteurs de croissance	Epidermal growth factor, hepatocyte growth factor, insulin-like growth factor, transforming growth factor β (TGF-β)
Facteurs angiogeniques et inhibiteurs	Vascular endothelium growth factor, fibroblast growth factor, platelet- derived growth factor, tissue inhibitors of metalloproteinases, angiostatin, endostatin
Proteines microbicides	Thymosine-β4, thrombocidines 1 et 2 (NAP-2)
Médiateurs de l'immunité	Précurseur du complément C3, précurseur du complément C4, Globuline β1H, facteur D, facteur H, inhibiteur C1, IgG

I.2.2.3.2 Les granules denses

Les granules denses, d'environ 250 nm de diamètre, sont un sous-type d'organites liés aux lysosomes que l'on trouve exclusivement dans les plaquettes. Ils sont moins nombreux (3 à 5 par plaquette) et plus petits que les granules α (Tableau 1). Cependant, ces granules contiennent des concentrations extrêmement élevées de cations, des polyphosphates, des

nucléotides, et des amines bioactives tels que la sérotonine et l'histamine (Tableau 3).

La membrane des granules denses contient des protéines de surface comme les organites liés aux lysosomes tels que le CD63 et LAMP-2 (CD107b) mais aussi des intégrines présentes sur la membrane plaquettaire y compris la GPIb (CD42) et αIIbβ3 (CD41/CD61) [41].

L'exocytose de ces granules est dépendante du Ca^{2+} et est observée lors de l'activation plaquettaire en particulier après la stimulation par le collagène ou l'ADP. La sécrétion du contenu des granules denses est le résultat de la fusion de leur membrane et de la membrane plasmique[42].

Tableau 3 : Contenu des granules denses (liste non exhaustive)[32,33].

Type de protéine	**Exemples**
Cations	CA^{2+}, Mg^{2+}, K^{+}
Phosphates	Polyphosphate, pyrophosphate
Amines bioactive	Sérotonine, histamine
Nucléotides	ADP, ATP, UTP, GTP

I.2.2.3.3 Les lysosomes

Les plaquettes contiennent peu de lysosomes primaires et secondaires (Tableau 1). Ces lysosomes contiennent de nombreuses hydrolases acides et cathepsines dans des réservoirs et expriment le CD63 et la LAMP-2 sur leur membrane. La fonction des lysosomes plaquettaires n'est pas bien étudiée. Ils peuvent jouer un rôle dans la digestion endosomale, comme observé dans les cellules nucléées. Les plaquettes contiennent également des peroxysomes[43]. Cependant, l'importance de cet organite en fonction plaquettaire est indéterminée.

Les lysosomes plaquettaires, comme les lysosomes dans d'autres types cellulaires, contiennent des enzymes impliquées dans la dégradation des protéines, des glucides et des lipides (Tableau 4).

Tableau 4 : Contenu des lysosomes[32,33].

Type de protéine	Exemples
Enzymes degradant les protéines	Cathepsines, elastase, collagenase, carboxypeptidase, proline carboxypeptidase
Enzymes degradant les glucides	Glucosidase, fucosidase, galactosidase, glucuronidase, mannosidase, hexosaminidase, arabinofuranosidase
Phosphatases	Phosphatase acide

I.2.2.3.4 Autres organites : les mitochondries, les glycosomes et les granules T

Les mitochondries plaquettaires sont peu nombreuses et structurellement simples. Elles

contribuent de manière significative au métabolisme énergétique de la cellule. Ainsi les mitochondries seules peuvent soutenir les besoins énergétiques des plaquettes. Les mitochondries contiennent également du calcium et sont considérées comme la source principale de calcium nécessaire à l'activation des plaquettes[44].

Un autre organite de forme ronde ou ovale et de taille similaire à celle des granules α a été aussi identifié dans les plaquettes, le glycosome[45]. Les glycosomes sont enfermés dans une membrane unitaire typique, et leur teneur en particules de glycogène est identique en apparence aux particules de glycogène se trouvant libres dans le cytoplasme ou condensées en masses de glycogène.

Récemment, un nouveau type granulaire a été identifié. Ces granules ont été nommés granules T car leur découverte a été basée sur la localisation de la protéine TLR-9[46].

I.2.2.4 Les vésicules extracellulaires

Les vésicules extracellulaires, y compris les microvésicules circulantes ou microparticules et les exosomes, dérivées de cellules ou de plaquettes sont présents dans le sang périphérique et sont des éléments importants impliqués dans l'activation du système de coagulation, le transport des macromolécules et la communication intercellulaire[47].

Les microparticules, ayant une taille entre 0,1 et 1 μm, sont de petites vésicules membranaires libérées par de nombreux types de cellules par bourgeonnement de la membrane plasmique en réponse à l'activation cellulaire ou à l'apoptose[48]. Les microparticules peuvent emballer et délivrer des facteurs de croissance, des ARNm, des miARN, et contenir des récepteurs de surface qui leur permettent de cibler des cellules et d'agir dans la communication cellulaire, en se liant et en fusionnant avec des membranes de cellules cibles ou encore d'être adsorbé via leurs récepteurs[49–51].

Dans le sang humain, les microparticules pourraient dériver des leucocytes, des cellules endothéliales, des cellules musculaires lisses, et des cellules cancéreuses [48]. Toutefois, près de 90% des microparticules circulantes sont d'origine plaquettaires [52].

Sinauridze et al.[53] ont rapporté que les microparticules plaquettaires (PMP) possèdent 50 à 100 fois plus d'activité procoagulante spécifique supérieure à celle des plaquettes activées. Les PMP sont capables d'améliorer l'adhérence des leucocytes et les cellules endothéliales [54]. Les PMP ont également un rôle pro-inflammatoire et sont impliquées dans plusieurs pathologies telles que le syndrome coronarien aigu, l'athérosclérose, les inflammations vasculaires ou également dans le développement et la progression du cancer [48,51,55–57].

Les exosomes sont définis comme de petites vésicules d'origine endocytaire d'un diamètre assez homogène de 30 à 100 nm. Ils sont délivrés par la plupart des types cellulaires après fusion des corps multivésiculaires de l'appareil de Golgi avec la membrane plasmique[58,59]. Ils diffèrent des MP non seulement par leur taille et leur origine mais aussi par leur composition. Ils contiennent très peu de phosphatidylsérine (PS) à leur surface et ne sont pas impliqués les réactions de la coagulation. Ils sont enrichis en tétraspanines comme le CD63, CD81 et CD9, en molécules du complexe majeur d'histocompatibilité et en d'autres protéines spécifiques comme Alix, TSG101 et HSP70[60]. Une ultracentrifugation supérieure ou égale à 100 000g est nécessaire pour les isoler. Leur forme régulière et arrondie permet de les isoler dans un gradient de sucrose dans une zone à faible densité de 1,10 à 1,21 g/mL, ce qui les différencient des autres vésicules à forme plus irrégulière qui sont retrouvées dans des zones de plus haute densité (>1,23 g/mL)[61] (**Tableau 5**).

L'isolement des microparticules et des exosomes se fait par ultracentrifugation différentielle. Des protocoles bien détaillés pourront être consulté dans les références[62,63]. Leurs caractérisation et quantification se font par cytométrie en flux, par microscopie électronique à transmission ou encore par résonance plasmonique de surface couplée à la microscopie à force atomique[64–66].

Tableau 5 : Caractéristiques différentielles des exosomes et des microparticules[59].

	Exosomes	Microparticules
Taille (diamètre)	30-100 nm	100-1000 nm
Densité de flottaison	1,10 – 1,21 g/mL	NA
Morphologie	Arrondies	Hétérogène
Composition lipidique	Acide lysobisphosphatidique (ALBP), faible exposition des PS, cholestérol, céramide, shingomyéline	Forte exposition des PS, Cholestérol
Marqueurs protéiques	Alix, TSG101, HSC70, CD63, CD81, CD9	Sélectines, intégrines, CD40, métalloprotéinases, annexine 2, caspases
Origine	Exocytose des corps multivésiculaires	Vésiculation de la membrane plasmique
Composition	Protéines, ARNm, miARN	Protéines, ARNm, miARN

I.2.2.5 Les récepteurs plaquettaires

Les récepteurs plaquettaires assurent l'interaction des plaquettes avec différentes cellules et matériaux sous-endothéliaux sont impliqués dans la réactivité des plaquettes en réponse à une large gamme d'agonistes et de protéines d'adhérence. Il s'agit de récepteurs constitutionnels

pré-exposés sur la surface plaquettaire, mais aussi des récepteurs présents sur les membranes des granules qui vont s'ajouter aux membranes plaquettaires après activation.

Etant donné que la fonction principale des plaquettes est l'hémostase, leurs principaux récepteurs ont un rôle direct dans ce processus, que ce soit dans l'activation ou l'adhérence plaquettaire pour assurer l'interaction avec les parois des cellules endommagées, des protéines structurales du sous-endothélium (exposées après attrition de l'endothélium vasculaire) ou avec d'autres plaquettes voisines pour contribuer à la formation de thrombus.

En outre, il est de plus en plus reconnu que les plaquettes font partie des cellules de l'immunité. En effet, les plaquettes expriment une gamme de récepteurs sans rôle direct identifié à ce jour dans l'hémostase mais qui pourraient être impliqués dans d'autres activités, telles que l'inflammation, la défense contre les agents pathogènes, la croissance tumorale et l'angiogenèse. (revue dans [67]).

I.3. Rôles des plaquettes sanguines

I.3.1. Rôle des plaquettes dans l'hémostase et la formation de thrombus

Le rôle des plaquettes dans l'hémostase est fondamental pour maintenir l'intégrité vasculaire, un système circulatoire fermé et soumis à une haute pression. Dans les conditions physiologiques normales, les plaquettes circulent à l'état de repos à proximité des cellules endothéliales qui tapissent les vaisseaux sanguins sans jamais former d'adhérences stables. En cas de rupture de cette homéostasie (lésion vasculaire) et grâce à leur grande capacité à reconnaitre les constituants de la matrice sous-endothéliale (MSE), les plaquettes adhèrent, s'activent, sécrètent leur contenu granulaire et s'agrègent entre elles pour former un thrombus. Ce dernier sera consolidé par le caillot de fibrine formé suite à l'activation de la coagulation à la surface des plaquettes activées.

Plusieurs modèles ont été utilisés pour traiter le rôle des plaquettes dans l'hémostase et particulièrement dans la formation d'un thrombus. Pour se rapprocher des mécanismes de la coagulation, nous avons choisi de présenter le modèle de trois phases : i) initiation ; ii) extension et iii) stabilisation (**Figure 3**).

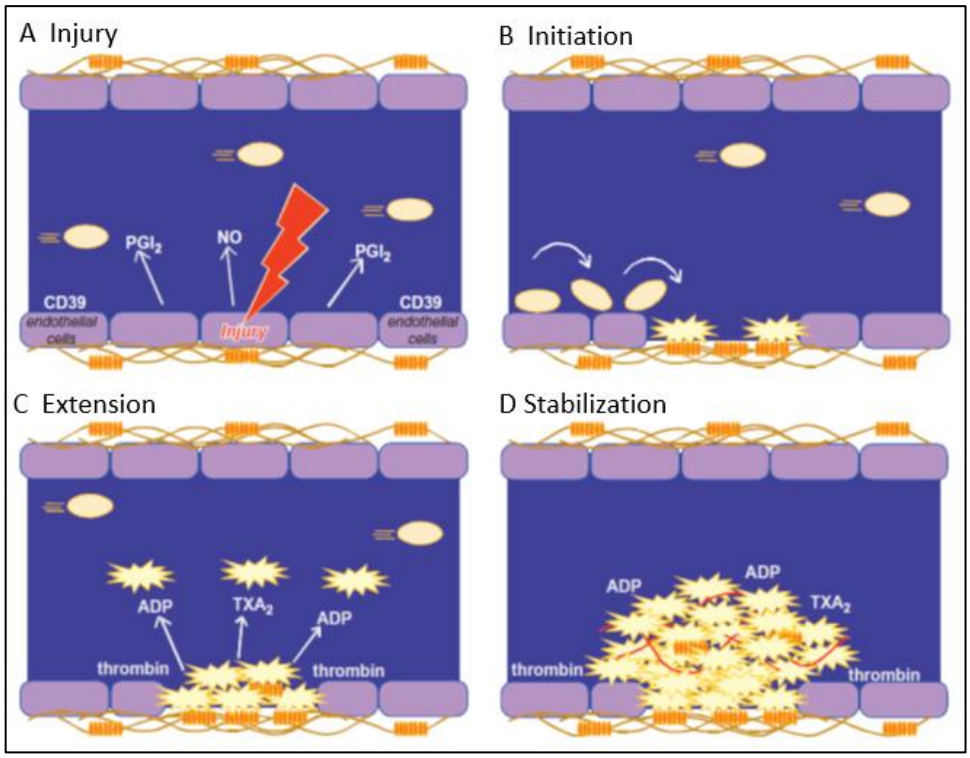

Figure 3 : Les trois phases de la formation du thrombus plaquettaire après une lésion vasculaire.

(A) A l'état normal l'activation et l'adhérence plaquettaire sont inhibées par des facteurs dérivées des cellules endothéliales. Ces inhibiteurs comprennent la prostaglandine PGI2 (prostacycline), le monoxyde d'azote (NO) et le CD39, une ATPase de la surface des cellules endothéliales qui peut hydrolyser des quantités infimes d'ADP. (B) Phase d'initiation : la mise à nu de la matrice sous-endothéliale entraine l'adhérence des plaquettes au facteur von Willebrand via la GPIb (CD42) et au collagène par la GPVI et la formation de la première couche de plaquettes activée. (C) Phase d'extension : la sécrétion d'agoniste solubles permet le recrutement et l'activation d'autres plaquettes. (D) Phase de stabilisation : le thrombus plaquettaire est consolidé par le caillot de fibrine. (D'après[68]).

La phase d'initiation, correspond à l'adhérence des plaquettes aux différentes structures de MSE et à la formation de la première couche de plaquettes activées. L'adhérence des plaquettes à la MSE est médiée principalement par le complexe GPIb-V-IX, la GPVI et l'intégrine α2β1 (CD49B/CD29)[69,70]. L'interaction de ces récepteurs avec leurs ligands respectifs (facteur von Willebrand pour la GPIb (CD42), le collagène pour la GPVI et l'α2β1), déclenche une signalisation dont la résultante est l'activation plaquettaire[71,72] (**Figure 4**). Cette activation se traduit par : un changement de forme, des modifications biochimiques (e.g. phosphorylations, réarrangement de Phospholipides), la sécrétion du contenu granulaire et l'activation de l'intégrine αIIbβ3 (connue aussi sous le nom de la GPIIb-IIIa) qui devient capable de fixer le fibrinogène et lier d'autres plaquettes.

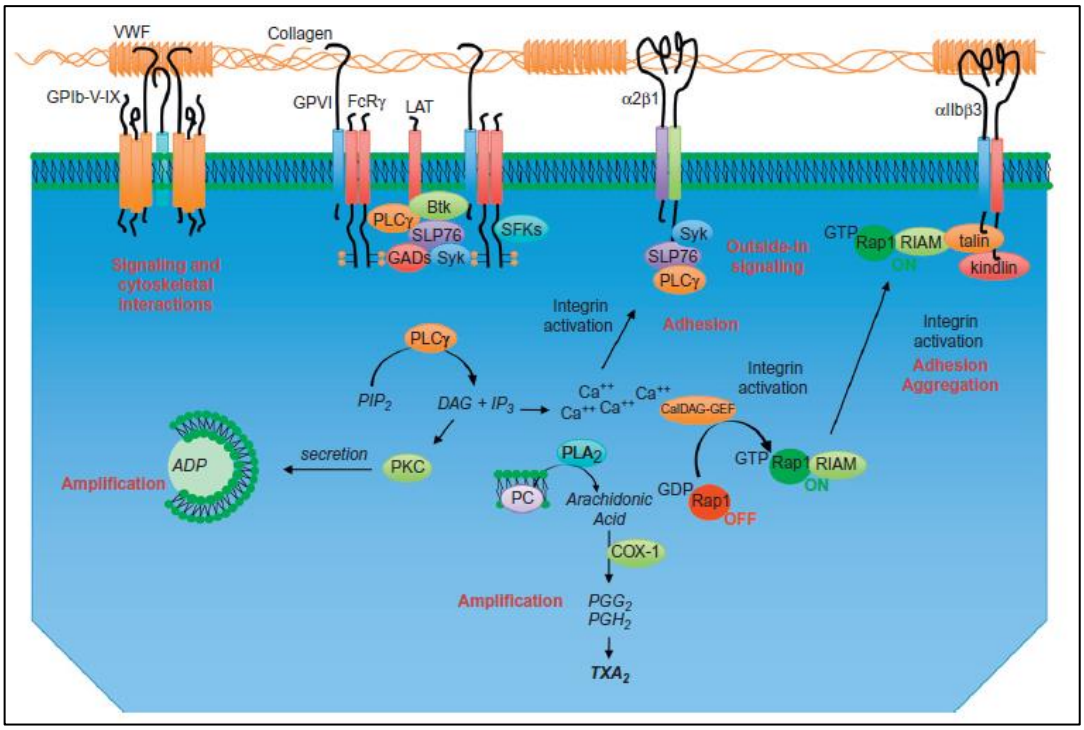

Figure 4 : Les différentes voies de signalisation déclenchées par l'adhérence des plaquettes au collagène du sous-endothélium.

L'adhérence au collagène de la GPVI suivie de sa condensation entraine la phosphorylation d'un résidu tyrosine au niveau du domaine cytoplasmique de FcRγ suivie par la fixation et l'activation d'une tyrosine Kinase la SyK. Une des conséquences de l'activation de la SyK et la phosphorylation et l'activation de la phospholipase Cγ, entrainant l'hydrolyse du phosphoinositid, la sécrétion de l'ADP, la synthèse et la libération du TxA2, et la génération d'un signal inside-out responsable de l'activation des intégrine α2β1 (CD49B/CD29) et αIIbβ3 (CD41/CD61). (D'après[68]).

Dans la phase d'extension, les plaquettes, passant de la zone de la lésion endothéliale, sont activées lorsqu'elles rencontrent les agonistes plaquettaires solubles (e.g. ADP, Thromboxane A2, sérotonine, Gas) libérés par la première couche de plaquettes activées. Toujours à la surface des premières plaquettes activées, les traces de thrombine (un puissant agoniste plaquettaire) qui se sont formées, participent aussi à l'activation et au recrutement d'autres plaquettes. La fixation de ces différents agonistes sur leurs récepteurs déclenche différentes voies de signalisation[33,73] (**Figure 5**). L'activation plaquettaire induite par ces différents agonistes déclenche, entre autres, une signalisation intracellulaire de type « *inside-out* » entraînant l'activation et la concentration (clustérisation) des αIIbβ3 (CD41/CD61)[74]. Sous sa forme activée l'αIIbβ3 permet l'agrégation plaquettaire en formant des ponts inter-plaquettaires *via* son principal ligand, le fibrinogène. Au cours de cette phase, les plaquettes activées participent activement à la régulation des réactions de la coagulation et la génération explosive de thrombine nécessaire pour la fibrino-formation[75]. Les plaquettes participent à la coagulation en exprimant à leurs surfaces les phospholipides essentiels pour l'assemblage des

complexes tenases et protrombinase et en sécrétant un grand nombre de molécules pro-coagulantes, principalement le facteur Va.

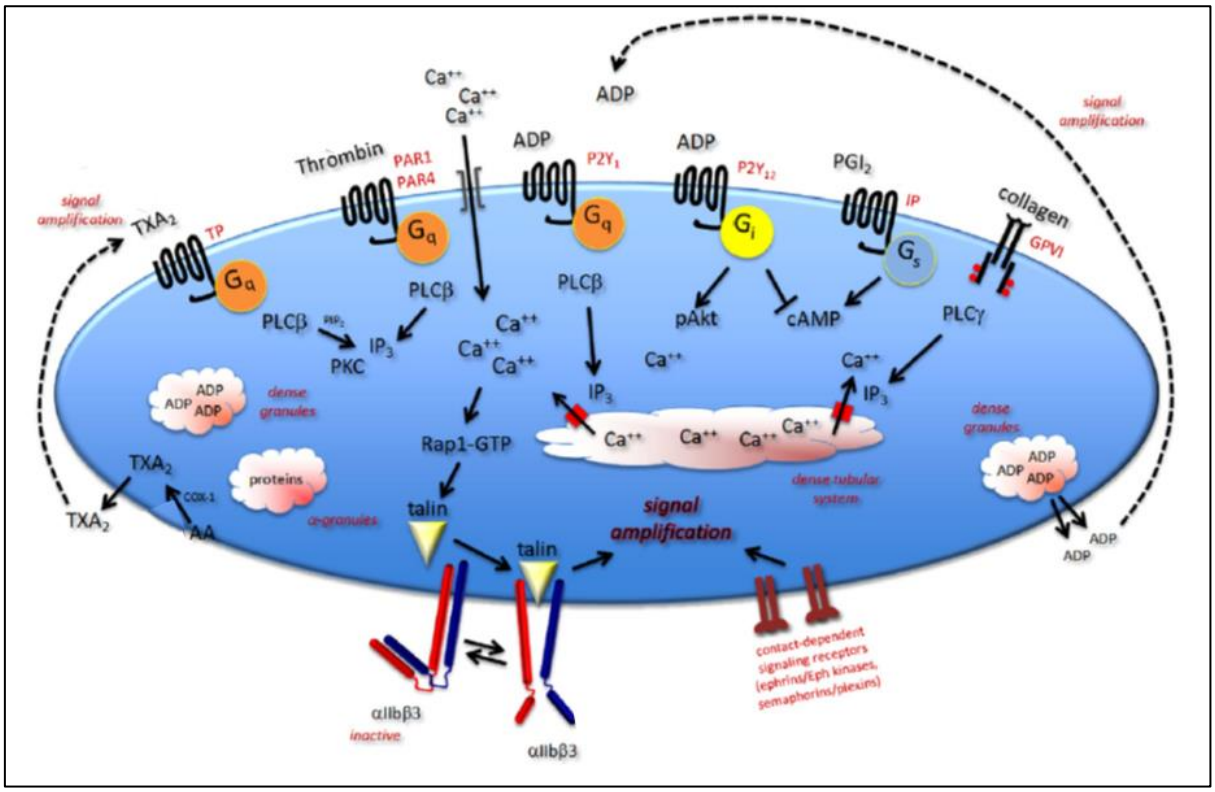

Figure 5 : Vue d'ensemble des principales voies de signalisation mises en jeu lors de l'activation des plaquettes.

AA : Acide Arachidonique ; TXA2 : Thromboxane A2, PLC : Phospholipase C ; ADP : Adéniosine iPhosphate ; PGI2 : Prostaglandine I2 ; PKC : protéine Kinase C ; cAMP : AMP cyclique ; IP3 : Iniositol 1,4,5 Phosphate ; PAR : Protease Activated Factor ; GP : Glycoprotéine (D'après[68]).

La phase de stabilisation correspond aux événements survenant après la formation du thrombus plaquettaire pour consolider et renforcer ce thrombus et prévenir sa désagrégation prématurée. Il y a des preuves croissantes que les plaquettes continuent à signaler, même après leur incorporation dans un thrombus, entre autres par la signalisation de type « *outside-in* ». Plusieurs plaquettes émettent des filopodes et des lamellipodes ce qui permet d'augmenter leur surface d'interaction avec les cellules adjacentes (plaquettes ou leucocytes) *via* le grand nombre de récepteurs qu'elles expriment. Le résultat final est la formation d'un thrombus hémostatique stable comprenant des plaquettes activées, des globules rouges et des leucocytes, noyés dans un réseau de fibrine réticulée.

Des observations conduites *in vivo* par imagerie intra-vitale, et des études conduites *in vitro*, démontrent que la formation du thrombus est un phénomène dynamique très complexe et que le modèle en trois phases ne reflète pas la réalité[76–78]. Il est aussi de plus en plus évident que

les plaquettes engagées dans la formation du thrombus sont hétérogènes et peuvent adapter leurs réponses en fonction des variables espace/temps (revu dans[79]). En effet, à chaque instant après la lésion endothéliale et à différentes zones du thrombus en cours de formation, des plaquettes avec différents degrés d'activation et par conséquent hétérogènes du point de vue morphologique, phénotypique et biochimique, sont impliquées.

I.3.2. Rôle des plaquettes dans l'inflammation, l'immunité innée et l'immunité adaptative

Les systèmes de l'hémostase, de l'inflammation et de l'immunité, à la fois innée et adaptative, sont intimement liés et on connaît de mieux en mieux leurs interactions. En effet, les plaquettes contribuent à l'initiation et l'amplification du processus inflammatoire. Les plaquettes, participent à l'inflammation à la fois en exprimant des récepteurs[67] qui facilitent l'adhérence des plaquettes avec d'autres cellules vasculaires et leucocytaires et en libérant une large gamme de cytokines et de chémokines[32]. Cet arsenal de récepteurs et de molécules solubles participe également à la défense de l'hôte contre différents types de pathogènes **(Figure 6)**.

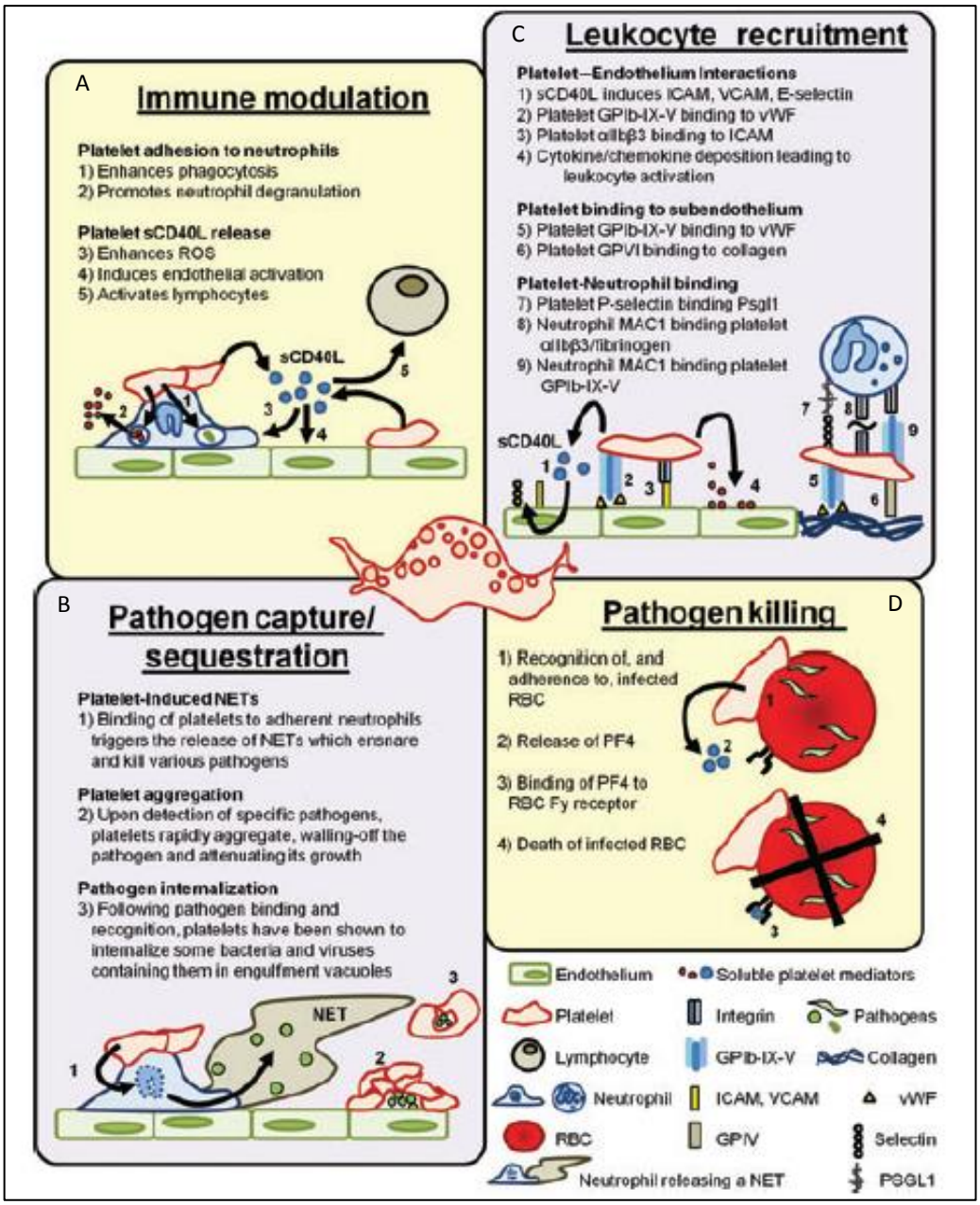

Figure 6 : Rôle des plaquettes dans l'inflammation et l'immunité[80].

Les plaquettes jouent un rôle central dans la conduite, la modulation de la réponse immunitaire et la défense de l'hôte. (A) Les plaquettes sont capables de moduler directement la fonction d'autres cellules telles que les cellules endothéliales, les neutrophiles et les lymphocytes. (B) Les plaquettes facilitent l'adhérence des neutrophiles aux sous-endothélium, la libération des chimiokines et des cytokines qui attirent et activent les leucocytes, induisant l'expression des molécules d'adhérence par l'endothélium. (C) Les plaquettes jouent un rôle actif dans la capture et la séquestration des pathogènes par la formation de NET (Neutrophil Extracellular Traps), enrobant les agents pathogènes au sein des agrégats de plaquettes et dans l'internalisation directe des agents pathogènes. (D) Grâce à la libération de médiateurs solubles, les plaquettes peuvent induire directement la mort des cellules cibles infectées.

I.3.2.1 Les interactions entre les plaquettes, l'endothélium et les leucocytes

Les plaquettes expriment de nombreuses molécules d'adhérence et des ligands qui facilitent les interactions entre les plaquettes, les leucocytes et l'endothélium. Les plaquettes expriment de grandes quantités de P-sélectine qui, lors de l'activation, sont rapidement mobilisées à partir des granules α à la surface des plaquettes où elles peuvent médier l'adhérence à des cellules exprimant le PSGL-1, principalement les neutrophiles, les monocytes et d'autres leucocytes, les cellules endothéliales, et d'autres plaquettes[81,82].

Pendant une aggression de l'endothélium par des endotoxines, l'adhérence et le roulement dit « rolling » des plaquettes sont médiés par la GPIba (CD42b) et la P-sélectine (CD62P) suivis par une immobilisation ferme sur l'endothélium vasculaire enflammé, immobilisation médiée par l'αIIbβ3[83,84]. Une fois adhérentes, les plaquettes expriment rapidement de grandes quantités de P-sélectine générant une surface ad hoc sur laquelle les neutrophiles peuvent s'attacher et rouler grâce à leur expression de PSGL-1[83,84]. Ce « rolling » initial est suivi d'une adhérence ferme des plaquettes adhérentes aux neutrophiles par Mac-1, liée au fibrinogène ; les neutrophiles vont se fixer à la GPIIblIIa et la GPIba plaquettaires[83,84]. Cette couche initiale de plaquettes-neutrophiles va servir de plateforme sur laquelle d'autres types de leucocytes peuvent adhérer. L'adhérence des plaquettes aux neutrophiles peut également être établie par le CD40L plaquettaires qui pourrait induire alors la régulation positive de l'expression de la E-sélectine, ICAM-1 et VCAM-1[85]. Outre la P-sélectine, les plaquettes expriment un certain nombre d'intégrines β1 et β3, y compris α5β1 (VLA-5 ou CD49e/CD29), α6β1 (VLA-6 ou CD48fB/CD29), α2β1 (GPIa / IIa, VLA-2, ou CD49b / CD29) et GPIIb/IIIa (αIIbβ3) [83,84]. Ces molécules médient l'adhérence des plaquettes à ICAM et JAM (« Junction Adhesion Molecule ») sur les leucocytes, l'endothélium et aux protéines de la matrice extracellulaire telles que la fibronectine, la laminine et le collagène[83,84]. Les plaquettes expriment également des molécules d'adhérence de la superfamille des Ig telles que ICAM-2, JAM-A, JAM-C et PECAM-1 (CD31)[83]. Ces molécules interagissent avec d'autres cellules par une combinaison d'interaction de type homophile (PECAM-1, JAM-A) ou hétérophiles (ICAM-2, JAM-A, JAM-C)[80].

Cet ensemble complexe de molécules d'adhérence et des ligands assure les interactions cellulaires même dans des conditions de flux sanguin élevé[86–88].

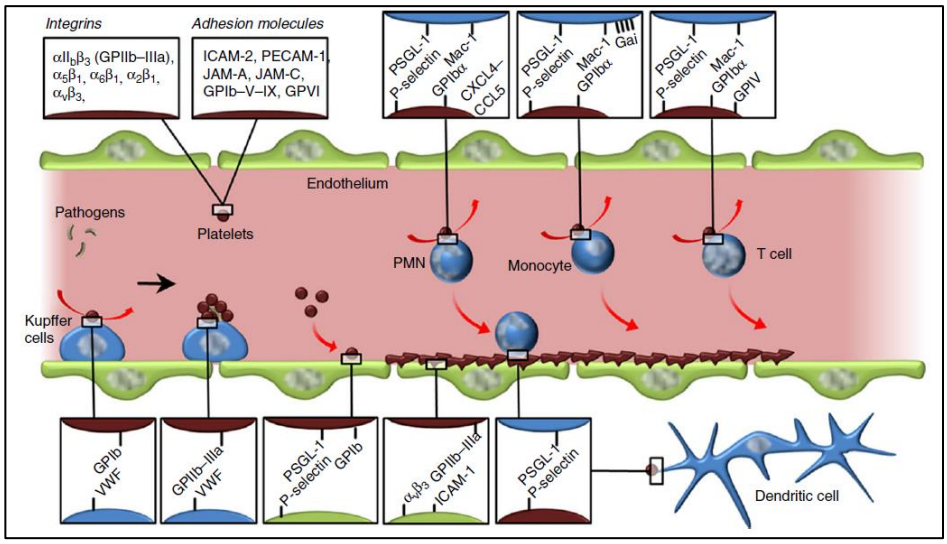

Figure 7 : Les plaquettes dans l'immunité : les récepteurs et ligands impliqués dans l'interaction plaquettes-leucocytes[89].

I.3.2.2 Les facteurs immunomodulateures plaquettaires

Outre les molécules d'adhérence exprimées par les plaquettes qui favorisent les interactions physiques de cellule à cellule, les plaquettes libèrent une panoplie de molécules solubles ayant des fonctions immunomodulatrices qui vont activer et recruter les leucocytes au site d'attrition, d'inflammation ou d'infection.

Ces molécules solubles, préformées et stockées dans les différents granules ou néosynthétisées dans le cytoplasme, sont responsables de l'activation, le recrutement et aussi l'inhibition d'une large gamme de cellules immunitaires et non immunitaires modulant ainsi la fonction inflammatoire ou immunitaire. Citons à titre d'exemples, les produits dérivant des granules α ayant un rôle chimiotactique tels que MIP-1 α, MCP-3 et RANTES qui sont connus pour recruter et activer les leucocytes[90–94]. Le PF4, la β-thromboglobuline et CXCL-5 peuvent moduler la chimiotaxie des neutrophiles[90,95]. Les plaquettes libèrent également des facteurs de croissance ayant des activités immunomodulatrices, tels que le PDGF et TGF-β et des médiateurs immunitaires contenus dans les granules denses tels que l'histamine et la sérotonine[32]. L'interleukine-1β produite et libérée par les plaquettes peut favoriser des réponses inflammatoires par les leucocytes et les cellules endothéliales[96,97].

Des molécules d'origine granulaire (granule α) exposées à la surface plaquettaire après activation pourraient également être clivées et joueraient un rôle actif dans l'immunomodulation (e.g. sCD62P, sCD40L, RANTES). En effet, les plaquettes ont été identifiées comme étant la plus grande source de CD40L soluble (sCD40L), une molécule qui a été décrite comme capable d'induire la production de ROS et l'augmentation de l'expression

des molécules d'adhérence par les neutrophiles, d'activer les macrophages, et d'induire une activation optimale des cellules T cytotoxiques *in vitro* et *in vivo*[98].

Les plaquettes peuvent aussi activer les lymphocytes B et potentialiser la production d'IgG après stimulation par le sCD40L confirmant ainsi le rôle des plaquettes comme un partenaire essentiel de l'immunité adaptative[93,99].

I.3.2.3 Rôle des plaquettes dans la défense de l'hôte

Les plaquettes modulent non seulement la réponse immunitaire, mais participent également à la capture et la séquestration de certains pathogènes infectieux. Cette fonction est assurée par l'expression d'un certain nombre de récepteurs de l'immunité innée, y compris un assortiment de récepteurs de reconnaissance des motifs conservés de pathogènes (PRR), dont les récepteurs de type Toll-like (TLR1 à 10)[80,90,100]. Les plaquettes expriment également des co-récepteurs et des molécules de signalisation associées à ces PRR[101]. Ces récepteurs et co-récepteurs sont fonctionnels et, lors de la liaison d'un ligand, induisent l'activation plaquettaire[102].

En plus des TLR, les plaquettes ont la capacité de détecter et de répondre à la présence d'agents pathogènes infectieux grâce à leurs récepteurs de type integrines (e.g. αIIbβ3 (CD41/CD61), GPIbα (CD42b)), les récepteurs de la sous-famille des Igs (TREM-1 ligand), les récepteurs Fc (e.g. FcγRIIa (CD32), FcεRII (CD23)) et les récepteurs de type C-lectines (CLEC-2 et DC-SIGN). Grâce à ces récepteurs, il a été rapporté que les plaquettes sont capables de lier voire d'internaliser, directement ou par l'intermédiaire de protéines plasmatiques (e.g. le fibrinogène, la fibronectine et le vWF), des bactéries (e.g. *S. epidermidis*, *S. aureus*, *H. pylori*, *S. sanguinis*, *S. gordonii* et *S. pneumoniae*) des virus (e.g. VIH, VHC, des adénovirus et des hantavirus) et des champignons/parasites (e.g. *C. albicans*, *A. fumigatus* et *P. falciparum*)[90,103].

Ainsi lorsque les plaquettes détectent un agent pathogène, elles sont susceptibles de s'activer et sont capables de mener une réaction inflammatoire par sécrétion des BRMs (« Biological Response Modifiers »), des peptides antimicrobiens (e.g. β-défensine, thrombocidines) contenus dans leurs granules ou des chémokines qui recrutent de nouvelles cellules immunitaires (e.g. RANTES, PF4) [90,103–106].

En outre, au cours d'une endotoxémie, les plaquettes activées par les lipopolysaccharides (LPS) se lient à des neutrophiles et induisent la production de NET (« Neutrophil extracellular traps »)[107,108]. Ces NET sont composés de chromatine décondensée libérée dans le milieu

extracellulaire (par les PMN) formant ainsi une surface adhésive recouverte de nombreuses protéines antimicrobiennes (β-defensine 1, myéloperoxydase, cathepsine). Les NET sont ainsi capables de piéger les pathogènes limitant ainsi la dissémination des agents infectieux[80,108,109].

II. La transfusion des plaquettes

Depuis l'étude de Hersh et al.[110] en 1958 démontrant, le rôle de la transfusion plaquettaire dans la réduction de la mortalité liée aux hémorragies, chez les patients atteints de leucémie aiguë et traités par chimiothérapie, les prescriptions de transfusions de plaquettes n'ont cessé d'augmenter. En effet, la transfusion de plaquette est largement utilisée en pratique clinique pour prévenir et traiter les hémorragies liées à un déficit quantitatif (Thrombocytopénie) ou qualitatif (Thrombopathies) de plaquettes. Elle a aussi rendu possible l'utilisation de chimiothérapie agressive (avec une forte action myélosuppressives) dans le traitement de différents types de cancers. Aujourd'hui, plus de 2 millions de produits plaquettaires ou de concentrés de plaquettes (CP) sont transfusés aux Etats-Unis et 3 millions en Europe, chaque année. Devant l'accroissement des demandes et à cause de la durée de vie limitée le plus souvent à 5 jours des CP, il est difficile aux banques de sang, malgré les grands efforts pour assurer un approvisionnement régulier et adéquat, de maintenir un stock optimisé.

Le but de cette partie est d'introduire trois points :

- Les différents types de produits plaquettaires disponibles, leurs méthodes de préparation et leurs caractéristiques.
- Les lésions de stockage des plaquettes.
- Les effets indésirables receveurs liés à la transfusion plaquettaire

II.1. Les différents types de concentrés plaquettaires

Deux grandes catégories de CP peuvent être préparées soit à partir de dons de sang total, soit à partir de dons d'aphérèse. Dans les deux cas, les CP proviennent de donneurs dont la sélection a été faite conformément aux bonnes pratiques de prélèvement[111] et aux critères de sélection des donneurs[112]. Comme pour tous les autres produits sanguins, les CP ne sont utilisés qu'après qualification biologique (qualification immuno-hématologique et dépistage des maladies infectieuses transmissibles majeurs : hépatite B et C, VIH-1, VIH-2, HTLV-I, HTLVII et la syphilis) du don à partir duquel ils sont préparés.

II.1.1. Préparation de CP à partir de dons de sang total

Les CP obtenus à partir de dons de sang total peuvent être préparés selon deux méthodes : La première dite la méthode de Plasma Riche en Plaquette (PRP) et la deuxième dite méthode de « *Buffy-Coat »* ou couche leuco-Plaquettaire (BC) (**Figure 8**). En Europe, notamment en France, et au Canada la

préparation des CP se fait par la méthode de BC, tandis que la méthode de PRP est utilisée aux Etats-Unis. Les CP obtenus par les deux méthodes sont délivrés sous forme de mélange de concentrés plaquettaires (MCP) déleucocyté (plus exactement leuco-réduit) obtenu en poolant plusieurs (4 à 7, souvent 5) CP provenant de dons différents de même groupe sanguin ABO.

Il est à signaler que dans certains pays à moyens économique réduits, comme la Tunisie, les CP sont préparés par la méthode de plasma riche en plaquette (PRP) à partir de sang total et sont utilisés sous forme de CP standards (non poolés) non déleucocytés.

II.1.1.1 Méthode de PRP

Cette méthode consiste à centrifuger le sang total à une faible vitesse. Cette première centrifugation permet d'obtenir le PRP. Ce dernier est ensuite transféré dans une poche dédiée pour le stockage des plaquettes et subit une deuxième centrifugation à forte vitesse. Cette deuxième centrifugation permet d'obtenir un culot constitué de plaquettes (Culot plaquettaire) et un surnageant constitué par du plasma pauvre en plaquette (PPP). La majeure partie du PPP est retirée et le culot plaquettaire est remis en suspension dans un faible volume du PPP restant (50 à 70 ml). La dernière étape consiste à pooler en moyenne cinq CP unitaires pour obtenir un MCP. Bien que plus délicate que pour les CP préparés par la méthode de BC, l'addition de solution additives (« Platelet Additive Solution » = PAS) aux CP préparé par la méthode de PRP est également possible.

II.1.1.2 Méthode de « *Buffy-Coat* »

Comme pour la méthode de PRP, la méthode de BC utilise un procédé de centrifugation différentielle en deux étapes, mais la séquence des étapes est inversée. La première étape consiste à centrifuger le don de sang total à forte vitesse. Cette centrifugation permet de sédimenter toutes les cellules et d'obtenir un surnageant formé de plasma pauvre en plaquette. Les plaquettes et les leucocytes sédimentent au-dessus des globules rouges en formant la couche leuco-plaquettaire ou « *Buffy-Coat* ». En moyenne cinq (systématiquement en France) BC obtenus de dons de même groupe ABO, sont *poolés* et dilués dans du plasma autologue ou dans une solution additive (PAS). Le pool est ensuite centrifugé à faible vitesse, le surnageant riche en plaquette est retenu et constitue le CP, alors que le sédiment des globules rouges et des leucocytes est éliminé.

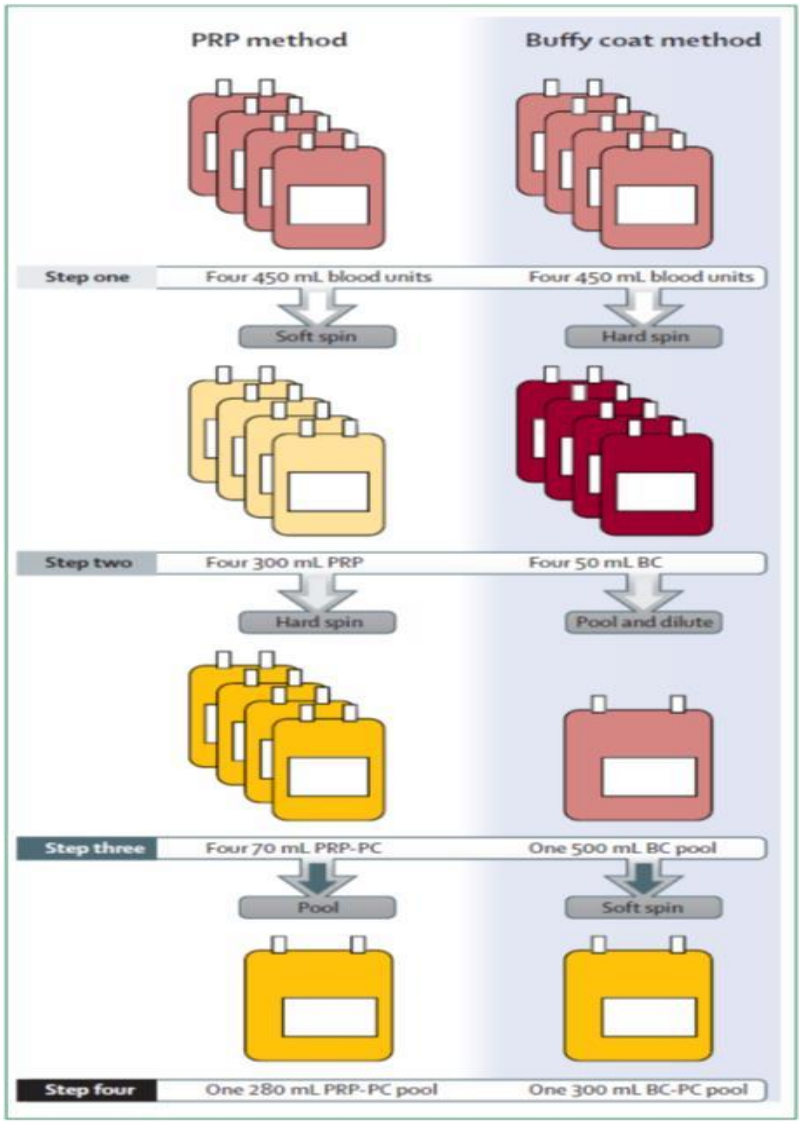

Figure 8 : Préparation des CP à partir de sang total par la méthode de PRP et la méthode de *Buffy-coat*[113].

II.1.2. Préparation des CP par aphérèse

L'une des avancées les plus importantes dans la préparation des produits sanguins est l'introduction dans les années 70 des séparateurs de cellules sanguines qui permettaient de recueillir de manière efficace et sélective les plaquettes dans un volume prédéfini de plasma, à l'aide d'un procédé appelé aphérèse[114,115]. Le sang du donneur prélevé à travers un cathéter passe à travers un dispositif de prélèvement fermé et stérile, monté dans un instrument d'aphérèse. Les plaquettes sont séparées des autres composants cellulaires par centrifugation différentielle et recueillies avec un volume prédéfini de plasma dans une poche dédiée ; les globules rouges et une partie plus ou moins importante de plasma sont retournés au donneur. Les instruments d'aphérèse sont actuellement entièrement automatisés et déterminent les paramètres de séparation (le volume de prélèvement, le nombre de cycle et le temps de

séparation) en fonction des données qui lui sont entrées et qui concernent, le poids, la numération plaquettaire et l'hématocrite du donneur.

En fonction du séparateur, la déleucocytation est assurée soit par un procédé intégré à la séparation, soit par une filtration du concentré en circuit clos à la fin du recueil. Les produits plaquettaires obtenus par plaquettophérèse sont appelés Concentrés de Plaquettes d'Aphérèse (CPA) – en France – ou Concentré Plaquettaires Unitaire (CPU) (en Tunisie).

II.2. Standards des CP (Normes)

Les PC préparés doivent répondre à des standards préétablis et qui permettent de contrôler les différentes étapes (prélèvement, préparation et stockage) précédant l'obtention d'un CP fini. Ces standards diffèrent d'un pays à un autre et selon la méthode de préparation des CP (**Tableau 5**). Les standards concernent généralement les paramètres suivants : Le volume, le nombre de plaquettes, le nombre de leucocytes résiduels et le pH.

Tableau 6 : Standards des CP aux USA et en Europe[113].

	Source	Total platelet count	Total leucocyte count	Total leucocyte count in leucocyte-reduced products	pH at end of allowable storage
PRP platelets from one unit of whole blood	AABB	$>55\times10^9$ in ≥90% of units	NA	$<0{\cdot}83\times10^6$ in ≥95% of units	>6·2 in ≥90% of units
PRP platelets from one unit of whole blood	CoE	$>60\times10^9$ in ≥75% of units	$<200\times10^6$	$<0{\cdot}2\times10^6$ in ≥90% of units	6·4–7·4
Buffy coat platelets from one unit of whole blood	CoE	$>60\times10^9$ in ≥75% of units	$<50\times10^6$	$<0{\cdot}2\times10^6$ in ≥90% of units	6·4–7·4
Apheresis platelets	AABB	$>300\times10^9$ in ≥90% of units	NA	$<5\times10^6$ in ≥95% of units	>6·2 in ≥90% of units
Apheresis platelets	CoE	$>200\times10^9$ in ≥90% of units	NA	$<1\times10^6$ in ≥90% of units	6·4–7·4

NA=not available.

Plusieurs autres paramètres peuvent être étudiés et permettent d'apprécier et de comparer l'effet des différentes méthodes de préparation ainsi que les conditions de stockage sur l'intégrité structurale et fonctionnelle, le degré d'activation, et le statut métabolique, des plaquettes. Ces différents paramètres ont fait l'objet de plusieurs études et seront détaillés dans la section II.4. Lésions de stockage des plaquettes.

II.3. Conservation des CP

II.3.1. Le métabolisme plaquettaire

Le métabolisme énergétique des plaquettes est actuellement bien connu. Il est admis que les adénines nucléotides sont réparties dans les plaquettes en au moins deux pools : le pool de stockage et le pool métabolique. Le pool de stockage, qui contient une quantité à peu près

égale d'ADP et d'ATP, est stocké dans les granules des plaquettes et est libéré lorsque les plaquettes sont stimulées par des agonistes des glycoprotéines de l'hémostase exposées à leur surface. Le pool métabolique, formé principalement par l'ATP généré par les voies métaboliques, fournit non seulement l'énergie nécessaire à l'activation plaquettaire mais aussi permet de maintenir l'intégrité structurale des plaquettes dans la circulation *in-vivo* et pendant le stockage des plaquettes.

Deux voies métaboliques sont à distinguer dans les plaquettes[116–118] :

II.3.1.1 La voie de phosphorylation oxydative

Cette voie, nécessitant de l'oxygène, permet de régénérer six molécules d'ATP pour une molécule d'oxygène. Il existe une grande variété de carburants qui peuvent alimenter les réactions du métabolisme oxydatif. Le Pyruvate qui est le précurseur du lactate dans la glycolyse, les acides gras libres dans le plasma et les corps cétoniques comme le bêta-hydroxybutyrate et acétoacétate, peuvent tous être transformés en acétyl-CoA qui se condense ensuite avec l'oxaloacétate pour former du citrate qui est ensuite introduit dans le cycle de Krebs (**Figure 9**). Ce dernier permet de récupérer l'énergie sous forme d'électrons à haut potentiel de transfert et une molécule de GTP ou d'ATP ; les électrons à haut potentiel de transfert, récupérés sur le NADH, peuvent ensuite circuler à travers la chaîne respiratoire mitochondriale pour permettre à leur tour la formation de molécules d'ATP supplémentaires par la phosphorylation oxydative. Les plaquettes sont capables d'utiliser l'ensemble de ces combustibles (cités plus haut) à un degré plus ou moins important selon les circonstances.

II.3.1.2 La voie de la glycolyse anaérobie

Cette voie, très active en l'absence d'oxygène, permet la régénération de deux molécules d'ATP à partir d'une molécule de glycose. En plus de l'ATP, la conversion du glucose en lactate produit un cation H+ qui, en l'absence d'un accepteur, est responsable d'une diminution du pH incompatible avec la viabilité des plaquettes et la voie anaérobie qui passe par la glycolyse anaérobie. Au cours du stockage dans les conditions optimales, le taux d'ATP ne chute que de 20% par rapport au taux basal[119]. Ceci suggère un turnover d'ATP efficace et compatible avec la durée de stockage préconisée de 5 jours. La glycolyse est l'une des voies métaboliques qui permet d'alimenter la régénération de l'ATP à partir de l'ADP. Lors de la glycolyse la conversion du glucose en lactate entraine la régénération d'une molécule d'ATP mais aussi un cation H+ responsable de la diminution de pH.

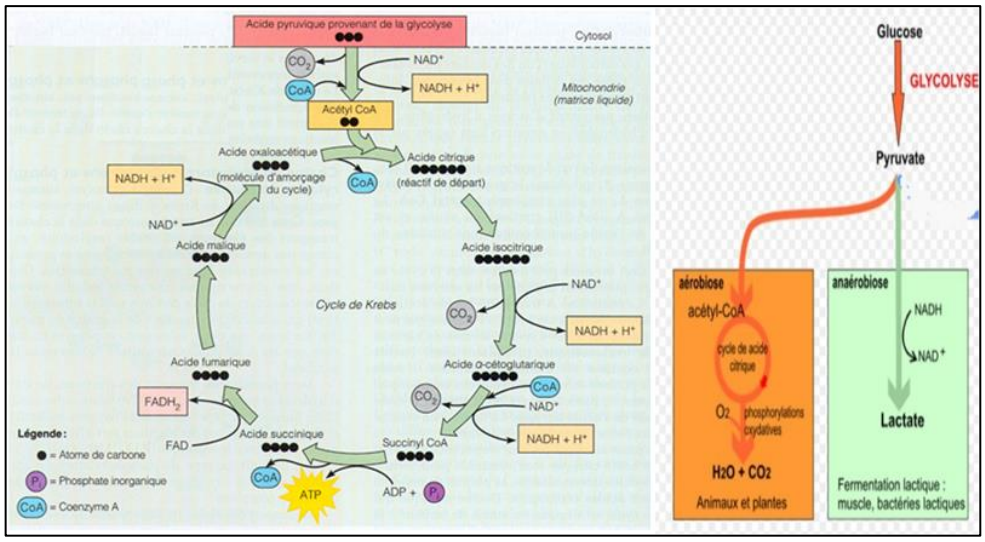

Figure 9 : Les voies métaboliques dans les plaquettes[120].

II.3.2. Conditions optimales de conservation des plaquettaires

La préservation de la viabilité et de l'intégrité structurale et fonctionnelle des plaquettes durant la période de stockage est l'un des objectifs principaux de la pratique transfusionnelle. Compte tenu des caractéristiques métaboliques des plaquettes (détaillées plus haut) il est primordial d'assurer un apport suffisant et régulier d'oxygène, d'éviter une diminution importante du pH et de fournir une quantité optimale et suffisante de source permettant la régénération des molécules d'ATP source importante d'énergie. *In vitro* ces paramètres peuvent être influencés par plusieurs variables :

II.3.2.1 Température de stockage

Les CP ont été initialement stockés au réfrigérateur comme les concentrés de globules rouges, jusqu'à ce qu'il ait été déterminé que la fonction et la viabilité plaquettaire étaient profondément compromises lorsqu'elles étaient stockées à une température basse. Actuellement, la température de stockage des CP est comprise entre +20°C et +24°C, ce qui a permis d'améliorer nettement la viabilité post-transfusionnelle des plaquettes. La température de 22°C±2°C permet de maintenir le métabolisme plaquettaire à un niveau bas et par conséquent de préserver l'intégrité structurale des plaquettes[119]. Des banques américaines utilisent néanmoins des plaquettes conservées à 4°C notemment en conditions d'urgence.

II.3.2.2 Agitation des CP

Les CP sont soumis à une agitation horizontale douce, constante et continue. L'agitation continue permet d'améliorer les échanges gazeux (O_2 et CO_2) à travers la membrane des

poches de stockage et d'éviter la sédimentation des plaquettes qui peut rendre l'O_2 inaccessible à toutes les plaquettes[121]. Connaissant le rôle de l'O_2 dans le métabolisme plaquettaire, ces conditions sont plus que nécessaires. Il a été démontré que la glycolyse anaérobie est régulée à la hausse dans les plaquettes stockées sans agitation, ce qui peut conduire à une augmentation de la production d'acide lactique et à une baisse de pH[122].

II.3.2.3 Les poches de stockage

L'une des avancées les plus significatives dans l'utilisation des CP a été le développement des contenants de stockage en plastique perméables aux gaz, qui permettent un échange adéquat d'O_2 et de CO_2. La première génération de poches de stockage était constituée de chlorure de polyvinyle (PVC) et d'un plastifiant le phtalate de 2-diéthyl-hexyle (DEHP). Ce type de poche n'était pas suffisamment perméable aux gaz et le métabolisme aérobie n'étaient pas maintenu, ce qui entraînait une surproduction d'acide lactique et une chute rapide de pH donc les CP ne pouvaient pas être conservés au-delà de 3 jours[123]. La deuxième génération de poches de stockage, composée de PVC et de plastifiants non-DEHP tels que butyryl-tri-hexyle citrate, était plus perméable aux gaz, permettant le stockage des plaquettes pendant 5 jours[124]. Actuellement, on dispose de poches constituées de polyoléfine qui possèdent une plus grande perméabilité à l'O_2 et au CO_2, et sont compatibles avec une durée de stockage qui peut aller jusqu'à 7 jours[125].

II.3.2.4 Les Solutions Additives de Plaquettes (PAS)

Anucléées, les plaquettes n'ont pas de mécanisme de protection et de réparation autonome, ni de réversion d'apoptose, et elles ont par conséquent besoin d'un milieu protecteur riche en protéines et en substances source d'énergie. C'est pourquoi les plaquettes contenues dans les CP sont originalement suspendues dans du plasma autologue anticoagulé avec du Citrate-Phosphate-Dextrose (CPD) pour les MCP et Acide citrique-Citrate trisodique-Dextrose (ACD) pour les CPA. Le plasma permet de bien maintenir le pH grâce au 20mM de bicarbonate qu'il contient, et de maintenir la viabilité grâce à sa contenance en substances (e.g. acides gras libres, corps cétoniques, acides aminés) pouvant être utilisées pour alimenter le cycle de Krebs en Acétyl-CoA et maintenir le métabolisme oxydatif. Actuellement, le milieu de suspension des plaquettes est composé de 1/3 de plasma et 2/3 de PAS. Ces solutions additives sont des solutions salines isotoniques auxquelles sont ajoutés de nombreux facteurs désignés pour diminuer la consommation d'O2, l'utilisation du glucose comme

source d'énergie, et la production de lactate. Les différents types de PAS ainsi que leurs compositions ont été largement détaillés par Gulliksson et al.[118].

La réduction de l'activation des plaquettes lors des étapes de préparation des CP, l'optimisation des conditions de stockage et l'inclusion éventuelle de composants clés dans le milieu de stockage des plaquettes, tels que le glucose, l'acétate, le citrate, le phosphate, le potassium et le magnésium, permettent de maintenir l'intégrité structurale et fonctionnelle des plaquettes pendant une longue durée (5 à 7 jours) et d'améliorer la survie des plaquettes *in-vivo* après transfusion.

II.4. Lésions de stockage des plaquettes

Les plaquettes préparées à des fins transfusionnelles subissent différents types de stress lors du prélèvement (contact avec des surfaces étrangères, aspiration), de la préparation (contrainte élevée de cisaillement infligés par la centrifugation), des transformations (filtration, irradiation, inactivation de pathogènes) et du stockage (diminution du pH, agitation, température). Confrontées à ces stress, les plaquettes vont répondre par des modifications morphologiques, biochimiques/métaboliques, phénotypiques et fonctionnelles. Ces modifications, regroupées sous le nom de lésions de stockage des plaquettes (LSP), sont pour la plupart responsables de l'activation plaquettaire, et par conséquence, aussi responsables de la détérioration continue et progressive de la fonction des plaquettes au cours du stockage des CP. C'est ainsi que les LSP sont définies comme étant l'ensemble des changements délétères conduisant à la détérioration progressive de la viabilité, de la structure et de la fonction plaquettaire et qui se produisent à partir du moment où le sang est prélevé du donneur jusqu'au moment où les plaquettes sont transfusées au receveur[126].

Qu'elles soient déclenchées par des facteurs extrinsèques (méthodes de préparation) ou intrinsèques (facteurs plasmatiques et plaquettaires, leucocytes résiduels), les LSP pourraient être responsables en grande partie non seulement de la réduction globale de l'efficacité thérapeutique de la transfusion des CP, mais également de l'induction des effets indésirables chez le receveur (EIR)[125,127,128]. Les modifications inflammatoires sont liées à la sécrétion du contenu granulaire riche en molécules inflammatoires et à la néoformation de molécules pro-inflammatoires[129,130].

L'impact des LSP sur la qualité des CP peut être évalué par des tests *in vivo* ou par des tests *in vitro*. Les tests *in vivo* sont destinés à évaluer l'efficacité clinique et la survie des plaquettes

en post transfusion. En cas d'hémorragie active, le meilleur indicateur de l'efficacité clinique des produits plaquettaire est l'arrêt de saignement. En cas de traitement prophylactique, l'efficacité clinique est appréciée par la mesure du temps de saignement, l'augmentation du chiffre de plaquettes (« Corrected Count Increment » ou CCI) ou encore par le suivi isotopique de la survie des plaquettes[119].

Les LSP peuvent aussi être évaluées par des tests *in vitro*. D'une grande variété, ces derniers peuvent être classés en trois grands groupes[119,131].

- Tests évaluant la viabilité *in vitro* des plaquettes : dans ce groupe sont inclus les tests permettant d'évaluer les changements morphologiques (indice de tournoiement, le volume plaquettaire moyen, le changement de forme en réponse à des agonistes, réponse au choc hypotonique), les modifications métabolique (e.g. pH, PO2, HCO3-, déplétion en ATP), la lyse (LDH), l'apoptose (e.g. ADN et potentiel mitochondrial), et l'étude de la fonction plaquettaire (e.g. test d'agrégation, PFA-100, thromboélastographie)
- Tests évaluant l'activation plaquettaire : marqueurs d'activation membranaire : la mesure de ces marqueurs se fait principalement par des techniques de cytométrie en flux et concerne la mesure de CD62p, CD63, l'Annexine V, CD40L, LIBS ; marqueurs solubles (e.g. sCD62p, sCD40L, RANTES, PF4, thromboglobuline, TNF, IL8) : ces dosages se font principalement par des techniques immunologiques (e.g. ELISA et LUMINEX) ; les microparticules sont en général dosées par cytométrie en flux après ultracentrifugation.
- Tests évaluant la mécanistique des LSP : Ces tests sont très spécialisés et ils concernent l'étude de l'omique plaquettaire : protéome, métabolome, transcriptome, phosphorylome, lipidome.

La littérature est très riche en études évaluant, à l'aide des tests cités ci-dessus, l'effet des différentes méthodes de préparation, les conditions de stockage et les manipulations post-préparation sur les LSP. Ces études s'accordent toutes sur les faits suivants : indépendamment des méthodes de production, l'activation plaquettaire est un phénomène inévitable ; l'intensité de la primo activation influence, de manière générale, les autres types de LSP ; l'activation plaquettaire est corrélée aux autres LSP ; les LSP sont temps dépendant et atteignent leurs maximums vers le 3ème jour de stockage[104,132] ; Les LSP ne sont pas formellement corrélées à l'efficacité clinique[128], mais ce point est toujours en discussion.

Il y a un intérêt croissant en faveur de l'étude des molécules inflammatoires et les BRM secrétés par les plaquettes au cours du stockage et leurs implications dans la survenue des EIR chez les receveurs[133–136].

II.5. Réactions post-transfusionnelles liées à la transfusion plaquettaire

Comme toute autre transfusion de produit sanguin labile (PSL), la transfusion de plaquettes n'est pas sans risque. Elle peut être associée à des réactions post-transfusionnelles plus ou moins graves communément appelées effets indésirables receveurs (EIR). Deux grands types de réactions post-transfusionnelles sont à distinguer : les réactions post-transfusionnelles hémolytiques (principalement liées à la transfusion des concentrés de globules rouges ou de composé riche en plasma) et les réactions post-transfusionnelles non-hémolytiques. Les réactions non-hémolytiques sont les EIR les plus fréquemment rencontrées avec les transfusions de CP et comprennent les allo-immunisations, les réactions fébriles non-hémolytiques (RFNH), les réactions allergiques, l'accident de détresse respiratoire aigu (TRALI), l'hypotension, la surcharge circulatoire, les infections virales et bactériennes et les accidents métaboliques[137,138].

Selon le rapport de l'ANSM de 2015 relatif aux transfusions réalisées en 2014, 2,1% des transfusions ont donné lieu à un EIR. Comme le montre la **figure 10,** la fréquence des EIR n'est pas également répartie et varie selon la nature des PSL. En effet, plus de 25% des EIR déclarés sont dus aux seuls CP qui ne représentent pourtant que 10% des PSL transfusés[139]. La majorité des EIR liés à la transfusion de plaquettes est de nature inflammatoire, avec dans l'ordre décroissant de fréquence les réactions allergiques, les RFNH et le TRALI.

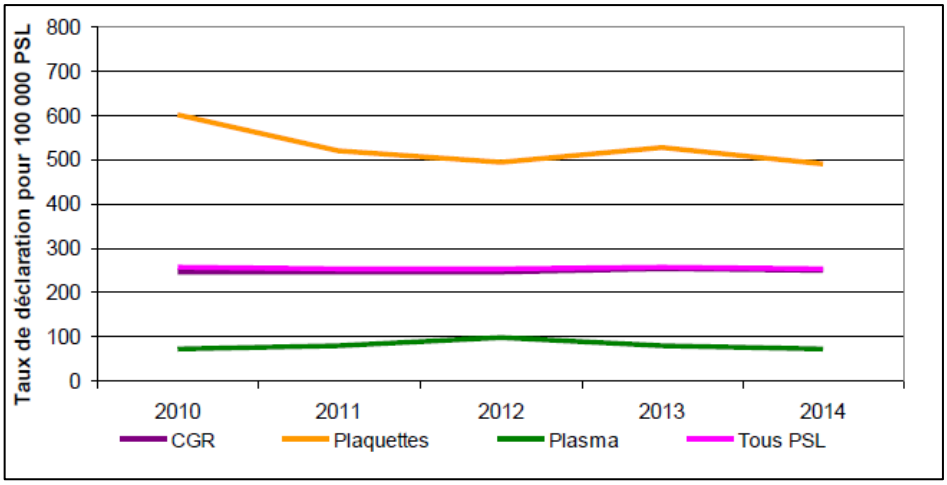

Figure 10 : Evolution des taux d'EIR declarés pour 100 000 PSL, par type de PSL, 2010 – 2014[139].

La survenue des EIR plaquettaires est sous l'influence de facteurs liés à la triade transfusionnelle donneurs (sexe, phénotype, génotype), PSL (qualité, LSP+++), et receveurs (état clinique, phénotype, génotype). A ce jour, les facteurs liés aux caractéristiques du produit plaquettaire sont les plus étudiés. On s'intéresse tout particulièrement à l'étude de l'association EIR inflammatoire/type et concentration des molécules libérées par les plaquettes sous l'effet des LSP. Plusieurs raisons expliquent l'intérêt croissant porté à ces types d'EIR :

- La transfusion de plaquettes est d'avantage une thérapeutique complémentaire incontournable dans la prise en charge de patients particulièrement vulnérables. Il est donc nécessaire d'éviter le plus que possible d'accroitre l'état pré-inflammatoire de ces patients.
- La persistance des EIR à composante inflammatoire malgré l'introduction systématique de la leucoréduction (les leucocytes et leurs produits (BRM) sont en effet des pourvoyeurs généreux d'EIR).
- La reconnaissance du rôle inflammatoire et immuno-modulateur des plaquettes. Comme nous l'avons bien mentionné plus haut, les plaquettes sont équipées de toute la machinerie nécessaire pour jouer un rôle dans l'inflammation transfusionnelle.
- La mise en évidence, en 2001, par Phipps et al[140], du rôle physiopathologique d'un BRM considéré comme une « cytokine-like », le CD40L appelée encore CD154, sous sa forme soluble (sCD40L) dans la survenue de RFNH ; ce sera rappporté aussi dans les TRALI par Khan et al[141]. Les travaux de notre laboratoire ont également confirmé le rôle primordial de cette molécule dans les EIR, mais ils ont aussi identifié d'autres BRM tels que l'OX40 ligand soluble, l'IL27, le MIP1α et l'IL13[132,135,136,142], en associations pathogènes.

II.6. Le CD40L

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Review

The Signaling Role of CD40 Ligand in Platelet Biology and in Platelet Component Transfusion

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Abstract: The CD40 ligand (CD40L) is a transmembrane molecule of crucial interest in cell signaling in innate and adaptive immunity. It is expressed by a variety of cells, but mainly by activated T-lymphocytes and platelets. CD40L may be cleaved into a soluble form (sCD40L) that has a cytokine-like activity. Both forms bind to several receptors, including CD40. This interaction is necessary for the antigen specific immune response. Furthermore, CD40L and sCD40L are involved in inflammation and a panoply of immune related and vascular pathologies. Soluble CD40L is primarily produced by platelets after activation, degranulation and cleavage, which may present a problem for transfusion. Soluble CD40L is involved in adverse transfusion events including transfusion related acute lung injury (TRALI). Although platelet storage

designed for transfusion occurs in sterile conditions, platelets are activated and release sCD40L without known agonists. Recently, proteomic studies identified signaling pathways activated in platelet concentrates. Soluble CD40L is a good candidate for platelet activation in an auto-amplification loop. In this review, we describe the immunomodulatory role of CD40L in physiological and pathological conditions. We will focus on the main signaling pathways activated by CD40L after binding to its different receptors.

Keywords: CD40 ligand; CD40; inflammation; signaling pathways; p38 mitogen-activated protein kinases (MAPK); nuclear factor-KappaB (NF-κB)

1. Introduction

CD40 ligand (CD40L)—otherwise known as CD154—is of particular interest for several reasons. It is easily detectable in plasma; it is essential to immunity at large and central to adaptive immunity, being among the seminal molecules that tether antigen (Ag)-specific T and B-lymphocytes in the synapse; and it is indispensable for the formation of germinal centers (GCs) in lymph nodes [1–4]. CD40L is thus crucial for cell signaling in both adaptive and innate immunity, as it is expressed by a large variety of cells that take a role in immune responses [1,4]. Further, CD40L has genetic and molecular polymorphisms, with pathogenic and pathologic consequences [5]. Intriguingly, its soluble form is principally generated by platelets, and it is responsible for transfusion associated hazards [6–8]. Together, those properties require the attention of pathologists and clinicians, as CD40L is more important in medicine than initially thought.

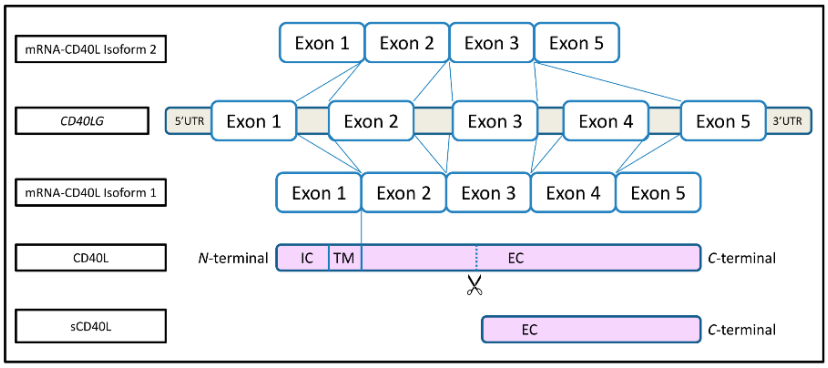
In this review, we will discuss the role of CD40L and its soluble form (sCD40L) in transfusion hazards. It is associated with high levels of inflammatory molecules such as chemokines, cytokines and biological response modifiers (BRMs) released by platelets during storage. sCD40L is a master pro-inflammatory BRM in transfusion [6–12]. Platelet sCD40L has been largely studied in inflammation and autoimmune disease [3,13–15], but the mechanism for its regulation is just beginning to be unraveled.

2. What Is CD40L?

CD40L is a 33 kDa type II transmembrane protein belonging to the Tumor Necrosis Factor (TNF) superfamily. The CD40L gene (*CD40LG*) encodes a 261 amino acid (AA) protein with a 22 AA cytoplasmic domain, a 24 AA transmembrane (TM) domain, and a 215 AA extracellular domain (Figure 1) [3]. CD40L is constitutively highly expressed by a panoply of hematopoietic and non-hematopoietic cells [1,3,4,16]. CD40L can be further expressed or overexpressed by activated cells, the most characteristic and best studied of which are activated and/or differentiated T cells [4]. Like other members of the TNF family, active CD40L at the cell surface or in its soluble form is composed of homotrimers [17]. This multimeric conformation of CD40L is of crucial importance for effective interaction with CD40 and the subsequent intracellular signaling [18]. Moreover, the

soluble forms of CD40L retain their ability to form trimers, which bind CD40 and deliver biological signals [18]. Membrane bound CD40L can be cleaved at methionine 113 of the extracellular domain and shed as a soluble form [19–21]. The principal isoform (isoform 1) is encoded by 5 exons. The second CD40L isoform (isoform 2) is poorly described (Figure 1). It is a truncated 240 AA protein lacking exon 4 in the *CD40LG* (extracellular domain), and the functional consequence of this is unknown [22]. Of note, membrane bound CD40L is expressed on B cells and dendritic cells (DCs). It is not expressed on non-activated T cells and platelets, but is weakly expressed on non-activated macrophages, neutrophils and endothelial cells [23]. It is highly expressed on activated T cells and platelets from which it can be cleaved as a soluble form, but it is not cleaved from B cells, DCs and macrophages. There is no up-regulation in neutrophils and endothelial cells, regardless of whether they are activated [1,3,4,23].

Figure 1. Sheme of the CD40 ligand gene structure and its different isoforms. Intracellular domain (IC), transmembrane domain (TM), extracellular domain (EC).



The main receptor for CD40L is CD40, which is constitutively expressed by antigen presenting cells (APCs) such as B cells, macrophages, and DCs [3,4,24]. CD40 is also expressed by platelets [25,26], neutrophils, endothelial cells [23] and T-cells [27–30]. Five distinct isoforms of CD40 are expressed with two isoforms predominating in human and mice [31–33]. Isoform 1 predominates and is membranous, but may be cleaved into a soluble form by a metalloproteinase, ADAM-17 [34]. In contrast, isoform 2 is produced as a soluble form resulting from alternative splicing [35]. It is hypothesized that the soluble forms act as competitive inhibitors for the membranous form, though this remains unclear [36]. Although CD40 is a type I TM protein that can form monomers, dimers and trimers, only the latter form fully activates cells [37–39].

CD40L can also bind to three integrins: the platelet glycoprotein αIIbβ3 (GPIIb/IIIa), otherwise known as receptor for fibrinogen and von Willebrand Factor [40,41]; α5β1 (CD49e/CD29), an integrin that binds to matrix macromolecules and proteinases and thereby stimulates angiogenesis [42–44]; and Mac-1, an integrin (otherwise known as CR3 (Complement Receptor 3), CD11b/CD18, or $\alpha_M\beta2$), mainly expressed by neutrophils, natural killer cells and macrophages to trigger a transduction signal and mediate inflammation [45]. The functional interaction of CD40L with α5β1 is independent of its binding to αIIbβ3 and CD40 [43,44]. Interactions between CD40L and α5β1 are not relevant in platelet physiology/physiopathology [45].

3. What Is the Function of CD40L?

The interaction between CD40 and CD40L is essential in the innate and adaptive immune systems, both in physiology and in physiopathology.

3.1. CD40/CD40L in Physiology

First characterized as a major marker on carcinoma cells, CD40 was next shown to be a key molecule shared by endothelial cells and most APCs, including B-cells, monocytes and DCs [46]. Interactions with CD40L are mandatory for the B-cell response to T-dependent Ags [2]. In particular, studies on patients with primary Ab immunodeficiencies targeting CD40 or CD40L have definitively established the requirement of these interactions for GC formation and the generation of memory B-cells and long-lived plasma cells [47]. More recent data on GC reactions and follicular helper T-cells (T_{FH}) show that the polarization of CD4 T-cells into T_{FH} is initiated by contact with DCs at the border of B-cell follicles and maintained by GC B-cells [48]. The expression of BCL6, the master regulator of T_{FH}, is dependent on CD40-CD40L and ICOS-ICOSL interactions outside follicles and within GCs [49]. CD40L-induced CD40 signaling in B-cells is crucial for inducing the expression of BCL6 and Ki67 in GC B-cells, allowing the proliferation of GC B-cells in the dark zone and expression of activation-induced deaminase (AID), a transcription factor required for somatic hypermutation (dark zone) and Ig class switching (light zone). CD40-CD40L interactions are further required for the selection of B-cell clones expressing high affinity BCR that takes place within the GC light zone. In physiological conditions, only selected B-cell clones differentiate into effector B-cells (memory and plasma cells). CD40 is also constitutively expressed by DCs and macrophages, and its triggering induces the expression of other co-stimulatory molecules and the release of cytokines that modulate T- and B-cell responses [24]. CD40 activation on macrophages also induces the release of nitric oxide and reactive oxygen species, contributing to the destruction of intracellular pathogens. Strikingly, CD40-induced CD40L signaling in CD8 T-cells rescues them from the exhaustion observed during chronic viral infections and is important to maintain their poly-functionality [50]. With CD40 being expressed on various B-cell lymphomas and carcinomas (nasopharynx, bladder, cervix, kidney and ovary), there is a renewed interest in CD40/CD40L in the control of tumor growth, leading to the development of new therapeutic strategies [51].

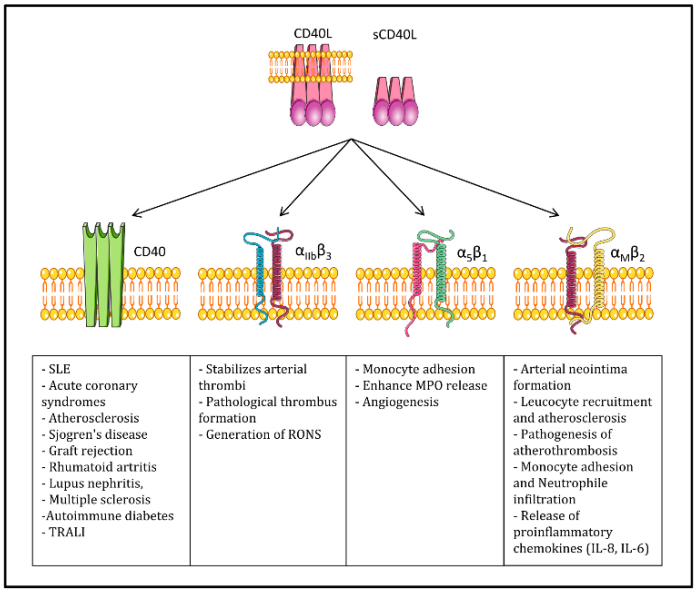
3.2. CD40L and Its Receptors in Inflammatory Pathologies

As already presented, in addition to the classical receptor CD40, CD40L also binds the αIIbβ3, α5β1, and Mac-1 (αMβ2) integrins and induces different biological responses. Figure 2 illustrates the pathological role of each dyad interaction.

The CD40-CD40L system is associated with both pro-thrombotic and pro-inflammatory effects. Soluble CD40L contributes to the pathophysiology of atherosclerosis and atherothrombosis [52]. Because of its autocrine, paracrine, and endocrine activities, sCD40L enhances platelet activation, aggregation, and platelet-leukocyte conjugation that may lead to atherothrombosis [13,53,54]. CD40L binding may result in the activation of CD40 expressing cells with interleukin production [23,55].

The interaction of CD40L with CD40 on endothelial and other vascular cells upregulates adhesion molecules such as E-selectin, VCAM-1, ICAM-1 and proinflammatory cytokines such as regulated on activation normal T cell expressed and secreted (RANTES), interleukin (IL)-6, and IL-8 as well as matrix metalloproteinase (MMP)-1, -2, -3, and -9 [56]. Soluble CD40L also stimulates the expression of tissue factor (TF) on monocytes and on endothelial cells [57,58]. After CD40L and CD40 interact on the endothelial surface, thrombomodulin expression is decreased, facilitating thrombin generation [59]. CD40L-CD40 interactions activate endothelial cells via either sCD40L *in vivo* or by a specific antibody to CD40. Membrane-bound CD40L, but not sCD40L, induces the upregulation of pro-inflammatory cytokines and cell adhesion factors in endothelial cells. However, both forms of CD40L activate both classical and alternative NF-κB pathways [60]. In addition, sCD40L induces endothelial dysfunction with decreased NO synthesis and augmented oxidative stress [61]. These events may further contribute to endothelium injury and accompanying atherogenesis. sCD40L may play a pathogenic role in triggering acute coronary syndromes [54,62]. The involvement of CD40-CD154 interactions in autoimmunity and allo-immunity is also well documented. In fact, many tissue injuries and immune mediated pathologies such as graft allo-rejections involve this signaling pathway [63]. CD40-CD40L interactions play a significant role in the production of auto-antibodies in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and other autoimmune diseases. An increased serum level of soluble CD154 was reported in SLE, RA, and Sjogren's disease, in correlation with the relevant auto-antibodies and with the clinical disease activity [14,64].

Figure 2. CD40L and its receptors: the binding of CD40L to CD40, αIIbβ3, α5β1, or Mac-1 (αMβ2) induces different inflammatory pathologies. Systemic lupus erythematosus (SLE), transfusion-related acute lung injury (TRALI), reactive oxygen and nitrogen species (RONS), Myeloperoxidase (MPO).



αIIbβ3 integrin was first identified as a receptor for CD40L by André *et al.* [65]. They showed that sCD40L can bind to αIIbβ3 integrin on activated platelets, thereby inducing platelet spreading and promoting platelet aggregation under high shear rates, as well as allowing stability of arterial thrombi [66]. The same group further reported that CD40L is a primary platelet agonist capable of inducing platelet activation, induction of fibrinogen binding and the formation of platelet microparticles by binding to its αIIbβ3 receptor and triggering outside-in signaling [67]. In addition, the engagement of αIIbβ3 by CD40L or other ligands that induce platelet adhesion upregulates CD40L surface exposure on platelets [68], enhancing the interaction of platelets with CD40+ cells, including ECs. Incubation of platelets with recombinant sCD40L led to enhanced P-selectin expression, aggregation, and platelet-leukocyte conjugation. The inhibition of either sCD40L or αIIbβ3 attenuated the generation of reactive oxygen and nitrogen species (RONS) by platelets [69].

Mac-1 is an important mediator of neutrophil and monocyte adhesion to the activated endothelium during inflammation. CD40L ligation to Mac-1 is involved in mediating CD40L/Mac-1-dependent monocyte and neutrophil adhesion and transmigration at the atherosclerotic lesion site, as well as neointimal formation during atherogenesis [42,70]. In transfusion, the sCD40L concentration increases in stored platelets compared to fresh platelets [9,10]. The neutrophil priming ability of stored platelets is significantly higher compared to fresh platelets [71]. Soluble CD40L and CD40-activated-neutrophils are essential to permit the adhesion and migration of neutrophils by Mac-1 secretion. This signal is the main system to recruit neutrophils into pulmonary tissue [72]. CD40+ neutrophils primed by CD40L+ activated platelets and sCD40L are recruited and over-stimulated by IL-6, IL-8 and IL-1β originating from alveolar macrophages and fibroblasts. In alveolar space, these neutrophils secrete ROS, proteases, PAF and elastase-α1-antitrypsin complexes that insult the pulmonary parenchyma [73]. In another study using the two-event TRALI mouse model, Hidalgo and colleagues demonstrated an increase in platelet interactions with adherent neutrophils in the systemic circulation [74]. These interactions were dependent on E-selectin expression on the endothelium interacting with E-selectin ligand on neutrophils, which ultimately led to the polarization of Mac-1 on the leading edge of the neutrophils. Circulating platelets interacted with the clustered Mac-1, although the platelet ligand mediating this interaction is not known [74]; could it be CD40L?

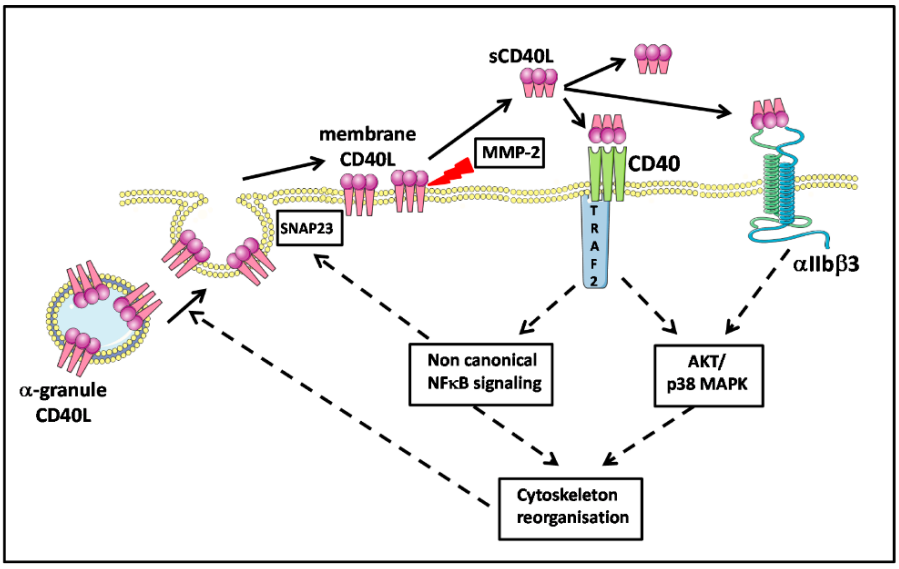
The α5β1 integrin is expressed by endothelial cells, smooth muscle cells, monocytes/macrophages and platelets. It is implicated in cell adhesion, migration, and proliferation as well as survival of many cell types. The binding of CD40L to a monocytic cell line expressing α5β1 integrin leads to the phosphorylation of the extracellular signal regulated kinases 1/2 (ERK-1/2) and expression of IL-8 mRNA in these cells [14]. However, unlike fibrinogen and vitronectin which are the natural ligands of α5β1, CD40L binds to the inactive rather than the active form of α5β1. Interestingly, CD40L/α5β1 interactions do not interfere with the binding of CD40L to CD40, indicating that CD40L can bind simultaneously to both receptors [43].

The role of α5β1 as a receptor for CD40L in α5β1-expressing-cells has not yet been investigated. Hassan *et al.* hypothesized the involvement of the CD40L/α5β1 dyad in angiogenesis and pathological conditions of the vascular system after the tethering of cells in inflamed tissues such as atherosclerotic lesion sites [75].

4. Platelet CD40L

The discovery in 1998 that platelets preferentially express many copies of CD40L on their surfaces upon activation was surprising because CD40L was thought to characterize immune reactive cells, and platelets were not yet acknowledged to display any immune function [25]. CD40L was then found in platelet cytoplasm [25,65,76,77], and years later more precisely identified as being docked in the platelet α-granules [78] (Figure 3). The discovery that, despite being non-nucleated cells devoid of DNA apart from mitochondrial DNA [79], platelets can retrotranscribe RNA using a spliceosome [80–83] and lead to detectable RNA messages for cytokines, questioned the possibility that CD40L is also produced *de novo* by activated platelets. Recently, some RNA-seq studies did not find CD40L mRNA in platelets [84–87]. This result suggests that a preformed protein is synthesized by megakaryocytes and stored in α-granules before platelet fragmentation [88–90].

Figure 3. Schematic overview of the regulation of platelet CD40L and the role of sCD40L in signaling after binding to platelet CD40 and αIIbβ3 inducing an auto-amplification loop. Synaptosomal-associated protein 23 (SNAP23), mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), protein kinase B (AKT) matrix metalloproteinase-2 (MMP-2), TNF receptor associated factor 2 (TRAF2).



After stimulation by different agonists, platelets undergo a degranulation process via a well characterized mechanism [91], and either export the α-granule molecules to the membrane in a fixed form or secrete them as a soluble form. Granules fuse with the platelet membrane and display their fixed CD40L on the surface. This process occurs within seconds to minutes after stimulation [25]. CD40L is thus expressed on the platelet surface only after activation, and this molecule is identical in terms of structure and physiological function to membrane bound CD40L

expressed in activated T-lymphocytes and other cells. It can notably generate signals for the recruitment and extravasation of leukocytes. It induces, through the engagement of CD40, the secretion of chemokines and the expression of adhesion receptors in endothelial cells [25]. It provides a powerful link between platelets and the immune system: CD40L expressed on activated platelets induces dendritic cell maturation, B-cell isotype switching, and augments CD8+ T-cell responses in both *in vitro* and *in vivo* models [92–95].

Platelets do not maintain CD40L on their surface for long. It is cleaved and released in a soluble form and may also be carried on the surface of microparticle-derived platelets [96]. Platelets are the major source of sCD40L in the circulation [65,97,98]. The normal range of sCD40L in the serum of a healthy adult is estimated at 0.79 to 4.7 ng/mL, by means of immunoassay techniques [99–101].

Of note, platelets constitutively express CD40 on their surfaces, both when resting and upon activation (Figure 3) [25,26,92]. This is surprising, as CD40 has long been considered to characterize APCs. Some sCD40L is reabsorbed on the platelet surface and principally binds CD40, a mechanism of recycling that must not be ignored when discussing platelet physiology and pathology.

5. Platelets, CD40L, and Molecular Signaling

5.1. Platelet Activation in Platelet Components and Molecular Signaling

Platelet activation and the signaling pathways involved in hemostatic conditions are well documented [102–104]. However, there is little information regarding the platelet components (PCs) prepared and processed for transfusion.

Several proteomic studies have investigated platelet changes after either resting (*ex vivo*) conditions or stimulation (*in vitro*) [105,106]. Most have tested activation markers such as shape change, glycolysis, supernatant pH levels, platelet CD62P and CD40L surface expression, reactivity to repeated activation by agonists, secretion of platelet granule products, cytoskeletal reorganization and expression of apoptotic markers [9,107,108]. Most of those studies, as well as the subsequent ones, were carried out with the purpose of improving the platelet physiology in the *ex vivo* conditions that lead to the possibility of storing platelets for a limited number of days and transfusing homologous donor platelets to a recipient patient. The signaling pathways involved in the "spontaneous" activation of platelets in PCs were investigated [105,109,110].

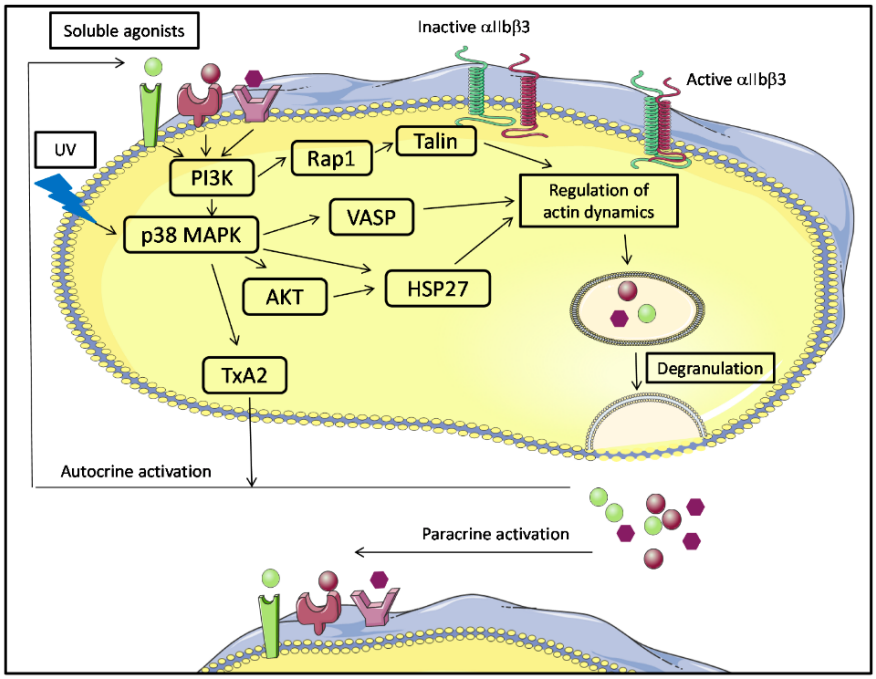
Schubert *et al.* [109] found evidence for a signaling pathway mediating PC storage lesions in which PI3-kinase-dependent Rap1 activation leads to integrin αIIbβ3 activation and platelet degranulation. This pathway involves two principal actors: Rap1, a small GTPase that modulates αIIbβ3 affinity, most likely through effects on the actin cytoskeleton [111], and Talin, an adaptor protein that links αIIbβ3 to the actin cytoskeleton. In hemostasis, this pathway is activated by soluble molecules after binding to different receptors, leading to the activation of the integrin αIIbβ3 [112].

Moreover, several studies identified the activation of the p38 MAPK signaling pathway during the aging of platelets not subjected to added stimulus [105,110], and/or after treatment of platelet concentrates with UV light with the intent of eradicating infectious pathogens. p38 MAPK is more

highly activated after UV exposure, a PI3-kinase-dependent mechanism that involves AKT, VASP and HSP27. AKT thus acts as a substrate for p38 MAPK. HSP27 is a substrate for AKT, and it regulates actin dynamics and degranulation. This confirms the earlier finding that MAPK activation stimulates platelet degranulation and TxA2 synthesis, which may in turn activate platelets via the TP receptor [113]. After degranulation, soluble factors (ADP, ATP, TxA2, Ca^{2+} and thrombin) are released and may act quickly to amplify autocrine activation of platelets as well as the activation of surrounding platelets (Figure 4) [88].

Platelets possess a variety of pathogen recognition receptors (PRRs) to sense bacterial and viral moieties and other receptors that could be involved in platelet activation in PCs [114–116]. Activated platelets can, consequently, secrete inflammatory cytokines and chemokines and other biological response modifiers (BRMs), including sCD40L, which could be a good candidate for such autocrine activation loops in platelets (Figures 3 and 4).

Figure 4. Principal signaling pathways inducing platelet activation in platelet components. Phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase B (AKT), Thromboxane A2 (TxA2), Vasodilator-stimulated phosphoprotein (VASP), Ras-proximate-1 (Rap1), Heat shock protein 27 (HSP27).



5.2. Platelet Membrane CD40L Regulation and Shedding

As already stated, CD40L was initially thought to be almost absent from the surface of non-activated platelets [117]. It has been recently reported, however, that resting platelets express very low levels of CD40L on their surface but can translocate massive amounts of CD40L to the

surface within minutes of activation. This CD40L can subsequently be cleaved and released as a soluble molecule into the circulation [92,118,119]. Such CD40L would thus be mobilized from the α-granules (Figure 3).

Knowledge regarding CD40L cleavage, either from T-cells or from platelets, remains incomplete. Cleavage from T-cells has been attributed to two types of MMPs. Two other MMPs are also responsible for the cleavage of platelet membrane bound CD40L.

MMPs constitute a large family of more than 25 functionally related endopeptidases mediating the proteolytic cleavage of most matrix proteins, as well as several non-matrix proteins including cytokines, chemokines, adhesion molecules and surface receptors [120]. ADAM10 has been proposed as a candidate MMP for CD40L cleavage and shedding from T-cells [121]. ADAM17 has been shown to be another candidate in an *in vitro* model of Jurkat E6.1 T-cells, where the inhibition of both ADAM10 and ADAM17 nearly completely inhibited CD40L shedding from the cells, suggesting that no other MMP besides ADAM10 and ADAM17 is involved [122]. The mechanisms that cleave activated platelet CD40L appear to be quite different. Not only MMPs but also the integrin αIIbβ3 are mandatory. MMP2 is the best candidate, based on different experimental approaches [123–126]. A novel enzyme has revealed itself as a potential candidate, at least in pathological situations, as demonstrated in a mouse model of sepsis. Here, MMP9 was involved in the shedding of CD40L after platelet-neutrophil interaction. Again, different experimental approaches confirmed a role for MMP9 [127–130].

Interestingly, the enzymatic regulation of CD40L cleavage from CD40L-positive cells appears cell-dependent. Platelets and T-cells use different proteases to cleave sCD40L from their cell surfaces (MM2/MMP9, and ADAM10/ADAM17, respectively), despite both cell types containing all four identified enzymes. Among the possible explanations are the existence and particularities of the cytoplasmic or granule reservoirs. Those distinct mechanisms have functional consequences on signaling pathways triggered upon CD40L/CD40 activation between these two cell types.

In platelet CD40L cleavage, the need for functional and complete αIIbβ3 remains intriguing [123,131]. For example, Glanzmann thrombocytopenia patients fail to properly release sCD40L upon platelet activation [123].

5.3. Platelet and CD40L Signaling

CD40L production by platelets is an interesting intersection between hemostasis and inflammation. Hemostatic activation of platelets (by ADP, thrombin, collagen, *etc.*) induces inside-out signaling and, consequently, activation of αIIbβ3. This leads to outside-in signaling and degranulation, followed by CD40L expression on the membrane surface. After activation, CD40L is shed and released in an active form that can activate different cell types, including platelets.

Soluble CD40L may activate platelets via two independent receptors, CD40 and αIIbβ3. After sCD40L binding, both receptors activate AKT and enhance platelet p38 MAP kinase phosphorylation. One study showed that this signaling pathway initiates the generation of inflammatory molecules such as reactive oxygen and nitrogen species [69].

Soluble CD40L binding to platelet αIIbβ3 (through its KGD sequence) enhances thrombus formation and induces platelet spreading via outside-in integrin signaling in an auto-amplification

loop [65,67]. This phenomenon also induces the generation of microparticles, especially through phosphorylation of tyrosine-759 in the cytoplasmic domain of the β3 chain [67].

Soluble CD40L may also activate platelets via the CD40 receptor, which is present on platelet membranes [25,26]. In this case, the mechanism is outside-in independent. The CD40L/CD40 activation in platelets involves a CD40-dependent TRAF2/Rac1/p38 MAPK signaling pathway and triggers phosphorylation of IkBα [132,133]. Thus, the sCD40L/CD40 interaction also triggers NF-κB pathway activation in platelets. In this case, NF-κB acts as a signaling molecule and not a transcription factor. IκB phosphorylates SNAP23, a key protein for the fusion of alpha granules and the plasma membrane [134]. IKKb blockade inhibits SNAP 23 phosphorylation and prevents SNARE complex formation (SNARE complex formation reviewed in [90,135,136]) and platelet degranulation [134]. These mechanisms are outlined in Figure 3.

6. CD40L and Platelet Component Transfusion

The sCD40L association with platelets has been popularized because of the description of transfusion hazards [6–12]. Before that, although well published, this association received little consideration. For more than a decade, sCD40L-linked associated hazards also received modest consideration, probably because the attention of transfusiologists focused on preventable hazards, and residual leukocytes were considered to be responsible for all symptoms of inflammation [137]. Transfusion-linked inflammation was not yet acknowledged, but classed as discomfort. Accidents were attributed to other causes, which were sometimes reported as unidentified. Soluble CD40L gained attention when progress was made in the field of hemostasis and thrombosis, which outlined the role of platelets and leukocytes in the formation of atheroma plaque deposition and led to the proposal that cardiovascular disease is inflammatory [16,99,138–143].

Platelets in an inventory are generally stored no longer than 5 days (ranging from 3 to 7 days depending on country regulations). During storage, and without the addition of any stimulus intended to activate them, those so-called “resting” platelets are exposed to a number of stresses, including the process of constituting a PC, exposure to plastics, preservatives and gases, rotation, and changes in temperature [107,144,145]. Platelets are extremely reactive to external signals and are designed to sense external danger. They are equipped with many types of receptors and danger sensors, and they respond to multiple signals [83,116]. Anticoagulant factors and bacterial residues can modify the status of platelets that are believed to be “resting”, but which in fact are lightly stimulated just above physiological steady state [83,116]. As platelets secrete more pro-inflammatory than anti-inflammatory BRMs, they begin to produce or secrete BRMs that are fairly detectable in the PC supernatant by day 3 [9,146]. Soluble CD40L is the most visible cytokine-like BRM which is thus made, and it is produced in amounts that are sufficient to activate CD40+ cells *in vitro*, including B-cells, dendritic cells, and macrophages [146,147]. It is therefore fully bioactive. The longer the PC is stored, the more BRMs are found, apart from some molecules with extremely short half-life [11,12,146]. CD40L has a short half-life outside the α-granule, but its secretion over day 3, for 2 to 4 days, still allows biological function [146]. In general, PC transfusion is safe and accomplishes what it is expected to do: prevent or stop bleeding in the allocated patient/recipient. In approximately 10% of cases, moderate intolerance symptoms are reported, which are referred to

as either febrile non-hemolytic transfusion reactions (FNHTRs) or allergic reactions (in fact, allergic-type reactions) [148]. In 2% of cases, the symptomatology is more severe, and presents more clearly as inflammatory [148]; such cases have been investigated by several groups, and there is a consensus on the responsibility of sCD40L that is found in excess in the PC or in the recipient's plasma [6,7,10–12]. Soluble CD40L does not carry the full responsibility, but it is chiefly to blame [7,11,12]. It is also responsible in part for the physiopathology of a severe transfusion hazard called TRALI (Transfusion-Related Acute Lung Injury) [10], despite one recent publication that disputed this [149]. An open question is why some PCs seem loaded with sCD40L. If platelets in PCs can be over-stimulated by some unexpected event in the process, it probably does not occur in all cases [7,150].

7. Concluding Remarks: Towards Molecular Medicine Based upon CD40L and CD40 Polymorphisms

As the CD40/CD40L molecular tandem is essential in many pathways of physiological but also pathological immune and inflammatory responses, its control is valuable in patient care. We and other groups have worked extensively during the past few years on the involvement of sCD40L in transfusion associated hazards, and we have recently obtained evidence that there are a number of *CD40LG* polymorphisms that may affect the behavior of platelets in a PC processed for the purpose of transfusion [151]. Combined with polymorphisms of *CD40*, this may affect the preferential decrease of inhibitory isoforms of the molecules and the increase of high affinity isoforms. Certain platelet donors may express high levels of sCD40L that are promptly cleaved [11,12,126], and/or certain recipients express high affinity CD40 receptors on both circulating cells and endothelial cells, favoring excess CD40/CD40L reactions and adverse events. Cell signaling through these interactions may prompt those cells to either synthesize or release copious amounts of bioactive BRMS with inflammatory potential. If proven, donor selection and/or patient investigation would allow better matching to prevent such adverse events. Serious adverse events would also benefit from the recent development of biologicals that target either CD40 or CD40L. In fact, Tanaka *et al.* [152,153] have succeeded to remove 80% to 90% of sCD40L in PCs using a column of adsorptive cellulose beads. However, there was a significant decrease in the recovery of platelets after adsorption. In other diseases, blockade of CD40/CD40L was performed using anti-CD40L Abs, but unfortunately these drugs have exhibited potentially adverse interactions with platelets in patients [154].

Molecular or personalized medicine is thus underway for patients presenting with high risk of potentially lethal acute inflammatory responses. If not yet implementable at a large scale, this may be forecast for the very near future.

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Author Contributions

Fabrice Cognasse, Sandrine Laradi and Olivier Garraud designed and supervised this manuscript. Chaker Aloui, Olivier Garraud and Yolande Richard wrote this manuscript. Antoine Prigent, Caroline Sut and Sofiane Tariket participated in the discussion and in the conception of the figures. Hind Hamzeh-Cognasse and Bruno Pozzetto reviewed the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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III. Omique des plaquettes

III.1. Généralités sur le génome humain

Le génome est le patrimoine génétique qui caractérise chaque individu. Codé par quatre bases d'acide désoxyribonucléique (ADN), le génome humain est réparti sur 46 chromosomes : 22 paires de chromosomes autosomaux homologues et 2 chromosomes sexuels. Un chromosome est constitué de parties codantes appelées gènes et d'autres non codantes. Un gène est lui-même composé de parties codantes appelées exons qui vont être transcrites en ARN messager (ARNm) puis traduites en protéines. Le projet du génome humain a montré un catalogue d'environ 22000 gènes dans un paysage de 3,2 milliards de paires de bases (pb). La partie codante ou exons des 22000 gènes englobe seulement environ 1,5% du génome entier.

Les études du patrimoine génétique ont montré que la séquence d'ADN transmise d'une génération à l'autre est généralement conservée. Toutefois, au cours de la réplication de l'ADN ou de la méiose cellulaire, il peut y avoir des mutations et/ou des recombinaisons chromosomiques. Ces phénomènes sont à l'origine de la grande diversité génétique entre les individus et les populations. Des modifications soudaines et transmissibles de la séquence d'ADN (par exemple une substitution, une insertion ou une délétion d'une base), en fonction de leurs positions, peuvent être silencieuses, c'est à dire n'avoir aucun effet sur la protéine résultante, ou au contraire avoir une incidence positive ou négative sur la protéine et donc sur l'individu. Ces modifications du génome ont été largement utilisées comme marqueurs pour étudier la transmission des « caractères » des parents aux descendances et aussi pour identifier les causes directes ou indirectes des « caractères » des individus et des populations dans des conditions physiologiques et pathologiques[143].

III.1.1. Marqueurs moléculaires en génétique humaine (variants et haplotypes)

Les avancées en biologie moléculaire ont permi l'identification de marqueurs génétiques reposant sur la variabilité de la séquence d'ADN à des positions parfaitement connues[144]. Les premiers marqueurs furent les *Restriction Fragment Length Polymorphisms* (RFLP), les *Variable Number of Tandem Repeat* (VNTR ou minisatellites) et les *Short Tandem Repeat Polymorphisms* (STRP ou microsatellites)[145].

Les plus récents sont les Single Nucleotide Polymorphisms (SNP). Ils font partie de la famille des RFLP, mais n'impliquent le changement (i.e. mutation, délétion, insertion) que d'un

nucléotide en un locus donné et sont de manière générale bialléliques (A, a). Chaque individu sera donc porteur au niveau d'un SNP d'un des trois génotypes possibles (l'un et/ou l'autre des deux génotypes homozygotes (AA et/ou aa) et/ou le génotype hétérozygote (Aa)[146].

Les SNP représentent une source d'information riche, abondante et facile à étudier et sont les marqueurs les plus utilisés dans les études d'associations génétiques. Le nombre de SNP augmente de jour en jour, car de plus en plus de génomes sont séquencés. Les SNP communs sont arbitrairement définies comme ayant une fréquence d'allèle minoritaire (MAF, de Minor Allele Frequency) de 0,05 (e.i. 5% dans la population qui a été échantillonnée). Des variants sont classés comme "rares" quand leur MAF est inférieur à 0,05 et supérieur à 0,01 ; et de variants "privés" quand leurs MAF est inférieure à 0,01. Ces variants rares et privés pourraient être nommés « mutations ». La grande majorité des variants connus ont une fonction neutre, mais certains modifient subtilement le risque de maladies.

La base de données principale qui fournit des informations sur les variations de séquences, les fréquences des allèles, les différences de fréquences entre les populations d'ethnies différentes et leurs conséquences fonctionnelles est dbSNP (http://www.ncbi. nlm.nih.gov/projects/SNP). Avec les avancées technologiques et les grands projets de séquençage, le génome humain ne cesse pas d'être mis à jour fournissant ainsi des estimations plus précises sur le nombre de SNP, un nombre exponentiellement croissant de variants rares et une estimation plus fine des fréquences alléliques dans différents groupes ethnique[143].

Plus récemment que les SNP, un nouveau type de marqueur génétique a été introduit en 2006. Il s'agit des variabilités du nombre de copies d'un gène (CNV, de « Copy Number Variants »). Ce marqueur est généralement défini comme des ajouts ou des suppressions dans le nombre de copies d'un segment particulier de l'ADN (plus de 1 kb de longueur) par rapport à la séquence d'un génome de référence[147,148]. L'étude des CNV a beaucoup d'intérêt dans la recherche de la diversité génétique dans les populations humaines et dans différentes maladies.

Les variants génétiques sont régulièrement répartis dans le génome (1/1000pb en moyenne) et ont tendance à être transmis ensemble[149]. Ainsi, il est possible d'étudier dans un premier temps uniquement les SNP représentant des blocs de déséquilibre de liaison, ou blocs de LD (« Linkage Disequilibrium »). Le LD correspond à la ségrégation conjointe non aléatoire sur un chromosome de deux ou plusieurs allèles provenant de deux ou plusieurs loci différents à une fréquence plus importante que ne le voudrait le hasard c'est-à-dire que l'information

recueillie sur un des SNP est valable pour tous les SNP du bloc (en fonction de la force du LD). Ceci permettra de génotyper un SNP par bloc de LD sans pour autant perdre de l'information génétique et de diminuer ainsi le nombre de SNP à étudier dans un gène dans le but de réduire le coût du génotypage[149].

III.1.2. Les techniques de génotypage

De nombreuses méthodes ont été développées pour identifier et caractériser les variations génétiques (découverte ou génotypage de variations connues). Ces techniques sont toutes basées sur la technique de réaction de polymérisation en chaîne (PCR, de « Polymerase Chain Reaction ») et ont beaucoup évolué avec le progrès technologique. Citons à titre d'exemple quelques techniques de criblage de variations inconnues : la SSCP (« Single Strand Conformation Polymorphism »), la DGGE (« Denaturing Gradient Gel Electrophoresis »), la dHPLC (« denaturing High Performance Liquid Chromatography ») et la HRM (« High Resolution Melt »). D'autres techniques sont plus spécifiques et permettent de génotyper les polymorphismes déjà connus telles que la PCR-RFLP (« Restriction Fragment Length Polymorphism »), l'ARMS-PCR (« Amplification Refractory Mutation System Polymerase Chain Reaction ») ou encore les techniques de PCR en temps réel telles que la Taqman. Néanmoins, la méthode la plus fiable pour identifier une variation reste le séquençage direct [145,150,151].

Outre ces techniques classiques, des méthodes de génotypage à haut débit automatisées ont été mises au point telles que la technologie du SNPlex[152] et la « Sequenom MassARRAY iPLEX Platform[153,154] » qui permettent de génotyper jusqu'à 48 SNP simultanément et à partir d'un même échantillon d'ADN. Par ailleurs, plusieurs plateformes (e.g. Illumina® et Affymetrix®) ont développé des puces à ADN pouvant génotyper jusqu'à 5 millions de SNP en parallèle et à partir du même échantillon d'ADN.
Actuellement, les techniques de séquençage nouvelle génération (NGS de « Next Generation Sequencing ») sont encore plus performantes, leur coût de revient décroit et les plateformes sont de plus en plus accessibles.

III.1.3. Les approches d'études génétiques

Le choix d'approche ou de stratégie d'une étude génétique dépend de l'étiologie du caractère étudié. L'étiologie d'une maladie par exemple représente l'ensemble des mécanismes directement liés à son apparition. D'un point de vue génétique, dans le plus simple des cas, la

modification d'un gène majeur est responsable à elle seule de l'apparition de la maladie. Il s'agit alors de maladie monogénique ou d'étiologie simple. Elle présente en général un très fort caractère héréditaire.

Lorsqu'une maladie présente des composantes génétiques et environnementales multiples, on parle alors de maladie multifactorielle ou d'étiologie complexe. Leur mode de transmission est bien moins évident et la coexistence d'effets combinés de facteurs génétiques et environnementaux rend difficile la prédiction de la maladie au regard d'un gène seul. On fait alors plutôt référence aux gènes impliqués en tant que gènes de prédisposition ou de susceptibilité[155].

Selon l'étiologie d'une maladie, deux types d'études sont proposées : les études familiales et les études cas-témoins. Les études familiales traitent conjointement un certain nombre de familles de façon à détecter la transmission préférentielle d'allèles chez les personnes atteintes par la maladie considérée. Il s'agit de vérifier la transmission d'une variation génétique des parents aux enfants et aussi de comparer les enfants entre eux. Aux études familiales s'ajoute une approche plus épidémiologique appelée cas-témoins. Une étude cas-témoins cherche à déceler une différence de distribution des variants génétiques entre une population de cas, constituée d'individus diagnostiqués avec une maladie ou un phénotype d'intérêt, et une population de témoins sélectionnés qui ne sont a priori pas porteurs de la maladie ou du phénotype. Pour ce deuxième type de stratégie d'étude, il est nécessaire d'assurer l'homogénéité des deux groupes en prenant soin de les assortir sur des covariables telles que l'âge, le sexe et l'éthnicité qui peuvent avoir une influence sur le phénotype observé et ainsi biaiser le facteur génétique recherché[156,157].

Les études d'association entre une variante génétique et une maladie ou un phénotype cherchent à déceler une association à un niveau populationnel. L'association peut être directe si le marqueur observé est un locus de susceptibilité, ou indirecte si celui-ci se trouve physiquement proche du locus de susceptibilité et que leurs allèles sont statistiquement associés en raison du déséquilibre de liaison (**Figure 11**).

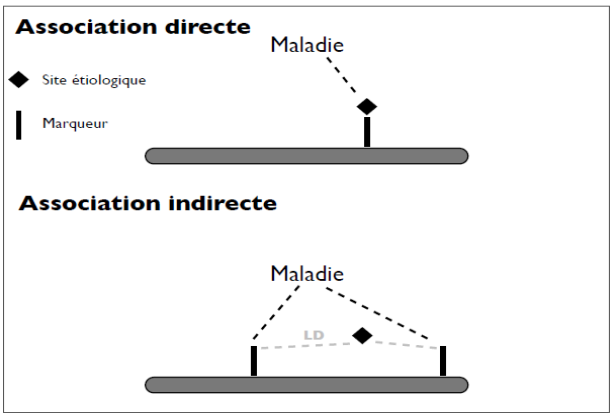

Figure 11 : Association génétique directe et indirecte.

Pour réaliser une étude d'association, deux approches sont proposées : "*Gènes-Candidats*" et "*Genome-Wide*". Les approches gènes-candidats consistent à sélectionner un ensemble de gènes dont les fonctions pourraient intervenir dans l'étiologie de la maladie étudiée, et à les tester directement par association. Le choix des gènes peut être guidé par des "a priori" biologiques tels que la fonction ou l'appartenance à une voie métabolique associée à une maladie, ou encore sur la base de la localisation dans une région chromosomique d'intérêt, suggérée par une précédente étude de liaison ou d'association. Même lorsque les connaissances a priori sont larges et que la physiopathologie de la maladie est relativement bien comprise, l'approche gènes-candidats n'identifiera qu'une fraction des déterminants génétiques. Dans le cas contraire, elle est alors inadaptée pour appréhender de façon exhaustive les causes génétiques des maladies.

Au contraire, une étude d'association genome-wide (ou systématique) investit une grande partie du génome sans aucun "a priori" sur l'identité des loci impliqués. Cette approche représente une stratégie impartiale, non dirigée et assez complète pouvant être mise en place en l'absence d'indices sur la fonction ou la position des loci de susceptibilités[155,157,158].

III.2. Analyses génétiques et génomiques sur les gènes codants pour des protéines plaquettaires

Le nombre d'études génétiques portant sur les protéines plaquettaires est très élevé. Des études ont identifié des polymorphismes associés aux phénotypes héréditaires des plaquettes tels que la numération, le volume moyen ou la réactivité spontanée des plaquettes ou en réponse à des agonistes des récepteurs d'activation plaquettaires[159–163].

Plusieurs gènes codant pour des protéines plaquettaires ont été étudiés dans des maladies monogéniques ou mendéliennes. Ces maladies ont une relation directe avec la

thrombocytopénie et ses différents types. En effet plusieurs gènes codants, le plus souvent, pour des protéines de surface telles que les glycoprotéines ou les integrines, mais aussi plusieurs autres, ont été identifiées[164–172]. D'autres études sur la thrombose et ses complications ont également identifié plusieurs variantes génétiques en causes[173–178].

Les études des maladies complexes ou multifactorielles ont également identifié des associations pour des polymorphismes portés par des gènes codants pour des protéines plaquettaires avec des maladies vasculaires ou cardiovasculaires[179–189].

Des thérapies antiplaquettaires ont été développées pour la prévention primaire ou secondaire des complications d'occlusions coronaire, cérébrale et des artères périphériques. Toutefois, il y a une contribution génétique à la variation inter-individuelle en réponse à plusieurs antagonistes[190–195]. Un grand espoir pour la médecine personnalisée est d'utiliser des tests génétiques pour identifier les patients qui bénéficieront de médicaments spécifiques (ciblés par exemple sur une particularité génétique de leur tumeur)[196].

III.3. Protéome des plaquettes

Le protéome est la totalité des protéines présentes dans une cellule à un moment donné. Il est sous contrôle régulier et varie d'une cellule à l'autre. Le protéome d'une cellule est spécifié par l'information codée par l'ADN nucléaire, qui est transcrit en ARN messager (ARNm) puis traduit en protéines qui peuvent subir des modifications post traductionnelles.

Concernant les plaquettes sanguines, plusieurs données indiquent que les mégacaryocytes les approvisionnent avec des milliers d'ARNm et de protéines. Comme dans les mégacaryocytes parents, les ARNm plaquettaires sont poly-adénylés et sont compétents pour la traduction. Les plaquettes circulantes ont la possibilité d'ajuster en permanence leur protéome par l'intermédiaire de la synthèse, de la modification post-traductionnelle et de la dégradation des protéines[197].

Bien que beaucoup moins sensibles que les analyses transcriptomiques, de nombreuses études protéomiques ont été réalisées sur les plaquettes.

III.3.1. Les approches d'études protéomiques

Plusieurs approches ont largement été utilisées pour étudier le protéome plaquettaire. L'étude du protéome combine souvent plusieurs techniques qui permettent tout d'abord la séparation des protéines. En fonction des objectifs de l'étude, une étape facultative de digestion

enzymatique, qui consiste à fragmenter les protéines en peptides, pourrait être envisagée. Les protéines ou peptides séparés sur gel d'électrophorèse sont ensuite identifis et quantifis directement par analyse bioinformatique de la photo du gel. En cas de digestion, les peptides sont séquencés par spectrométrie de masse et quantifiés d'une façon absolue ou semi quantitative selon l'approche utilisée (**Figure 12**).

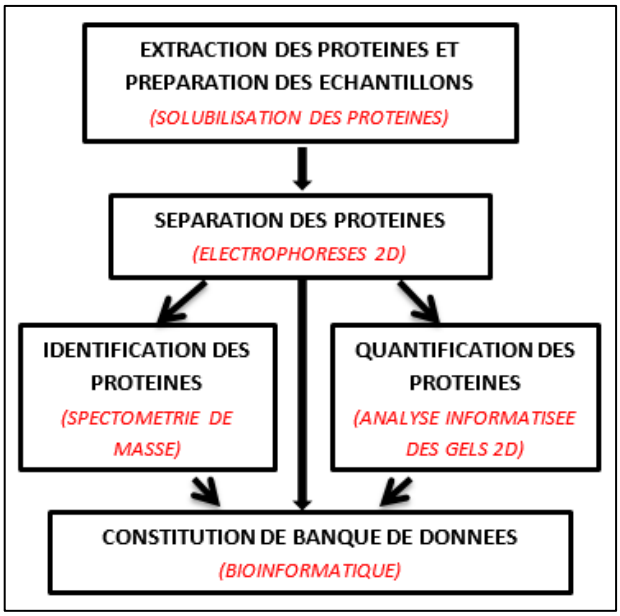

Figure 12 : Les grandes étapes de la protéomique

III.3.1.1 Les approches électrophorétiques

La technique d'électrophorèse bidimensionnelle (2D) est largement utilisée pour séparer les protéines plaquettaires pour le séquençage ultérieur[198]. L'électrophorèse 2D combine deux étapes de séparation : la première consiste à séparer les protéines en fonction de leurs points isoélectriques et la deuxième consiste à les séparer sur gel de polyacrylamide dodécyl sulfate de sodium (SDS-PAGE) dans un deuxième sens de migration selon leurs poids moléculaires. Les spots protéiques sont ensuite colorés par des colorants qui les rendent visibles tels que le bleu de Coomassie et le nitrate d'argent ou qui les rendent fluorescents par des fluorochromes tels que le SYPRO Ruby et le Cyanin. En fonction de l'utilisation ultérieure des gels 2D, ces colorants ou intercalants fluorescents peuvent également être mélangés avec les échantillons avant la migration électrophorétique. Après la migration, l'électrophoregramme est directement photographié. L'analyse de la photo à l'aide d'outils bioinformatiques permet de quantifier l'intensité des spots protéiques et d'identifier les protéines différentiellement exprimées entre deux conditions différentes ; tel est le cas de la technique DIGE (« Differential In-Gel Electrophoresis ») utilisée pour étudier le protéome plaquettaire durant

le stockage [126,199]. Les spots peptidiques peuvent ensuite être séquencés afin d'identifier la séquence peptidique exacte et ce en utilisant la spectrométrie de masse[200]. Les approches 2D, couplées à la spectrométrie de masse, sont couramment utilisées pour détecter les changements du protéome entre les plaquettes au repos et activées[201].

III.3.1.2 Les approches non électrophorétiques ou « gel-free »

Les approches « gel-free » sont basées sur la digestion protéolytique des protéines. Ces approches centrées sur les peptides emploient diverses méthodes chromatographiques pour séparer et sélectivement enrichir les peptides. Des techniques utilisent des marqueurs ou « label » et d'autres dites label-free ne nécessitent pas de marquage. Parmi les techniques utilisant des marquages isotopiques, nous citons la SELDI (« surface-enhanced laser desorption/ionization »), l'ICAT (« isotope-coded affinity tags »), la quantification relative et absolue par marquage isotopique (iTRAQ : « isotope tags for relative and absolute quantitation »), et la COFRADIC (« combined fractional diagonal chromatography »). La technique SELDI utilise des puces à protéines. Elle a été utilisée pour comparer les protéomes de plaquettes dans les modèles de cancer chez la souris[202]. Les techniques de marquage d'affinité isotopique ont été utilisées pour augmenter la sensibilité de la détection de petits fragments peptidiques et pour recueillir des informations quantitatives concernant les concentrations de protéines. Parmi celles-ci, l'ICAT marque sélectivement les résidus de cystéine des fragments de peptides qui sont digérés par la trypsine et l'iTRAQ étiquette chaque fragment digéré par la trypsine. Par conséquent, iTRAQ fournit généralement plus d'informations que l'ICAT en ce qui concerne le changement absolu de la composition protéique. Thon et Devine ont utilisé avec succès l'ICAT et l'iTRAQ pour examiner l'expression différentielle des protéines de plaquettes pendant le stockage[126]. Martens et coll ont utilisé la COFRADIC pour détecter 641 protéines dans les plaquettes, avec un recouvrement de 40% des protéines caractérisées par des méthodes 2- DE[203]. Avec les progrès de la haute résolution et de la sensibilité des technologies de spectrométrie de masse, diverses méthodes nécessitant une quantité moindre de protéines et produisent des résultats avec un indice de confiance élevé sont d'actualité comme la quantification label-Free pour laquelle le principe est basé sur la chromatographie liquide suivie par la spectrométrie de masse en tandem (LC/MS-MS)[204].

Ces techniques générant un grand nombre de protéine, sont classées parmi les techniques de haut débit nécessitant ainsi une validation des résultats par une technique classique d'étude de protéines comme le Western blot mais aussi l'ELISA et la cytométrie en flux[205].

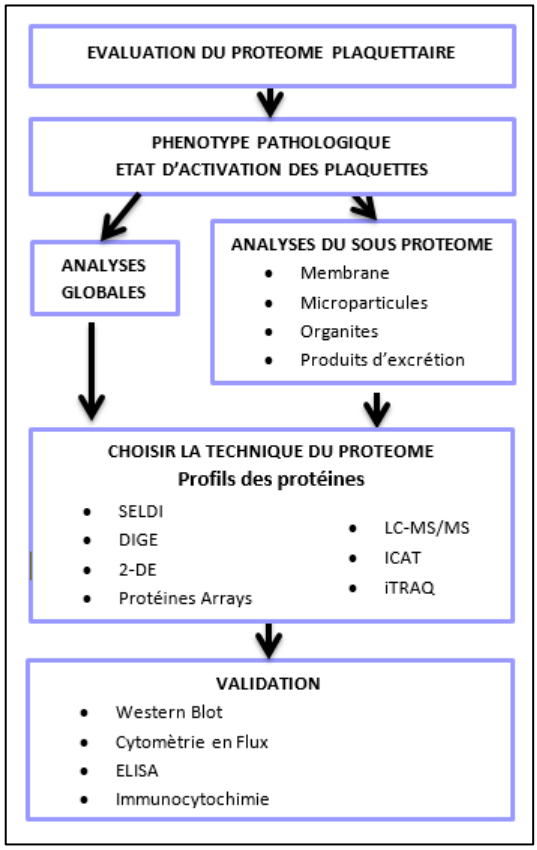

Figure 13 : Les principales stratégies et techniques utilisées pour étudier le protéome plaquettaire

III.3.2. Les études protéomiques sur des produits plaquettaires

De nombreuses études protéomiques ont été menées sur le protéome global des plaquettes. Ces études ont fourni des informations détaillées sur le répertoire de protéines et de phosphoprotéines exprimées par les plaquettes au repos et activées[197,201]. Ainsi, ces études ont également permis de caractériser les empreintes protéomiques des plaquettes destinées à la transfusion en comparant les techniques de préparation, les éventuelles altérations liées aux procédés d'inactivation ou de réduction de pathogènes ou également l'influence de la durée de stockage[206–208] ou encore caractériser et comparer le protéome plaquettaire des sujets malades et sains[209].

D'autres chercheurs se tournent plus souvent vers les analyses subcellulaires[39,210,211]. Ce type d'approche, qui est souvent désigné comme « sous-protéome », réduit la complexité des mélanges protéomiques, augmentant ainsi la probabilité d'une identification réussie de

protéines. L'analyse de sous-protéomes permet également de définir l'origine des protéines à l'intérieur des plaquettes.

III.3.2.1 Protéome des membranes plaquettaires

Le protéome membranaire des plaquettes comprend toutes les protéines qui sont associées à ou incorporées dans la bicouche lipidique, comprenant les protéines qui sont liées à la membrane plasmatique et aux membranes intracellulaires des organites[211].

Jusqu'à récemment, très peu de protéines membranaires ont été identifiées par les méthodes classiques de protéomique. Moebius et al.[212] ont été les premiers à utiliser des procédés d'enrichissement des protéines de la membrane. En éliminant les protéines abondantes du cytosquelette, ils ont identifié 83 protéines membranaires incluant des protéines déjà connues (e.g. GPIIb-IIIa, le complexe GPIb-IX-V) et d'autres inconnues (e.g. G6b-A). En outre, ce groupe a identifié de nombreux domaines transmembranaires contenant des protéines localisées dans des compartiments intracellulaires tels que les mitochondries et le réticulum endoplasmique rugueux[212]. En 2007, Senis et al.[213] ont identifié 136 protéines transmembranaires dans les plaquettes humaines.

L'un des inventaires les plus complets de l'analyse des protéines membranaires des plaquettes à ce jour a été développé par Lewandrowski et al.[214]. Ils ont identifié 1282 protéines dans les plaquettes et ont déterminé que plus de la moitié d'entre elles sont d'origine membranaire. D'autres études ont également caractérisé le protéome membranaire[215,216].

III.3.2.2 Protéome des granules plaquettaires

En 2007, Maynard et al ont identifié plus de 200 protéines originaires des granules α purifiés. Un grand nombre de protéines avait été précédemment attribué aux granules α et la grande majorité (65%) a été détectée comme associée à l'excrétion plaquettaire. Ils ont également trouvé 44 protéines qui n'étaient pas déjà connues pour être présentes dans les granules α[38]. Plus récemment, la même équipe de recherche a comparé la signature protéomique des protéines des granules α de sujets sains et de patients atteints du syndrome de plaquettes grises[217]. D'autre part, Ruiz Hernández et al. [218] ont récemment caractérisé le protéome des granules denses. Ce groupe a identifié 40 protéines, y compris des protéines associées à l'actine, des enzymes de la glycolyse et des protéines régulatrices qui n'avaient pas été précédemment attribuées aux granules denses[218].

III.3.2.3 Protéome des microparticules plaquettaires

Les microparticules sont de petites vésicules membranaires produites par la plupart des types cellulaires, y compris les mégacaryocytes et les plaquettes[219,220]. Au cours des dernières années, il est devenu évident que les microparticules participent activement à la coagulation sanguine et à l'inflammation[221,222]. Les microparticules plaquettaires représentent plus de 90% des microparticules du plasma des individus sains, et il est bien documenté que les plaquettes activées génèrent des microparticules[219,223,224]. Les plaquettes contribuent également au pool des microparticules du plasma qui augmentent dans des conditions pathologiques telles que le syndrome coronarien aigu, le diabète et le sepsis[225–227]. En 2005, Garcia et al.[219] ont caractérisé la signature protéomique des microparticules provenant de plaquettes activées. Ils ont identifié 578 protéines dans les microparticules dérivées des plaquettes. En outre, la majorité des protéines des microparticules dérivées des plaquettes sont aussi présentes dans les microparticules isolées de la circulation sanguine[219,228].

III.3.2.4 Le secrétome des plaquettes

Le protéome du produit d'excrétion des plaquettes, souvent désigné sous le nom de « sécrétome », comprend toutes les protéines libérées par les plaquettes. Les protéines d'excrétion sont dérivées des granules α, des granules denses, des microparticules et des exosomes.

Les études protéomiques ont confirmé l'identité des protéines précédemment connues comme secrétées par les plaquettes. En outre, elles ont permis d'identifier de nouvelles protéines sécrétées par les plaquettes[39,229].

En 2004, Coppinger et coll[39] ont effectué la première analyse protéomique des protéines libérées par les plaquettes activées. Ils ont identifié plus de 300 protéines dans le produit de libération des plaquettes[39]. Le même groupe a déterminé que l'aspirine modifie les modèles d'expression de protéines des plaquettes activées[230].

En utilisant une approche protéomique différente, Piersma et al.[231] ont augmenté le nombre détecté de protéines sécrétées par les plaquettes à 716 protéines. Une grande partie de leur liste de protéines identifiées dans le secrétome se chevauche avec d'autres listes obtenues à partir d'études protéomiques faites sur des microparticules et des granules α[38,219,231].

III.4. L'ARN plaquettaire

L'ensemble des 22 000 gènes codant pour des protéines est composé d'à peu près de 700 000 exons[232,233]. L'épissage alternatif lors de la transcription induit une augmentation de près de dix fois le nombre de transcrits ; jusqu'à présent, 190 000 transcrits de gènes uniques ont été identifiés. Ce nombre devrait augmenter avec l'augmentation exponentielle des ensembles de données générées par le séquençage de l'ARN obtenu à partir de populations de cellules relativement pures. Le nombre de transcrits par gène varie largement et varie d'un seul transcrit aux milliers de transcrits alternatifs résultant ainsi à plusieurs isoformes de protéines[232]. En plus de l'ARN codant pour des protéines, il existe plusieurs autres types d'ARN non codants. Ces ARN non codants représentent une grande quantité d'informations génétiques et ont clairement un rôle dans le développement, la physiologie et les maladies[233]. Les ARN non codants les mieux étudiés sont les microARN (miARN)[234]. Il existe au moins neuf autres types d'ARN non codants ayant différentes tailles et différentes fonctions[235].

Concernant les plaquettes sanguines, malgré l'absence d'ADN génomique, elles contiennent un réticulum endoplasmique rugueux et des ribosomes[236] qui peuvent utiliser des ARNm hérités du mégacaryocyte comme matrice pour la synthèse de novo de protéines[237–239]. Une observation surprenante a été la mise en évidence de l'existence de l'épissage d'ARNm dans les plaquettes humaines[240] suggérant que la régulation de la traduction de l'ARNm plaquettaire est plus complexe qu'on ne le pensait précédemment. Ces observations confirment l'existence d'un contrôle post-transcriptionnel de l'expression génique dans les plaquettes humaines.

III.4.1. Les techniques d'investigation de l'ARN plaquettaire

Les études de l'ARN plaquettaire et surtout de l'ARNm et des miARN ont débuté par l'utilisation des techniques classiques telles que le Northern Blot[126], la transcription inverse (RT-PCR) et la PCR quantitative (qPCR)[241,242]. Avec le progrès technologique, plusieurs autres techniques ont permis d'évaluer globalement le transcriptome des plaquettes, incluant la technique SAGE (« Serial Analysis of Gene Expression ») [243–245], les microarrays (puces à ARN)[243,244], et le séquençage nouvelle génération (RNA-seq)[246,247].

Sans doute, les approches microarray et SAGE ont joué un rôle central dans la caractérisation initiale du transcriptome plaquettaire[243,244]. L'approche RNA-seq, cependant, a dépassé ces techniques, car elle nécessite des quantités minimales de matériel de départ, a une sensibilité

accrue par rapport à d'autres méthodes, et elle fournit une identification objective de l'ensemble de la transcription (régions non traduites ; introns et exons), de nouveaux gènes et des séquences intergéniques[246,248].

III.4.2. Le transcriptome plaquettaire

Plusieurs études transcriptomiques ont été réalisées sur l'ARN plaquettaire. Les premières furent descriptives portant sur la découverte du contenu plaquettaire en ARN. Les études par microarray et par SAGE ont estimé que les plaquettes peuvent contenir jusqu'à 32% du génome humain sous la forme d'ARNm[243–245,249–252]. La curiosité des chercheurs a continué avec l'apparition des approches RNA-seq. Ils ont trouvé que la signature plaquettaire est plus complexe que l'on imaginait et que les plaquettes contiennent presque tous les types d'ARN[246,247,253].

L'équipe d'Osman et al. s'est intéressée à l'influence des réducteurs de pathogènes sur le transcriptome des plaquettes destinées à la transfusion[254,255]. D'autres équipes ont réalisé des études d'expression différentielle pour comparer le transcriptome des patients avec ceux de témoins[256–260].

III.4.3. Les MicroARN plaquettaires

Les microARN (miARN) sont de petits fragments d'ARN non codants, de 18-24 nucléotides qui jouent un rôle fondamental dans la régulation post-transcriptionnelle des gènes en ciblant l'ARN messager (ARNm), induisant sa dégradation ou la répression de la traduction[261]. La plupart des miARN caractérisés ont une préférence à se lier aux régions 3' non traduites (3' UTR) de leurs ARNm cibles[261] régulant ainsi près de 60% des ARNm codants pour des protéines[262]. Une revue de Ouellet et al.[263] résume bien la voie de biosynthèse des miARN et leurs composantes protéiques, ainsi que les processus de régulation pour exercer une variété de fonctions biologiques chez les eucaryotes.

Dans les plaquettes, on estime que les miRNA pourraient être transportés par les exosomes et les microparticules[264–266].

Une étude basée sur la qPCR et réalisée par Osman et Falker a permis la détection de 281 miARN, parmi lesquels 6 miARN étaient différentiellement exprimés en réponse à l'activation plaquettaire par la thrombine[242]. Une étude similaire par Nagalla et al. [267] utilisant des microarrays, a identifié 284 miARN exprimés par les plaquettes parmi lesquels certains

sont différentiellement exprimés selon la réactivité plaquettaire à l'épinephrine. La même étude a montré que les miARN sont fonctionnels et régulent l'expression des ARNm plaquettaires en se fixant sur la partie 3'UTR.

Une étude plus récente utilisant le séquençage nouvelle génération a montré que parmi les 2500 miRNA identifiés chez l'homme (http://www.mirbase.org version 21), les plaquettes humaines en expriment 492. Ils ont également détecté des isoformes de miARN confirmant ainsi l'existence de la machinerie régulatrice de la biosynthèse des miARN dans les plaquettes[268].

D'autres études ont également montré que les plaquettes secrètent différents miARN en fonction des agonistes utilisés et ont même identifié des miARN comme marqueurs d'hyperréactivité[269–271].

Des études surprenantes ont montré que les miARN plaquettaires transportés par les microparticules peuvent jouer un rôle régulateur sur des cellules endothéliales distantes[272–274]. Comme les études protéomiques et transcriptomiques, les miARN plaquettaires ont ont été investigués dans différents types de maladies. En effet, des miARN plaquettaires différentiellement exprimés ont été identifiés chez des patients (e.g. maladie drépanocytaire[275], maladies cardiovasculaires[276]).

III.5. Relation entre le protéome et le transcriptome plaquettaires

Des analyses exhaustives du protéome présentent plusieurs inconvénients dont la capacité limitée pour identifier les protéines faiblement abondantes. Ainsi, les chercheurs ont récemment utilisé la transcriptomique dans les plaquettes pour prédire l'expression de protéines. Cette approche est communément appelée « omiques intégrées ou ''integreted omics'' » car elle utilise la combinaison d'approches transcriptomiques et protéomiques[213,277,278].

Bien que le degré de corrélation entre les transcrits et leurs protéines correspondantes varie entre les études, la majorité des transcrits de plaquettes sont représentés par la protéine correspondante. Dans une comparaison côte à côte du transcriptome et du protéome, McRedmond et al.,[250] ont constaté que près de 70% des transcrits et des protéines correspondantes sont co-exprimées par les plaquettes. Toutefois, plusieurs équipes de recherche ont constaté qu'il n y a pas de corrélation entre le transcriptome et le protéome plaquettaire mais plutôt qu'ils sont complémentaires[277,279,280].

Bien que les analyses RNA-Seq et d'autres types d'analyses d'ARNm sont des outils puissants, il convient de noter que le transcriptome ne signifie donc pas toujours le protéome. Cette discordance peut provenir de la présence de protéines qui sont synthétisées par les mégacaryocytes, puis l'ARNm est dégradé avant d'entrer dans les plaquettes. L'inhibiteur tissulaire de métalloprotéinase-3 (TIMP-3) est un excellent exemple de ce type de regulation d'ARN[281]. De plus, l'analyse de l'ARN ne prend pas en compte les protéines absorbées à partir du plasma. Dans d'autres cas, les ARNm mais pas leurs protéines correspondantes, sont transférés aux plaquettes. Ces ARNm sont traduits en protéines lors de l'activation des plaquettes et d'autres ne sont jamais traduits[239,281]. Enfin, l'analyse de l'ARN incorpore une étape d'amplification, ce qui augmente les risques d'amplification des ARN provenant d'une éventuelle contamination des leucocytes. Ainsi, une extrême prudence doit être prise pour éliminer les leucocytes des préparations de plaquettes (e.g. utilisation de billes magnétiques anti-CD45), suivi par la confirmation que les transcrits et les protéines proviennent bien des plaquettes sanguines.

Problématique et objectifs

Notre équipe de recherche a été parmi les premières à montrer et à confirmer le rôle des plaquettes sanguines comme cellules de l'immunité et de l'inflammation, capables de secréter, pendant le stockage, des facteurs de croissance, des cytokines, des chemokines et d'autre facteurs responsables de la modification de la réponse immunitaire (BRM). Ainsi, nos travaux s'inscrivent dans l'étude des aspects inflammatoires des plaquettes destinées à la transfusion et leurs rôle dans la survenue d'EIR de type RFNH .

Cognasse et al.[132], Hamzeh-cognasse et al.[135], Nguyen et al.[136] ont identifié des molécules proinflammatoires sur-exprimées dans des CP ayant induit un EIR (e.g. sCD40L, sOX40L, IL27, IL13).

D'après les études de l'équipe de Blumberg et al.[133] et de notre équipe, la sur-expression de ces molécules n'induit pas systématiquement un EIR. Ceci nous a incité a émettre l'hypothèse de l'existence d'éventuels polymorphismes au niveau de ces molécules (chez les donneurs) et aussi au niveau de leurs récepteurs (chez le receveur) qui seraient responsables d'une tolérance ou d'une réaction lors de l'intéraction de ces couples recepteurs/ligands.

Pour vérifier cette hypothèse, nous avons débuté nos explorations pas l'investigation du gène *CD40LG* étant donné que la proteine correspondante, CD40L, était la première molécule identifiée comme un acteur principal dans la physiopathologie des EIR, ce qu'ont confirmé plusieurs équipes de recherche.

Trois grands axes ont été développés dans le cadre de ma thèse de sciences :

- Dans la première partie, nous nous sommes intéréssé à l'étude des polymorphismes génétiques du gène *CD40LG* chez une population de donneurs de sang dont le CP a ou n'a pas induit un EIR. Nous avons également étudié d'autres gènes en relation avec CD40L tels que le gène de son récepteur principal *CD40* et aussi le gène de l'integrine *ITGA2*.
- Dans la seconde partie, nous avons étudié les lésions de stockage des CP. Pour cela, nous avons exploré les profils de sécretion plaquettaire et leucocytaires au cours du stockage ainsi que l'influence des leucocytes résiduels dans des CP non-leucoréduits, tels que délivrés en Tunisie.

  Nous avons également investigué l'effet des BRM plaquettaires et celui de l'ADN mitochondrial dans la survenue d'EIR dans des CP leucoréduits, tels que ceux délivrés en France.

- Dans la troisième partie du travail, nous avons appliqué les approches de protéomique et de transcriptomique de dernière génération (LC-MS/MS et RNA-seq, respectivement) afin d'analyser les CP ayant induit un EIR dans le but de mieux comprendre la physiopathologie des EIR.

Publications

I. Article 1 : Etude d'association entre CD40LG et les EIR

Sci Rep. 2014 Nov 28;4:7239. doi: 10.1038/srep07239.

Are polymorphisms of the immunoregulatory factor CD40LG implicated in acute transfusion reactions?

Aloui C[1], Sut C[2], Prigent A[1], Fagan J[3], Cognasse F[1], Granados-Herbepin V[4], Touraine R[4], Pozzetto B[2], Aouni M[5], Fendri C[5], Hassine M[6], Chakroun T[7], Jemni-Yacoub S[7], Garraud O[1], Laradi S[1].

Le but de cette étude était de :

Cribler le gène CD40LG dans deux populations indépendantes de donneurs de sang.

Génotyper les polymorphismes identifiés.

Effectuer une étude d'association génétique entre deux groupes de donneurs de sang, le premier dans le groupe dont les concentrés plaquettaires ont induit un EIR et le deuxième, servant de contrôle dans le groupe dont les concentrés plaquettaires n'ont pas induit un EIR.

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Are polymorphisms of the immunoregulatory factor *CD40LG* implicated in acute transfusion reactions?

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The CD40 ligand (CD40L/CD154), a member of TNF superfamily, is notably expressed on activated CD4+ T-cells and stimulated platelets. CD40L is linked to a variety of pathologies and to acute transfusion reactions (ATR). Mutations in this gene (*CD40LG*) lead to X-linked hyper-IgM syndrome. Some *CD40LG* polymorphisms are associated with variable protein expression. The rationale behind this study is that CD40L protein has been observed to be involved in ATR. We wondered whether genetic polymorphisms are implicated. We investigated genetic diversity in the *CD40LG* using DHPLC and capillary electrophoresis for screening and genotyping (n=485 French and Tunisian blood donors). We identified significant difference in the *CD40LG* linkage pattern between the two populations. Variant minor alleles were significantly over-represented in Tunisian donors (P<0.0001 to 0.0270). We found higher heterogeneity in the Tunisian, including three novel low frequency variants. As there was not a particular pattern of *CD40LG* in single apheresis donors whose platelet components induced an ATR, we discuss how this information may be useful for future disease association studies on *CD40LG*.

The CD40 ligand gene (*CD40LG*), located on the long arm of human X chromosome at position q26.3-q27.1, harbors five exons spanning about 12 kilo base pairs (kb) (http://www.ncbi.nlm.nih.gov/gene/959). The CD40L (CD154), which is a member of the tumor necrosis factor (TNF) superfamily, is a 33 kDa type II membrane glycoprotein. It is mostly expressed on activated CD4+ T-cells and stimulated platelets; presence of this protein is often a characterizing feature of antigen presenting cells[1–3]. CD40L is also expressed on non-leukocyte and non-immune cells such as endothelial cells[4]. CD40L expression is post-transcriptionally regulated in part through mRNA stability[5].

The costimulatory molecule CD40L and its receptor CD40 have essential roles in adaptive immunity; it also plays a role in inflammatory responses, which are important in innate immunity[6]. The normal interaction between T and B lymphocytes via CD40 and CD40L induces B cell activation, proliferation, differentiation, survival and immunoglobulin isotype switching, thereby regulating B cell commitment to mature plasma or memory B cells[3,7]. Deficient CD40L expression might induce a reduced antigen response, such as that seen in X-linked hyper-IgM syndrome (HIGM1)[8], whereas polymorphisms in this ligand have been associated with various pathologies as autoimmune and infectious diseases[9,10]. CD40L is cryptic in unstimulated platelets, but is translocated to the cell surface within seconds or minutes after *in vitro* activation, where it is involved in *in vivo* thrombus formation[11]. CD40L expressed on the surface of activated platelets interacts with CD40 to trigger inflammatory responses and expression of tissue factor in endothelial cells[12]. Approximately 95% of the soluble fragment of CD40L (sCD40L) found in plasma is derived and cleaved from platelets, which are important players in inflammation[13], in addition to their roles in hemostasis, thrombosis and platelet regulatory functions[14–16]. The contribution of platelets and their secretory products has been observed in tissue pathology[17,18].

Interaction between platelet-derived CD40L and target cells such as a blood transfusion recipient's B-lymphocytes and vascular endothelial cells is considered a highly inflammatory process in transfusions, where it is known to be responsible for adverse events, such as febrile non hemolytic transfusion reactions (FNHTR),

Table 1 | Minor allele frequencies of *CD40LG* SNPs in the French (n=211) and Tunisian population (n=274) identified by DHPLC

SNP	Marker ID	Position Chr X (Location)	Allele	Minor Allele Frequency (MAF)		Statistic		PCR Fragment
				French population	Tunisian Population	χ^2	p value	
1	c.-3525_-3526insCAAACAAA[a]	135726882 (5′ UTR)	$(CAAA)_8$	0.003	0.003	0.017	1[b]	*CD40LG*-5′UTR
2	**rs201992677 [-/CAAA]**	**135726882 (5′ UTR)**	**$(CAAA)_7$**	**0.175**	**0.364**	**28.141**	**<0.0001**	***CD40LG*-5′UTR**
3	**rs3092952 A>G**	**135726950 (5′UTR)**	**G**	**0.175**	**0.354**	**25.040**	**<0.0001**	***CD40LG*-5′UTR**
4	**rs1126535 T>C**	**135730555 (exon 1)**	**C**	**0.154**	**0.224**	**4.998**	**0.027**	***CD40LG*-E1**
5	rs147739883 C>T	135732387 (IVS 1)	T	0	0.003	0.828	1[b]	*CD40LG*-E2
6	c.8140A>G[a]	135738547 (exon 4)	G	0	0.003	0.828	1[b]	*CD40LG*-E4
7	**rs3092923 T>C**	**135741185 (IVS 4)**	**C**	**0.116**	**0.326**	**39.424**	**<0.0001**	***CD40LG*-E5A**
8	rs148594123 G>A	135741443 (exon 5)	A	0.027	0	9.793	0.0002[b]	*CD40LG*-E5B
9	**rs3092921 C>T**	**135743000 (3′UTR)**	**T**	**0.113**	**0.314**	**37.392**	**<0.0001**	***CD40LG*-3′UTRB**
10	c.*2367C>G[a]	135743941 (3′UTR)	G	0	0.003	0.828	1[b]	*CD40LG*-3′UTRC
11	**rs3092920 G>T**	**135743991 (3′UTR)**	**T**	**0.103**	**0.286**	**33.203**	**<0.0001**	***CD40LG*-3′UTRC**

[a]Novel mutations identified in this study.
[b]Fisher Exact Test (n<5).
Alleles are denoted as ancestral/derived and are referred to by their dbSNP ID. Polymorphisms in boldface were included in the LD and haplotype analysis.

atypical allergy and hypotension. Such reactions can be benign or severe signs of inflammation, while others such as ATR and transfusion-related acute lung injury (TRALI) are generally thought to result, at least in part, from elevated sCD40L levels[19–23].

However, the vast majority of transfusions, despite inducing high levels of pro-inflammatory molecules, proceed without harm to the patient. This fact gives rise to the hypothesis of genetic susceptibility in the donor population relating to cytokines and/or chemokines and in recipients to the relevant receptor involved (both in physiology and pathophysiology). For this, we wondered whether genetic *CD40LG* polymorphisms are implicated.

To determine the dispersion of genetic variation in populations that are distinct but have certain common ancestries, we sought to examine *CD40LG* among populations of volunteer blood donors, in central Tunisia and in metropolitan France (whose platelet component induced or not ATR). We hypothesized that variability within a population would reveal high levels of genetic polymorphism or segregation of particular haplotypes[24,25].

Denaturing High Performance Liquid Chromatography (DHPLC) and capillary electrophoresis were performed to analyze polymorphisms in the *CD40LG*, and to estimate the allele frequencies and *CD40LG* haplotype structures and patterns of linkage disequilibrium (LD) around a 17 kb region that includes the *CD40LG*. This study, besides, aimed to provide population genetic data that could be used for future studies on the *CD40LG* and its association with pathology.

Results

Nucleotide polymorphism analysis. DHPLC analysis found nine SNPs and two variable number tandem repeats (VNTRs) in 10 of the 11 amplicons investigated herein. Homogeneity was tested for males and females in each population (Supplementary Table S1). All genotype frequencies were in Hardy-Weinberg Equilibrium (HWE) in the studied populations for all of the typed SNPs. Table 1 summarizes the allele frequencies of the 11 variants identified.

All the amplicons contained an average of one SNP. However, *CD40LG*-5′UTR and *CD40LG*-3′UTR harbored 3 polymorphisms (Table 1). At the genotyping stage, we observed that 11% of the samples from females that displayed one peak at the screening analysis, showed multiple peaks when mixed with the wild homozygous control sample, thus presenting the mutant homozygous state.

Three variants were novel: a non-synonymous c.8140A>G in exon 4, c.*2367C>G in the 3′UTR in the Tunisian population at low frequency (MAF=0.003 for both), and one insertion in the 5′UTR (c.-3525_-3526insCAAACAAA) in one male from each of the study populations. Seven polymorphisms were transitions, three were A/G and four C/T, while two were transversions (one C/G, one G/T). Eight of the 11 variants were previously reported in the NCBI database. Only three variants were located in the coding region: rs1126535 in exon 1, c.8140 A>G in exon 4 and rs148594123 in exon 5. The others were in the non-coding region with three variants in the 5′UTR, one in IVS4 and three in the 3′UTR (Table 1; Supplementary Figure S1).

Variant minor alleles with MAF>0.05 were significantly over-represented in the Tunisian individuals (P<0.0001 to 0.027), in comparison with the French individuals (Table 1). In addition, we compared MAFs for the two populations with those extracted from the 1000 genomes project (Figure 1). In the present French population, all the allele frequencies were close to those belonging to the Utah residents with Northern and Western European ancestry (CEU) population (Figure 2). Regarding the Tunisian study, all the allele frequencies of the SNPs fell between those belonging to African ancestry (African Ancestry in Southwest US, ASW; Luhya in Webuye, Kenya, LWK; Africans "Yoruban in Ibadan", Nigeria, YRI) and those with European origins, the CEU group, British from England and Scotland (GBR) and Tuscan in Italy (TSI), with the exception of rs1126535 for which the frequency is very close to that of TSI.

LD and block haplotype analysis. The haplotype structure of the French and Tunisian populations with YRI, CEU, TSI and Japanese in Tokyo (JPT) populations is shown in Figure 2. There was a marked difference in the pattern of LD between the two populations. Using the solid spine algorithm[26] in the Haploview software, two haplotype blocks were identified across the 17 kb region in both cohorts, with evidence of disruption in LD between SNP4 and SNP7. Block 1, spanning 3 kb, encompassed the 5′UTR and the first exon and contained three SNPs (rs201992677, rs3092952, rs1126535), while block 2 encompassed the rest of the gene. In block 1, there were three haplotypes in the French cohort with an AAT predominant one (0.852), which is also the most frequent among the four haplotypes found in the Tunisian cohort (0.607). The second most frequent haplotype was GGC; it was more prevalent in the Tunisian population than in the French one (0.214 versus 0.123). In block 2, there was one additional haplotype in the Tunisian cohort, even though the high-frequency haplotype was TCG for both populations (0.889: French cohort and 0.633: Tunisian cohort). Considering the resulting haplotype blocks, the SNPs defined five and eight core haplotypes in the French and Tunisian cohorts, respectively. The most frequent one was AAT/TCG in both populations (Figure 2).

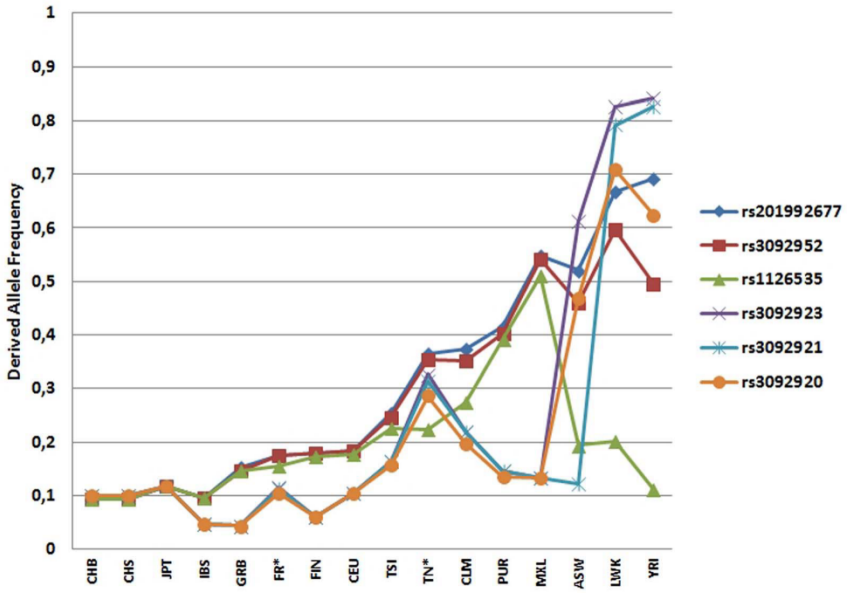

Figure 1 | Derived allele frequencies for six variants with minor allele frequencies in the *CD40LG* from French (FR) and Tunisian (TN) donors and those extracted from the 1000 genomes database. African Ancestry in Southwest US (ASW), Han Chinese in Beijing (CHB), Han Chinese South (CHS), Japanese individuals in Tokyo (JPT), Iberian populations in Spain (IBS), British from England and Scotland (GBR), Finnish from Finland (FIN), Utah residents with Northern and Western European ancestry (CEU), Tuscan in Italy (TSI), Colombian in Medellin (CLM), Puerto Rican in Puerto Rico (PUR), Mexican Ancestry in Los Angeles, CA (MXL), African Ancestry in Southwest US (ASW), Luhya in Webuye, Kenya (LWK), Africans ''Yoruban in Ibadan'', Nigeria (YRI) taken from the 1000 genomes project. *Present study.

rs201992677 [-/CAAA], described in the 1000 Genomes data but not in HapMap studies, was in high linkage disequilibrium with rs3092952 with D'=1 and r^2=0.9 in the Tunisian dataset and D'=1, r^2=1 in the French dataset. Surprisingly, all the male donors possessing the 'G' minor allele (rs3092952) were also found to carry seven CAAA repeats (rs201992677); in two cases, eight CAAA repeats were identified, which constitute a novel VNTR (c.-3525_-3526insCAAACAAA), instead of the most frequently found six CAAA repeats. Notably, three major 'A' type alleles of rs3092952 were unusually associated with seven CAAA repeats (rs201992677) in females originating from Tunisia, but not from France.

(CA)n microsatellite analysis. Allele frequencies were calculated in the French and Tunisian donors and the data revealed no deviation from HWE; we had previously verified the different allele frequencies for (CA)n repeats between males and females (P=0.297 and 0.137 for the French and the Tunisian donors, respectively). Twelve different alleles (from 18 to 31 CA repeats, corresponding to PCR products from 98 to 124 bp) were detected in the French cohort while 17 alleles (with 16 to 33 repeats, corresponding to PCR products from 94 to 128 bp) were observed in the Tunisian cohort. The $(CA)_{26}$ allele was the central or median allele and the most represented one in both groups, albeit more frequent in French than in Tunisian donors (Figure 3).

The allele distribution of the donors carrying 26 CA repeats, and less than or more than 26 CA repeats between the French and the Tunisian cohorts were compared by univariate statistical analysis. The allelic distribution between the French and the Tunisian donors was significantly different for allele groups of repeats as shown in Table 2.

Given the large difference between the two studied populations, we conducted a preliminary study in a group of 30 French single apheresis platelet donors whose platelets (in platelet components) induced an ATR. However, we did not observe any allelic or genotypic association with neither SNPs nor CA repeats in 3′UTR, nor haplotype of *CD40LG* (Supplementary Table S2 and S3).

Discussion

DHPLC is a high-throughput semi-automated method with good accuracy for detecting base pair changes, either transversions or transitions, and can be used as a screening or a genotyping strategy that also allows identification of new polymorphisms, including more than one in the same fragment[27]. By this strategy, three new polymorphisms, albeit some at a very low frequency, have been identified in the Tunisian and French study groups where there are no population *CD40LG* reports, yet. We screened and genotyped 11 variants including two VNTRs. Of the three SNPs located in the coding region, the novel c.8140 A>G in exon 4 found in a Tunisian donor resulted in a missense mutation (ATA to GTA) in which isoleucine was substituted for valine in the expressed protein at amino acid position 127 (p.I127V) of the extracellular portion[28]. This mutation is predicted to be benign by *PolyPhen-2* with a score of 0.001 (http://genetics.bwh.harvard.edu/ggi/pph2/). The rare minor allele 'A' of the exonic rs148594123 inducing p.G219R was retrieved in exclusively eight healthy French including 4 females at the heterozygous A/G state and 4 'A' hemizygous males, although we did not find the hypomorphic mutation p.G466X in XIAP, the adjacent gene of *CD40LG* with which cosegregation has been described as resulting in HIGM1[29]. rs147739883, which was found in a Tunisian male, has been previously detected only in the Kenyan population (LWK). In addition, the minor 'C' allele of rs1126535 in exon 1 is associated with susceptibility to severe malaria[30] and would increase the risk of reduced bone mineral density and osteopenia/osteoporosis (as a medium risk) for this synonymous SNP as it could affect splicing regulation[31].

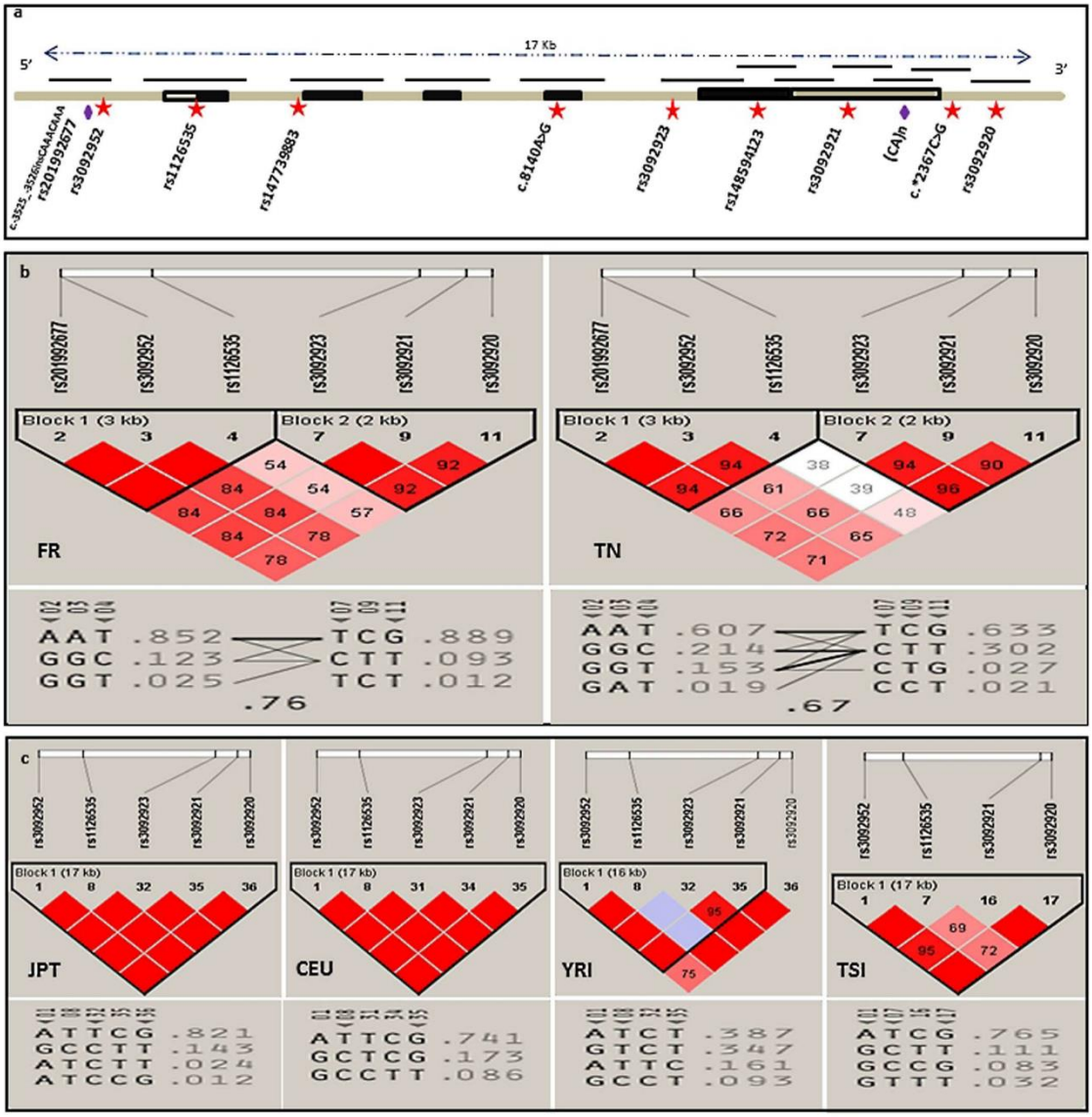

Figure 2 | Genomic structure of the *CD40LG* and the haplotype blocks of the French (FR) and Tunisian (TN) populations (this study) compared with those of JPT, CEU, YRI and TSI (HapMap data). (a) Genomic structure of the *CD40LG* gene and the position of the identified polymorphisms. Exons are represented by boxes, with filled boxes denoting translated regions. Upward facing lines indicate amplified fragments, and polymorphisms are shown as red stars for SNPs and purple lozenges for microsatellites. (b) *CD40LG* LD and haplotype diversity in French and Tunisian populations. (c) *CD40LG* LD and haplotype diversity in JPT, CEU, YRI and TSI populations. The numbers in boxes represent the pairwise D' value between adjacent SNPs. For (b) and (c), haplotype frequencies are shown below the LD diagrams. Triangular pointers highlight SNPs which could be tagged for future association analysis.

Among the three SNPs located in the 5′UTR, rs3092952 has been shown to provide an active role in *CD40LG* expression; the minor 'G' allele induces increased expression of the membrane and soluble forms of CD40L, thereby leading to enhanced CD40 interactions; this SNP is implicated in myocardial infarction and vascular disorders[32]. The variant 'C' allele of rs3092923, located in IVS4, is described as conferring a marginal reduced risk of follicular lymphoma[33]. CD40L regulation by the 5′UTR is related to the G allele of rs3092952[32], which is itself in high LD with the variable number of tandem CAAA repeats located a few bases upstream (rs201992677); therefore, could an increase in expression of the protein also be partially related to the presence of the CAAA repeats? as has been described in transcriptional regulation of the insulin-like growth factor 1[34].

We defined a core region of 17 kb in *CD40LG*. There is less than 2 kb in distance between haplotype blocks 1 and 2, whereas Chadha

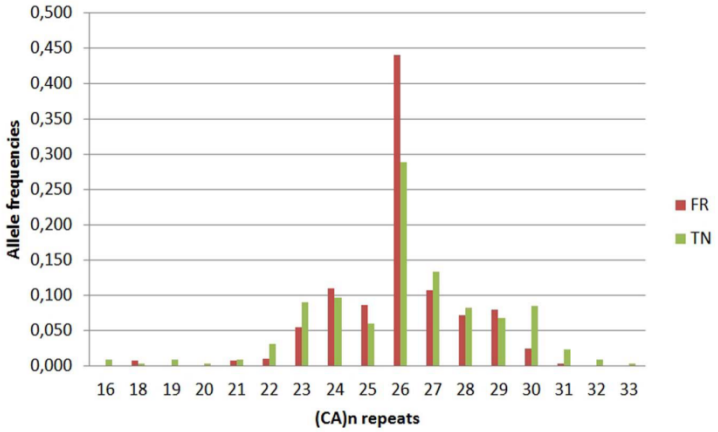

Figure 3 | Frequency distribution of the number of (CA)n repeats in the *CD40LG* in the French (FR) and Tunisian (TN) populations. Allele frequencies correspond to the number of the group of test alleles divided by the total number of alleles.

et al. described a distance of 4 kb[35]. We compared the results obtained for the haplotype structure of the SNPs, and the LD patterns with frequencies for Caucasians (CEU, TSI), Africans (YRI) and Asians (JPT). The LD structure of *CD40LG* on chromosome X and haplotypes in the corresponding LD blocks differed between the two populations studied. The Tunisian haplotypes were found to be intermediate between the European populations (French in the present study and TSI) and the African populations.

However, considering the HapMap data, the structure of LD in *CD40LG* and the haplotypes in the CEU European group, were found to be similar to those of the Asian (JPT) population, regardless of the allele frequency differences at each SNP. Our results for the French population were closer to those obtained with a European English cohort by Chadha *et al.*[35], and also from TSI than to those of CEU (Figure 2). As already described, these results do not always correspond with the data (allele frequencies and LD) downloaded from HapMap Phase II and III; this may be explained by local variations within a country and/or by genotyping errors[36].

It is worth noting that the French and Tunisian populations, as with any other North African population, do not exist in the HapMap or 1000 genomes datasets. Our results indicate moderate transferability of tagging SNPs on *CD40LG* from the European-Caucasian population to the French population. For the Tunisian population, this transferability is hardly conceivable. This approach has often been attempted in other populations (e.g. Thai and Spanish), and with other genes, with the aim of genotyping hotSNPs with which to assist future genetic association studies[25,37].

The distribution of the frequencies of the CA repeat alleles in the populations studied appears similar to that obtained in others studies, although the major allele identified was 24 to 27 CA repeats. As discussed by Stewart *et al*, this difference is most likely explained by differences in the systems used for determining the repeat numbers: i.e. denaturing polyacrylamide gel and silver staining[38], allele-specific oligonucleotide hybridization of membrane-fixed DNA products[39] (major allele with 24 repeats), denaturing polyacrylamide gel and autoradiography[40] (26 repeats), capillary electrophoresis using a CEQT 8000 Genetic analysis[41] (27 repeats), or a CEQ 2000 genetic analysis system, as in the present study (26 repeats)[42].

Table 2 | Allelic distributions of the *CD40LG* $(CA)_n$ repeat polymorphism in the French and Tunisian cohorts (291 and 353 alleles respectively)

	French cohort	Tunisian cohort	
$(CA)_n$	N[a] (frequency)	N[a] (frequency)	P-value[b]
<26 CA	80 (0.275)	109 (0.309)	0.00019
26 CA	128 (0.440)	102 (0.289)	
>26 CA	83 (0.285)	142 (0.402)	

[a]Number of alleles.
[b]Bonferroni correction : P-value is significant when <0.016.

Different selection pressures may explain the extreme variability of this 3′UTR and its association with immune disorders. For example, polymorphisms of longer $(CA)_n$ alleles are seen in autoimmune processes such as seen in patients with systemic lupus erythematosus[43], those with a high risk of thromboembolic disease[44], or rheumatoid arthritis[39,45]. However, no association was found in the context of alloimmunity (e.g. rejection of renal transplantation) with either acute graft rejection or patient death, but a strong association was shown with long-term graft failure[41]. Shorter CA repeats are also linked to rare inherited HIGM1 with an informative usefulness in linkage analysis[40]. Is there any infectious selection process that could explain the more frequently observed longer alleles in the Tunisian population in relation to the need for enhanced inflammation[46]? It appears that the number of repeats increases over generations, but would this bear any relationship to ancestral population? This would reinforce the supposed old origins of the Tunisian population[47].

CA dinucleotide is the most common repeat sequence in the human genome. Human CD40L is encoded by an unstable mRNA; this instability is conferred by a portion of its 3′UTR including a dual cis-acting element comprising a polypyrimidine-rich region and CA repeats[48]. It was shown that this CA repeats region is a binding site of nuclear factors and thus could modify the affinity of interaction with *CD40LG* mRNA and consequently, the translation efficiency[48–50].

The $(CA)_{24}$ allele (corresponding to $(CA)_{26}$ in our study) appears to confer more stability on its mRNA, and longer alleles confer higher expression levels[43,45,48].

To the best of our knowledge, ours is the first description of this microsatellite marker in Tunisians. This microsatellite has a distribution characteristic of each population as described in other studies[38–40]. In the present French and Tunisian study, the $(CA)_{26}$ allele showed a statistically significant difference in frequency between the two populations (0.438 versus 0.291, respectively), while a previous French study found a frequency of 0.319. The frequency of heterozygosity was 0.70 (this study) and 0.79 (DiSanto *et al.*)[40] for the French, and 0.82 for the Tunisian (this study). This result reflects genetic diversity in the Tunisian population, confirmed by the allelic

distribution of (CA)n alleles, which has also been reported in the Spanish population, demonstrating that there was also a highly polymorphic marker (of 15 alleles) in Spaniards versus 17 in Tunisians[38]. The dinucleotide repeat (CA)n is a marker that is highly polymorphic, and therefore useful for genetic studies on the CD40L molecule in relation to immunity.

Both French and North African populations have a tumultuous history with various ancestors but the Tunisian population appears to be significantly more heterogeneous as demonstrated by Y-STR diversity in the Sousse population[51]; the three novel polymorphisms are also in favor of the extreme heterogeneity in the studied cohort (Figure 1)[35,52,53]. Heterogeneity in haplotype frequencies is often higher in Africans such as the YRI group, in comparison to the European populations who left the African continent and experienced bottlenecks during their migrations, reducing SNPs diversity (Figure 2). The intermediate Tunisian *CD40LG* haplotype structure might be explained by its African location and multilayered history[53].

As variants repartition is different between the 2 populations, these findings added to the current knowledge base in the studied French and Tunisian populations, may ultimately lead to better investigate the *CD40LG* associated diseases. Their prevalence were found to be often in support with genetic polymorphisms repartition (Table 1 and 2), e.g. cardiovascular disorders[32,54,55], systemic lupus erythematosus[43,56,57], rheumatoid arthritis[45,58], although exact Tunisian prevalence is difficult to clarify for lack of national epidemiological registers. In addition to CD40L polymorphisms, other genetic markers were found differently associated in the studied populations[59] whereas others were similarly associated[60]. On the other hand, this technical strategy could be used for HIGM1 investigation (we covered 182 of all the 183 mutations in *CD40LG* that cause HIGM1 (http://www.hgmd.org/ updated in June 2014). It is less expensive than actual used techniques, especially in developing countries such as Tunisia where HIGM syndrome is frequently found[46,61,62].

Although several studies have found an association between ATR and high level of sCD40L in the transfused blood component[19–23], we did not find significant differences in *CD40LG* genetic pattern in donors whose platelets induced or not an ATR in the French population. This would be in favor of a CD40L release mechanism not due to a genetic regulation. It is noteworthy that all the previous studies have been performed with small effectives. We will have to extend the *CD40LG* study to a more important cohort of donors including donors whose donated platelets induced TRALI. It will be also interesting to investigate CD40L receptor (CD40 gene) in the recipients presenting ATR.

In conclusion, we identified polymorphisms known as regulatory within the *CD40LG* i.e. rs3092952 in the 5′UTR, (CA)n repeats in the 3′UTR, some rare and novel mutations, and polymorphisms involved in several diseases. These polymorphisms could modulate immune responses via interactions between CD40L and its various receptors[63]. We found a major difference between Maghreb (Tunisian) and European (French) populations in their *CD40LG* haplotype patterns. Surprisingly, we did not find any difference in *CD40LG* genetic pattern in apheresis platelet donors whose blood components prepared for transfusion purpose induced or not an ATR. Our findings provide useful information for future disease-association studies on the *CD40LG* in the context of inflammation and auto-immunity.

Methods

Ethical considerations. Informed and written consent was obtained from all the healthy donors who participated in this study, according to a protocol approved by the Ethics Committees for scientific research at Saint-Etienne (France), F. Bourguiba (Monastir, Tunisia), and F. Hached (Sousse, Tunisia) University Hospitals. All analyses were performed anonymously.

Studied populations. The cohort contained 485 healthy volunteer human blood donors, acting as non-profit donors (211 French donors who presented at the Auvergne-Loire Regional Branch of the French National Blood System EFS collection sites (130 males and 81 females) and 274 Tunisian donors who presented at the Monastir Blood Bank or the Regional Blood Transfusion Centre of Sousse blood collection sites (195 males and 79 females). Blood donors at both locations were unrelated and randomly chosen to enter this study, based on the timing of their donations, and no selection bias was applied to them (Supplementary Table S4). For comparison, the allele and genotype information for other populations were downloaded from HapMap Phase II and III (http://www.hapmap.org, August 2010) and from the 1000 genomes project (http://www.1000genomes.org). Thirty French single apheresis platelet donors whose donated blood components induced an ATR (FNHTR) were also tested (18 males and 12 females) (Supplementary Table S4).

DNA samples. EDTA-treated blood samples were collected from all individuals. DNA was purified from peripheral blood leukocytes using a FlexiGene DNA kit (Qiagen, Paris, France) according to the manufacturer's instructions. DNA concentrations and purity were assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA).

SNPs and microsatellite analysis. The nomenclature used herein for reporting sequence alterations abides by the original Human Genome Variation Society guidelines. NC_000023.10 was used as the reference sequence. All information about the SNPs detected was extracted from the public Single Nucleotide Polymorphism database dbSNP built 138 (http://ncbi.nlm.nih.gov/SNP/).

DHPLC analysis: screening and genotyping. To screen for the maximum number of polymorphisms potentially involved in CD40L protein expression or reported to be associated with immune disorders or inflammation[32,45,48,64], PCR was performed with eleven primer pairs with corresponding annealing temperatures (Table 3, Supplementary Data S1 for PCR protocols). To promote stable heteroduplex formation, PCR products were denatured for 5 min at 96°C, and then gradually cooled at a rate of 0.5°C per 10 sec over 150 cycles.

At the screening stage, 5 µL of each PCR product was automatically analyzed by DHPLC using the Wave system (Transgenomic, Ltd. Glasgow, UK)[65,66]. PCR products were injected into a preheated, fully equilibrated chromatographic column (DNASep Column, Transgenomic Ltd.). A linear gradient of 5% triethylammonium acetate (TEAA) (Buffer A) and 25% acetonitrile-5%TEAA (Buffer B) was generated for each amplicon. Each DHPLC run included a DNA loading step (5% drop for loading Buffer B), a linear separation gradient (2% Buffer B slope per min, 4.5 min), a wash step (75% acetonitrile; Buffer D, 0.5 to 1 min) and an equilibration step (0.9 to 1.2 min). The eluate was detected by an ultraviolet light detector at 260 nm. DHPLC conditions were optimized via fragment melting profile analysis, using the Wave system software Navigator connection WADMIN (Transgenomic, Ltd.).

The melting point (and profile) of each fragment was analyzed according to Fixman and Friere's implementation of Poland's algorithm[67], which calculates the probability that a base is in the helical duplex conformation (at 75–90%) or in the non-helical, single-stranded form. Each amplicon was, therefore, assessed at two or three column temperatures (Table 3). The flow speeds, temperatures, retention times and initial Buffer B concentrations of the elution gradient for DHPLC analysis were determined by the software provided. If the sample contains heteroduplex molecules, these will denature at lower concentrations of acetonitrile and will be visualized as peaks (or sometimes a single peak) with shorter retention times than that of a homoduplex. Control samples sequenced previously (homozygous and heterozygous for a known SNP, displaying profiles with single or multiple peaks) were run in parallel.

At the genotyping stage, all the samples from females presenting with one peak at the screening stage were mixed in equal volumes with a reference sample. Such samples were run again using the same DHPLC conditions and the same controls described above. Samples presenting with one peak at the screening stage had the same genotype of the homozygous control if the same single peak that was observed was homozygous for the other allele if multiple peaks were seen. Distorted multiple peak elution profiles differing from the reference profiles indicated the presence of a different polymorphism from the reference one or an additional SNP in the fragment; additional sequencing was applied in these cases.

For the male samples, because the gene of interest is on the X chromosome, a preliminary step was required. This involved mixing the PCR product from each sample with the PCR product of one of the controls (1V/1V) that was previously sequenced and for which no polymorphism for the fragment had been found and was, therefore, automatically genotyped.

DNA sequencing. To identify the type and position of the genetic variants, DNA samples with abnormal DHPLC patterns were re-amplified as described above, and then subjected to direct sequencing in both forward and reverse directions using the same PCR primers. PCR products were purified by Amicon Centrifugal filters (Millipore, Molsheim, France). Sequencing reactions were performed using the GenomeLab DTCS Quick Start mix (Beckman Coulter, Brea, CA). The resulting products were purified with Beckman Coulter's CEQ DTCS kit according to the manufacturer's instructions, subjected to analysis with a CEQ 2000 XL-Beckman Coulter sequencing machine, and assembled using CEQ 8000 Beckman Coulter software (Beckman Coulter, Inc. CA). For each DHPLC profile displayed, 10 samples were sequenced. However, all the heteroduplex profiles for the CD40L-5′UTR fragment were sequenced because of more than one polymorphism.

Table 3 | PCR fragments, primer sequences, annealing temperatures (Ta)°C and conditions of Denaturing High Performance Liquid Chromatography (DHPLC) analysis of *CD40LG*

Fragment *CD40LG*- (bp)	Primer sequences (5'>3')		Annealing temperature (°C)	DHPLC analysis		
	Forward primer	Reverse primer		Column temperature (°C)[a]	Buffer B %	Time shift (min)
5'UTR (285)[b]	TACTTGGGAGGCTGAGGCAG	TTGACCCCTGCCCACATTT	62	*[56.9]*, [57.9]	63.4	0
E1 (362)[b]	GCGCTCTTAACTAATCCTGAG	TCTTCTATTTGGTTTCTACCATC	55	*55.1*, *56.1*, [57.1]	64.1	0
E2 (283)[b]	TGCCGTGGAAATGAATGTAG	CCCGATCTAGCAAATGTAGTAAA	58,5	*55.6*, [56.6]	62	0
E3 (209)	AAACCCCACAGCAGAGCCC	CCTGATGCAACAACACTGGGT	58	*[56.8]*, *57.8*	59	0
E4 (310)[b]	TCAGTGGGAGAGATGTCAGACC	CCCAGGGGAAAAGAGGAGTTTA	58	*[56.3]*, *58.3*	62.5	0
E5A (383)[b]	GAACCATGCTCTGCTTCACCT	CACTTGGCTTGGATCAGTCA	58	*58.3*, [59.3]	64.5	0
E5B (432)[b]	GAGAGAATCTTACTCAGAGCTGC	ATGTCTGCATCAGTGGGGTT	58,5	*57.2*, [60]	65.4, 63.4	0 0.4
E5C (356)	TCATAATACAGCACAGCGGT	TTCCCTCTGCATCTTCACT	57,4	*57.1*, [58]	64	0
3'UTRA (304)[b]	ATGCAGAAGGGAAATGGGG	GTTAGAAAGGGGGATTGAGAAG	58	*58.9*, [60.9]	62.6	0 1
3'UTRB (339)[b]	GTTGCAGGAGATTGAAAGAAC	TAGTGGCTTGAAGATGCTGC	55	*[58.5]*, [58.9]	63.6	0
3'UTRC (244)[b]	CCAAGACCTTGTCCCATTCACC	CAGTCATTTTTTACTCCATGAGTGC	59	*[56.5]*, [57.3]	60.6	0

[a]Column temperature selection was based on the melting domains present in the PCR fragments.
The column temperature recommended by the Navigator connection Wadmin software is shown in italics, while the temperature at which a polymorphism was accurately identified is shown within square brackets.
[b]SNPs were identified in these PCR fragments.

Capillary electrophoresis: genotyping 3'UTR CA repeats. Samples were resolved by capillary electrophoresis separation, using a CEQ 2000 XL genetic analysis system (Beckman Coulter, Inc. CA, USA). To 0.5 μL of PCR product were added 0.25 μL of a 600 bp standard and 40 μL of sample loading solution (Beckman Coulter). Specifically designed labeled oligonucleotides were excited to fluoresce using a diode laser. The length of the amplified fragment was estimated with reference to the internal ladder and the number of repeats was calculated by analogy with 10 sequenced samples, using the CEQ 8000 Beckman Coulter software provided.

Statistical analysis. Allele and genotype frequencies were calculated and compared using XLSTAT™ (Addinsoft, Paris, France) computer software. The $\chi 2$ test was used to compare the allelic distribution between males and females and the allelic frequencies between French and Tunisian populations, using the Bonferroni correction or Fisher's exact test, where appropriate. Statistical significance was taken as $P<0.05$. For each polymorphism, the quality of the genotype data was assessed by testing for HWE, using the Haploview program http://www.broad.mit.edu/mpg/haploview/[26]. For analysis of the 3'UTR CA repeats, HWE was tested by comparison of the observed and expected genotype frequencies. Haploview 4.2 was also used to calculate the pairwise linkage disequilibrium (LD) of the polymorphisms for which the minor allele frequency (MAF) was >0.05. LD assessment and *CD40LG* haplotype definition, was determined using Lewontin's standardized disequilibrium coefficient D', the squared correlation coefficient r^2, while the block definition was based on the solid spine method[26]. For practical purposes, we introduced rs201992677 [-/CAAA] to the LD analysis, arbitrarily calling 'A' the wild type allele with six CAAA repeats, and 'G' the variant allele with seven repeats.

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Author contributions

S.L., F.C. and O.G. initiated and completed the project. S.L. and C.A. designed the experiments. C.A. and C.S. performed the experiments. M.H., T.C., S.JY., M.A., C.F., B.P. and C.A. contributed to the recruitment of the blood donors. J.F., V.G.H. and R.T. did the DHPLC analysis. C.A. and A.P. analyzed the data. S.L. drafted the manuscript. All authors reviewed the manuscript.

Additional information

Accession codes: We submitted the three novel mutations in DDBJ database with following accession numbers

> AB897730 52b053e41553421871002993.*CD40LG*1
> AB897731 52b053e41553421871002993.*CD40LG*2
> AB897732 52b053e41553421871002993.*CD40LG*3

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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SUPPLEMENTARY INFORMATION

Supplementary Data S1: PCR protocols

This text describes the PCR protocols and primers used.

Primer design for DHPLC and capillary electrophoresis is provided.

Statistical results of the tests for homogeneity in males and females for each population.

Supplementary Figure S1: Detection of *CD40LG* polymorphisms

This Figure presents:

DHPLC and direct sequencing profiles obtained from analysis of PCR fragments i.e., wild homoduplex-type and aberrant heteroduplex elution profiles at the temperature used for accurate identification, and sequence data corresponding to the mutant DHPLC profile.

Patterns obtained by separation over capillary electrophoresis for CA repeat microsatellite in the 3' UTR.

Supplementary Table S1 Test for homogeneity between males and females in *CD40LG* in the French (n=211) and Tunisian populations (n=274)

Supplementary Table S2 Minor allele frequencies of *CD40LG* SNPs in the French donors whose PC did not induced an ATR (n=211) vs donors whose PC induced an ATR (n=30)

Supplementary Table S3 Allelic distributions of the *CD40LG* (CA)n repeat polymorphism in the French cohort whose single apheresis platelet components (PC) induced or not an ATR (40 and 291 alleles respectively).

Supplementary Table S4 Demographic distribution of the French and Tunisian cohorts.

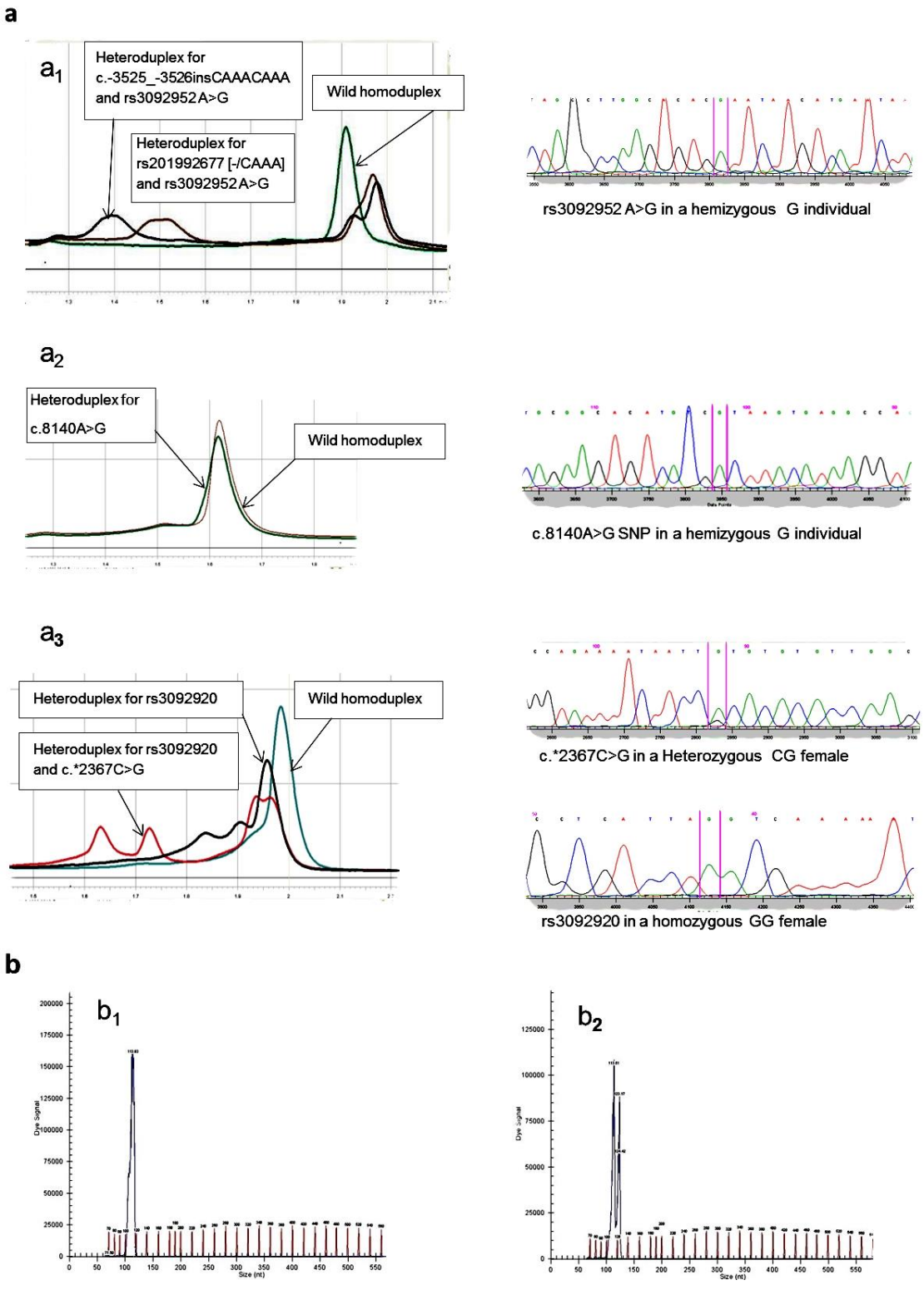
Supplementary Data S1

PCR amplification

Eleven primer pairs (**Table 1**) were designed using the following software: Geneious (http://geneious.com), Netprimer (http://premierbiosoft.com/netprimer/index.html) and Amplify3 (http://engels.genetics.wisc.edu/amplify/). These were designed to explore the entire exonic sequence and intron/ exon boundaries of the *CD40LG* gene, as well as 285 bp of the 5′UTR and 1353 bp of the 3′UTR region, to screen for the maximum number of polymorphisms that are potentially involved in CD40L protein expression or are reported to be found in association with immune disorders or inflammation. Large exon 5 was split into three overlapping parts. The sizes of the PCR fragments were between 244 and 432 bp.

PCRs were performed in 25 µL volumes for the female samples and 18 µL volumes for the male samples. Reactions contained HotStartTaq mix including 1X PCR buffer, 1.5 mM $MgCl_2$, 0.1U of HotStart Taq DNA polymerase, (Qiagen, Paris, France), 200 µM of each dNTP, 0.4 µM of each primer and 100 ng of genomic DNA. Reactions were cycled for 15 min at 95°C, followed by 35 cycles for 30 s at 94°C, 30 s at the annealing temperature (55-58°C), 30 s at 72°C, and 10 min at 72°C as a final extension. Amplifications were carried out in a C1000 Touch™ Thermal Cycler (Biorad, Hercules, USA). Oligonucleotide primer pairs were synthesized by Eurofins MWG Operon (Ebersberg, Germany). The corresponding annealing temperature of the PCR cycles for each primer pair is shown in Table 3.

Fragments of the 3′UTR containing the microsatellite (CA)n were PCR amplified, for both male and female samples at 58°C as the annealing temperature with the reverse 5′-AAGAAGAGAACTGACTAGCAACGG-3′ primer (Eurofins MWG Operon, Ebersberg, Germany). The forward 5′-CCCCAGTCTCTCTTCTCAATCC-3′ primer was designed with a fluorescent tail at the 5′end, using WellRed dye-labeled D4-PA oligonucleotides (Sigma-Aldrich, St Quentin-Fallavier, France).



Supplementary Figure S1

Detection of *CD40LG* polymorphisms.

a DHPLC and direct sequencing profiles obtained from analysis of PCR fragments including examples of wild homoduplex type and aberrant heteroduplex elution profiles at the temperature used for accurate identification (left panel) and examples of sequence data corresponding to the mutant

DHPLC profile (right panel): (**a_1**) c.-3525_-3526insCAAACAAA, rs201992677 [-/CAAA] and rs3092952 detection in *CD40LG*-5'UTR (**a_2**) c.8140A>G detection in *CD40LG*-E4 even with subtle differences in the pattern, (**a_3**) rs3092920 G>T and c.*2367C>G detection in *CD40LG*-3'UTRC.

b CA microsatellite repeats in the 3'UTR, using 600 bp as the internal size standard. The patterns obtained were from capillary electrophoresis separation: (**b_1**) Peak 114 is the allele peak for one hemizygous $(CA)_{26}$ repeat, while double peaks of 110 and 120 correspond to the heterozygous $(CA)_{24}$ / $(CA)_{29}$ (**b_2**).

Supplementary Table S1 Test for homogeneity between males and females in *CD40LG* in the French (n=211) and Tunisian populations (n=274)

			French population				Tunisian population			
SNP	Marker ID (allele)	Alleles	Allele Frequencies		Statistic		Allele Frequencies		Statistic	
			Male	Female	χ^2	P-value	Male	Female	χ^2	P-value
1	c.-3525_-3526insCAAACAAA	$(CAAA)_{6/7}$	0,992	1,000	1,250	0,4	0,995	1,000	0,813	0,999[a]
		$(CAAA)_8$	0,008	0,000			0,005	0,000		
2	rs201992677 [-/CAAA]	$(CAAA)_6$	0,791	0,852	1,858	0,173	0,655	0,614	0,624	0,430
		$(CAAA)_7$	0,209	0,148			0,345	0,386		
3	rs3092952 A>G	A	0,792	0,852	1,774	0,183	0,656	0,633	0,211	0,646
		G	0,208	0,148			0,344	0,367		
4	rs1126535 T>C	T	0,808	0,877	2,623	0,105	0,774	0,778	0,009	0,926
		C	0,192	0,123			0,226	0,222		
5	rs147739883 C>T	C	1,000	1,000	0,000	1,000	0,995	1,000	0,813	0,999[a]
		T	0,000	0,000			0,005	0,000		
6	c.8140A>G	A	1,000	1,000	0,000	1,000	0,995	1,000	0,813	0,999[a]
		G	0,000	0,000			0,005	0,000		
7	rs3092923 T>C	C	0,138	0,099	1,105	0,293	0,303	0,354	1,069	0,301
		T	0,862	0,901			0,697	0,646		
8	rs148594123 G>A	G	0,969	0,975	0,100	1,000	1,000	1,000	0,000	1,000
		A	0,031	0,025			0,000	0,000		
9	rs3092921 C>T	C	0,869	0,901	0,737	0,391	0,708	0,658	0,991	0,320
		T	0,131	0,099			0,292	0,342		
10	c.*2367C>G	C	1,000	1,000	0,000	1,000	1,000	0,994	1,238	0,447[a]
		G	0,000	0,000			0,000	0,006		
11	rs3092920 G>T	G	0,900	0,895	0,019	0,890	0,749	0,671	2,589	0,108
		T	0,100	0,105			0,251	0,329		

[a]Fischer Exact Test (n<5)

Supplementary Table S2 Minor allele frequencies of *CD40LG* SNPs in the French donors whose PC did not induced an ATR (n=211) vs donors whose PC induced an ATR (n=30)

SNP	Marker ID	Position Chr X (Location)	Allele	Minor Allele Frequency (MAF)		Statistic		PCR Fragment
				Donors whose PC induced an ATR	Donors whose PC did not induced an ATR	χ^2	p-value	
2	rs201992677 [-/CAAA]	135726882 (5' UTR)	$(CAAA)_7$	0.175	0,143	0.007	0.9312	*CD40LG*-5'UTR
3	rs3092952 A>G	135726950 (5'UTR)	G	0.175	0,143	0.007	0.9312	*CD40LG*-5'UTR
4	rs1126535 T>C	135730555 (exon 1)	C	0.154	0,143	0.113	0.7369	*CD40LG*-E1
7	rs3092923 T>C	135741185 (IVS 4)	C	0.116	0,119	0.149	0.6999	*CD40LG*-E5A
8	rs148594123 G>A	135741443 (exon 5)	A	0.027	0	1.058	0.3037	*CD40LG*-E5B
9	rs3092921 C>T	135743000 (3'UTR)	T	0.113	0,119	0.149	0.6999	*CD40LG*-3'UTRB
11	rs3092920 G>T	135743991 (3'UTR)	T	0.103	0,119	0.069	0.7928	*CD40LG*-3'UTRC

Supplementary Table S3 Allelic distributions of the *CD40LG* $(CA)_n$ repeat polymorphism in the French cohort whose single apheresis platelet components (PC) induced or not an ATR (40 and 291 alleles respectively)**.**

	Donors whose PC induced an ATR	Donors whose PC did not induced an ATR	
$(CA)_n$	N[a] (frequency)	N[a] (frequency)	P-value[b]
<26 CA	16 (0.400)	80 (0.275)	0.223
26 CA	13 (0.325)	128 (0.440)	
>26 CA	11 (0.275)	83 (0.285)	

[a]Number of alleles

[b]Bonferroni correction : P-value is significant when <0.016

Supplementary Table S4 Demographic distribution of the French and Tunisian cohorts.

	Tunisian cohort		French cohort			
			whose PC did not induced an ATR		whose PC induced an ATR	
	n= 274		n= 211		n=30	
Gender	Male	Female	Male	Female	Male	Female
	195 (71.168%)	81 (29.562%)	130 (61.611%)	81 (38.388%)	18 (60%)	12 (40%)
Age (years) (mean ± SD)	33.404 ± 9.128	23.302 ± 9.225	49.200 ± 11.947	44.568 ± 12.347	46.766 ± 15.949	38.819 ± 16.032

II. Article **2 : Tertra-ARMS-PCR : article méthodologique**

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Development of a highly resolutive method, using a double quadruplex tetra-primer-ARMS-PCR coupled with capillary electrophoresis to study CD40LG polymorphisms.

Aloui C[1], Sut C[1], Cognasse F[1], Granados V[2], Hassine M[3], Chakroun T[4], Garraud O[5], Laradi S[6].

N'ayant trouvé aucune association génétique des polymorphismes étudiés avec l'apparition d'EIR post-transfusion plaquettaire (premier article), et vu l'existence d'association d'autres polymorphismes introniques du gène CD40LG avec de nombreuses pathologies inflammatoires et autoimmunes, nous avons développé une technique multiplexe, la quadruplexe Tetra-ARMS-PCR, pour compléter la première étude et vérifier une éventuelle association de ces polymorphismes introniques avec les EIR.

Ce papier a été publié comme étant un papier méthodologique dans lequel nous avons validé et confirmé la structure haplotypique du gène CD40LG dans les deux populations étudiées. Des résultats non publiés ont montré l'absence d'association de 6 polymorphismes supplémentaires du gène CD40LG avec la survenue d'EIR.

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Development of a highly resolutive method, using a double quadruplex tetra-primer-ARMS-PCR coupled with capillary electrophoresis to study *CD40LG* polymorphisms

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ABSTRACT

Polymorphisms in the CD40 ligand gene (*CD40LG*) are associated with various immunological disorders such as tumors, autoimmune and infectious diseases. The aim of this study was to develop a highly optimized double quadruplex tetra-primer amplification refractory mutation system PCR (double quadruplex T-ARMS-PCR) coupled with capillary electrophoresis to allow genotyping of eight relevant candidate *CD40LG* SNPs and to establish haplotypes.

After conducting the double quadruplex T-ARMS-PCR, the genotypes obtained through agarose electrophoresis were compared with those obtained through capillary electrophoresis. This strategy was applied to analyze the genetic patterns of *CD40LG* in two distinct cohorts of blood donors (211 French and 274 Tunisian).

The T-ARMS-PCR method was rapid, inexpensive, reproducible and reliable for SNP determination. Regarding the separation technique, capillary electrophoresis allows traceable and semi-automated analysis while agarose electrophoresis remains a cost-effective technique that does not require specialized or costly equipment. Using these methods, we identified significantly different genetic heterogeneity between the two investigated populations ($p \leq 0.0001$) and we also extensively characterized their haplotypes.

The obtained genotype distribution and the optimized quadruplex T-ARMS-PCR technique coupled with capillary electrophoresis provides valuable information for studying pathologic inflammation leading to various diseases in which *CD40LG* might be a candidate gene.

1. Introduction

The human CD40 ligand (CD40L) is a member of the TNF superfamily. Its gene (*CD40LG*) is located on the long arm of the X chromosome at position q26.3–q27.1. This gene harbors five exons spanning approximately 12 kilobase pairs (kb). *CD40LG* encodes a 33 kDa type II membrane glycoprotein, which plays a central role in adaptive and innate immunity and is involved in both physiological and pathological (exacerbated) inflammation. CD40L is overexpressed on activated CD4+ T-cells, activated platelets, certain subsets of B lymphocytes, dendritic cells, follicular dendritic cells and subsets of other leukocytes [1]. It is also expressed on many other cell types, such as the endothelial cells [2] that line vessels and engage in partnerships with platelets during primary hemostasis. CD40L expression is regulated via sequence variations in the 5′ UTR [3] and through mRNA stability via a CA repeat microsatellite in the 3′ UTR [4,5]. Deficiencies in CD40L expression may cause a reduced antibody response, as observed in X-linked hyper-IgM syndrome (HIGM1), whereas polymorphisms in this ligand

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have been associated with various pathologies, such as acute coronary syndrome (cardiovascular disorder) [3], Kawasaki disease (autoimmune disorder) [6] and severe malaria (infectious disease) [7]. Elevated sCD40L levels have been associated with transplant rejection [8] and transfusion, inducing benign or severe adverse events [9–11].

We previously reported the development of a denaturing high performance liquid chromatography (DHPLC) for *CD40LG* screening and genotyping of exonic and intronic polymorphisms but mostly those located at exon–intron junctions. However, introns have been implicated in regulating gene expression and DNA–protein interactions, therefore, intronic variations may affect these functions [12]. In addition, information from multiple single nucleotide polymorphisms (SNPs) genotypes is expected to provide better genetic information that may eventually link the risk of a disease with a haplotype. These factors influenced our decision to examine SNPs based on their previously reported associations with inflammatory disorders.

We introduced a multiplex PCR to establish an efficient typing of *CD40LG*, simultaneously targeting eight relevant SNPs reported to be associated with inflammatory pathologies. Therefore, we used a highly optimized double quadruplex tetra-primer amplification refractory mutation system PCR (T-ARMS–PCR), which allowed us to calculate allele frequencies and *CD40LG* haplotype structures at a very reasonable cost. The T-ARMS–PCR enabled us to perform large scale surveys in extended populations (blood donors from metropolitan France and Central Tunisia) to determine the dispersion of genetic variation in populations that are distinct but also have common ancestries. This technical approach will provide relevant information regarding *CD40LG* SNPs that may be associated with various immunological-inflammatory disorders.

2. Material and methods

2.1. Subjects

2.1.1. Ethics statements

Written informed consent was obtained from all donors who participated in this study according to a protocol approved by the ethics committees for scientific research of the Saint-Etienne (Saint-Etienne, France), F. Bourguiba (Monastir, Tunisia) and F. Hached (Sousse, Tunisia) University Hospitals. All analyses were performed anonymously.

2.1.2. Study populations

We investigated the same cohort as previously described [13]. Briefly, 485 healthy blood donors consisting of 211 French volunteers (130 males and 81 females) from the Auvergne-Loire Regional Branch of the French National Blood Bank and 274 Tunisian volunteers (195 males and 79 females from the Monastir Blood Bank and the Regional Blood Transfusion Centre of Sousse) were used. Genotype information from reference populations used for further comparisons was downloaded from the 1000 Genomes Project (www.1000genomes.org).

2.2. DNA extraction

EDTA-treated blood samples were collected from all individuals. DNA was purified from peripheral blood leukocytes using the FlexiGene DNA kit (Qiagen, Paris, France) according to the manufacturer's instructions. DNA concentrations and purity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.3. SNP selection

For the first quadruplex T-ARMS-PCR group, rs975379, rs3092929 and rs3092920 were chosen because these SNPs were previously studied in systemic lupus erythematosus, lymphoma, diabetic atherosclerosis, systemic sclerosis and rheumatoid arthritis [14–18]. One variant, rs3092945, showed a significant association with reduced risk of severe malaria in C hemizygous males [19] and with an increased risk of developing respiratory distress in CC females [7].

For the second quadruplex T-ARMS-PCR group, rs3092948 and rs3092927 were chosen because these SNPs were previously investigated in immunological disorders, such as coronary syndrome [14] and systemic lupus erythematosus [15]. The rs715762 variant was selected due to the significant link between the A hemizygous variant and an increased risk of non-Hodgkin lymphoma in males [16]. An inverse association was also found between the A allele of rs3092933 and the risk of follicular lymphoma in women [16].

2.4. Quadruplex T-ARMS-PCR

Genotyping of the polymorphisms was performed via multiplex PCR amplification, followed by fragment analysis. PCR was performed using two sets of 16 in-house-designed primers (Set 1 and 2) to amplify four mutant and/or four wild amplicons and four mandatory consensus gene-specific internal control products in each set, as illustrated in Fig. 1. Information about known SNPs was obtained from the public Single Nucleotide Polymorphism Database (dbSNP), build 138 (http://ncbi.nlm.nih.gov/SNP/). Primers were designed, and their melting temperatures were determined based on the genomic sequence of *CD40LG* (NC_000023.10) using several websites and bioinformatics software packages, including Geneious (Biomatters Limited, Auckland, New Zealand, www.geneious.com) to organize the sequence and estimate primer localization; NetPrimer (Lifesciences, Palo Alto, CA, USA, www.premierbiosoft.com/) for in silico tests to detect the Tm, dimers and hairpins; Amplify 3 (University of Wisconsin, Madison, WI, USA http://engels.genetics.wisc.edu/amplify/) to check secondary PCR products in situ; and the UCSC Genome Bioinformatics Site (University of California, Santa Cruz, CA, USA, http://genome.ucsc.edu/) to align the selected primers with the whole genome. The specificity of the T-ARMS-PCR specific primer sequences was enhanced by introducing an additional mismatch at the penultimate or third nucleotide from the 3′ end of the primer when necessary (Table 1) [20,21].

Each of the two groups of T-ARMS-PCR primers used for the amplification of twelve different sized fragments was pooled in a single 25 μL reaction volume containing 12.5 μL of master mix from the Multiplex PCR kit (Qiagen, Paris, France) and optimized concentrations of each primer. Table 1 shows the primer sequences for the 2 sets as well as their respective optimized melting temperatures and the final reaction concentrations used to amplify 100 ng of genomic DNA. Multiplex PCR was performed using a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA).

For each primer set, a temperature gradient was preliminarily carried out to determine a stringent annealing temperature, and an optimized touchdown PCR protocol was performed as follows: for both PCR groups, an initial denaturation step of 95° for 10 min, followed by 7 cycles of 94 °C for 30 s, 70 °C minus 1 °C per cycle for 30 s, and 72 °C for 30 s. Then, we performed 29 cycles of 94 °C for 30 s, annealing for 30 s at temperatures of 65 °C and 63 °C for multiplex T-ARMS-PCR groups I and II, respectively, and extension at 72 °C for 45 s, followed by a final extension cycle at 72 °C for 10 min.

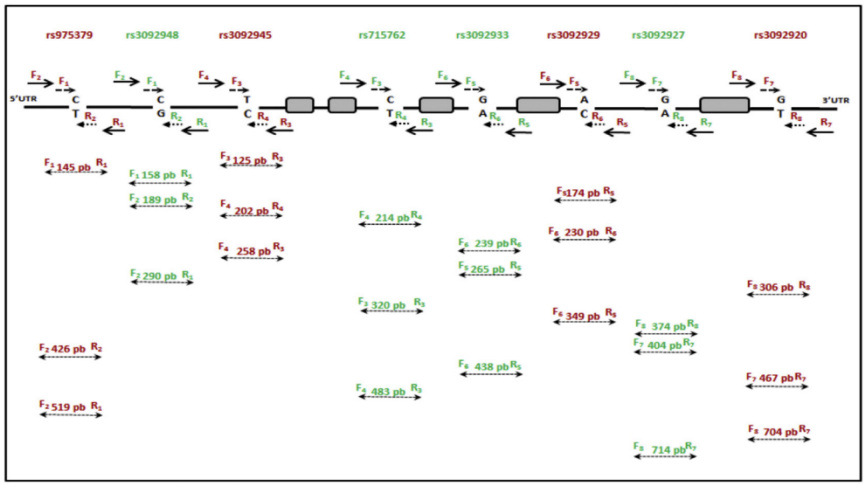

Fig. 1. Schematic illustration showing the positions of the primers use in the proposed double quadruplex T-ARMS-PCR sets I and II for the analysis of *CD40LG*. Exons: gray boxes; introns: lines; quadruplex T-ARMS sets I and II are indicated in red and green, respectively. The primer sequences are shown in Table 1. ➡: Wild-type allele primers; ⇢: Mutant allele primers; →: Consensus outer primers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Primers for the two quadruplex T-ARMS-PCR groups.

	SNP	Primer	Sequence[a] (5′ → 3′)	Tm (°C)	C[b] (μM)	Length (bp)	
						Specific bands	Control bands
Group I	rs975379 C>T	F1-rs975379	TTCTTCCAGAATTCCCTTCAAAGCATG***C***C	72.7	0.2	145	519
		R1-rs975379	CCAGGTCCCCAAGGTGAGCTTTCTTG	72.6	0.12		
		F2-rs975379	CACTTCTCTACTGTGGATGCTTACCCAGTGC	72	0.08	426	
		R2-rs975379	CCCACCCCAAACACAGCAGGAGAA	72.7	0.12		
	rs3092945 T>C	F3-rs3092945	CCTATTTTCCCTATTCTGAACTGTTACATCAGCA***T***	69.1	0.2	125	258
		R3-rs3092945	AGACTCTGGAATTGAAGTTAAAGTAAAAATCGTGAC	68.1	0.14		
		F4-rs3092945	ATAAGCAAGACAGGTGCAAGTGCCTCCT	70.9	0.1	202	
		R4-rs3092945	GTGTACACTGTTCCAATCCATTAGATAATTGTT***A***G	65.9	0.2		
	rs3092929 A>C	F5-rs3092929	ACTGGGAGCATCCCTCCTCCTAACC***T***A	70	0.1	174	349
		R5-rs3092929	TGTAACGTCTTGGGACCTATTGATCTTTACAG	68.4	0.1		
		F6-rs3092929	TCTTCAAAGTGAGTTCAAATGCACAGATGGG	72.5	0.1	230	
		R6-rs3092929	CATGAGGCCCATTTCCACACAGTGAAG***C***G	77.8	0.1		
	rs3092920 G>T	F7-rs3092920	GCAAAAATTTGCTACACTGAAAGGATCCTCATTA***G***	70.8	0.2	467	704
		R7-rs3092920	GCACCCCTGTTCTGACAGCTTGCTAGAG	72.5	0.2		
		F8-rs3092920	GCACCCTGCACTTAGCAAGTGCTTCACA	74.3	0.1	306	
		R8-rs3092920	ATGAGTGCATTTTTATCATCCAGATATTTTTGACA	69.2	0.1		
Group II	rs3092948 C>G	F1-rs3092948	CAGAAGGATGAGAGAAAGAGAAGGCTTCAA***C***	69.2	0.2	158	290
		R1-rs3092948	CCTCTCCTTTGTAGATTACTGAATTGTACCATCCT	68.8	0.1		
		F2-rs3092948	CCTGTTAGGGAGAGACAGGCATGAGAGG	71.2	0.1	189	
		R2-rs3092948	CAGAATGCCCTGCTTGTACCTCAATT***C***	69.2	0.1		
	rs715762 C>T	F3-rs715762	AGAACACTGAGTCAAATACCCTTGGGCC	70.5	0.1	320	479
		R3-rs715762	AGAACCTGAAAGCAAGCAGCGGATTT	70	0.1		
		F4-rs715762	GCTAGCATGAAGTCACTGCAGTGACTGTG	71.3	0.1	214	
		R4-rs715762	CCTAGAAATGACCCCTAAGTGAGCCG***C***A	72.4	0.14		
	rs3092933 G>A	F5-rs3092933	GGCAGCCTGATCCGTCTTTGAATT***G***	71.1	0.1	265	438
		R5-rs3092933	AACAGTGGAAGACATCAGGGATCTTTAGCC	70.1	0.1		
		F6-rs3092933	TTATTTGCCCGGTTCTTAAAGTGAGAGCAT	69.7	0.3	239	
		R6-rs3092933	GACTTCTCTGGAGTAGTATAGTAATCTTGGTATTATTAGA***A***T	63.9	0.3		
	rs3092927 G>A	F7-rs3092927	TGTGTGTGCATATGTGTATGTGTGTGACA***T***G	72.3	0.08	404	714
		R7-rs3092927	GGGGCTTTATGTCACCCTTTTGACATCTAA	70.3	0.12		
		F8-rs3092927	TCTGTAAAGATCAATAGGTCCCAAGACGTTACA	69	0.2	374	
		R8-rs3092927	TTTTTTCTCTCTCTGTCCATCTCTCTTTCTC***G***CT	71	0.2		

[a] Specific nucleotides are underlined; mismatches are presented in bold and italics.
[b] Final reaction concentration.

2.5. Separation of PCR products

2.5.1. Basic agarose gel electrophoresis

The multiplex PCR products were loaded into a 2% agarose gel with GelRed® for separation via electrophoresis, and the products were visualized with UV light. A DNA size marker of 100–3000 bp (NIPPON Genetics EUROPE, Dueren, Germany) was used to identify product sizes determined by the Gel Doc™ (Bio-Rad, Hercules, CA, USA).

2.5.2. Capillary electrophoresis separation

The samples were also resolved via capillary electrophoresis separation using the CEQ 2000 XL genetic analysis system (Beckman Coulter Inc., Brea, CA, USA) and LPA-1 separation gel, with a capillary temperature of 50 °C, a voltage of 2 kV and an injection time of 30 s. The samples were initially denatured at 90 °C for 120 s, and fragments were separated at 4.8 kV, using an extended run time of 2700 s to capture longer reads. PCR was carried out according to the previously described protocol, except that the primers were designed with a fluorescent tail at the 5′ end using WellRED dye-labeled D2, D3 and D4 –PA oligonucleotides (Sigma–Aldrich, St Quentin-Fallavier, France). A 0.38 μL aliquot of a 600 bp size marker and 40 μL of sample loading solution (Beckman Coulter Inc., Brea, CA, USA) were added to 1 μL of the 1/10 diluted PCR products. The PCR products were excited to fluoresce using a diode laser. The length of the amplified fragments was estimated in reference to the internal ladder using the provided CEQ 8000 Beckman Coulter software.

2.5.3. Method validation

To confirm the specificity of each amplicon and the allele-specific primers, thirty randomly selected DNA samples corresponding to specific quadruplex-T-ARMS-PCR patterns were re-amplified as described above, and then subjected to Sanger sequencing in both the forward and reverse directions using the same common outer PCR primers for each SNP. The PCR products were purified with Amicon Centrifugal Filters (Millipore, Molsheim, France). Sequencing reactions were performed using the GenomeLab DTCS Quick Start Mix (Beckman Coulter, Brea, CA, USA). The resulting products were purified with the Beckman Coulter's CEQ DTCS kit according to the manufacturer's instructions, followed by analysis with a CEQ 2000 XL-Beckman Coulter sequencing machine and assembled using the CEQ 8000 Beckman Coulter software (Beckman Coulter, Inc., Brea, CA, USA).

2.6. Statistical analysis

Statistical analyses and haplotype frequency estimations were conducted using the Haploview 4.2 program [22] available at http://www.broad.mit.edu/mpg/haploview/. The quality of the genotype data for each polymorphism was assessed through Hardy–Weinberg equilibrium testing. Linkage disequilibrium (LD) assessment and the *CD40LG* haplotype frequency estimation were conducted using the Lewontin's standardized disequilibrium coefficient D′ and the squared correlation coefficient r^2. Blocks were defined based on the Solid Spine method. Haploview 4.2 was used to compare the allelic distribution between males and females, the allelic distribution between French and Tunisian populations and the haplotype frequencies between the two populations. Statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Validation of quadruplex T-ARMS-PCR

A total of 485 DNA samples were typed in the multiplex T-ARMS-PCR assays, followed by separation of the PCR products via both typical agarose gel electrophoresis and polyacrylamide gel capillary electrophoresis.

We started with a pool of seven samples for optimizing each SNP (4 primers). Then, we carried out the amplification of four SNPs together in one tube and four others in a second tube, to perform a "double quadruplex T-ARMS-PCR".

Multiplex PCR usually leads to the preferential amplification of one target sequence over another [23]. To overcome the difficulty of specificity and efficiency of the PCR products, we had to modify the primer concentrations (from 0.08 to 0.3 μM) and enhance the specificity of the inner specific primers by modifying the 3′ end sequence by introducing an additional mismatch at the penultimate base of the wild primer (T-ARMS-PCR group I: F1-rs975379, F5-rs3092929, F7-rs3092927; T-ARMS-PCR group II: F5-rs3092933) and/or of the mutant primer (T-ARMS-PCR group I: R4-rs3092945, R6-rs3092929; T-ARMS-PCR group II: R4-rs715762, R6-rs3092933) or even at the third nucleotide (R8-rs3092927) (Table 1). The R8-rs3092927 primer was modified the most: its concentration changes in reaction were unsuccessful so we designed a modified primer in two successive steps: a base in −1 then in −2 positions relative to the 3′ end. Therefore, several series of PCR tests were conducted.

Eventually, the amplified fragments, including both the allele-specific amplicons and the internal control bands, showed identical lengths to those predicted based on the locations of the designed primers. The common outer primer pairs were always present, which determined the control bands or peaks (depending on the separation method) (Fig. 2).

Completely concordant results were obtained for the T-ARMS-PCR assays, regardless of the separation technique, confirming each homozygous and heterozygous SNP profile. Sanger DNA sequencing confirmed the obtained genotypes for 30 tested samples. The SNP rs3092920 was previously genotyped via dHPLC and produced an identical result [13].

Fig. 2A shows the agarose electrophoregram for 8 different samples using the quadruplex T-ARMS-PCR group I primers, which represents possible genotypes for the 4 polymorphisms. The bands were sufficiently clear with minimal background and allowed for an accurate genotype assignment.

Following agarose separation, we used a second technique to identify polymorphisms based on peak size and color. Fig. 2B shows a peak pattern obtained with one fluorescent amplified sample after separation via capillary electrophoresis. This profile corresponds to lane 7 of the agarose gel after the migration of the T-ARMS-PCR group I.

The T-ARMS-PCR method has become a commonly used technique for SNP genotyping. Triplex and quadruplex PCR methods are more limited because they require primer design based on specific sequence characteristics; for example, a sequence with a high TA percentage requires longer primers (e.g., R9-rs3092933), increasing the risk of non-specific amplification and/or the formation of dimers [24,25]. The technical requirements of the *CD40LG* T-ARMS-PCR were very feasible to achieve, and involved an optimal 3.0 mM concentration of $MgCl_2$, without the need for improvements with BSA, betaine or DMSO, as reported by others [21,24,26–30]. Nevertheless, we had to combine modified sequences and concentrations of the different primers with touchdown PCR protocols.

The double quadruplex T-ARMS-PCR assay resulted in rapid, accurate and reproducible detection of 8 SNPs in as few as 2 single-tube PCRs that detected each SNP separately, or in groups of two or three depending on the goal. This method leads to simultaneous genotyping of polymorphisms even when large numbers of samples are assayed.

This method has been used in several clinical and research laboratories. For example, *GALT* gene mutations were detected via

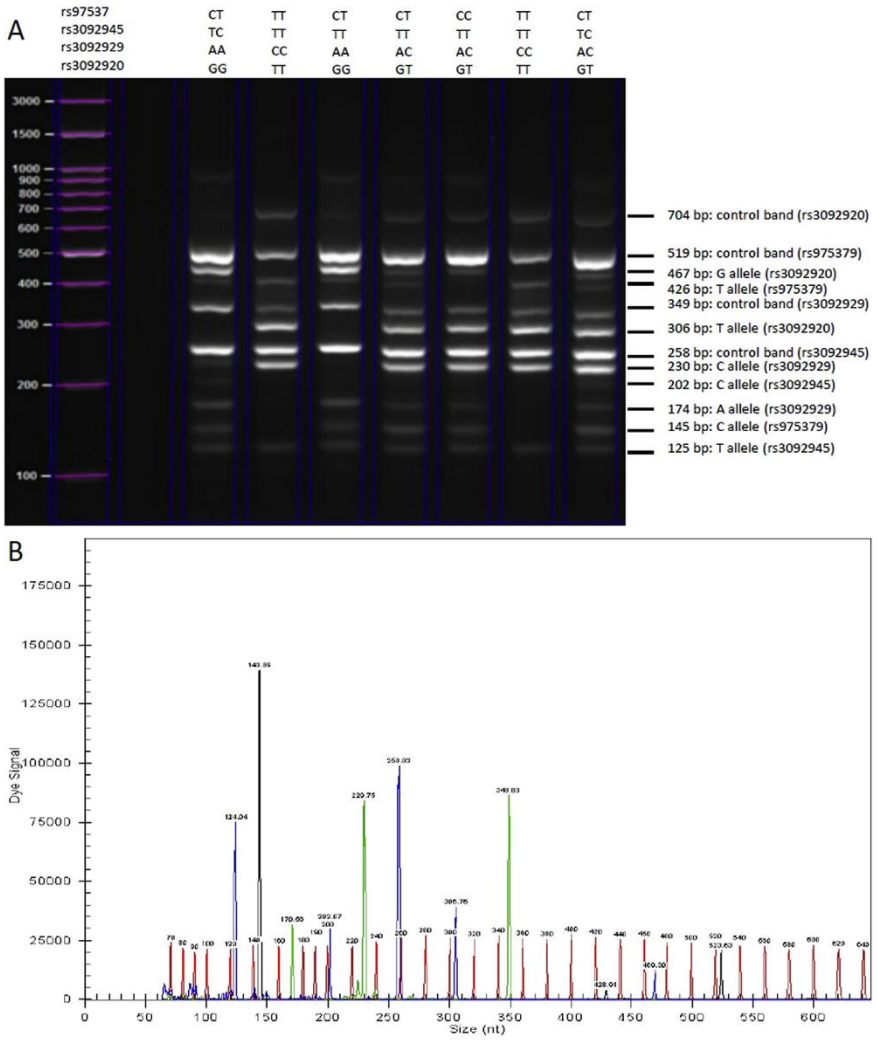

Fig. 2. Analysis of *CD40LG* via the quadruplex T-ARMS-PCR I and II method. (A) Agarose gel electropherogram showing the detection of rs975379, rs3092945, rs3092929 and rs3092920 (quadruplex-T-ARMS-PCR using set 1 primers). MW: Molecular weight DNA ladder, B: negative control. (B) Electropherogram for sample 7. The pattern was obtained via capillary electrophoresis separation.

classic ARMS-PCR [31]; genotyping for type 1 diabetes was conducted through T-ARMS-PCR [32]; the folate-homocysteine metabolic pathway was investigated using triplex T-ARMS-PCR [24]; and *β-globin* gene mutations were analyzed via quadruplex T-ARMS-PCR [33]. These assays require minimal hands-on effort and use standard PCR reagents, in addition to agarose gels for electrophoresis or polyacrylamide cartridges for capillary electrophoresis for which the cost is approximately 3-fold higher. Nevertheless, this cost is lower than the cost of capillary electrophoresis using universal-tailed mismatch amplification mutation assays, which are less expensive than the cost of analysis using TaqMan Assays [34]. Overall, the quadruplex T-ARMS-PCR with capillary electrophoresis separation remains more interesting because it allows traceability, semi-automation and high ease, however, agarose gel separation is advantageous in terms of cost. In addition, when multiple T-ARMS-PCRs are used the amplicon lengths for genotyping are required to differ by 2 bp for separation via capillary electrophoresis [35,36]. However, in agarose gel electrophoresis the limit of resolution is at least 20 bp because of the limitations regarding size discrimination between PCR products.

Several methods exist for interrogating SNPs that use high-tech instrument methods, including enzyme-based techniques [37], genome-wide association studies based on DNA arrays [38,39], pyrosequencing [40], matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry [41] and next-generation sequencing (NGS) [42], are elegant, accurate and

suitable for the detection of multiple polymorphisms, but require specialized and costly equipment. Other methods are more commonly used, such as probe analysis (i.e., TaqMan real-time PCR assays) [43] and Sanger sequencing, but these techniques are also costly due to the required equipment and reagents. Because the typical restriction fragment length polymorphism method of PCR (RFLP-PCR), which is only useful when a SNP creates or abolishes a restriction site, is laborious, time-consuming, and demands additional steps, making this method less useful for genotyping polymorphisms in large numbers of samples. Based on the findings described here and with respect to high-tech methods of SNP genotyping, T-ARMS-PCR meets our expectations and sufficiently addresses our scientific questions within financial constraints.

3.2. Distribution of CD40LG polymorphisms in the studied populations

The genotype distribution for all investigated polymorphisms was found to be in Hardy–Weinberg equilibrium, showing p-values greater than the standard cut-off value of 0.05.

All minor allele frequencies (MAFs) were significantly overrepresented among Tunisian individuals ($P < 0.0001$) compared with French individuals, except for the rare minor allele rs715762 (Table 2). In addition, we compared the MAFs in the two populations with those from the 1000 Genomes Project (Fig. 3). All allele frequencies in the French population (present study) were similar to those in the Caucasian population (CEU). All allele frequencies for the SNPs in the Tunisian population fell between the frequencies found in populations of African ancestry (ASW, LWK, YRI) and those of European origin (CEU, GBR, TSI), whereas the frequency of the rare allele rs715762 was similar to the French study population. Our results indicated a moderate transferability of tagging SNPs for *CD40LG* from the European-Caucasian population to the French population. For the Tunisian population, the transferability of tagging SNPs for *CD40LG* was nonexistent.

3.3. Linkage disequilibrium and haplotype analysis

Fig. 4 illustrates the diversity of the haplotype structures observed in the French and Tunisian populations, including the eight studied polymorphisms in addition to those studied

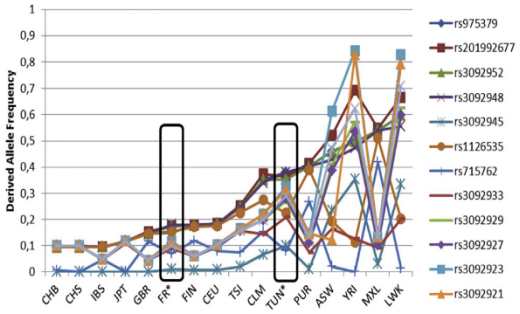

Fig. 3. Derived allele frequencies for the studied *CD40LG* polymorphisms, including minor allele frequencies in French (FR) and Tunisian (TN) donors and in data extracted from the 1000 Genomes database. African Ancestry in the Southwest US (ASW), Han Chinese in Beijing (CHB), Han Chinese South (CHS), Japanese individuals in Tokyo (JPT), Iberian populations in Spain (IBS), British from England and Scotland (GBR), Finnish from Finland (FIN), Utah residents with Northern and Western European ancestry (CEU), Tuscan in Italy (TSI), Colombian in Medellin (CLM), Puerto Rican in Puerto Rico (PUR), Mexican Ancestry in Los Angeles, CA (MXL), African Ancestry in Southwest US (ASW), Luhya in Webuye, Kenya (LWK).

previously [13]. There was a marked difference in the pattern of LD between the two populations. Two haplotype blocks were identified across *CD40LG*, with evidence of disruption in LD observed between rs715762 and rs3092933. Block 1, spanning 9.8 kb encompassing the 5′ UTR and the first two exons, contained seven SNPs (rs975379 and rs3092945 in quadruplex T-ARMS-PCR group I, rs3092948 and rs715762 in quadruplex T-ARMS-PCR group II, and rs201992677, rs3092952 and rs1126535 from previous data), while block 2 encompassed the remainder of the gene sequence. Considering the resulting haplotype blocks 1 and 2, the SNPs defined twelve and five core haplotypes (with frequency >1%) in the French and Tunisian cohorts, respectively. Therefore, the haplotype distribution was also significantly different between the two populations (Table 3).

These findings regarding the allele and haplotype frequencies for the *CD40LG* polymorphisms clearly reflect the high diversity of this gene sequence among different ethnic groups. Consequently,

Table 2
Allele frequencies for *CD40LG* polymorphisms in the French and Tunisian populations.

	SNP	X Chr position (Location)	Allele	French population		Tunisian population		χ^2	p-value[a]
				Allele frequency	HW p-value	Allele frequency	HW p-value		
Group I	rs975379 C>T	135723584 (5′ UTR)	C	0.85	0.2819	0.62	0.551	33.056	<0.0001
			T	0.15		0.38			
	rs3092945 T>C	135729609 (5′ UTR)	T	0.99	0.8652	0.9	0.9888	16.25	<0.0001
			C	0.01		0.1			
	rs3092929 A>C	135738923 (IVS 4)	A	0.89	0.7933	0.73	0.8186	20.618	<0.0001
			C	0.11		0.27			
	rs3092920 G>T	135743991 (3′ UTR)	G	0.9	0.5782	0.71	0.8186	28.176	<0.0001
			T	0.1		0.29			
Group II	rs3092948 C>G	135727761 (5′ UTR)	C	0.82	0.2819	0.62	0.551	32.146	<0.0001
			G	0.18		0.38			
	rs715762 C>T	135733413 (IVS 2)	C	0.93	0.5965	0.92	0.2775	0.412	0.553
			T	0.07		0.08			
	rs3092933 G>A	135737217 (IVS 3)	G	0.91	0.2714	0.79	0.881	16.486	<0.0001
			A	0.09		0.21			
	rs3092927 G>A	135739378 (IVS 4)	G	0.89	0.5782	0.72	0.8186	29.165	<0.0001
			A	0,11		0,28			

HW: Hardy–Weinberg.
[a] Fisher's exact test between Tunisian and French allele frequencies.

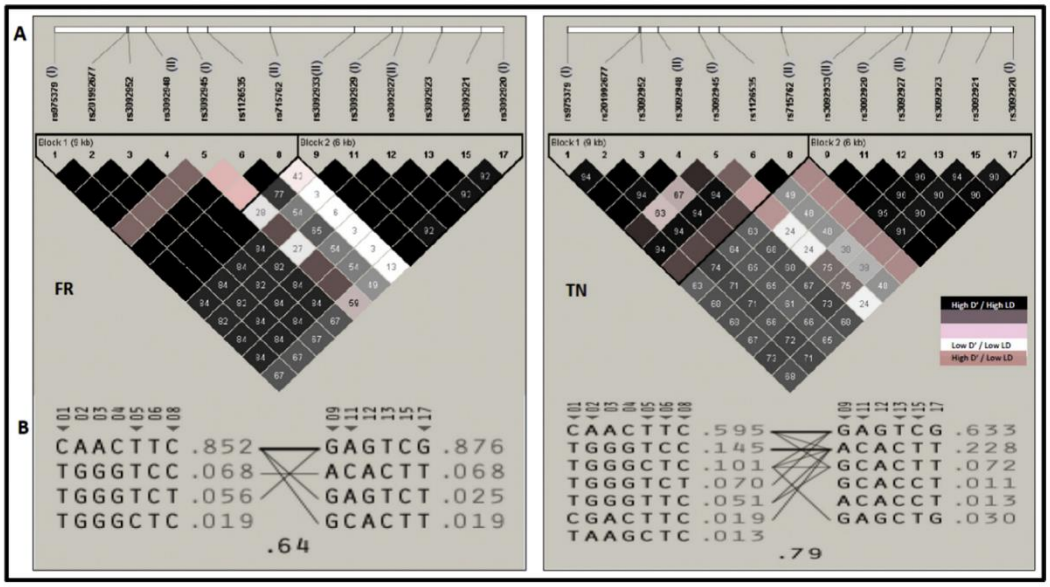

Fig. 4. *CD40LG* LD/block structure and haplotype diversity in the French (FR) and Tunisian (TN) populations: (a) Pairwise D′ values and haplotype blocks were estimated with Haploview 4.2 software. The number representing the intersection of each pair of SNPs represents the pairwise D′ value between the two SNPs. (I) or (II) indicates the quadruplex T-ARMS-PCR group in which the polymorphism was analyzed; the other SNPs were previously studied (data not shown). (b) Haplotype diversity is shown below the LD diagram. The marker numbers are specified by haplotype tagging SNPs highlighted with a triangular pointer. Haplotype frequencies are shown next to each haplotype.

Table 3
Haplotype distribution of *CD40LG* between the French and Tunisian populations.

	Haplotype	Haplotype frequency (French)	Haplotype frequency (Tunisian)	χ^2	P-value
Block 1	CAACTTC	0.852	0.595	26.481	2.6613E-7
	TGGGTCC	0.068	0.145	5.081	0.0242
	TGGGTCT	0.056	0.070	0.27	0.6033
	TGGGCTC	0.019	0.101	9.806	0.0017
	TGGGTTC	0.006	0.051	5.775	0.0163
Block 2	GAGTCG	0.876	0.633	25.737	3.9128E-7
	ACACTT	0.068	0.228	16.328	5.3265E-5
	GCACTT	0.019	0.073	5.503	0.019
	GAGCTG	0.000	0.028	4.61	0.0318
	GAGTCT	0.025	0.000	3.968	0.0464

there are no records in the HapMap or 1000 Genomes databases for French and Tunisian populations (nor for any North African populations). Therefore, the present investigation provides novel information at the French level, regarding the migratory pathways for Tunisians and also for the population of Tunisia. This may lead to a better understanding of genetic patterns that are significant to immunological disorders, including cardio-vascular, auto-immune, infectious diseases, even in transplantation and in transfusions.

In conclusion, the double quadruplex T-ARMS-PCR detected 8 SNPs using 2 single tube PCR reactions. This method detected each SNP separately or in groups of two or three, depending on the research interest. It also provided simultaneous genotyping for polymorphisms within a large sample size. This method has significant potential to be widely applied to *CD40LG* screening because this gene is very important in the complex inflammation process. Overall, quadruplex T-ARMS-PCR coupled with peak separation by capillary electrophoresis permits high ease, semi-automated and traceable quadruplex T-ARMS-PCR for Laboratory Accreditation. This method may be expanded to other genes or combined for several SNPs in different genes. This methodological strategy revealed discrepancies in the prevalence of *CD40LG* polymorphisms that may be explained by ethnic and geographic differences.

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Authors' contributions

SL, FC and OG initiated and completed the project. SL and CA designed the experiments. CA carried out the design of primers,

participated in the collection and analysis of the samples and performed the statistical analysis. CS developed the optimization of the quadruplex T-ARMS-PCR method. VG optimized the capillary electrophoresis, MH and TC participated in the collection of the samples. SL and CA drafted the manuscript. OG coordinated the study. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest relevant to this manuscript.

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III. Article 3 : sCD40L et régulation génétique

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Levels of human platelet-derived soluble CD40 ligand depend on haplotypes of CD40LG-CD40-ITGA2.

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Cette étude a été réalisée avec des prélèvements d'ADN et de surnageants plaquettaires de donneurs de sang provenant de la banque de sang de Sousse, Tunisie.

La première raison qui nous a incité à faire cette étude est l'existence de deux marqueurs génétiques associés à la concentration de CD40L soluble ou à la quantité d'ARN messager[282,283]. Une seule étude (Malarstig et al. 2006[284]) constitue la référence unique concernant le sCD40L.

Notre but était donc de vérifier ces deux marqueurs déjà connus et d'étendre l'étude aux autres polymorphismes déjà optimisés dans le papier « méthode[150] ».

Nous avons également testé les haplotypes génétiques à la recherche d'une éventuelle association.

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Levels of human platelet-derived soluble CD40 ligand depend on haplotypes of *CD40LG-CD40-ITGA2*

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Increased circulating soluble CD40 ligand (sCD40L) is commonly associated with inflammatory disorders. We aimed to investigate whether gene polymorphisms in *CD40LG*, *CD40* and *ITGA2* are associated with a propensity to secrete sCD40L; thus, we examined this issue at the level of human platelets, the principal source of sCD40L. We performed single polymorphism and haplotype analyses to test for the effect of twelve polymorphisms across the *CD40LG*, *CD40* and *ITGA2* genes in blood donors. *ITGA2* presented a positive association with rs1126643, with a significant modification in sCD40L secretion (carriers of C allele, P = 0.02), unlike the investigated *CD40LG* and *CD40* polymorphisms. One *CD40LG* haplotype (TGGC) showing rs975379 (C/T), rs3092952 (A/G), rs3092933 (A/G) and rs3092929 (A/C) was associated with increased sCD40L levels (1.906 µg/L (95% CI: 1.060 to 2.751); P = 0.000009). The sCD40L level was associated with the inter-chromosomal CD40LG/CD40/*ITGA2* haplotype (ATC), displaying rs3092952 (A/G), rs1883832 (C/T) and rs1126643 (C/T), with increased sCD40L levels (P = 0.0135). Our results help to decipher the genetic role of *CD40LG*, *CD40* and *ITGA2* with regard to sCD40L levels found in platelet components. Given the crucial role of sCD40L, this haplotype study in a transfusion model may be helpful to further determine the role of haplotypes in inflammatory clinical settings.

Clinical interest in CD40 ligand (CD40L, CD154) regulation is commonly reported in various inflammatory disorders[1,2] and notably in relation to adverse events (AEs) after platelet transfusion. CD40L is mainly expressed on the surface of T cells, certain subsets of other leukocytes, endothelial cells and activated platelets[3–5]. CD40L binds to its preferred receptor CD40, thereby driving adaptive immune responses[6].

Cell surface CD40L can be proteolytically cleaved by matrix metalloproteinases (MMPs) to generate soluble CD40L (sCD40L), which is biologically active as an important proinflammatory molecule and is also classified as a "Biological Response Modifier"[7,8]. Circulating sCD40L is known to be mainly derived from activated platelets *via* MMP-2[9,10], which accounts for nearly 95% of the sCD40L in the plasma. sCD40L release increases in platelet components (PCs) under storage conditions and is directly responsible for febrile non-haemolytic transfusion reactions and other immediate transfusion adverse events (AEs)[11–15].

Thus, we hypothesized the existence of a genetic risk factor in relation to the donor. In an initial study, we investigated the coding sequences, exon-intron junctions and regulatory regions of *CD40LG*, but we did not find any particular genetic pattern of *CD40LG* in two groups of individuals regardless of whether their donated platelets induced an AE[16], despite the fact that two *CD40LG* polymorphisms are involved in *CD40LG* regulation, namely, sequence variations in the 5′ UTR of *CD40LG* (rs3092952)[1] and a CA microsatellite in the 3′ UTR that affects mRNA stability[17,18].

In the present study, we characterized the secretion of sCD40L in PCs destined for transfusion on day 0 of preparation (D_0) and on the day of delivery (D_{del}) in order to assess a possible genetic association between regulatory polymorphism and enhanced *in vitro* sCD40L release in PCs during storage. In most blood transfusion services, PC delivery is allowed from D_0 to D_5, with an average of D_4 at worst and D_3 at best. Moreover, sCD40L might also be modulated by independent genetic markers such as rs1883832 in the promoter region of the CD40 receptor[19] and/or the C807T polymorphism (rs1126643) in the coding region of the platelet receptor for collagen (*ITGA2*).

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The latter was previously associated with some individual variation in platelet expression levels of GPIa/IIa (integrin $\alpha 2\beta 1$), the preferential platelet receptor for collagen, which plays a crucial role in platelet adhesion and activation. This marker has further been defined as an independent predictor for the release of sCD40L[20,21].

Therefore, the present study sought to highlight a possible genetic association (single markers and haplotypes) between 10 single nucleotide polymorphisms (SNPs) of *CD40LG*, rs1883832 of *CD40* and rs1126643 of *ITGA2*, which display enhanced *in vitro* sCD40L release in PCs during storage.

Results

The genotype distribution for all investigated polymorphisms was found to be in Hardy-Weinberg equilibrium.

Correlation between sCD40L levels and single polymorphisms. Relevant *CD40LG*, *CD40* and *ITGA2* polymorphisms were assessed.

No significant correlation was detected between the investigated *CD40LG* polymorphisms and sCD40L levels in the PCs, neither at D_0 nor at D_{del}, for all ten investigated CD40LG polymorphisms (Table 1).

There was also no association found between sCD40L levels and rs1883832 of the *CD40* gene, although this polymorphism has been shown elsewhere to regulate CD40L expression[19,22].

However, there was a significant association with rs1126643 of *ITGA2*; the platelets of C-allele carriers (CC and CT) secreted elevated levels of sCD40L upon storage in shelf-life conditions–that is, with no deliberate stimulation–compared with the non-C carriers (TT homozygous), $P = 0.08$ at D_0 and $P = 0.02$ at D_{del} (Table 1).

Haplotype association with sCD40L levels. Five *CD40LG* haplotypes accounted for 97.6% of all potential combinations, including rs975379 (C/T), rs3092952 (A/G), rs3092933 (A/G) and rs3092929 (A/C). The association between *CD40LG* haplotypes and CD40L secretion leading to sCD40L is reported in Table 2. One haplotype (H_4: TGGC; frequency: 2.6%) was associated with the largest increase in sCD40L levels at the day of PC delivery, i.e., 1.906 μg/L (95% CI: 1.060 to 2.751; $P = 0.000009$), compared with the reference haplotype H_1 (CAGA). None of the other four CD40LG haplotypes was associated with any difference in sCD40L secretion and plasma levels.

Eight haplotypes accounted for 100% of potential multigene *CD40LG* (rs3092952)/*CD40* (rs1883832)/*ITGA2* (rs1126643) combinations. These haplotypes were tested for association with PC sCD40L levels. A significant association was shown for haplotype H_C at the time of preparation [H_C: ATC; frequency: 10.8% with increased sCD40L level, i.e., 0.189 μg/L (95% CI: −0.182 to 0.560; $P = 0.0135$)], compared with the most common haplotype H_A (ACC; Table 3). It is interesting to note that haplotype H_G (GTC), which differs from H_C (ATC) by the first allele (A instead of G of the rs3092952 polymorphism), was also associated with a small non-significant increase in sCD40L levels at D_{del}, i.e., 0.553 μg/L (95% CI: 0.235–0.87; $P = 0.071$). No other haplotype was consistently associated with sCD40L levels.

These results are reported after full adjustments for covariates, i.e., gender, age, number of platelets (10^9/L) and the number of days of storage (inventory condition) prior to delivery. The major contributing factor exerting a positive effect on sCD40L levels was the platelet count ($P = 0.000406$ and $P < 0.000001$ at D_0 and D_{del}, respectively) for the *CD40LG* haplotype analysis. The same covariate was identified in the inter-chromosomal haplotype analysis ($P = 0.001457$ and $P < 0.000001$ at D_0 and D_{del}, respectively).

Discussion

SNP association studies did not reveal any association with sCD40L levels measured in PCs at either D_0 or D_{del}. These results were consistent with our previous findings[16] showing that no particular pattern of *CD40LG* in individuals who donated platelets by single apheresis and those in whom PCs induced an adverse transfusion reaction. It does seem, in light of the results and limitations of this study, that there are no so-called "regulator polymorphisms of *CD40LG*", at least in healthy individuals.

Our findings did disagree with the observations of Malarstig *et al.*, who showed in a large cohort of patients with cardiovascular disease that carriers of the G allele of rs3092952[1] had a 10% higher sCD40L level. However, to the best of our knowledge, this finding has yet to be consistently reproduced and despite this apparent correlation, rs3092952 did not confer an increased risk of cardiovascular adverse events. Regarding CA repeats in the 3′-UTR of *CD40LG*, our results corroborate those of Bugert *et al.*, indicating that neither the sizes of the alleles nor the genotypes of the CA repeat polymorphism were associated with plasma sCD40L levels[23] (Table 1). Dai *et al.* found no association between CA repeats and mRNA expression in CD4 + T cells[24]. Only studies by Perez-Aciego *et al.* found that CD40L expression (membrane and mRNA) decreases in CD4 + T cells, but only in those with 24 CA alleles[25,26]. Taken together, these results indicate that the regulation of membrane CD40L expression and sCD40L levels is complex and implies not only genetic variations in *CD40LG*. Furthermore, one cannot exclude the possibility of environmental influences, cell-related post-translational regulation, catabolic regulation and/or polygenic control.

Extensive literature reported the association of the *CD40* -1C/T polymorphism (rs1883832) with CD40L expression. However, published results are difficult to compare because either the T or the C allele is implicated, with relatively equal frequencies between them[19,22]. The present study found no significant association with this polymorphism. Some of our unpublished data, however, confirm a positive correlation between surface protein CD40L expression and the rs1883832 genotypes present in the CD40 gene in T lymphocytes, in line with the findings of Zhang B *et al.*[22].

The only genotype associated with sCD40L levels in PCs was found with C-allele carriers (CC and CT) of *ITGA2*, with a positive association ($P = 0.02$). This finding is discordant with the work of Antoniades *et al.*, who defined the T allele as an independent predictor for the release of sCD40L in healthy subjects but only in the subgroup with von Willebrand factor greater than or equal to the median[21]. Thus, again, an association with a specific

Gene	Polymorphism	Genotype	N	D_0: Median (1st–3rd Quartile)	p-value	D_{del}: Median (1st–3rd Quartile)	p-value
CD40LG	rs3092952	AA+A	69	560.34 (336.82–916.4)	0. 99	854. 78 (534. 26–1148. 66)	0. 33
		AG	43	496.44 (303.98–967.47)		781. 84 (472. 23–1266. 56)	
		GG+G	30	589.45 (362.42–791.13)		1121. 455 (564. 897–1353. 59)	
	CACA	26+/other	37	498.36 (336.82–1123.88)	0. 55	746. 72 (431–1266. 56)	0. 3
		Only 26	24	444.4 (230.86–898.925)		682. 74 (515. 15–1253. 45)	
		No 26	81	556 (361.88–908.76)		924. 54 (584. 18–1251. 6)	
	rs975379	CC+C	70	547.5 (340.265–914.49)	1	853. 91 (535. 15–1144. 325	0. 35
		CT	42	502.08 (301.73–973.085)		799. 8 (471. 455–1266. 56)	
		TT+T	30	589.45 (362.42–791.13)		1121. 455 (564. 898–1353. 59)	
	rs3092948	CC+C	66	589.93 (340.265–920.075)	0. 9	865. 64 (526. 025–1187. 405)	0. 47
		CG	44	494.97 (306.23–961.855)		799. 8 (473. 005–1266. 56)	
		GG+G	32	527.18 (360.925–765.35)		1084. 295 (573. 613–1347. 33)	
	rs3092945	CC+C	6	875.95 (531.935–1022.295)	0. 31	1186. 315 (694. 273–1256. 79)	0. 25
		TC	22	364.16 (272.27–834.385)		678. 66 (471. 455–1079. 625)	
		TT+T	114	581.25 (358.115–914.49)		900. 52 (542. 5–1340. 11)	
	rs715762	CC+C	127	546.48 (345.99–924.24)	0. 6	853. 04 (517. 89–1255. 06)	0. 32
		CT	8	370.36 (271.215–706.9)		851. 46 (515. 415–1158. 05)	
		TT+T	7	642.6 (430.32–930.08)		1199. 83 (940. 05–1350. 46)	
	rs3092933	AA+A	14	693.37 (391.3–791.13)	0. 9	944. 07 (516. 2–1226. 195)	0. 56
		GA	37	546.48 (362.84–927.18)		932. 62 (560. 54–1594. 4)	
		GG+G	91	498.36 (295.56–949.43)		839. 96 (536. 1–1200. 075)	
	rs3092929	AA+A	80	495.34 (284.07–898.925)	0. 34	847. 37 (574. 81–1234. 04)	0. 55
		AC	38	494.97 (359.945–986.99)		835. 4 (452. 68–1256. 1)	
		CC+C	24	693.37 (408.945–918.54)		1037. 81 (571. 483–1275. 85)	
	rs3092927	AA+A	22	693.37 (419.265–892.89)	0. 3	1084. 295 (558. 508–1310. 51)	0. 58
		GA	39	496.44 (372.19–987.97)		853. 04 (456. 06–1245. 64)	
		GG+G	81	492.32 (288.94–893.1)		839. 96 (556–1223. 2)	
	rs3092920	GG+G	77	498.36 (288.94–893.1)	0. 44	876. 5 (556–1266. 56)	0. 83
		GT	39	546.48 (381.58–1060.56)		818. 48 (453. 36–1245. 64)	
		TT+T	26	632.57 (382.26–834.965)		980. 34 (516. 2–1256. 79)	
CD40	rs1883832	CC	82	553.41 (338.47–910.575)	0. 93	895. 93 (591. 49–1244. 88)	0. 62
		CT	52	531.86 (312.11–922.77)		865. 64 (467. 36–1271. 075)	
		TT	8	451.22 (377.53–1087.42)		544. 175 (386. 4–1472. 04)	
ITGA2	rs1126643	CC	53	534.66 (299.48–868.98)	0. 08	876. 5 (490. 7–1224. 72)	0. 02
		CT	64	647.6 (361.455–1009.2)		998. 95 (639. 67–1621. 995)	
		TT	25	401.28 (218.04–657.18)		691. 5 (454. 72–942. 56)	

Table 1. Frequency distribution of *CD40LG*, *CD40* and *ITGA2* genotypes, considering sCD40L level, on the day of preparation (D_0) and on the day of PC delivery (D_{del}). The association analysis between sCD40L level and rs1126643 in ITGA2 on the day of delivery is detailed as follows: CC vs CT = 0.095; CC vs TT = 0.167; TT vs CT = 0.006.

allele (T or C) seems to be an ambiguous result, similar to other polymorphisms, e.g., *CD40*[19,22]. This polymorphism may affect either mRNA stability or a regulatory genetic region, with a subsequent change in the density of the expressed molecule on the platelet surface and, consequently, an alteration of platelet adhesion and activation, although this polymorphism does not alter the functional status of the protein[20]. This possibility may explain why the association between the *ITGA2* genotype and sCD40L levels was only found on the day of delivery, suggesting the requirement for sustained platelet activation over time, due to the preparation and storage process[27,28].

We identified a highly significant association with the *CD40LG* haplotype at the day of PC delivery (H_4: TGGC; P = 0.000009; Table 2). This result may be explained by progressive sCD40L release during platelet storage[13,27]. Moreover, we also identified one inter-chromosomal CD40LG/CD40/ *ITGA2* haplotype, H_C (ATC) from the rs3092952, rs18828232 and rs1126643 genotypes, associated with sCD40L levels (P = 0.0135; Table 3). This result highlights the importance of the association of several polymorphisms in different genes that are involved in the complex regulation of this immuno-modulatory molecule that is released after platelet activation. Notably, the frequency of H_C was nearly 11% of all the investigated *CD40LG/CD40/ ITGA2* haplotypes.

H_G was also associated with a relative increase in sCD40L levels at D_{del}, although the difference did not reach statistical significance. This similarity could be linked to the similarity of the two haplotypes, H_G (GTC) and H_C (ATC), given that they show a difference in only the allele of the first rs3092952 polymorphism, which presents a regulatory function; however, that function was not identified in our study, which considered the polymorphism alone.

CD40LG haplotype (rs975379, rs3092952, rs3092933, rs3092929)			Haplotype interaction with sCD40L			
	Haplotype	Frequency (N = 142)	sCD40L mean (95% CI) at D_0	P-value	sCD40L mean (95% CI) at D_{del}	P-value
H_1	CAGA	0.578	−0.122 (−0.776–0.531)	reference	0.470 (−0.098–1.040)	reference
H_2	CAGC	0.057	0.172 (−0.922–0.127)	0.484	0.203 (−0.978–1.384)	0.594
H_3	TGGA	0.105	−0.037 (−0.690–0.615)	0.698	0.353 (−0.336–1.043)	0.639
H_4	**TGGC**	**0.026**	−0743 (−1.357 −1.208)	0.927	**1.906 (1.060–2.751)**	**0.000009***
H_5	TGAC	0.210	−0.123 (−0.829–0.583)	0.997	0.509 (−0,272–1.291)	0.843
Other		0.024				

Table 2. Frequency distribution of *CD40LG* haplotypes in PCs and their interaction with sCD40L level, considering sCD40L level, on the day of preparation (D_0) and on the day of PC delivery (D_{del}). Data represent the mean and relative 95% CI of the difference in platelet supernatant sCD40L levels (μg/L) observed in one copy of each haplotype configuration compared with the reference haplotype. Haplotypes (Hn) are indicated in ACGT format with rs975379 (C/T), rs3092952 (A/G), rs3092933 (A/G) and rs3092929 (A/C). The data are boldfaced if the corresponding haplotypes show a significant P-value (<0.05). All data were adjusted for gender, age and platelet count. Data on D_{del} were further adjusted for the number of days before delivery.

CD40LG (rs3092952)/ *CD40* (rs1883832)/ *ITGA2* (rs1126643)			Haplotype interaction with sCD40L			
	Haplotype	Frequency (N = 142)	sCD40L mean (95% CI) at D_0	P-value	sCD40L mean (95% CI) at D_{del}	P-value
H_A	ACC	0.277	−0.118 (−0.461–0.226)	reference	0.248 (−0.345–0.531)	reference
H_B	ACT	0.226	−0.097 (−0.423–0.228)	0.869	0.293 (−0.444–0.631)	0.768
HC	**ATC**	**0.108**	**0.189 (−0.182–0.560)**	0.0135*	0.242 (−0.195–0.678)	0.975
H_D	ATT	0.033	−0.201 (−1.083–0.681)	0.857	0.301 (0.188–0.789)	0.827
H_E	GCC	0.161	−0.084 (−0.398 −0.231)	0.828	0.390 (0.009 −0.771)	0.350
H_F	GCT	0.093	−0.002 (−0.343–0.340)	0.394	0.145 (−0.426–0.717)	0.715
H_G	GTC	0.058	−0.141 (−0.820–0.539)	0.936	0.553 (0.235–0.872)	0.071
H_H	GTT	0.044	−0.201 (−0.108–0.681)	0.993	0.130 (−0,694–0.954)	0.754

Table 3. Frequency distribution of *CD40LG/CD40/ ITGA2* haplotypes in PCs and their interaction with sCD40L levels. Data represent the mean and relative 95% CI of the difference in platelet supernatant sCD40L levels (μg/L) observed in one copy of each haplotype configuration compared with the reference haplotype. Haplotypes (Hn) are indicated in ACGT format with rs3092952 (A/G), rs1883832 (C/T) and rs1126643 (C/T). The data are boldfaced if the corresponding haplotypes show significant P-values (<0.05). All data were adjusted for gender, age and platelet count. Data on D_{del} were further adjusted for number of days before delivery.

The apparent controversies between individual polymorphisms and haplotype analysis are explained by their different biological values and a higher informative analysis was attributed to haplotype investigation[29]. Sequential nucleotide variants may catch subtle changes in protein function, regardless of the presence of nucleic acid changes in the coding region[29,30].

We identified two haplotypes associated with high levels of plasma sCD40L. However, a large fraction of CD40L is known to be carried by extracellular vesicles, including microvesicles and exosomes, after platelet activation[31]. As most methods used to assay sCD40L (i.e., ELISA and Luminex technologies) don't distinguish between free sCD40L and microvesicle-carried-CD40L[32,33], we believe that the levels of sCD40L measured in our study are not an underestimate.

To the best of our knowledge, this is the first study to suggest the haplotypic and polygenic control of sCD40L release from platelets stored for transfusion. This highly informative *in vitro* model contributes to a better understanding of the genetic role of *CD40LG*, *CD40* and *ITGA2* with regard to sCD40L platelet secretion and the subsequent levels in PCs and, consequently, the role of sCD40L in AEs. However, we cannot exclude that the biological response may also be affected by pathophysiologic aspects and co-morbidities in the transfusion recipients. The replication of the associations we found, as well as additional functional studies in pathological situations with inflammatory disorders, is required to confirm our findings in a larger number of individuals and must be extended to other clinical settings (e.g., *in vivo* models).

Methods

Subjects. *Ethics statement.* The study was carried out in accordance with the Helsinki Declaration and approved by the ethical committee of the F. Hached University Hospital, Sousse, Tunisia. Informed and written consent was obtained from all the healthy donors who participated in this study.

Study population. The studied cohort comprised 142 volunteer blood donors, including 52 males and 90 females, 26 ± 10 years of age (mean ± SD), who donated whole blood at the Transfusion Centre of Sousse. Individual PCs were derived from each donation as described[34]. None of the blood donors were family-related; donors entered the study randomly, on the sole basis of the timing of their donations; no selection criteria specific to this study were applied.

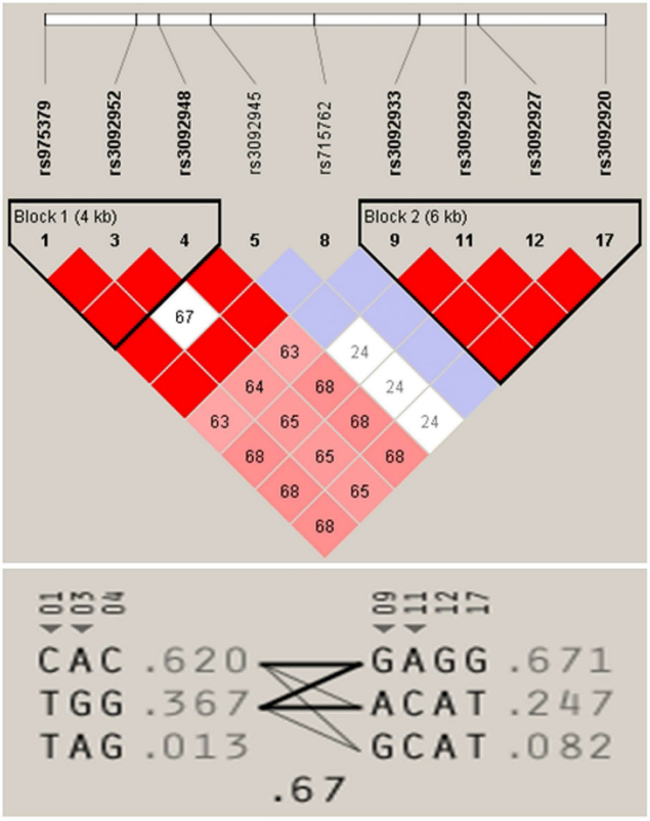

Figure 1. Representative LD plot of the two haplotype blocks at *CD40LG* and the tagged SNPs used for the haplotype association.

Genotyping. Genomic DNA was obtained from peripheral venous blood using the FlexiGene DNA Kit (Qiagen, Paris, France). For *CD40LG*, rs3092952 A/G and CA repeats were genotyped as previously described using denaturing High Performance Liquid Chromatography (dHPLC) and capillary electrophoresis, respectively[16]. The remaining 8 *CD40LG* polymorphisms were genotyped via multiplex PCR amplification in two groups of quadruplex Tetra primer Amplification Refractory Mutation System-PCR (T-ARMS-PCR) followed by fragment analysis (the first group included rs3092945 C/T, rs975379 C/T, rs3092929 A/C and rs3092920 G/T; the second group included rs3092948 C/G, rs3092927 A/G, rs715762 C/T and rs3092933 A/G)[35–39]. Each of the two groups of primers used for the amplification of twelve fragments of different sizes was pooled in a single 25-µL reaction volume, which contained 12.5 µL of master mix from the Multiple × PCR Kit (Qiagen, Paris, France), according to the manufacturer's recommendations. PCR conditions included an initial denaturation step of 95 °C for 10 min, followed by 7 cycles of 94 °C for 30 s, 70 °C minus 1 °C per cycle for 30 s and 72 °C for 30 s. Then, we performed 29 cycles of 94 °C for 30 s, followed by annealing for 30 s at 65 °C and 63 °C for multiplex T-ARMS-PCR groups I and II, respectively[40].

Genotype data were integrated with two additional SNPs, mapping at *CD40* (rs1883832 C/T) and at *ITGA2* (rs1126643 C/T), genotyped by single T-ARMS-PCR under the same technical conditions, with annealing temperatures of 67 °C and 67.5 °C for *CD40* and *ITGA2*, respectively (Supplementary Tables S1 and S2).

Soluble CD40L assay. Non-leukodepleted, individual PCs were prepared according to the standard protocol of the blood bank of Sousse[34]. Under sterile conditions, 4 mL was derived from PCs, first on the day of blood donation and platelet bag preparation (D_0) and again on the day of delivery (D_{del}). The samples were centrifuged at 180 g for 10 min and supernatants were collected and frozen at −80 °C and then thawed immediately before being assayed at room temperature. Soluble CD40L in PC supernatants was measured using a commercially available method by Luminex Technology according to the manufacturer's instructions (Milliplex Map Kit Millipore, Darmstadt, Germany).

Statistical analysis. All statistical analyses were performed with XLSTAT™ software (Addinsoft, Paris, France) using non-parametric methods. The Hardy-Weinberg equilibrium test was used to control the genotyping results. We used the Kruskal-Wallis test to evaluate the association between the genetic polymorphisms and sCD40L levels.

To test for the prevalence of genotypes of the CA repeat polymorphism, we defined three types as referred to Table 1 as follows: (i) genotype 'only 26' with the 26 CA allele exclusively (this group comprised females with two 26 alleles and males with one 26 allele, because the gene is X-linked); (ii) genotype '26+' with any allele other than 26 in addition to one 26 allele (heterozygous females); and (iii) genotype 'no 26' with no 26 allele (any genotype without the 26 allele).

For haplotype association, we used Haploview 4.2 software and employed the algorithm proposed by Gabriel[41] in order to choose tagged SNPs based on our previous study[16]. The haplotype tagger function was used to identify redundant SNPs, which were considered redundant if the pairwise LD (r^2) was $\geq$0.8. Haplotypes with a frequency $\leq$1% were excluded from the analysis (Fig. 1).

The consequences of displaying haplotypes on the propensity for platelet secretion of sCD40L were evaluated using THESIAS v3.1 software[42]. The association of each haplotype with sCD40L levels was measured using a regression parameter and the 95% confidence interval (CI), where the effect of each haplotype is compared with the most frequent haplotype, termed the 'reference' in the regression model. Adjustments were performed for different covariates, such as gender, age, platelet count and storage length prior to delivery (considered when testing for sCD40L at D_{del}).

All significance thresholds were set at $P < 0.05$.

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Author Contributions

S.L., F.C. and O.G. initiated and completed the project. S.L. and C.A. designed the experiments. C.A. carried out the primer design, participated in the sample collection and performed the statistical analysis (Haploview and THESIAS programs). C.S. and J.F. performed the genotyping. A.P. and S.T. participated in the interpretation of the results. T.C. participated in the sample collection. S.L. and C.A. drafted the manuscript. O.G. coordinated the study. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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Supplementary Table S1. *CD40LG* primers for the 2 quadruplex T-ARMS-PCR groups

	SNP	Primer	Sequence* (5'->3')	Tm (°C)	C† (μM)	Length (bp)	
						Specific bands	Control bands
Group I	**rs975379** C>T	F1 rs379	TTCTTCCAGAATTCCCTTCAAAGCATG***C***C	72.7	0.2	145	519
		R1 rs379	CCAGGTCCCCAAGGTGAGCTTTCTTG	72.6	0.12		
		F2 rs379	CACTTCTCTACTGTGGATGCTTACCCAGTGC	72	0.08	426	
		R2 rs379	CCCACCCCAAACACAGCAGGAGAA	72.7	0.12		
	rs3092945 T>C	F3 rs945	CCTATTTTCCCTATTCTGAACTGTTACATCAGCAT	69.1	0.2	125	258
		R3 rs945	AGACTCTGGAATTGAAGTTAAAGTAAAAATCGTGAC	68.1	0.14		
		F4 rs945	ATAAGCAAGACAGGTGCAAGTGCCTCCT	70.9	0.1	202	
		R4 rs945	GTGTACACTGTTCCAATCCATTAGATAATTGTT***A***G	65.9	0.2		
	rs3092929 A>C	F5 rs929	ACTGGGAGCATCCCTCCTCCTAACC***T***A	70	0.1	174	349
		R5 rs929	TGTAACGTCTTGGGACCTATTGATCTTTACAG	68.4	0.1		
		F6 rs929	TCTTCAAAGTGAGTTCAAATGCACAGATGGG	72.5	0.1	230	
		R6 rs929	CATGAGGCCCATTTCCACACAGTGAAG***C***G	77.8	0.1		
	rs3092920 G>T	F7 rs920	GCAAAAATTTGCTACACTGAAAGGATCCTCATTAG	70.8	0.2	467	704
		R7 rs920	GCACCCCTGTTCTGACAGCTTGCTAGAG	72.5	0.2		
		F8 rs920	GCACCCTGCACTTAGCAAGTGCTTCACA	74.3	0.1	306	
		R8 rs920	ATGAGTGCATTTTTATCATCCAGATATTTTTGACA	69.2	0.1		
Group II	**rs3092948** C>G	F1 rs948	CAGAAGGATGAGAGAAAGAGAAGGCTTCAAC	69.2	0.2	158	290
		R1 rs948	CCTCTCCTTTGTAGATTACTGAATTGTACCATCCT	68.8	0.1		
		F2 rs948	CCTGTTAGGGAGAGACAGGCATGAGAGG	71.2	0.1	189	
		R2 rs498	CAGAATGCCCTGCTTGTACCTCAATTC	69.2	0.1		
	rs715762 C>T	F3 rs762	AGAACACTGAGTCAAATACCCTTGGGCC	70.5	0.1	320	479
		R3 rs762	AGAACCTGAAAGCAAGCAGCGGATTT	70	0.1		
		F4 rs762	GCTAGCATGAAGTCACTGCAGTGACTGTG	71.3	0.1	214	
		R4 rs762	CCTAGAAATGACCCCTAAGTGAGCCG***C***A	72.4	0.14		
	rs3092933 G>A	F5 rs933	GGCAGCCTGATCCGTCTTTGAAT***T***G	71.1	0.1	265	438
		R5 rs933	AACAGTGGAAGACATCAGGGATCTTTAGCC	70.1	0.1		
		F6 rs933	TTATTTGCCCGGTTCTTAAAGTGAGAGCAT	69.7	0.3	239	
		R6 rs933	GACTTCTCTGGAGTAGTATAGTAATCTTGGTATTATTA GA***A***T	63.9	0.3		
	rs3092927 G>A	F7 rs927	TGTGTGTGCATATGTGTATGTGTGTGACA***T***G	72.3	0.08	404	714
		R7 rs927	GGGGCTTTATGTCACCCTTTTGACATCTAA	70.3	0.12		
		F8 rs927	TCTGTAAAGATCAATAGGTCCCAAGACGTTACA	69	0.2	374	
		R8 rs927	TTTTTTCTCTCTCTGTCCATCTCTCTTTCTC***G***CT	71	0.2		

* Specific nucleotides are underlined; mismatches are presented in bold and italics

† Final reaction concentration

The specificity of the T-ARMS-PCR specific primer sequences was enhanced by introducing an additional mismatch at the penultimate or third nucleotide from the 3' end of the primer when necessary.

Supplementary Table S2. *CD40G and ITGA2* primer sequences for T-ARMS-PCR

	SNP	Primer	Sequence* (5'->3')	Tm (°C)	C† (μM)	Length (bp)	
						Specific bands	Control bands
CD40	**rs1883832** C>T	F1 CD40	GCCGCCTGGTCTCACCTCGC***T***	72.7	0.20	263	573
		R1 CD40	ACCCCAGCCCGGGAGAAGAGAGA	72.4	0.11		
		F2 CD40	GGTCGCAGGAGCAGGCTAGCTCC	71.5	0.20	355	
		R2 CD40	GCACTGCAGAGGCAGACGAACCGT***G***	75.9	0.20		
ITGA2	**rs1126643** C>T	F1 ITGA2	AATATGGTGGGGACCTCACAAACACATT***C***	70.9	0.22	235	459
		R1 ITGA2	CCCAGCTGCCTTCTCAAAGTATTCAAGAC	70.3	0.42		
		F2 ITGA2	GGAAGTGATGCCTTAAAGCTACCGGC	69	0.47	284	
		R2 ITGA2	CCAAAACTTACCTTGCATATTGAATTGCTCC***A***	71	0.28		

* Specific nucleotides are underlined; mismatches are presented in bold and italics

† Final reaction concentration

The specificity of the T-ARMS-PCR specific primer sequences was enhanced by introducing an additional mismatch at the penultimate or third nucleotide from the 3' end of the primer when necessary.

IV. Article 4 : ADN mitochondrial et EIR

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Platelet components associated with adverse reactions: predictive value of mitochondrial DNA relative to biological response modifiers.

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Dans cette étude, nous avons dosé des BRM plaquettaires et de l'ADN mitochondrial dans des surnageants plaquettaires de deux groupes ; le premier ayant induit un EIR chez des patients transfusés et le deuxième étant le groupe contrôle.

Nous avons également examiné si ces marqueurs pourraient être considérés comme des marqueurs prédictifs de survenue d'EIR.

TRANSFUSION COMPLICATIONS

Platelet components associated with adverse reactions: predictive value of mitochondrial DNA relative to biological response modifiers

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BACKGROUND: Biological response modifiers (BRMs), secreted by platelets (PLTs) during storage, play a role in adverse events (AEs) associated with transfusion. Moreover, mitochondrial DNA (mtDNA) levels in PLT components (PCs) are associated with AEs. In this study we explore whether there is a correlation between pathogenic BRMs and mtDNA levels and whether these markers can be considered predictors of transfusion pathology.

STUDY DESIGN AND METHODS: We investigated a series of reported AEs after PC transfusion, combining clinical observations and mathematical modeling systems.

RESULTS: mtDNA was consistently released during the first days of PC storage; however, mtDNA release was earlier in "pathogenic" than in nonpathogenic PCs. PC supernatants with high levels of mtDNA along with soluble CD40 ligand (sCD40L) were significantly associated with occurrences of AEs. The fact that mtDNA did not associate with the 14 BRMs tested suggests the role of mtDNA in PC transfusion-linked inflammation is independent of that of BRMs, known to be associated with AEs. We present evidence that PLTs generate distinct pathogenic secretion profiles of BRMs and mtDNA. The calculated area under the curve for mtDNA was significantly associated with AEs, although less stringently predictive than those of sCD40L or interleukin-13, standard predictors of AE. The established model predicts that distinct subtypes of AEs can be distinguished, dependent on mtDNA levels and PC storage length.

CONCLUSIONS: Further work should be considered to test the propensity of mtDNA in PLT concentrates to generate inflammation and cause an AE.

Transfusion is a safe process; adverse events (AEs) are uncommon and occur in less than 2% of procedures. Platelet component (PC) transfusion accounts for close to 10% of the transfused blood components in France but generate more than 25% of all AEs, according to a comprehensive national hemovigilance survey.[1] This is despite a systematic leukoreduction to below 10^6 white blood cells/PC (in mean 3.5×10^5) that

ABBREVIATIONS: AAE = atypical allergic event; AE(s) = adverse event(s); ATR (s) = acute transfusion reaction(s); AUC = area under the curve; BRM(s) = biological response modifier(s); FNHTR = febrile nonhemolytic transfusion reaction; MDC = macrophage-derived chemokine; MIP = macrophage inflammatory protein; mtDNA = mitochondrial DNA; sCD40L = soluble CD40 ligand; PC(s) = platelet component(s); SDA = single-donor apheresis.

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is performed within 24 hours of collection and frequently processed using a cell separator. It has been consistently shown that long PC storage (longer than 4 days) increases the risk of AEs manifesting in the recipient.[2-4] Implicated factors include microparticles, oxygenated moieties of membrane lipids, inflammatory mediators (such as histamine, serotonin, and ADP/ATP), and biological response modifiers (BRMs) that themselves comprise cytokines, chemokines, growth factors, inhibitory factors, and related molecules.[5-8] West and colleagues[9] recently described that mitochondria are central participants in innate immunity. We recently reported[10] that mitochondrial DNA (mtDNA) levels in PCs were significantly associated with, on the one hand, reports of AEs and, on the other hand, specific BRM secretion profiles.[9] BRMs in particular are either shed from the platelet (PLT) membranes or released or eventually secreted de novo,[11,12] and there is good evidence that proinflammatory factors accumulate over time in stored PCs. One such factor identified to date is the soluble CD40 ligand (sCD40L).[13,14] It is even considered a master inflammatory factor in transfusion.[12,14-17] Significantly elevated levels of sCD40L were consistently found in cases presenting with AEs, but additional experimental investigations suggested that PLT-secreted factors other than sCD40L are also involved in clinical AEs. Thus, PLT-derived sOX40L and interleukin (IL)-27, which accumulate in PC supernatants, were also found in association with sCD40L in PCs leading to AEs but not in PCs that did not lead to declared pathology.[18] Based on this information, we have developed matrix and statistical models, using decision trees to predict the risk of hazardous outcomes. We then generated an "accident prediction model" that proved to be dependent on the level of a given BRM within the indicated component. Three factors proved to be significantly associated with AEs: sCD40L, IL-13, and macrophage inflammatory protein (MIP)-1α. Furthermore, their relative concentrations were further predictive of the clinical presentation (febrile nonhemolytic transfusion reaction [FNHTR] or atypical allergic event [AAE]).[2,6,12,18-20]

mtDNA has been recognized as a highly potent inflammatory trigger; it is reportedly found outside the cells in blood in various pathologies.[21] We first reported very recently its close association with PC transfusion pathology.[10] We thus felt that it would be valuable to explore whether mtDNA correlates with otherwise acknowledged markers of PC transfusion-linked inflammation, such as BRMs, and whether they can be considered predictors of transfusion pathology. Our novel approach combines clinical reports, biological data, and mathematical or statistical models. This method, aimed at deciphering pathogenic situations, would ideally help to prevent the delivery of risky PCs to patients at risk of manifesting severe inflammation.

MATERIALS AND METHODS

Cases and controls

We previously reported on the methods for collecting single-donor apheresis (SDA) PCs in our blood establishment. Apheresis PLTs were collected from regular anonymous blood donors who volunteered to provide blood for research purposes and signed a consent form, approved by the ethical committees of Etablissement Français du Sang.[18] Apheresis PLTs were collected from regular anonymous blood donors (Regional Blood Bank, EFS Auvergne-Loire, http://www.dondusang.net) who volunteered to provide blood for research purposes and signed a consent form, approved by the ethical committees of Etablissement Français du Sang.[18] PCs are identified with barcodes and none of the investigators can reconcile any single donor and his or her given barcode (only the blood service physician can in case of control sampling is needed for the donor, regarding a potential infectious risk). Further, recipients' data are anonymized with hospital-attributed barcodes. None of the authors can access the patient's file. All needed data are provided anonymously by the physician in charge. Thus, this study is completely anonymized. The transfusions were conducted as part of routine care in the close-by university clinics; the clinics' physicians were in charge of reporting the AEs or ATRs, describing the symptoms, and forwarding the PC identification number, along with a PC sample if some was leftover. The investigators' role here was 1) to design the study, 2) to educate clinicians and nurses in clinics to report on AEs to them as well, and 3) to educate labwork technicians to save the bags (shipped back to them according to the procedures in force) and to ship them to the laboratory research facilities. All samples harbored the hospital barcode number to identify the recipient, but in no way the clear identification of the patient in this case. This procedure protects the anonymity, according to the French regulation (CNIL).

Blood was collected on ACD-A with Amicus (Software Version 2.5, Fenwal, Inc., Lake Zurich, IL) or Trima (Gambro BCT, Lakewood, CO) cell separators. All PCs were automatically resuspended in 35% autologous donor plasma and 65% PLT additive solution (AS; InterSol, Fenwal, la Châtre, France; or SSP+™, MacoPharma, Mouveaux, France) and stored at 22 ± 2°C with gentle rotation and shaking (60 rpm) for a maximum of 5 days (after collection was completed) before being issued for transfusion. All collection procedures were performed as described previously and are the same ones for control SDA-PC and PCs that resulted in AEs.[18]

For this study, we selected 59 "control" SDA-PCs that were matched with each AE sample for the same storage time, collection processor, and nature of PLT AS, but were not associated with any declared AE. All collection procedures were performed in one of the two regional authorized settings. The samples were immediately shipped to

the unique processing platform of this blood establishment and distributed after qualification, which was generally between 3 and 5 days after collection (>75% by Days 4 and 5). The preparation procedure and the incident or accident declaration strictly conformed to the national protocols, as described in the previously reported study.[18] After the occurrence of an AE, immediately after declaration, the incriminated PC bags were shipped back to the blood establishment facility for immediate processing. If it was an overnight AE, the processing time never exceeded 12 hours. PLTs were sampled at the delivery time point for control PCs or at the time of incident declaration for the AE PCs.

In this study, we identified 42 PCs that were associated with Grade 3 AEs from 2008 to 2011 from more than 23,250 SDA-PCs produced and delivered during this period. All considered cases were scored as 3 (severe) according to the ISBT scaling system,[22] that is, necessitating medical assistance, with no Grade 4 (i.e., lethal) cases observed in this survey. The cases with accountability grades of 3 ("probable") and 4 ("certain")—in terms of accountability according to this international scale—were retained for the survey, and the "unlikely" and "possible" cases were discarded. Only patients who received only one blood component, for instance a PC, in the current transfusion episode were enrolled in this survey.

The diagnosis of inflammatory-type AEs was made on the immediate observation and report of 1) FNHTRs, generally manifesting with fever, rigors, and/or chills; 2) AAEs, which commonly involve erythematous rash, urticaria, and/or pruritus or more severe reactions with angioedema, but further discharged of typical allergic biology on the basis of serum tryptase, histamine, or IgE; and 3) in rare occasions, hypotension with tachy- or bradycardia. Those pathologies are, in general, considered associated with inflammatory cases.[23]

BRM measurement in PC supernatants

Control SDA-PC and PCs that resulted in AEs were shipped almost immediately to the blood establishment research facilities. The supernatants were collected within 12 hours and frozen (−80°C) until assay, with a maximum of 12 hours of elapsed time in the case of night AEs. We tested Gro-α, sCD40L, 6-Ckine (CCL21), CXCL9, IL-13, IL-15, IL-23, IL-33, MIP-1α, interferon (IFN)-γ, macrophage-derived chemokine (MDC), CCL19, CCL20, BCA-1, RANTES, sCD62P, and TSLP in the PC supernatants using Luminex technology, according to the manufacturer's instructions and as described previously.[18] Data (expressed in pg/mL) were adjusted to 10^9 PLTs.

mtDNA quantification in PLT concentrates

mtDNA was extracted from eluted anti-TOM22 processed samples with the DNA microextraction kit (QIAamp, QIAgen, Chatsworth, CA) according to the manufacturer's protocol and quantified by real-time quantitative polymerase chain reaction (PCR; CFX96 Touch, real time system, Bio-Rad, Marnes-la-Coquette, France) with the probe PCR kit (Rotor-Gene, QIAgen). Primers and probes (Integrated DNA Technologies, Coralville, IA) were used for specific amplification of human mtDNA (forward 5′-ACGCCTGAGCCCTATCTATTA-3′, reverse 5′-GTTGACCTGTTAGGGTGAGAAG-3′ and probe 5′/56 FAM/TGACAAGCG/ZEN/CCTATAGCACTCGAA/3IABkFQ/-3′). The quantitative PCR cycling condition consisted of an initial step of 95°C for 3 minutes followed by a two-step amplification of 95°C for 3 seconds and 60°C for 10 seconds (40 cycles). mtDNA extracted from PLTs was used for generation of standard curve (0.2 to 0.0016 ng/μL).

Statistical analysis

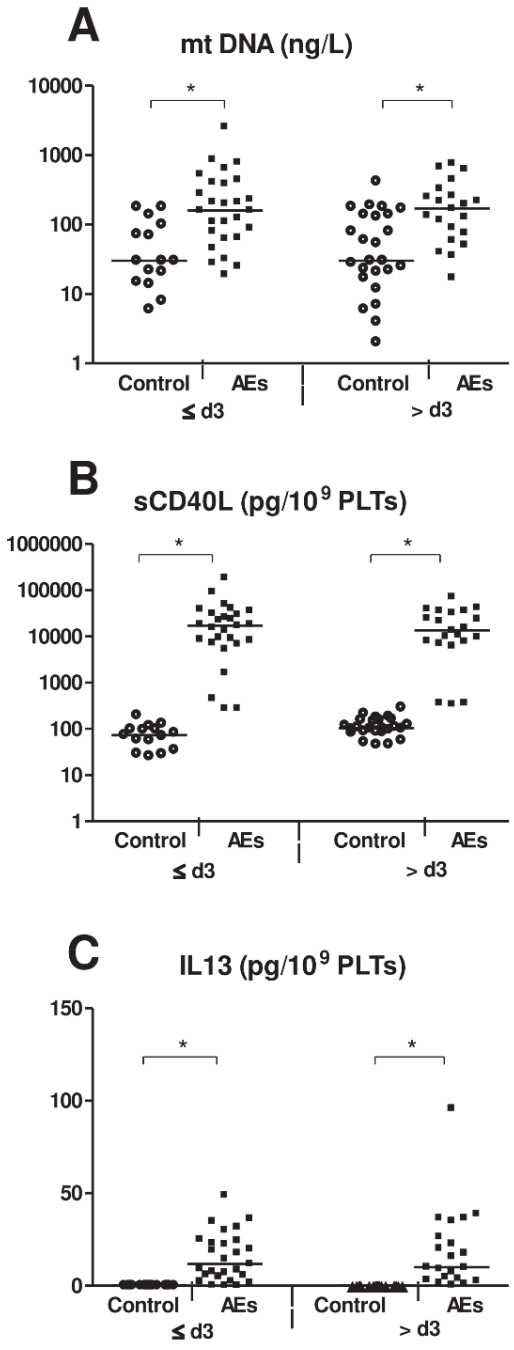
The concentrations of soluble factors and mtDNA were compared between the two groups using the nonparametric Kruskal-Wallis test, and analysis of variance (ANOVA) tests were performed to compare these concentrations per storage day. The p values were corrected for multiple comparisons using the Bonferroni correction. For each factor, the difference was considered significant if the p value was less than 0.05. Correlations between the variables were assessed using Pearson coefficients. A correlation was considered significantly different from zero when the p value was less than 0.05. Receiver operating characteristic curves were used to determine the cut-off values of soluble factor assays (Supplemental Data Fig. S1, available as supporting information in the online version of this paper), and the area under the curve (AUCs) were used to calculate the discriminatory ability of every candidate factor and classify them as potential sources of AEs. For each factor, a two-sample z-test was performed to test the null hypothesis. If the calculated p value was below the significance level ($\alpha = 0.05$), then the AUC was considered significantly different from 0.5 (null hypothesis, meaning no discriminating power). All statistics were calculated using computer software (XLSTAT, Addinsoft, Paris, France).

Decision-tree learning

We previously[18] adapted decision-tree learning for modeling of a policy of risk prevention for severe inflammatory outcomes in PC transfusion. Decision-tree learning is used in statistics, data mining, and machine learning and uses a decision tree as a predictive model that maps observations about an item to conclusions about the item's target value.

In this study, we analyzed a new matrix including mtDNA with a set of 101 training samples (from 42 AEs PCs and 59 control PCs) with 18 attributes (age of the blood sample, age of the donor; PLT count; and levels of Gro-α, sCD40L, 6-Ckine, CXCL9, IL-23, MIP-1α, IL-13,

IFN-γ, IL-15, MDC, IL-33, CCL19, CD62P, RANTES, and mtDNA). The matrix is designed to evaluate the risk associated with a given BRM if present, absent, or present in excess (three classes—AAEs, FNHTRs, and control).



RESULTS

mtDNA, sCD40L, and IL-13 found in leftover PCs that produced AEs

Over the investigation period, 42 inflammatory-type AEs were reported, which excluded obvious or later acknowledged cases of transfusion-transmitted bacterial infections, transfusion-related acute lung injury (TRALI), severe and documented (IgE dependent) allergy cases, and cases obviously linked to an antigen and antibody conflict. The variables linked to the PCs (42 "pathogenic" and 59 "nonnoxious") were described and characterized previously.[18]

Three days were considered to be the threshold for distinguishing "fresh" versus "old" PCs, and these groups were used for comparison of the amounts of proinflammatory BMRs. Interestingly, 80% of the surveyed AEs occurred with more than 3-day-old PCs ($p < 0.05$).[6,18,19,24]

mtDNA (Fig. 1A), sCD40L (Fig. 1B), and IL-13 (Fig. 1C) were significantly ($p < 0.05$) more elevated in SDA-PCs that produced AEs in the recipients than in the matched control PCs. sCD40L and IL-13 were tested as reference markers because of their previous association with AEs in both our experience and the experiences of several other groups.[6,12-15,25]

Elevation of mtDNA in supernatants of stored PCs over time

The levels of mtDNA were elevated on Day 4 (122.55 ± 52.64 ng/L) compared with Days 1 to 3 in control, nonpathogenic PCs (Fig. 2A), with a decrease on Day 5 (21.3 ± 7.15 ng/L) and return to baseline (Day 1) level (8 ± 8.93 ng/L). A similar profile was observed in PCs that were associated with AEs, but the peak of mtDNA was on Day 3 (484 ± 313.45 ng/L) and not Day 4 (233.95 ± 109.75 ng/L; Fig. 2A). The ANOVA tests performed to compare the levels of mtDNA per storage day show a significant ($p < 0.05$) variation during the storage concerning the control, nonpathogenic PCs but nonsignificant variation for PCs that were associated with AEs. Moreover, the p values were corrected for multiple comparisons using the Bonferroni correction and we noted only a significant difference for the levels of mtDNA on Day 5 compared with Day 4 in control, nonpathogenic PCs (Fig. 2A).

Fig. 1. Distribution (PCs delivered before 3 days [≤d3]or from 3 to 5 days [>d3]) of concentrations of the mtDNA (ng/L; A), sCD40L (pg/10^9 PLTs; B), and IL-13 (pg/10^9 PLTs; C) in the supernatants from 42 AE PCs and 59 control PCs. The data are represented with the median of the scattergram values. Concentrations in the control and AE samples were compared using two-tailed t test (*p < 0.05).

As PCs were collected by means of two distinct types of separators, we sought to examine the possibility that one process activates PLTs more than the other with respect to the release of mtDNA. No consistent difference between the Amicus and Trima processes was observed for either nonpathogenic (Fig. 2B) or pathogenic (Fig. 2C) PCs. No consistent difference between the Amicus and Trima processes was observed, with the exception of a higher Gro-α concentration on Days 3 and 4 in PCs prepared with the Amicus system and higher CCL19 concentration in PCs prepared with the Trima system; however, these variations were resolved by Day 5. The data were also similar regarding the PLT ASs used. We found no meaningful difference between the two processes in the soluble factors in the PCs that produced AEs.

PCs associated with serious AEs display characteristic profiles of secreted PLT factors and mtDNA

The fact that certain BRMs can be grouped into profiles and/or associate with clinical inflammatory reactions in PC transfusion has already been reported.[19] In this study we examined the possibility that at least some among the series of BRMs tested here are significantly linked with soluble mtDNA, as this factor was also identified in sterile inflammatory conditions; mtDNA was found positively correlated with pathogenic PC transfusions but its discovery was not at the time focused on transfusion and the sample size was not sufficient to give a significant result.[10] We thus investigated a potential correlation between PLT-soluble factors and mtDNA. mtDNA was significantly associated with none of the BRM tested, indicating that the mtDNA participation in PC transfusion-linked inflammation is BRM independent (Table 1).

Results from "learned decision trees"

AEs (n = 42) were divided as follows: we observed that in the not more than 3-day storage group, the majority of reported ATRs were AAEs (53%), followed by FNHTRs (34%) and hemodynamic trouble (13%). In the more than 3-day group, we observed that the majority of reported ATRs were FNHTRs (56%), followed by AAEs (37%) and hemodynamic trouble (7%). FNHTRs were less frequent (34% vs. 56%), whereas AAEs were more frequent (53% vs. 37%) when the PCs were "fresher." Because several AEs are defined at the same time as FNHTRs and hemodynamic trouble, we combined both samples for the analysis below.

Each group was analyzed with respect to mtDNA content (Supplemental Data Fig. S2A, available as supporting information in the online version of this paper), sCD40L (Supplemental Data Fig. S2B), and IL-13 (Supplemental Data Fig. S2C). A trend toward more AE cases with elevated mtDNA, sCD40L, and IL-13 levels was observed in patients

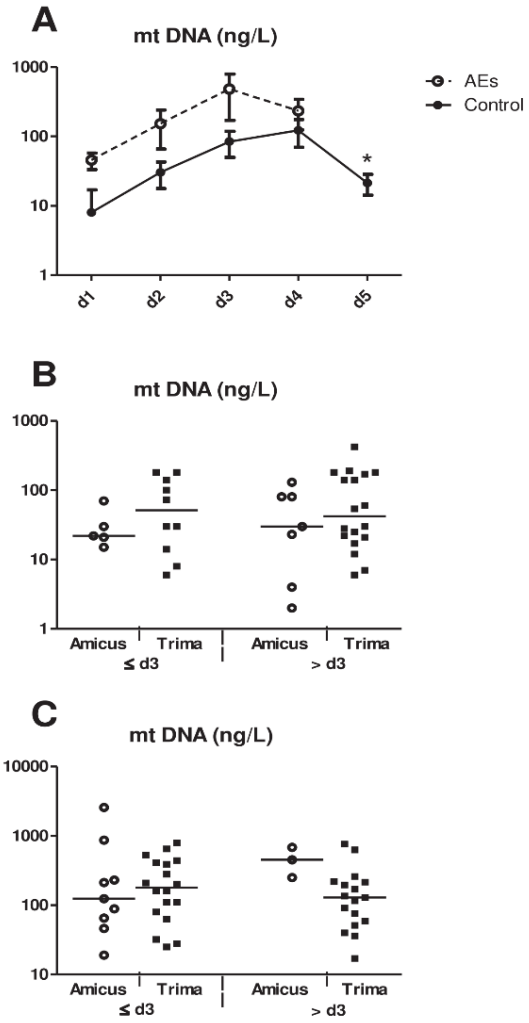


Fig. 2. Release of extracellular mtDNA during PLT storage for 5 days (d). The data are represented with the median of the scattergram values. (A) Factors that increased over 5 days of storage in the supernatant from control and pathogenic PCs samples. Differences of release of extracellular mtDNA during PLT storage for 5 days in the control PCs prepared by the Amicus and Trima processes during storage for control (B) and pathogenic (C) PC samples (no pathogenic PC samples stored for 5 days are included in this study). Release of extracellular mtDNA on Days 2 to 5 versus Day 1 in the same group were compared using ANOVA. The p values were corrected for multiple comparisons using the Bonferroni correction (*$p < 0.05$).

presenting with FNHTRs or hemodynamic trouble, than with AAEs, although this result did not reach significance.

We next calculated the AUCs, which represent the level of prediction of a given factor regarding the possible occurrence

TABLE 1. Pearson correlation of 14 soluble factors and extracellular mtDNA in the supernatants from 42 AE PCs and 59 control PCs

	Soluble factor														
Soluble factor	Gro-α	sCD40L	6-Ckine	CXCL9	IL-23	MIP-1α	IL-13	IFN-γ	IL-15	MDC	IL-33	CCL19	CD62P	RANTES	mtDNA (ng/L)
Gro-α	1.00														
sCD40L	**0.86***	1.00													
6-Ckine	0.09	0.08	1.00												
CXCL9	**0.29***	**0.27***	0.02	1.00											
IL-23	**0.27***	**0.14***	**0.63***	0.05	1.00										
MIP-1α	**0.74***	**0.66***	0.16	**0.29***	**0.38***	1.00									
IL-13	**0.63***	**0.62***	0.19	0.17	**0.43***	**0.91***	1.00								
IFN-γ	**0.59***	**0.54***	0.13	**0.29***	**0.32***	**0.90***	**0.86***	1.00							
IL-15	**0.64***	**0.64***	0.17	**0.13***	**0.33***	**0.89***	0.97	**0.87***	1.00						
MDC	**0.67***	**0.52***	**0.24***	**0.43***	**0.56***	**0.75***	**0.71***	**0.70***	**0.63***	1.00					
IL-33	**0.23***	0.15	**0.74***	0.10	**0.92***	**0.31***	**0.37***	**0.25***	**0.30***	**0.51***	1.00				
CCL19	**0.24***	0.14	0.45	**0.38***	**0.58***	**0.25***	**0.23***	**0.22***	0.13	**0.58***	**0.58***	1.00			
CD62P	**0.58***	**0.52***	0.12	0.21	**0.25***	**0.47***	**0.42***	**0.39***	**0.41***	**0.51***	**0.27***	**0.24***	1.00		
RANTES	**0.59***	**0.40***	**0.33***	0.20	**0.46***	**0.60***	**0.55***	**0.43***	**0.50***	**0.65***	**0.48***	**0.41***	**0.51***	1.00	
mtDNA	0.04	−0.05	0.05	0.05	−0.03	0.08	−0.04	0.00	−0.04	−0.05	−0.03	0.02	−0.05	0.10	1.00

* The bold values were different from 0 at a significance level of $\alpha = 0.05$.

TABLE 2. Discriminatory ability of CD40L, IL-13, and mtDNA to classify PCs as belonging to either the adverse effect or control group*

Factor	AUC	p value	Threshold
sCD40L	0.986	<0.0001	289.5 pg/10^9 PLTs
IL-13	0.961	<0.0001	0 pg/10^9 PLTs
mtDNA	0.798	<0.0001	20.34 ng/L

* All AUCs with a p value of <0.0001 (z test) were considered different from 0.5.

of AEs. The discriminatory ability of each factor was compared with 0.5 (random classifier), with a p value of less than 0.0001 (z test). When the AUC was different from 0.5, the AE occurrence was not random. High PLT supernatant levels of sCD40L, IL-13, and mtDNA displayed significant association with the occurrence of AEs compared with the control group. The cutoff value of each soluble factor test with the optimal sensibility and specificity is presented in Table 2.

To obtain a descriptive, interpretive model of the functional relationship between a given set of BRMs and mtDNA, selected by a cross-validated committee method, a decision tree was set up with the Weka platform. The decision tree describes several paths leading to leaves that assign a class to a new case, such as that depicted in Fig. 3. AEs are excluded when IL-13 is null. But when IL-13 is detectable (>0 pg/mL/10^9 PLTs), and depending on the mtDNA amount, there is a significant risk of AEs. Interestingly, the model further predicts that distinct AE subgroups could be distinguished, depending on the mtDNA level and the PLT storage length (Fig. 3).

DISCUSSION

For this study, we selected 1) 59 "control" SDA-PCs that were matched with each AE sample for the same storage time, collection processor, and nature of PLT AS, but were not associated with any declared AE; and 2) identified 42 PCs that were associated with Grade 3 AEs. One limitation of the study is the low number of donors or patients studied and the fact that we performed our study in a single regional French blood bank. The second limitation of this study concerns the absence of evaluation of pellet fraction of PLT concentrates, which contain inflammatory mediators and functional receptor. PLT receptors can be valuable in predicting AEs. These aspects would merit further investigation. Finally, this study investigates the PCs associated with adverse reactions, particularly the mtDNA and BRMs level.

BRMs and mtDNA generated in PCs during storage have been implicated as possible mediators of FNHTR and AAEs and may participate in the process of inflammation.[3,6,10,13,14,18,19,26-28] In this report we observed that mtDNA is released during the first days of storage and accumulates and then reduces, in both groups (controls and patients).

We observed an increase in the detectable concentration of mtDNA during PC storage on Day 4. After Day 4, the concentration of mtDNA reached the initial level. It is possible that this is due to degradation of mtDNA during storage, making detection impossible. Interestingly, the hypothetical mtDNA degradation during storage seems to occur earlier in PCs that are associated with reported AEs than in their counterparts not associated with AE. This phenomenon may depend on the level of mtDNA in PC samples or the mtDNA brittleness in PLTs in the different groups (patients and controls).

PLT supernatants with significant levels of sCD40L, IL-13, and mtDNA displayed significant association with the occurrence of an AE compared with PCs with no pathogenic outcome. The AUC of a factor was then used to

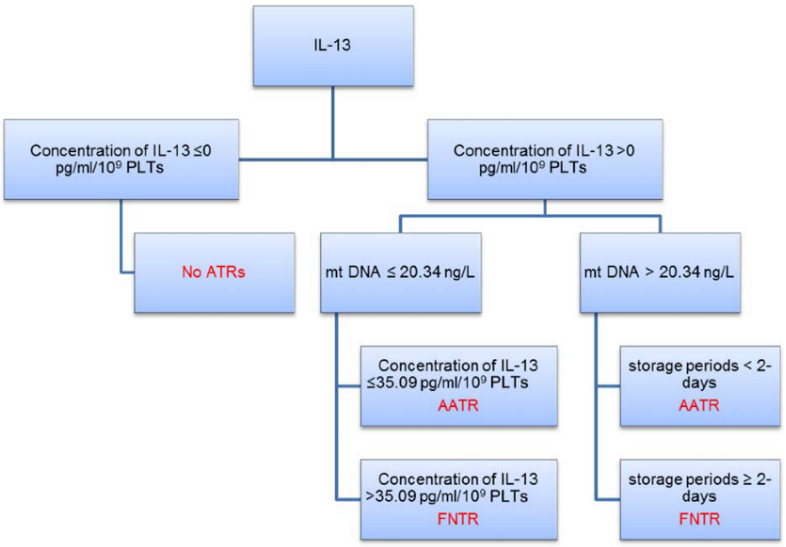


Fig. 3. Decision tree. Assays from a set of 101 training samples with 18 attributes (age of the blood sample; age of the donor; PLT count; and levels of Gro-α, sCD40L, 6-Ckine, CXCL9, IL-23, MIP-1α, IL-13, IFN-γ, IL-15, MDC, IL-33, CCL19, CD62P, RANTES, and mtDNA). We could predict the risk associated with a given cytokine if present, absent, or present in excess (three classes—AAEs, FNHTRs, and control), which allows the forecasting of unfavorable outcomes in patients. The success rate of the IL-13 model was the highest: 83%.

illustrate its performance to classify the PCs into two categories (control or AE associated) and to select the best cutoff values for the BRMs in the PC supernatants associated with AEs. Also, it appears that the AUC relative to mtDNA is less stringently predictive than those for IL-13 or sCD40L, but the p value is still highly significant (z test; <0.0001). This slight difference in prediction strength may be due to the independence of mtDNA from BRMs; this attribute thus renders the information extremely valuable (Supplemental Data Fig. S1).

This work also demonstrates that BRMs and mtDNA, as secreted or released products, are not stochastic but rather display significant associations with AEs. Moreover, no significant correlation was found between any tested BRM and mtDNA in reported AEs. Besides, this study illustrates that the association of certain BRMs with mtDNA are not only active for triggering an inflammatory response in the recipient when their amounts exceed a threshold, but are also critical in the clinical distinction of the AE symptoms.

Because it is expected that conditions that render a PC preparation potentially able to produce an AE can be identified before the PC being issued, we developed a statistical model to predict the safety or the pathogenicity of a given PC. Even if these findings cannot be implemented in practical clinical medicine, they reinforce the idea that PLTs have BRM and mtDNA secretion programs that are not simply stochastic but also contribute in altering the recipient's physiology and ultimately precipitating pathology. Some of the major challenges for proteomic studies is the knowledge of mining biologically useful information from the in vitro, ex vivo, or in vivo data. Furthermore, recent statistical methods for big data analysis need to be performed in transfusion context. The mathematical model algorithm seems to be used poorly in the transfusion context as blood involves a large amount of biological data involving complicated parameters from the classic triad of transfusion: the donor or labile blood components and processing attributes or recipient.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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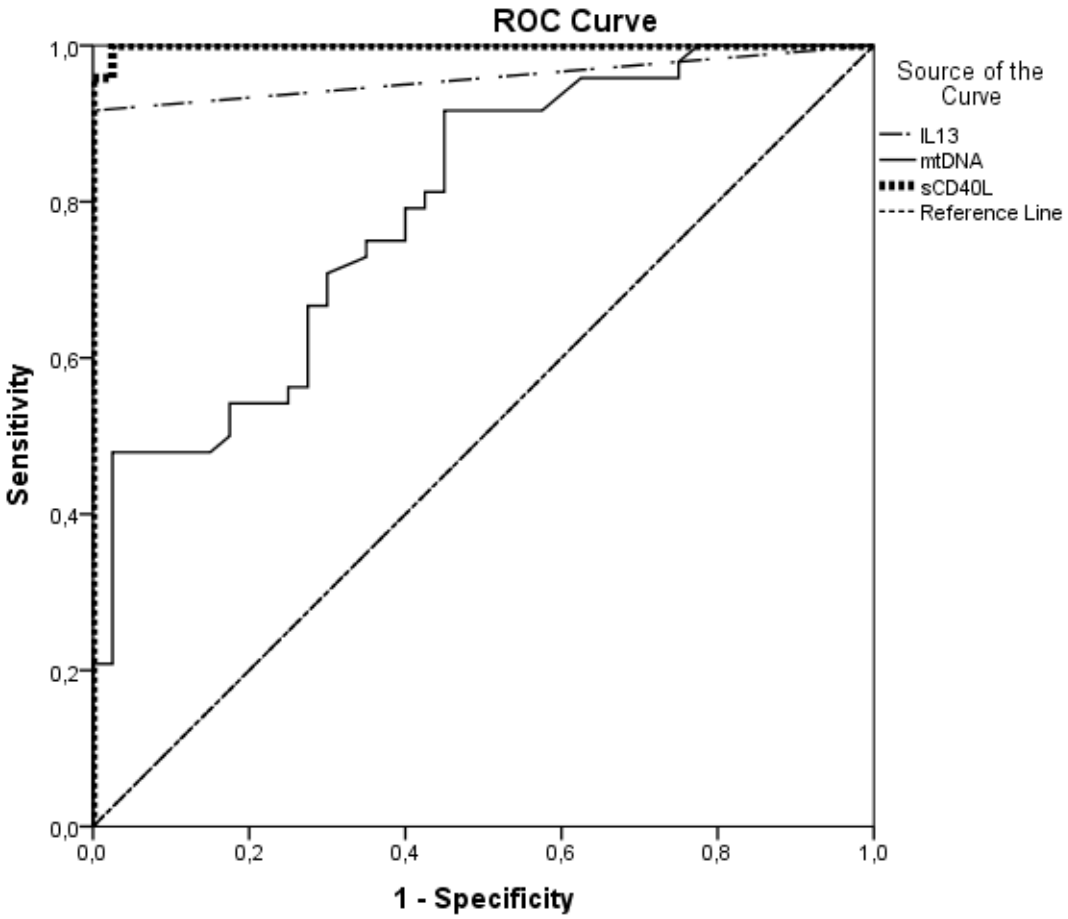
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Areas under the ROC curves: IL-13; mtDNA, sCD40L.

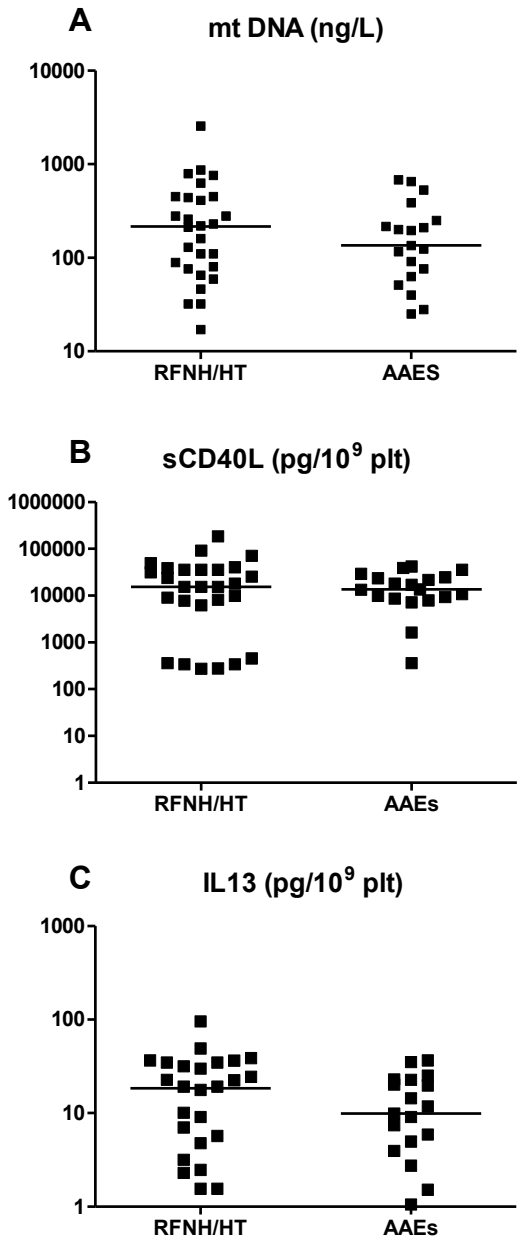
Fig. S2. Distribution of clinical observations of AEs resulting from a platelet transfusion with regard to mitochondrial DNA (ng/L) (A), sCD40L (pg/10^9 platelets) (B) and IL-13 (pg/10^9 platelets) (C). FNHTR, febrile non-hemolytic transfusion reaction (fever or chill) combined with hemodynamic trouble (HT); AAEs, atypical allergic AEs (erythematous rash, urticaria, and/or pruritus or more severe reactions with angioedema); excluding ALI (and TRALI), Transfusion-Associated Circulatory Overload, myocardial infarctions, and pulmonary embolism. The data are represented with the median of the scattergram values.

Supplemental data sFig 1



sFig 1. Areas under the ROC curves: IL13; mtDNA, sCD40L

Supplemental data sFig 2



sFig 2. Distribution of clinical observations of AEs resulting from a platelet transfusion with regard to mitochondrial DNA (ng/L) **(A)**, sCD40L (pg/10^9 platelets) **(B)** and IL-13 (pg/10^9 platelets) **(C)**. FNHTR, febrile non-hemolytic transfusion reaction (fever or chill) combined with hemodynamic trouble (HT); AAEs, atypical allergic adverse events (erythematous rash, urticaria, and/or pruritus or more severe reactions with angioedema); excluding ALI (and TRALI), Transfusion-Associated Circulatory Overload, myocardial infarctions, and pulmonary embolism. The data are represented with the median of the scattergram values.

V. Article 5 : Intéraction leucocytes-plaquettes et lésions de stockage

Leukocyte cytokines dominate over platelet cytokines overtime in non-leukoreduced platelet components.

Chaker Aloui[1,2], Tahar Chakroun[3], Antoine Prigent[1], Saloua Jemni-Yacoub[3], Fabrice Cognasse[1,2], Sandrine Laradi[1,2] and Olivier Garraud[1,4]

BLOOD TRANSFUSION

Impact Factor (2014): 2.372

Dear **Mr. Chaker ALOUI**,
I am pleased to inform you that your manuscript **076-16** entitled **Leukocyte cytokines dominate over platelet cytokines overtime in non-leukoreduced platelet components.** has been accepted on **06/06/2016** for publication in Blood Transfusion.
Your manuscript will now be edited by our scientific editor and you will then be sent the galley proofs for your approval.
Your work will be first pre-published on our web site and afterwards published in the printed issue of the Journal.

Thank you for your collaboration.
Yours sincerely,

Luisa Stea
Editorial Office
Blood Transfusion

Cette étude a été réalisée dans le cadre d'une collaboration entre l'Etablissement Français du Sang de Saint-Etienne et le Centre de Transfusion de Sousse, Tunisie.

Notre objectif était d'étudier l'influence des leucocytes résiduels et de leurs sécrétions – connues pour être abondantes – sur le programme sécrétoire des plaquettes dans des concentrés plaquettaires unitaires non-leucoréduits.

Pour ceci, nous avons caractérisé la cinétique de 6 BRM au cours du stockage ainsi que les interactions plaquettes-leucocytes.

ORIGINAL ARTICLE

Leucocyte cytokines dominate platelet cytokines over time in non-leucoreduced platelet components

Chaker Aloui[1,2], Tahar Chakroun[3], Antoine Prigent[1], Saloua Jemni-Yacoub[3], Fabrice Cognasse[1,2], Sandrine Laradi[1,2], Olivier Garraud[1,4]

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Background. Leucoreduction of blood components, including platelet components, is strongly encouraged but not yet universal, especially outside high income countries. As both leucocytes and platelets secrete copious amounts of pro-inflammatory cytokines/chemokines under various conditions and during storage, we investigated the potential of the respective secretory programmes of these cells in order to evaluate their subsequent pathophysiological effects.

Material and methods. A total of 158 individual non-leucoreduced platelet components were obtained from Tunisian donors and tested for characteristic biological response modifiers (BRM) of leukocytes (IL-1β, IL-8), platelets (sCD62P, sCD40L) and both cell types (TNF-α, RANTES) in the presence or absence of thrombin stimulation and after different periods of storage (up to 5 days). BRM levels were determined using enzyme-linked immunosorbent assays and Luminex technology. Platelet-leucocyte aggregate formation during storage was analysed using flow cytometry.

Results. Leucocyte- and platelet-associated BRM had clearly distinct profiles both at the onset (day 0) and termination (day 5) of the observation period but altered during the intermediate period so that their respective importance was inverted; in fact, the profiles were merged and indistinguishable on days 2-3. The leucocyte-derived BRM largely dominated over platelet-derived ones and further altered the BRM platelet secretion programme.

Discussion. This study contributes substantial, new information on leucocyte/platelet interactions and their likely role in transfusion when leucodepletion cannot be performed or is only partially achieved.

Keywords: transfusion, biological response modifiers, leucocyte/platelet interaction.

Introduction

Leucoreduction has become the standard for blood component processing in Europe and in many high income countries[1-5]. Systematic leucoreduction has demonstrated advantages of reducing alloimmunisation, carriage of viruses and transfusion-related immunomodulation[6,7]. Leucoreduction consistently lowers the amount of pro-inflammatory cytokines and chemokines, released predominantly from granulocytes and monocytes, which are largely responsible for transfusion-associated disorders (chills, moderate fever, rigors, etc.)[8]. However, platelets themselves, especially when ageing (stored for more than 3 days), also release substantial quantities of cytokines and chemokines[9,10]. Despite accounting for only 10% of transfused blood components, in settings of stringent leucoreduction, platelet transfusions are responsible for almost 25% of recorded adverse events and 50% of serious adverse events[11]. We and others have extensively evaluated the evolution of platelet-derived biological response modifiers (BRM) in leucoreduced platelet components (PC), and explored their involvement in the development of platelet transfusion adverse events[12-14]. The results cannot, however, be extrapolated to PC prepared from non-leucoreduced blood donations, as occurs in many lower income countries in which systematic leucoreduction is not performed. This is particularly the case when PC comprise whole blood-derived platelet-rich plasma (PRP), as in Tunisia, among other countries[15]. PC obtained from such a production process contain residual leucocytes. The coexistence of platelets and leucocytes in a potential platelet-activating environment initiates an interaction that can influence the secretory programmes of both types of cells. Certain cytokines/chemokines and, in general, BRM secreted by leucocytes and platelets in blood components are common, but the majority are acknowledged to be fairly specific to one or other of the types of cells[16,17].

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Through the assessment of BRM characteristic of leucocytes (interleukin-1β [IL-1β], interleukin 8 [IL-8]), platelets (sCD62P, sCD40L) and both cell types (tumour necrosis cell factor-α [TNF-α], regulated on activation, normal T cell expressed and secreted [RANTES]) and the measurement of platelet-leukocyte aggregates, we investigated the mutual influence of leucocyte- and platelet-released BRM in stored PC (individual whole blood-derived PRP), with two clearly distinct objectives: Firstly, to further explore the pathophysiological process that can lead to adverse events, including severe ones such as transfusion-related acute lung injury and severe febrile non-haemolytic transfusion reactions, as there are still gaps in the fine understanding of the pathogenic sequences[18]; and secondly to identify strategies that could reduce the impact of BRM in PC transfusions and that could be applied, despite limited resources, in medium-income countries.

Materials and methods

Platelet component preparation

The PC were prepared from whole blood donations collected from healthy donors. Informed consent was obtained from all participants and the *ad hoc* Ethics Committee of the Regional Blood Transfusion Centre at "F. Hached" Hospital (Sousse, Tunisia) approved the protocol. Blood was collected into triple blood bags (JMS Co. Ltd, Ang Mo Kio, Singapore) with acid-citrate-dextrose as the anticoagulant. The PC were isolated by a standard, two-step centrifugation method, as previously described by Bouslama *et al.*, and could be issued if they met all required testing parameters and quality controls[15]. Plasma removal and platelet additive solutions were not used and the PC were stored as unitary PC (UPC).

Testing protocols and sampling procedures

The samples used in this study were obtained by methods that preserved the sterility of the UPC so that they remained valid for distribution to patients. For each studied UPC, two samples were obtained. The first one was obtained immediately after the production process (day 0) as follows: after 1 hour of stirring, a 3 mL sample was moved into a quick transfer bag (VSE 0000A, MacoPharma, Mouvaux, France) that was aseptically connected to the bag containing the PC using a sterile connection device (Terumo Europe, Middle East & Africa, Leuven, Belgium). The second sample was obtained on a specified day (day 1, 2, 3, 4 or 5) of the UPC storage using the "stripping" method[19]. Each sample was subsequently divided into two aliquots, one for functional stimulation and analysis and one for determining the final concentrations of platelets, residual leucocytes, and contaminating red blood cells using a Beckman Coulter AcT 10 Hematology Analyzer (Beckman Coulter Inc., Paris, France).

A 600 µL volume from each sample was added to an equal volume of fixative solution (Thrombofix® Platelet Stabilizer 6607130, Beckman Coulter Inc.). This solution stabilises platelets and prevents their activation. The mixture was incubated for 1 hour at room temperature and then centrifuged at 450 *g* for 10 minutes. The supernatants were stored at −80 °C (unstimulated samples).

To a second 600 µL aliquot, we added 50 µL of thrombin receptor activator peptide (TRAP; Peptide SFLLRN, Sigma-Aldrich Chemie, Saint-Quentin Fallavier, France; 66.85 µM) for 30 minutes at room temperature and then added 650 µL of Thrombofix. One hour later, the mixture was centrifuged and the supernatants were frozen at −80 °C until further use (stimulated samples) (Online Supplementary Figure S1).

Quantification of biological response modifiers

The frozen PC supernatants were assayed using human enzyme-linked immunosorbent assays (ELISA) to measure sCD62P and RANTES (R&D Systems Europe Ltd., Abingdon, UK). The absorbance at 450 nm was measured with an ELISA microplate reader (Multiskan, Thermo Scientific, Illkirch, France). sCD40L, IL-8, IL-1β and TNF-α were measured by the multiplex method using Luminex technology, according to the manufacturer's instructions (Milliplex Map Kit Millipore, Darmstadt, Germany).

Analysis of platelet-leucocyte aggregates

Platelet-leucocyte aggregates were assessed in six supplementary UPC by means of flow cytometry. Samples of 5 mL, without volume replacement, were taken from each UPC on days 0, 1, 2, 3, and 5 for analysis. Samples were diluted 100-fold in phosphate-buffered saline and analysed under unstimulated conditions using Thrombofix® fixative solution. One hundred microlitres of each aliquot were incubated, for 15 minutes in the dark at room temperature, with 5 µL of a mouse monoclonal antibody to human CD41 conjugated to fluorescein isothiocyanate (FITC) (R&D system) and 5 µL of allophycocyanin (APC)-conjugated mouse monoclonal antibody to the human leucocyte common antigen CD45 (BD Biosciences, Paris, France). Mouse IgG1 FITC and APC isotype controls (BD Biosciences) were used at saturating concentrations as the negative controls. Stained samples were diluted 4-fold in phosphate-buffered saline and analysed in a flow cytometer (EPICS XL, Beckman Coulter). Platelet-leucocyte aggregates were identified as particles positive for both CD45 and CD41 and expressed as the percentage of total leucocytes.

Statistical analyses

XLSTAT (Addinsoft, Paris, France) and GraphPad Prism version 5.00 for Windows (San Diego, CA, USA) were used for statistical evaluation of the data. The Kolmogorov-Smirnov test was used to check for the normal distribution of the data. To compare paired or unpaired data of the PC samples during storage, we used the Wilcoxon signed-rank or Kruskal-Wallis tests, respectively, for non-parametric comparisons of cytokine levels at the different studied conditions. Principle component analysis for the visualisation of BRM correlations was performed using Spearman's correlations. P-values less than 0.05 are considered statistically significant.

For statistical reasons, samples stored for 1 or 2 days were grouped together, as were those stored for 3 or 4 days.

Results

Characteristics of the unitary platelet components

All 158 UPC, which originated from an equal number of donations from healthy volunteers (52 males and 106 females), were assessed for changes in platelet concentration, leucocyte contamination and pH. No significant changes in these parameters were observed between day 0 and day 5 and the samples were comparable between units and over time (Online Supplementary Figure S2).

The release (secretion) of a pre-defined set of BRM was tested for each UPC at day 0 and at the time of the units' issue. Soluble CD62P and sCD40L were selected for measurement because they are characteristic BRM of platelets; RANTES and TNF-α, because they are principally characteristic of platelets, although not completely absent from leucocytes; and IL-1β and IL-8, because they are chiefly characteristic of leucocytes. Online Supplementary Table SI shows the respective contributions of platelets and leucocytes to the overall amount of the six selected BRM. Importantly, platelets have been reported to secrete all six analysed BRM, although their release profiles probably differ from those of leucocytes[17,20].

Kinetics of release of biological response modifiers depending on time of storage

Figure 1 shows that the BRM secretion profiles can be grouped in pairs: sCD62P and TNF-α increased almost linearly over time, while sCD40L and RANTES peaked at days 3+4 and declined slightly, but not significantly, on day 5. IL-1β and IL-8 were nearly undetectable at day 0, began to be detectable on days 1+2, and were secreted fairly abundantly after day 2. There was a non-significant increase of IL-1β on day 5 over days 3+4, whereas there was a sustained and statistically significant increase of IL-8 on day 5 over days 3+4.

As reported, BRM are not specific but simply characteristic of certain cell types. Thus, we sought to investigate whether their secretion profiles could be determined. This could not be done based on the individual secretion assays for technical reasons, so we used a statistical (mathematical) model. Correlations between BRM release during storage were analysed through principle component analysis (Figure 2 and Table I). On day 0, two secretion profiles, separated by TNF-α, could be observed (Figure 2A): IL-1β and IL-8 in the upper right quadrant and RANTES, sCD62P and sCD40L below. There was a statistical correlation between TNF-α and the other five BRM (Table I). Moreover, IL-1β and IL-8 were statistically correlated but IL-8 did not correlate with sCD62P, RANTES and sCD40L, while IL-1β had no correlation with sCD62P but did exhibit a correlation with the other two BRM. On days 1+2, sCD62P dissociated from sCD40L and IL-1β dissociated from IL-8. The graphic representation (Figure 2B) clearly shows that sCD40L and IL-1β exchanged positions. On days 3+4, sCD62P re-associated with sCD40L, IL-1β and TNF-α, while RANTES dissociated from IL-1β and TNF-α. Indeed, RANTES secretion clearly polarised and opposed another polarisation: that of IL-8 and sCD62P (Figure 2C). Finally, on day 5, there were again two profiles with TNF-α returning to an intermediate position, similar to that on day 0, still dividing the RANTES+sCD40L+sCD62P and IL-1β+IL-8 secretion, but with these now in the opposite positions from day 0 (Figure 2D). This strongly suggests that platelet and leucocyte BRM secretion profiles are distinct from one another on days 1 and 5, while there are intricate associations in between, with possible cascade interactions (not assessed here).

Kinetics of platelet-leucocyte aggregate formation

As shown in Figure 3, the formation of platelet-leucocyte aggregates varied very little during the first 2 days of PC storage, then increased on days 3 and 5 although the differences were not statistically significant.

Reactivity of platelets to PAR1 activation in a platelet-rich plasma suspension with leucocytes

We next examined whether the presence of leucocytes in PRP suspensions, representing UPC, altered platelet reactivity to a thrombin analogue, TRAP (SFLLRN), which signals through platelet-expressed platelet-activating receptor 1 (PAR1). Because the present investigation was focused on platelet/leucocyte interactions, we measured BRM secretion in response to non-leucodepleted UPC, comprising platelets and contaminating leucocytes.

There is little evidence from the literature that cells other than platelets express PAR1 and are sensitive to

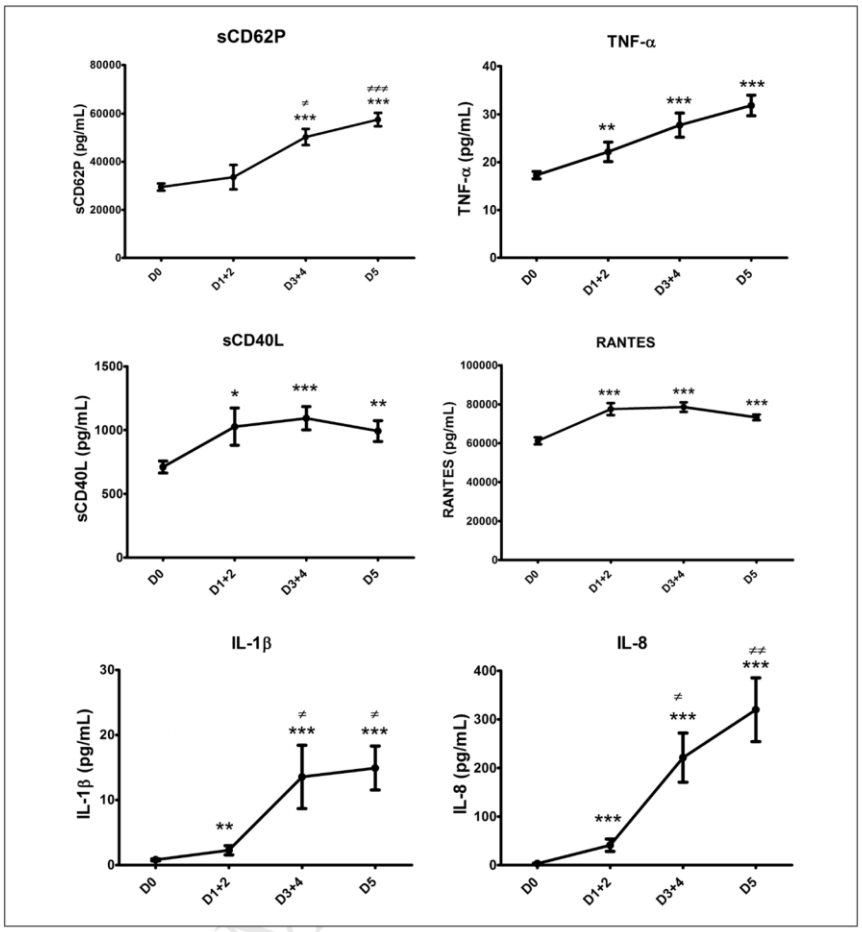

Figure 1 - Kinetics of BRM release depending on the day of storage.
* or ≠: p<0.05; ** or ≠≠: p<0.001; *** or ###: p<0.0001. Asterisks and hashtags represent significant differences against D0 and D1, respectively, using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. Data are presented as mean ± SEM; n=158 , 29, 65 and 64 for D0, D1+2, D3+4 and D5, respectively. D: day; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T cell expressed and secreted; IL: interleukin; SEM: standard error mean.

thrombin[21-24]. Despite our attempts, we failed to identify PAR1 on leucocytes in this series of experiments, but we do not rely unquestionably on these findings, as the working conditions (reagents) and controls appeared variable and did not lead to clear-cut data (not shown).

As shown in Figure 4, the exposure of platelets to TRAP during UPC storage still resulted in sustained production of sCD62P, TNF-α, sCD40L and RANTES. Furthermore, IL-8 and IL-1β secretion was elicited at the onset of the UPC process but only in minute amounts (although significantly different from the controls). However, while the secretion of these cytokines was more abundant beyond day 3, it was independent of TRAP stimulation; thus, one can hypothesise that minute mounts were mobilised from platelets early after stimulation, while the later, large amounts derived from the leucocytes in the stored PC. The responses of all BRM decreased after day 0 and remained stable (Figure 5).

Discussion

In this study, we evaluated the secretion of six BRM over time from platelets and residual leucocytes in non-leucodepleted individual PRP, constituting PC for transfusion in a medium income country (Tunisia). As was expected and in contrast to what is usually observed in PC obtained from leucodepleted-apheresis or from buffy coats, we found that there was a non-negligible production of BRM attributable to leucocytes, i.e., IL-1β and IL-8, and to a lesser extent, TNF-α. All three of these BRM can be produced by stored platelets in PC, but generally in less copious amounts[17,25]. Notably,

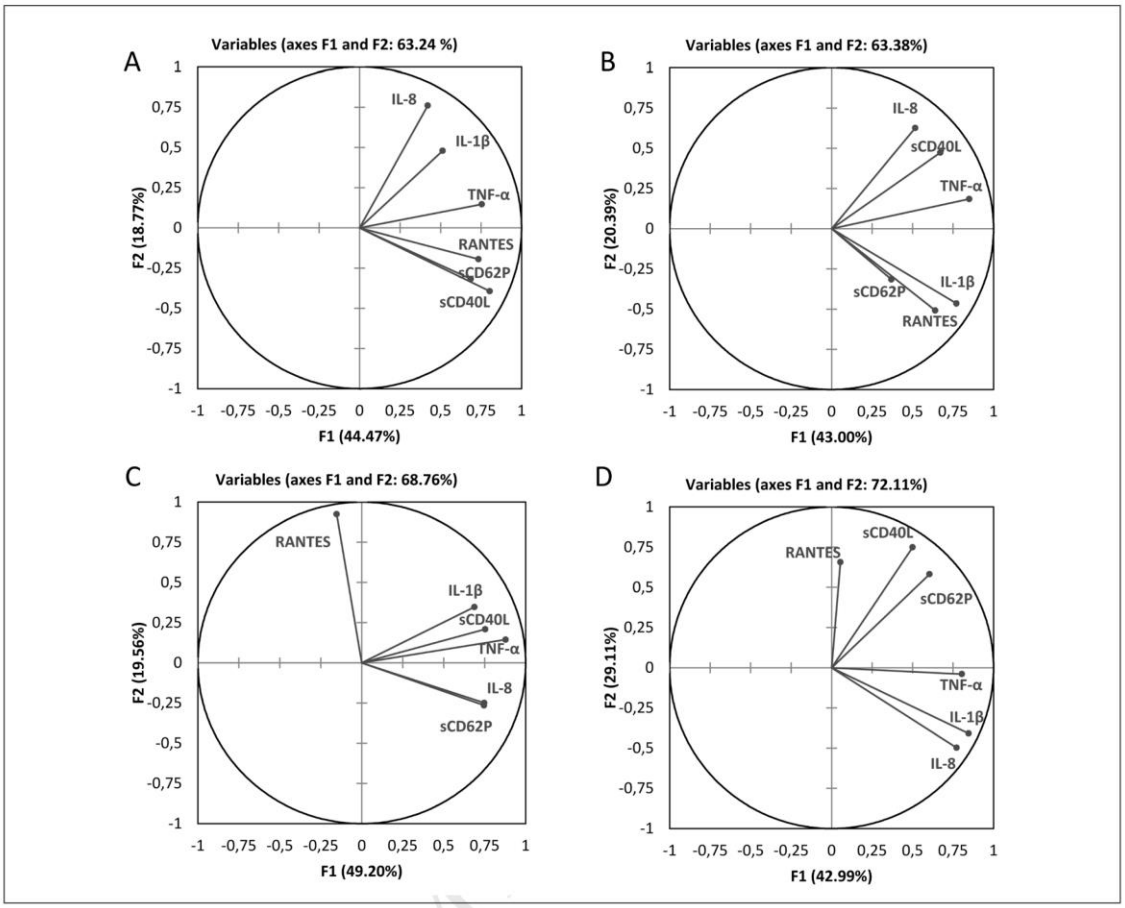

Figure 2 - A two-dimensional correlation monoplot of the coefficients of the first two principal components, showing relationships between the BRM during storage.
The correlation monoplot has vectors pointing away from the origin to represent the original variables. The angle between the vectors is an approximation of the correlation between the variables. A small angle indicates that the variables are positively correlated, an angle of 90 degrees indicates the variables are not correlated, and an angle close to 180 degrees indicates the variables are inversely correlated. The length of the line and its closeness to the circle indicate how well the variable is represented in the plot. A, B, C and D illustrate correlations at D0, D1+2, D3+4 and D5, respectively. D: day; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T cell expressed and secreted; IL: interleukin.

the profile of platelet-secreted BRM, as demonstrated by the "standard" sCD62P and sCD40L as well as by RANTES, was largely comparable to what is commonly observed[26-28]. This led to the first impression that non-leucodeleted PC carry both platelet- and leucocyte-BRM-associated inflammatory risks[13,29-31]. Because a canonical property of cytokines/chemokines (and likely mediators, which largely constitute the BRM under investigation in this study) is their action in cascades (along with redundancy), we explored the influence - if any - of leucocyte-derived BRM on the platelet secretory programme to assess whether there is indeed a cumulative inflammatory risk. In fact, most leucocyte-derived BRM have ligands on platelets and most platelet-derived BRM have ligands on leucocytes[8,32-34]. We showed a clearly individualised BRM profile at the onset of PC processing, with sets of statistically associated leucocyte BRM and platelet BRM on day 0, with TNF-α, produced by both cell types, in an intermediate position and associated with both. On day 5, just prior to the expiry date of the PC, polarised sets of leucocyte- and platelet-BRM (with an intermediate association with TNF-α) were observed again, although in inverted positions from day 0 (leucocyte-BRM secretion was copious and seemed to dominate the picture). In the period between days 0 and 5, there was an interplay between the leucocyte- and platelet-BRM associations, suggesting that products of one cell type influence the secretion of products by the other type and *vice versa*. One limitation of this study is that BRM could not be

Table I - Spearman's correlations between BRM levels during PC storage.

Variables	sCD62P D0	RANTES D0	CD40L D0	IL-1β D0	TNF-α D0	IL-8 D0
sCD62P D0	*1/0*	*0.387/<0.0001*	*0.514/<0.0001*	0.144/0.071	*0.417/<0.0001*	0.141/0.078
RANTES D0		1/0	0.596/<0.0001	0.359/<0.0001	*0.307/<0.0001*	0.152/0.057
CD40L D0			1/0	0.208/0.009	0.552/<0.0001	0.063/0.428
IL-1β D0				*1/0*	*0.272/0.001*	*0.287/<0.0001*
TNF-α D0					*1/0*	*0.392/<0.0001*
IL-8 D0						*1/0*
Variables	**sCD62P D1+2**	**RANTES D1+2**	**CD40L D1+2**	**IL-1β D1+2**	**TNF-α D1+2**	**IL-8 D1+2**
sCD62P D1+2	*1/0*	0.144/0.453	−068/0.723	0.347/0.065	0.155/0.419	0.268/0.159
RANTES D1+2		*1/0*	0.275/0.147	*0.579/0.001*	*0.382/0.041*	0.00/1.00
CD40L D1+2			*1/0*	0.283/0.135	*0.570/0.001*	*0.399/0.033*
IL-1β D1+2				*1/0*	*0.560/0.002*	0.099/0.608
TNF-α D1+2					*1/0*	*0.439/0.018*
IL-8 D1+2						*1/0*
Variables	**sCD62P D3+4**	**RANTES D3+4**	**CD40L D3+4**	**IL-1β D3+4**	**TNF-α D3+4**	**IL-8 D3+4**
sCD62P D3+4	*1/0*	−0.235/0.06	*0.490/<0.0001*	*0.304/0.014*	*0.555/<0.0001*	*0.475/<0.0001*
RANTES D3+4		*1/0*	0.049/0.699	0.095/0.449	−0.045/0.719	*−0.249/0.045*
CD40L D3+4			*1/0*	*0.361/0.003*	*0.647/<0.0001*	*0.396/0.001*
IL-1β D3+4				1/0	*0.600/<0.0001*	*0.441/<0.0001*
TNF-α D3+4					*1/0*	*0.526/<0.0001*
IL-8 D3+4						*1/0*
Variables	**sCD62P D5**	**RANTES D5**	**CD40L D5**	**IL-1β D5**	**TNF-α D5**	**IL-8 D5**
sCD62P D5	*1/0*	0.244/0.052	*0.644/<000.1*	*0.249/0.047*	*0.328/0.008*	0.188/0.137
RANTES D5		*1/0*	*0.334/0.007*	−0.085/0.502	−0.080/0.526	−0.119/345
CD40L D5			*1/0*	0.085/0.501	*0.418/0.001*	−0.0330.791
IL-1β D5				*1/0*	*0.586/<0.0001*	*0.875/<0.0001*
TNF-α D5					*1/0*	*0.484/<0.0001*
IL-8 D5						*1/0*

Values are presented as correlation matrix/p-value. The italic values were statistically different from 0 at a significance level of p<0.05. D: day; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T cell expressed and secreted; IL: interleukin.

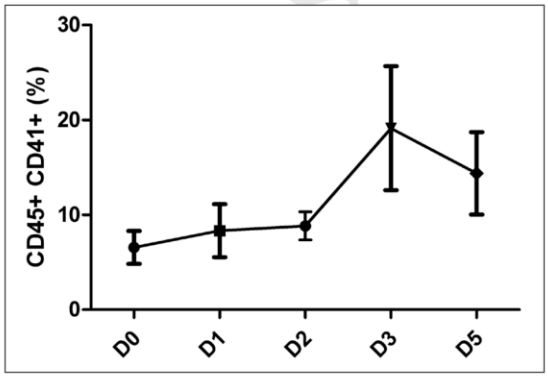

Figure 3 - Leucocyte-platelet aggregate formation during PC storage. Complexes were detected by flow cytometry using allophycocyanin (APC)-conjugated anti-CD45 (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated anti-CD41 (R&D system). Data are presented as mean ± SEM; n=6. PC: platelet concentrate; D: day; SEM: standard error mean.

measured along storage in the same PC. However, we found no significant variations in platelet and leucocyte contents between the PC groups analysed each day of storage.

Recently, there has been a flurry of publications highlighting the importance of platelet-leucocyte aggregates, which have proven to be essential in both physiology (haemostasis and clot stability[35,36]) and pathology (formation of neutrophil extracellular traps in sepsis, to cite one[37]). An intricate programme for platelet-leucocyte aggregate formation compared to the programmes for solely leucocytes or platelets would not be a complete surprise. We examined this issue closer, still in the context of UPC for transfusion. However, another limitation of this study is that this investigation could not be conducted on the series of samples described above, and data were obtained from pilot experiments carried out on an additional series of volunteer donations (n=6).

We obtained evidence that platelet-leucocyte aggregate formation increased between days 3 and 4, although differences were not statistically significant. As this increase paralleled the increase of sCD62p (indicating platelet activation) and the secretion of IL-1β and IL-8, our data may indicate not only the leucocyte origin of these BRM but also the probable mutual influence of both cell types (Figures 1 and 3). This platelet-leucocyte aggregate formation does not seem to alter the capacity of platelets to respond to external stimuli when needed and probably aids haemostasis.

We next examined the capacity of platelets, bathed in leucocyte-derived BRM, to react to haemostatic signals. The platelets maintained their secretory potential of platelet-associated BRM over time from day 0 to day 5 when they were exposed to TRAP (thrombin). On day 3 and later, IL-1β and IL-8 levels were high, irrespectively of TRAP stimulation, suggesting that these are platelet independent. While this specific point was not addressed in this study and it is likely that the ageing of PC is detrimental considering the cumulative risks posed by the inflammatory molecules that will be infused along with the therapeutic components (the platelets), ageing does seem compatible with continued reactivity of platelets to thrombin.

The present study examined the BRM responses of platelets in PRP as non-leucoreduced PC for transfusion. It confirmed that pro-inflammatory cytokines tend to accumulate in ageing PC stored under normal conditions; these cytokines create an inflammatory risk to the recipient and may also reduce the efficacy of the PC, as deduced from numerous other studies[8,38-42]. Furthermore, in non-leucodepleted conditions, leucocyte-derived BRM also accumulate with peaks reached gradually at the time the PC approaches its expiry date, but with considerable levels already by day 3 and onwards. These

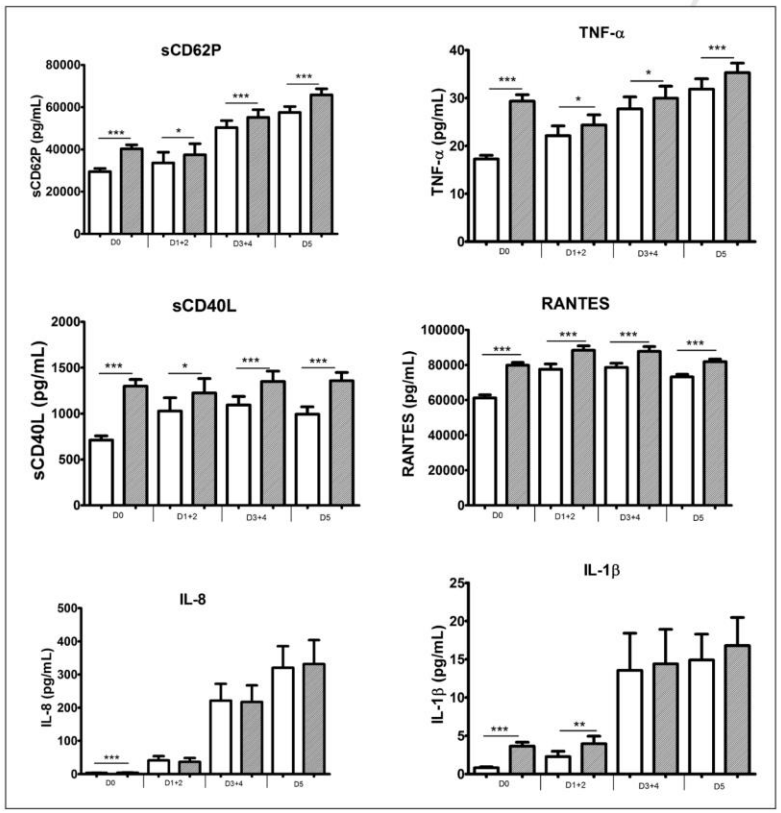

Figure 4 - Secretory profiles of BRM in response to TRAP stimulation.
Comparisons used the mean of the Wilcoxon sign ranked test for paired samples. White boxes represent the unstimulated condition and grey boxes represent the TRAP-stimulated condition. Data are presented as mean ± SEM; n=158, 29, 65 and 64 for D0, D1+2, D3+4 and D5, respectively. * $p<0.05$; ** $p<0.001$; *** $p<0.0001$. BRM: biological response modifier; TRAP: thrombin receptor activator peptide; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T cell expressed and secreted; IL: interleukin.

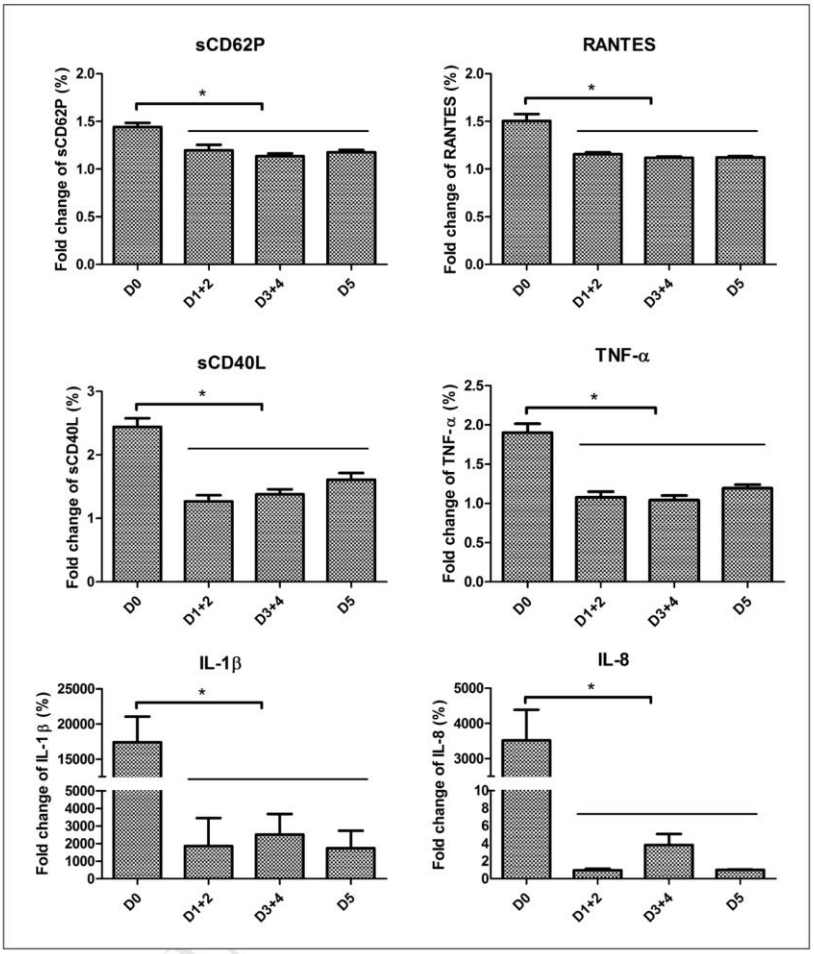

Figure 5 - Fold-changes of BRM levels in response to TRAP stimulation depending on storage time.
Data are presented as mean ± SEM; n=158 , 29, 65 and 64 for D0, D1+2, D3+4 and D5, respectively. BRM: bilogical response modifier; TRAP: thrombin receptor activator peptide; RANTES: regulated on activation, normal T cell expressed and secreted; TNF: tumour necrosis factor; IL: interleukin; D: day; SEM: standard error mean.

data confirm both data collected in the past by our group, albeit under different conditions, and those collected by others. In addition to the aforementioned evidence, we have contributed substantial novel information on leucocyte/platelet complexes and their likely role in transfusion when leucodepletion cannot be performed or is only partially achieved. Platelet-secreted and leucocyte-derived BRM are independent (as observed clearly on day 0), but their concomitant presence in the PC induces an interplay and a loss of independency was observed from day 1 to day 4. The variables became independent again by day 5, most likely because the amounts of leucocyte-derived BRM exceeded the platelet-secreted BRM so greatly that the leucocyte profile dominated.

Conclusions

This study confirms, in a novel transfusion circumstance, the interdependence of the BRM secretory profiles of leucocytes and platelets when bound in platelet-leucocyte aggregates. This interplay may have consequences for transfusion quality and safety, as already outlined. The data provide information for pathophysiological models of leucocyte/platelet interactions (at least via their secretory products). Leucocytes in PC are undesirable in general, and create higher risks as the shelf-life of the PC is prolonged. The leucocytes present in non-leucoreduced PC secrete BRM over time and are agonists with platelet-derived BRM. Strategies to reduce pro-inflammatory BRM should be sought. Alternatively, pathogen reduction technologies

for whole blood are under development and hold some promise with regards to reducing both leucocyte-mediated effects (as deduced from early studies[43]) and, potentially, allo-immunisation (antigen presentation). To what extent this strategy could be cost-efficient compared to conventional PC processing involving leucoreduction remains to be evaluated.

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Authorship contributions

OG, SL and FC participated in the study design, study supervision, and data interpretation; CA, TC and SJY participated in sample collection, experiment analyses and data interpretation. CA and AP performed the statistical analysis. OG, SL and CA wrote the manuscript. All Authors approved the final content of the manuscript.

The Authors declare no conflicts of interest.

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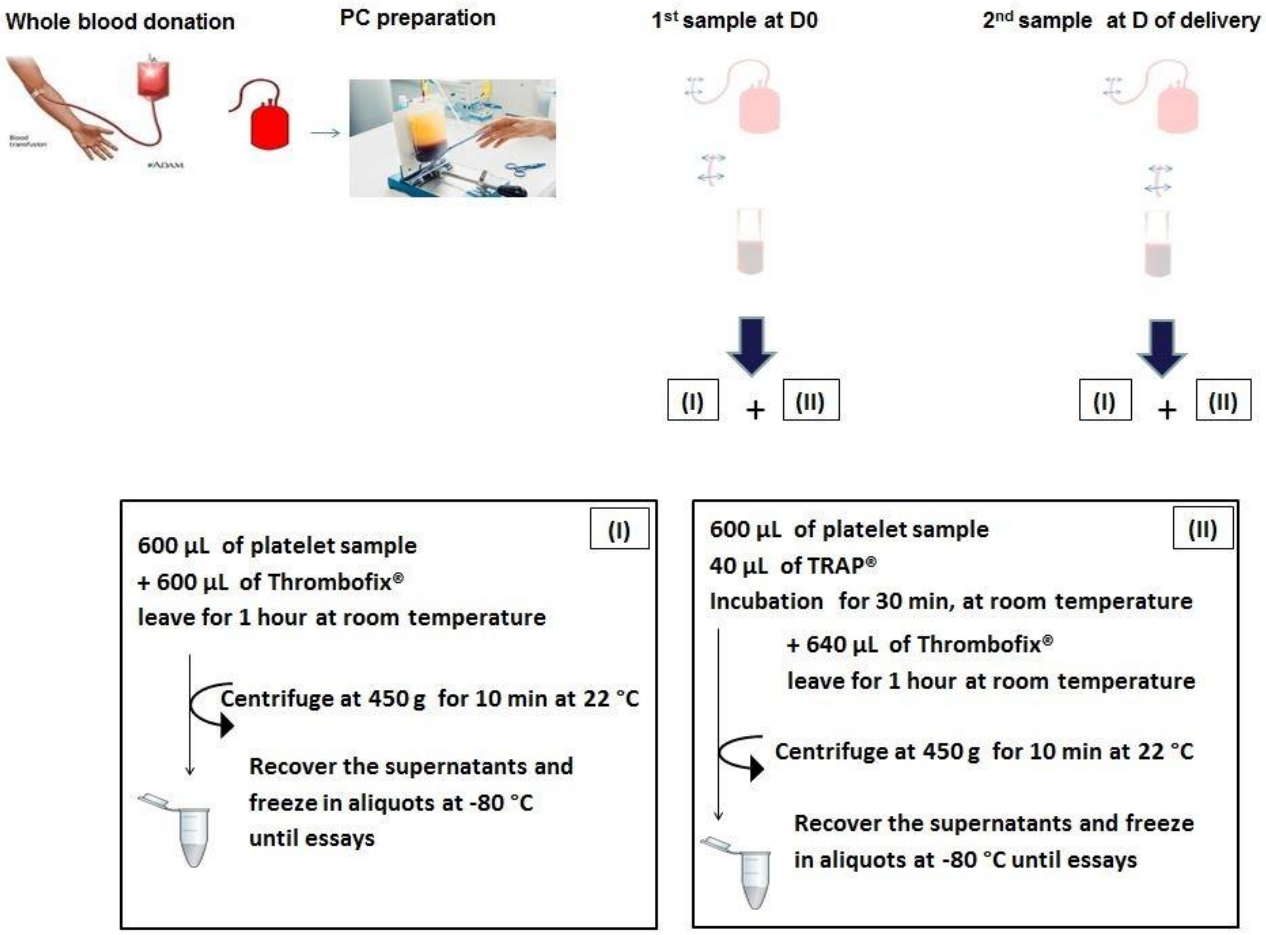
Online Suoolementary Content

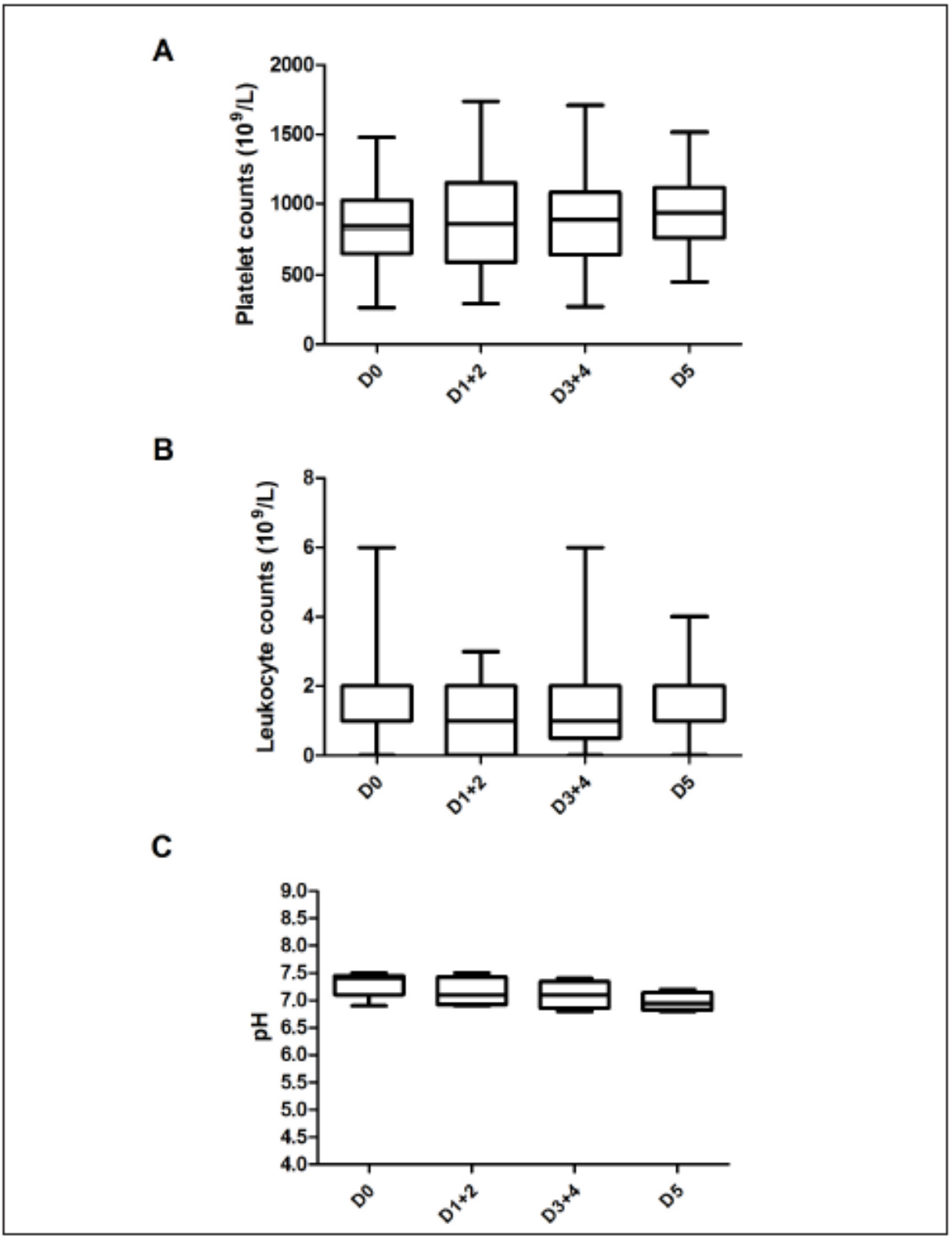


Online Supplementary Figure S1 – Diagram of the study design. PC: platelet concentrate; D: day; TRAP: thrombin receptor-activating peptides.

Online Supplementary Table SI - The main cells that secrete BRM and the relative amounts secreted.

	Platelets	Monocytes/Macrophages	T lymphocytes
sCD40L	+++		+
sCD62P	+++		
RANTES	+++		++
TNF-α	+	+++	++
IL-1β	+	+++	+
IL-8	+	+++	

BRM: biological response modifier; RANTES: regulated on activation, normal T cell expressed and secreted; TNF: tumor necrosis factor; IL: interleukin.



Online Supplementary

Figure S2 - Information about the platelet counts (A), the leucocyte counts (B) and the pH (C) of UPC, depending on storage time.

No significant variations were seen (Kruskal-Wallis test). Data are shown as boxplots with first and third quartiles as limits of the boxes and whiskers representing the minimum and the maximum of the data. n=158, 29, 65 and 64 for D0, D1+2, D3+4 and D5, respectively. UPC: unitary platelet concentrate; D: day.

VI. Article 6 : Omiques des concentrés plaquettaires impliqués dans des EIR

Integrated proteome and transcriptome analysis reveal cell death and inflammatory response as the most significant biological disorders in platelet components involved in acute transfusion reactions.

Chaker Aloui[1,2], Jocelyne Fagan[2], Fabrice Cognasse[1,2], Stéphane Claverol[3], Franc Salin[4], Isabelle Lesur[4,5], Sandrine Laradi[1,2] and Olivier Garraud[1,6].

Cet article est en préparation.

L'objectif de cette étude était de mieux comprendre les mécanismes moléculaires qui se déroulent au sein de la poche et ainsi de définir en détail les voies de signalisation qui auraient été particulièrement activées ou inhibées lors de la préparation et du stockage des CP impliqués dans un EIR.

Integrated proteome and transcriptome analysis reveal cell death and inflammatory response as the most significant biological disorders in platelet components involved in acute transfusion reactions.

Chaker Aloui[1,2], Stéphane Claverol[3], Franc Salin[4], Isabelle Lesur[4,5], Jocelyne Fagan[2], Halim Benamara[2], Christiane Mounier[6], Fabrice Cognasse[1,2], Olivier Garraud[1,7], Sandrine Laradi[1,2]

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Running head: Omics of platelet components involved in transfusion reactions

Abstract

Background: Blood platelets destined for transfusion release inflammatory molecules during preparation and storage that are occasionally associated with transfusion adverse events (AEs), despite the implementation of leukoreduction. The rationale of this study is to decipher the transcriptome and the proteome of Platelet Components (PCs) involved in severe and immediate adverse events referred to as acute transfusion reactions (ATRs).

Methods: We analyzed 6 leukoreduced PCs involved in ATRs versus 6 matched PC controls. We performed a Label-Free nLC-MS/MS method for quantitative proteomic analysis of the platelet pellets and the supernatants respectively. The platelet RNA transcriptome was acquired by RNA-Seq using the IonProton platform. Differentially expressed genes /proteins were analyzed by the GeneCodis3 and the Ingenuity Pathway Analysis (IPA) bioinformatics software.

Results: From the platelet pellet and the supernatant proteome studies, 1115 and 234 proteins, respectively, were identified from which 490 and 104, respectively, were differentially expressed within the two studied groups. For the transcriptome study, out of 19.143 identified genes from which 39 genes were differentially expressed. The bioinformatics interpretation of the differentially expressed proteins/genes revealed platelet activation and apoptosis as the most significant biological functions associated with ATRs. Moreover, mitochondrial dysfunctions and inflammatory disorders were among the most relevant disease mechanisms involved in ATRs after platelet transfusion.

Conclusion: The integrated proteome and transcriptome data of PCs revealed a significant association with platelet activation, apoptosis and inflammatory mechanisms which may be involved in platelet transfusion reactions. This study provides novel insights into the molecular mechanisms underlying this disease, thereby aiding to understand their mechanisms and could give path to prevent ATRs.

Introduction:

Platelets are key mediators of hemostasis. Thus, platelet concentrates (PCs) are an important component of supportive care for thrombocytopenic patients. Despite decades of improvements, preparation of PCs still represents one of the major challenges to the blood banks, in light of standard platelets storage limitations. Indeed, routine storage results in

decreased PLT morphology scores (loss of disk shape) and responses to agonist, increased hypotonic shock response, volume and density heterogeneity, PLT activation marker expression, release of PLT α-granules, cytosolic proteins and inflammatory mediators, increased pro-coagulant activity, and altered supernatant pH and glycoprotein expression [1–5].

It was shown that high levels of platelet-released inflammatory mediators commonly known as Biological Response Modifiers (BRSs) could induce adverse events (AEs) in recipients although leukoreduction. Indeed, platelet transfusion accounts for near 25% of the recorded adverse events (AEs)—and 50% of serious AEs (SAEs)—despite they represent only 10% of the delivered blood components[6]. We and others have largely evaluated the evolution of platelet derived BRMs in leukoreduced PCs, and showed the potential implication of these latter on the physiopathology of platelet transfusion ATRs[7–9]. However, the molecular mechanisms underlying the platelet release of these BRMs is still unclear.

"Omics" represent an ideal strategy to investigate *in vitro* changes of stored PLTs [10,11]. Owing to their anucleate nature, platelets have limited protein synthesis capacity[12] and a rather stable proteome that, in comparison to other cell types, is less affected by biological variability issues[10,11,13]. Translational application of proteomics and transcriptomics to PLT-related transfusion medicine issues has focused on many aspects of PLT product preparation, including the effects of preparation and storage (cell processing and pathogen inactivation) on *in vivo* viability[14–23].

In this view, we aimed in this study to decipher the proteome and the transcriptome signature of PCs involved in transfusion adverse events in order to better understand the mechanisms of this pathology.

Materials and methods:

Ethics statement

The study was approved by the Ethics committee of the University Hospital of Saint-Etienne (ID RCB 2014-A00405-42) and informed consents were obtained from all participants.

Sample collection and inclusion criteria

ATRs were characterized by cliniciants. Investigated PCs in these trials were exclusively delivered to hemato-oncology patients who were receiving prophylactic transfusion protocols in a setting of post-chemotherapy thrombocytopenia. In this study, we investigated

inflammatory-type ATRs which excluded obvious or later acknowledged cases of bacterial infections, TRALI, severe and documented allergy cases, and cases obviously linked to an Ag/Ab conflict.

Once the clinicians note that there is an ATR after a transfusion of either an apheresis or a pooled buffy coat PC, they reported it immediately to the Hemovigilance and the delivery services of the blood bank. Then we collect as quickly as possible the rest of the PC bag.

In the research laboratory, the collected sample (5 ml) was shared into two samples. The first one (4 ml) served to extract the total RNAs. The second one (1 ml) was centrifuged at 1500 rpm for 10 min and the supernatant was collected and stored at -80°C. The platelet pellet was washed twice with sterile PBS. The pellet was then resuspended in 80µL of sterile PBS and 20 µl of Laemmli buffer were added and the both were incubated at 100°C for 3 min and stored at -80°C.

Based on the characteristics of 6 collected PCs involved in the ATRs, we chose 6 "matched controls" for sex, ABO group, type of the PC (apheresis or pooled *buffy coat* PC, additive solution, and delivery day (Table 1). All samples were prepared by the identical protocol; Therefore, artifacts caused by preparation should affect all samples similarly.

Table 1. Caracteristics of the study cohort. ATR: sample involved in acute transfusion reaction; CTR: control sample; M: male; F: female; UV: ultraviolet.

ID	Preparation	ABO group	Sexe	Delivery date	Additive solution	Type	Grade	Imputability	Symptoms of the induced ATR
ATR1/ CTR1	Apheresis	A+	M	D4	Intersol	Amotosalen : UV irradiated	2	3	Rash, urticaria vasovagal response
ATR2/ CTR2	Apheresis	A+	F	D5	Intersol	Non-irradiated	1	1	chills, fever
ATR3/ CTR3	Pooled Buffy coat	A+	4M, 1F	D1	Intersol	Non-irradiated	1	3	chills
ATR4/ CTR4	Pooled Buffy coat	A+	4M, 1F	D2	Intersol	Non-irradiated	1	2	chills, fever
ATR5/ CTR5	Pooled Buffy coat	O+	4M, 1F	D2	Intersol	Non-irradiated	1	1	chills, fever
ATR6/ CTR6	Apheresis	A+	M	D3	Plasma 100%	Non-irradiated	1	1	urticaria

Proteome analysis

1. Sample preparation for Label Free analysis

Ten µg of each protein sample were solubilized in Laemmli buffer and deposited onto a 10% acrylamide SDS-PAGE gel. After colloidal blue staining, protein profiles were cut into 4 bands and each band was cut again in 1 mm x 1 mm gel pieces. Gel pieces were sustained in 25 mM ammonium bicarbonate 50% ACN, rinsed twice in ultrapure water and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature, covered with the trypsin solution (10 ng/µL in 40 mM NH_4HCO_3 and 10% ACN), rehydrated at 4°C

for 10 min, and finally incubated overnight at 37°C. Gel pieces were then incubated for 15 min in 40 mM NH_4HCO_3 and 10% ACN at room temperature. The supernatant was collected, and an H_2O/ACN/HCOOH (47.5:47.5:5) extraction solution was added onto gel pieces for 15 min. The extraction step was repeated twice. Supernatants were dried in a vacuum centrifuge and were resuspended in 100 µl of water acidified with 0.1%HCOOH. Samples were stored at -20°C.

2. nLC-MS/MS analysis

Peptide mixture was analyzed on an Ultimate 3000 nanoLC system (Dionex, Amsterdam, The Netherlands) coupled to a Electrospray Q-Exactive quadrupole Orbitrap benchtop mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Ten microliters of peptide digests were loaded onto a 300-µm-inner diameter x 5-mm C18 PepMapTM trap column (LC Packings) at a flow rate of 30 µL/min. The peptides were eluted from the trap column onto an analytical 75-mm id x 15-cm C18 Pep-Map column (LC Packings) with a 4–40% linear gradient of solvent B in 108 min (solvent A was 0.1% formic acid in 5% ACN, and solvent B was 0.1% formic acid in 80% ACN). The separation flow rate was set at 300 nL/min. The mass spectrometer operated in positive ion mode at a 1.8-kV needle voltage. Data were acquired using Xcalibur 2.2 software in a data-dependent mode. MS scans (m/z 300-2000) were recorded at a resolution of R = 70000 (@ m/z 200) and an AGC target of 1×10^6 ions collected within 100 ms. Dynamic exclusion was et to 30 s and top 15 ions were selected from fragmentation in HCD mode. MS/MS scans with a target value of 1×10^5 ions were collected with a maximum fill time of 120 ms and a resolution of R = 35000. Additionally, only +2 and +3 charged ions were selected for fragmentation. Others settings were as follows: no sheath nor auxiliary gas flow, heated capillary temperature, 260°C; normalized HCD collision energy of 25% and an isolation width of 3 m/z.

3. Database search and results processing

Data were searched by SEQUEST through Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc.) against the *Homo sapiens* Reference Proteome Set (Uniprot version 2015-07; 68482 entries). Spectra from peptides higher than 5000 Da or lower than 350 Da were rejected. The search parameters were as follows: mass accuracy of the monoisotopic peptide precursor and peptide fragments was set to 10 ppm and 0.02 Da, respectively. Only b- and y-ions were considered for mass calculation. Oxidation of methionines (+16 Da), propionamide (+71 Da) and carbamidomethylation of cysteines (+57 Da) were considered as variable modifications. Two missed trypsin cleavages were allowed. Peptide validation was performed

using Percolator algorithm[24] and only "high confidence" peptides were retained corresponding to a 1% False Positive Rate at peptide level.

4. Label-Free Quantitative Data Analysis

Raw LC-MS/MS data were imported in Progenesis QI 2.0 (Nonlinear Dynamics Ltd, Newcastle, UK). Data processing includes the following steps: (i) Features detection, (ii) Features alignment across the samples, (iii) Volume integration for 2-6 charge-state ions, (iv) Normalization on ratio median, (v) Import of sequence information, (vi) ANOVA test at peptide level and filtering for features $p < 0.05$, (vii) Calculation of protein abundance (sum of the volume of corresponding peptides), (viii) ANOVA test at protein level and filtering for features $p < 0.05$. Noticeably, only non-conflicting features and unique peptides were considered for calculation at protein level. Quantitative data were considered for proteins quantified by a minimum of 2 peptides.

Transcriptome analysis

1. RNA-seq library preparation and sequencing

Total RNA was extracted using the *mir*Vana™ miRNA Isolation Kit (Ambion by Life Technologies, Thermo fisher Scientific, Paris, France). RNA was then treated with DNase I (Qiagen, Paris, France). The quantity of the RNA was measured by using spectrophotometry, and the integrity of the RNA was confirmed by electrophoretic separation by using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California).

RNA-seq libraries were prepared from 2 ng of total RNAs without ribosome depletion as recommanded by Bray et al.[25], and mRNA was enriched with Dynabeads® mRNA Purification Kit. The mRNA fragmentation with RNase III and following steps were carried out according to the Ion Total RNA-Seq Kit v2 recommendation (#4476286E, Life Technologies). Samples were then indexed with Ion Adaptor Mix (#4476286E, Life Technologies). Emulsion PCR was performed using the One Touch system (Life Technologies). Beads were prepared using the One Touch 2 Template Kit v3 (#4488318). Sequencing was performed using the Ion Proton 200 sequencing kit v3 (#4488315) on the P1 Ion chip. Data were collected using the Torrent Suite v4.0 software.

2. Read mapping, gene expression quantification and statistical comparison

Sequencing was performed using the Ion Torrent Proton System (Ion Torrent Torrent Suite v4.0 software, Life Technologies). Reads were mapped against the 42,164 human genes from

the GRCh38.p5 genome version available in ENSEMBL (ftp://ftp.ensembl.org/pub/release-82/gff3/homo_sapiens/) using the CLC Genomics Workbench Reference Mapping function in CLC Genomics Workbench V7.0.3, with the following parameters: similarity 80%, minimum alignment length 90% and maximum number of alignments 10. Gene counts were then generated using HTSeq[26] with the Intersection_nonempty parameter. Differential expression comparing the ATR versus the control group was computed with the DESeq2 R Bioconductor package[27] (FDR 0.05; P-value <= 0.05).

Data Access

The proteome data are available at the PRIDE database with the dataset identifier PXD003510. Raw Ion Torrent sequence reads have also been submitted to the Sequence Read Archive of NCBI (Accession no. xxxxxxxxx).

Data interpretation: Functional annotation and Pathway Analysis

Differentially expressed proteins ($p<0.05$; absolute Fold Change ≥ 2) and genes ($p< 0.05$; false discovery rate, FDR<0.05) were used for the biological interpretations. Pathways and Gene ontology (GO) analysis was performed using the GeneCodis3 online resource (http://genecodis.cnb.csic.es) using the molecular function, cellular component, and biological process terms. The Ingenuity Pathway Analysis Release (Marsh 2016) (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), based on the most robust experimentally-validated gene–gene relationships were used to delineate the top canonical pathways, the predicted diseases and the biological functions.

Results:

Proteome analysis

1. **Platelet pellet proteome**

Peptide mapping identified 1723 proteins with a minimum of one peptide per protein. After stringent filter for identifying proteins with more confidence (a minimum of two peptides), we identified 1115 proteins from which 490 were differentially expressed: 339 up-regulated and 151 down-regulated.

Of note, one ATR sample and its homologous control were discarded from the proteome study because data did not map with the other samples. After checking, it has been found that

the sample was stored in 100% plasma without additive solution and its control which are different from the other samples (Table 1).

Bioinformatics elaboration for functional GO term enrichment via GeneCodis3 is reported for up-regulated and down-regulated proteins, separately in Figure 1. It is worth noting that increases and decreases in the levels of different proteins involved in the same biological processes were observed.

The top enriched biological processes with evident relationship with platelets of the up-regulated proteins was as follow: blood coagulation (GO:0007596) with 31 proteins (p = 4.38E-16), platelet activation (GO:0030168) with 21 proteins (p= 5.42E-13), apoptotic process (GO:0006915) with 25 proteins (p = 5.36E-09) and platelet degranulation (GO:0002576) with 11 proteins (p = 6.21E-09) (Figure 1, and supplementary Table S1).

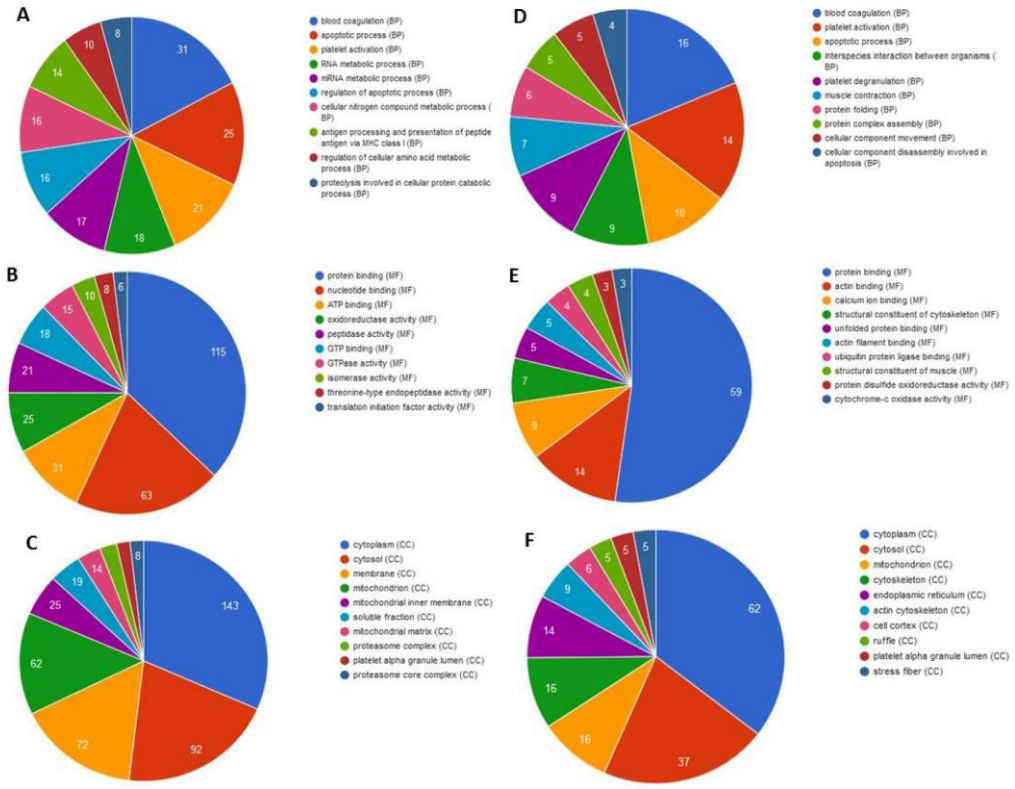


Figure 1. Enrichment Analysis of GO in the platelet pellets: Biological Process, Molecular Function and Cellular Component for the up-regulated (A, B and C) and down-regulated proteins (D, E and F), respectively.

Pathway analysis was done by the two integrated GeneCodis pathways (i.e. KEGG and Panther pathway) and IPA®. The common enriched pathways between the three software were Inflammation mediated by chemokine and cytokine signaling pathway, Integrin signaling pathway, Regulation of the cytoskeleton pathway. It is worth noting that the IPA software is the most up to date one using robust experiment results for the interpretation and it classified the mitochondrial dysfunction pathway as the most enriched one (- log p = 18) (Figure 2).

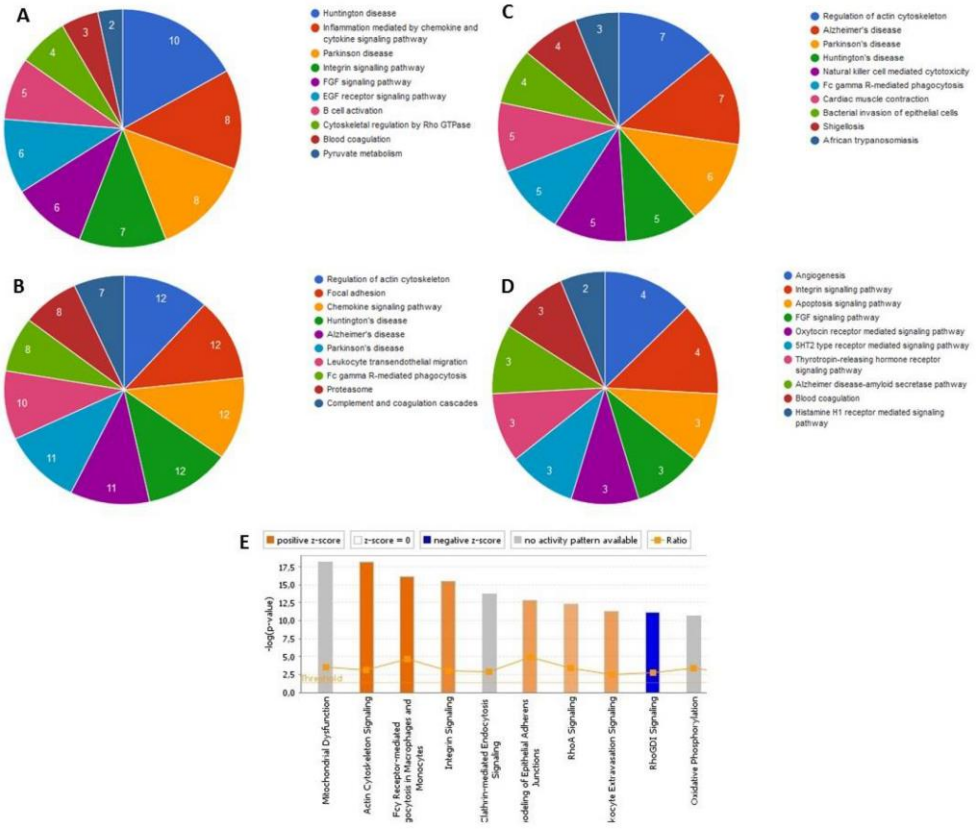
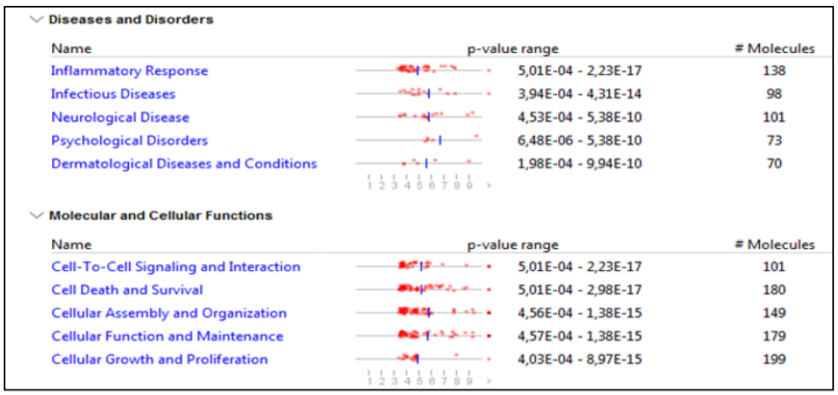


Figure 2. Enrichment Analysis in the platelet pellets of (A) KEGG Pathways, (B) Panther Pathways for up-regulated proteins; (C) KEGG Pathways, (D) Panther Pathways for down-regulated proteins; (E) Top 10 Enriched Canonical Pathways of differentially expressed genes (up and downregulated together) using the IPA software.

Based on the differentially expressed proteins, the IPA software predicted that 138 proteins are involved in Inflammatory Response (p-value range [5.01e-4 to 2.23e-17]) and classified it as the top one enriched Diseases and disorders (Table 2).

Table 2. Top diseases and biofunctions of the differentially expressed proteins (platelet pellets study) predicted by the IPA software



Diseases and Disorders

Name	p-value range	# Molecules
Inflammatory Response	5,01E-04 - 2,23E-17	138
Infectious Diseases	3,94E-04 - 4,31E-14	98
Neurological Disease	4,53E-04 - 5,38E-10	101
Psychological Disorders	6,48E-06 - 5,38E-10	73
Dermatological Diseases and Conditions	1,98E-04 - 9,94E-10	70

Molecular and Cellular Functions

Name	p-value range	# Molecules
Cell-To-Cell Signaling and Interaction	5,01E-04 - 2,23E-17	101
Cell Death and Survival	5,01E-04 - 2,98E-17	180
Cellular Assembly and Organization	4,56E-04 - 1,38E-15	149
Cellular Function and Maintenance	4,57E-04 - 1,38E-15	179
Cellular Growth and Proliferation	4,03E-04 - 8,97E-15	199

2. Supernatant proteome

Peptide mapping identified 332 proteins with a minimum of one peptide per protein. After stringent filter for identifying proteins with more confidence (a minimum of two peptides), we identified 234 proteins from which 104 were differentially expressed: 68 up-regulated and 36 down-regulated.

The bioinformatics analysis of the differentially expressed proteins in the supernatant proteome showed very similar results as the platelet pellets study; blood coagulation (p = 1.01026e-21), platelet activation (p = 3.03983e-20) and platelet degranulation (p = 4.16107e-23) was the most enriched biological processes (Figure 3 and supplementary Table S2).

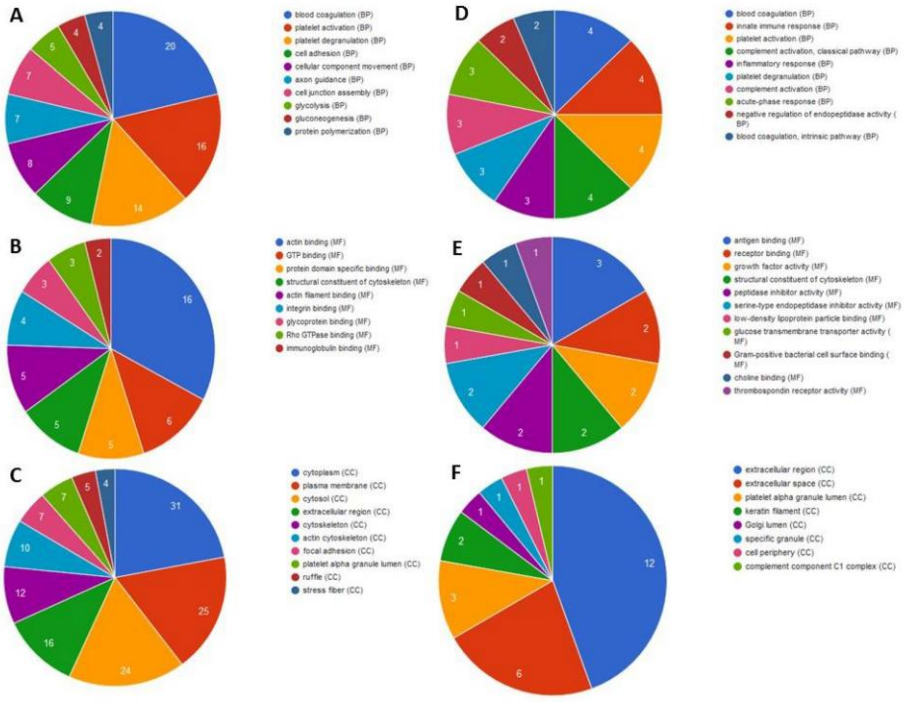


Figure 3. Enrichment Analysis of GO in the supernatant fraction: Biological Process, Molecular Function and Cellular Component for the up-regulated (A, B and C) and down-regulated proteins (D, E and F), respectively.

Pathway analysis of the proteins identified in the supernatants is also coherent with the platelet pellet study. The top common enriched pathways, between the three software, are Integrin signaling pathway, Regulation of the cytoskeleton pathway and Blood coagulation (Figure 4).

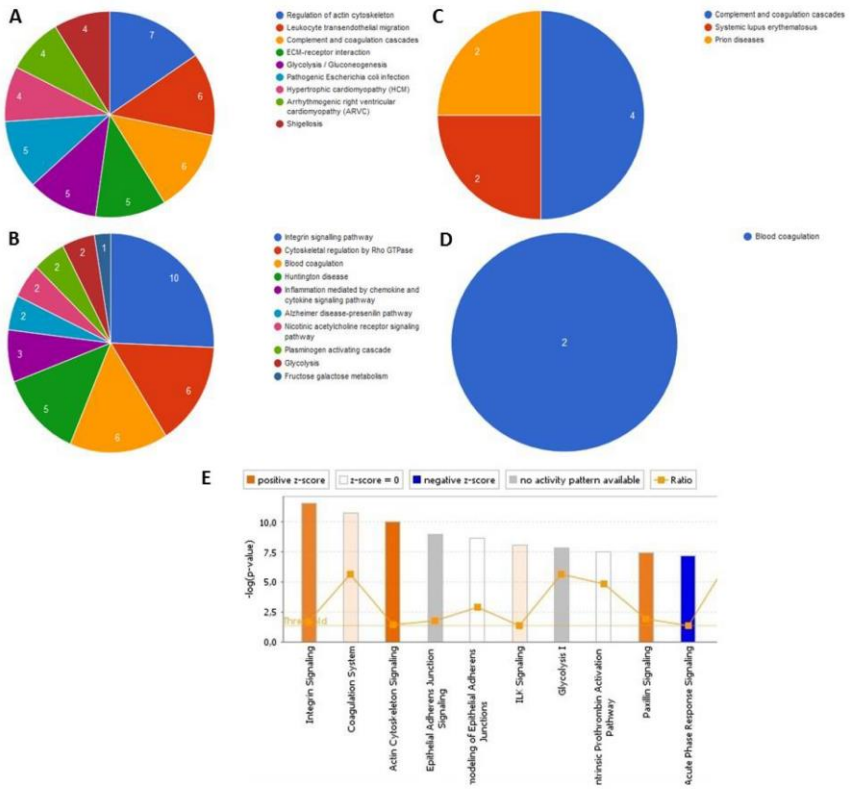


Figure 4. Enrichment Analysis in the supernatant fraction of (A) KEGG Pathways and (B) Panther Pathways for up-regulated proteins; (C) KEGG Pathways and (D) Panther Pathways for down-regulated proteins; (E) Enriched Canonical pathways of differentially expressed genes (up and down-regulated) using the IPA software.

As Diseases and Disorders, the IPA software predicted that 45 proteins are involved in Inflammatory Response (p-value range [2.69e-03 to 1.95e-22]), 27 proteins are involved in Hematological disease (p-value range [1.68e-03 to 1.19e-13]) and 62 proteins are involved in Organismal Injury and Abnormalities (p-value range [2.85e-03 to 1.19e-13]) (Table 3).

Table 3. Top diseases and biofunctions of the differentially expressed proteins (supernatant proteome study) predicted by the IPA software.

Diseases and Disorders

Name	p-value range	# Molecules
Inflammatory Response	2,69E-03 - 1,95E-22	45
Hematological Disease	1,68E-03 - 1,19E-13	27
Hereditary Disorder	1,86E-03 - 1,19E-13	40
Organismal Injury and Abnormalities	2,85E-03 - 1,19E-13	62
Immunological Disease	1,68E-03 - 2,09E-13	28

Molecular and Cellular Functions

Name	p-value range	# Molecules
Cell-To-Cell Signaling and Interaction	2,54E-03 - 1,95E-22	28
Cellular Assembly and Organization	2,47E-03 - 8,42E-14	35
Cellular Function and Maintenance	2,47E-03 - 8,42E-14	36
Cellular Movement	2,87E-03 - 2,09E-13	35
Cell Morphology	2,78E-03 - 2,46E-13	32

Network analysis using the IPA software showed that the most enriched one was the complement with their three pathways (i.e. classical, lectin, and alternate pathway) (Figure 5).

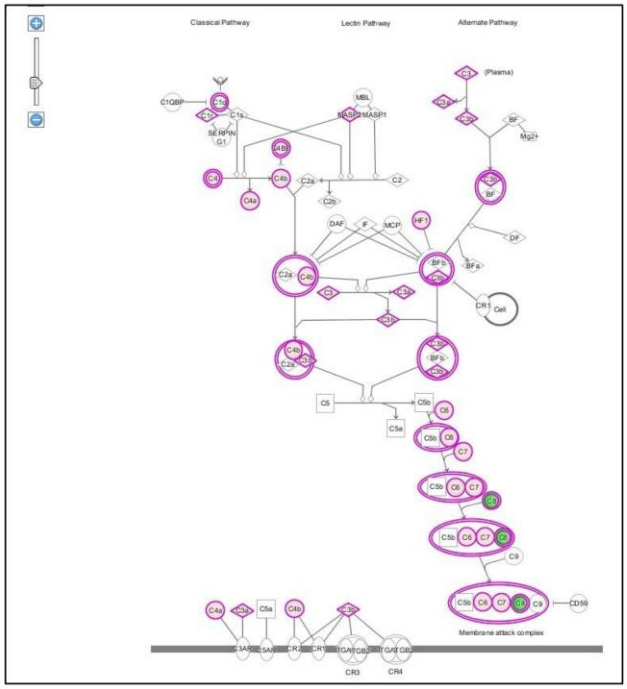


Figure 5. The complement network as the most enriched one in the supernatant fraction. Up-regulated proteins are shown in red; down-regulated proteins are shown in green; undetected proteins in our study are shown in grey.

Common up-regulated proteins identified in both supernatants and platelet pellets

From the 68 and 339 up-regulated proteins in supernatants and platelet pellets, respectively, 9 proteins were identified (ACTR2, CAP1, RSU1, RAP1B, TPM4, PKM, FLNA, VWF, FGB). Functional enrichment of biological process identified that 7 of these proteins are related to Response to stress (GO:0006950; p = 0.0439); 5 in platelet degranulation (GO:0002576; p =

0.000237) and 4 in platelet activation (GO:0030168; p = 0.000237). Cellular component analysis showed that 8 proteins are carried by extracellular exosomes (GO:070062; p = 0.000251) (supplementary Table S3).

Transcriptome

For the transcriptome study, the aligned reads mapped to 19143 genes with a minimum of one read. The comparison between the two groups identified 39 differentially expressed genes with a p-value <0.05 and a FDR <0.05. Fourteen genes were up-regulated in the ATR group and 25 genes were up-regulated in the control group.

The interpretation of the differentially expressed genes showed that the results are closer to those of the supernatants study (Table 4).

Table 4. Top diseases and biofunctions of the differentially expressed genes (transcriptome study) predicted by the IPA software

Diseases and Disorders		
Name	p-value range	# Molecules
Developmental Disorder	3,65E-02 - 5,66E-04	3
Hereditary Disorder	3,90E-02 - 5,66E-04	3
Immunological Disease	4,67E-02 - 5,66E-04	5
Organismal Injury and Abnormalities	4,67E-02 - 5,66E-04	18
Inflammatory Response	4,07E-02 - 8,64E-04	3
Molecular and Cellular Functions		
Name	p-value range	# Molecules
Cellular Function and Maintenance	4,23E-02 - 7,04E-06	6
Cell Morphology	4,81E-02 - 8,64E-04	5
Cell Signaling	1,97E-02 - 8,64E-04	2
Cell-To-Cell Signaling and Interaction	4,48E-02 - 8,64E-04	4
Cellular Assembly and Organization	3,90E-02 - 8,64E-04	5

Top enriched network predicted the Stromal Interaction Molecule 1 (STIM1) as the major regulator of the differentially expressed genes (Figure 6).

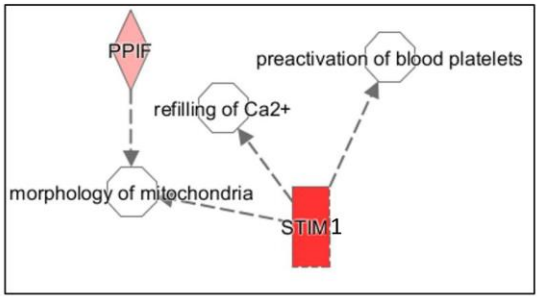


Figure 6. Network analysis of the transcriptome data (IPA software).

Discussion:

PCs can be obtained either by apheresis from a single donor, or by pooling of whole blood-derived buffy coats from many donors (five according to the French process). PCs are stored under agitation at 22±2°C for five days, in bags allowing for oxygen exchange, followed (in some centers) by pathogen inactivation to inhibit bacterial growth[28]. These gold standard of storage conditions are based on extensive research[29]. However, even under these optimized conditions, platelets lose viability and function over the storage period. This gradual loss of quality is known as the platelet storage lesion (PSL). The PSL shares features of both activation and apoptosis and is characterized by P-selectin exposure, surface receptor shedding, loss of mitochondrial membrane potential, caspase activation, externalized phosphatidylserine (PS), and microparticulation[29]. However, most transfusions are conducted without ATRs.

Previous studies on PCs investigated proteome or transcriptome alterations during storage, after pathogen reduction treatment and in some diseases (e.g. chronic kidney disease, cardiovascular disease). They found that both the platelet proteome and transcriptome profiles are gender, ethnic and storage dependent [14–23]. In this study, we sought to characterize the differentially expressed proteins and genes between ATR and control groups regardless the type of the PCs, the day of storage and the gender of donors. It's true that our groups involved both apheresis and pooled buffy coat PCs and both gender, but we chose rigorously the control group in order to avoid these biases.

Our results reveal that the three most enriched biological processes are blood coagulation, platelet activation and apoptotic process in addition to other less enriched but also significant (e.g. platelet degranulation, cellular component movement, cell junction assembly, actin cytoskeleton organization, regulation of cell shape, microtubule-based movement) (Figure 2 and 4, supplemental Tables 1 and 2). These 3 pathways share many common proteins. It was shown that highly activated platelets are procoagulant and are also apoptotic. Highly activated platelets are referred to as procoagulant platelets coated by adhesive proteins and coagulation factors[29]. The procoagulant platelet functions are closely linked to apoptosis[30]. Platelets are known to be able to engage in a program of cell death. The programmed apoptosis of platelets is the major process for clearance of aging platelets[31,31]. Platelets have the elements of the two apoptosis channels (intrinsic and extrinsic[32]) activating caspases[33] which cleave cellular substrates, including those that mediate DNA fragmentation. In our study we identified an enrichment of the intrinsic also called "mitochondrial" apoptosis

pathway. In the platelet pellet fraction, 25 proteins were up-regulated in the ATR group among them the most famous one to have a pro-death activity is the Bcl2-associated X protein (Bax) which was found to be 50 times more abundant in the ATR group.

The procoagulant function of activated platelets leading to apoptosis is also initiated by high and sustained levels of cytosolic Ca2+[34]. This issue was confirmed by the current transcriptome study which found the regulation of apoptotic process as the most enriched Biological process. The highest identified transcript in the ATR group were peptidylprolyl isomerase F (PPIF), Janus kinase 3 (JAK3) and stromal interaction molecule 1 (STIM1). STIM1 is the most overexpressed in PCs involved in ATRs. The IPA software analysis predicted that the STIM1 is the most important molecule to be a marker for regulation of the others. STIM1 has been identified as an intraluminal Ca2+ sensor and is located near Ca2+ stores, both in mitochondria: in the dense tubular system (the analog of the endoplasmic reticulum (ER) in platelets) and in the acidic stores (lysosomal-like organelles). Ca2+ mobilization, especially Ca2+ entry, is a crucial platelet activation-associated event. In fact, Platelets are non-electrically excitable cells, and store-operated calcium entry (SOCE) is a major pathway for calcium influx in platelets. This STIM transmembrane protein communicates information about the amount of stored Ca2+ to plasma membrane channels and consequently regulates platelet function[35]. Activation of platelets by all stimulatory agents leads to an increase of cytosolic Ca2+ levels, which triggers many intracellular signaling processes important for the physio-pathological expression of functional responses (e.g. hemostasis, thrombosis) [36]. In addition, Ca2+ is involved in essential cellular functions including mitochondrial energy metabolism and cell death. STIM is closely associated with platelet activation and/or apoptosis and play an imminent role in cell death[37]. Hawkins et al.[38] and Prakriya et al.[39] demonstrated a relationship between oxidative stress and STIM1-mediated Ca2+ entry. This sustained Ca2+ increase enhances mitochondrial Ca2+ loading and influences mitochondrial function, which over time can trigger cell death. The authors demonstrated that S-glutathionylation of the ER-resident protein STIM1 affects the mitochondrial homeostasis via CRAC activation and an elevation in cytosolic Ca^{2}+; cysteine 56 was revealed to be a sensor for oxidant-dependent activation of STIM1. Thus, SOCE and STIM1 are involved in the regulation of cellular energy production via regulation of mitochondrial shape and bioenergetics and thus play a role in oxidative stress. Common up-regulated proteins in both supernatants and platelet pellets fractions are shown to be involved in the Response to stress (GO:0006950). The relationship between this result and the high

expression of STIM1 could explain the fact that Oxidative stress stimulates STIM1 puncta formation and store-independent calcium entry. Upon store depletion, STIM1 oligomerizes, redistributes, and binds to the plasma membrane–localized protein Orai1/CRACM1 to form the CRAC channel and trigger Ca2+ entry and refilling of the ER stores[39]. An explanation of the identification of Mitochondrial Dysfunctions as the most canonical pathway predicted by the IPA software is that sustained cytosolic Ca2+ or aberrant mitochondrial Ca2+ uptake lead to irreversible mitochondrial dysfunction and bioenergetic collapse[40,41]. Ultimately, this reduction in mitochondrial bioenergetics will decrease mitochondrial energy production and will result in cell death.

In the same line with the Mitochondrial Dysfunctions, Boudreau et al.[42], Cognasse et al.[43] , and Yasui et al.[44], identified higher circulating mitochondrial DNA (mtDNA) in PCs involved in ATR. Zhang and colleagues[45] and Sun and colleagues[46] recently reported that intercellular mitochondria, consisting of formyl peptides and mitochondrial DNA (mtDNA), can activate human neutrophils and induce inflammation, once released from the cells due to trauma. Consequently, mitochondria are now recognized as one of the important Damage-associated molecular patterns (DAMPs). DAMPs, also known as alarmins, are molecules released by stress cells undergoing necrosis that act as endogenous danger signals to promote and exacerbate the inflammatory response[47]. The best known DAMPs are high-mobility group box-1 (HMGB1), S100 proteins, heat shock proteins, and serum amyloid A. Increased serum levels of these DAMPs have been associated with many inflammatory diseases, including sepsis, arthritis, atherosclerosis, lupus, and Crohn's disease[44].

Although the mechanisms that induce ATR are poorly understood, a number of studies have demonstrated the importance of neutrophils and monocytes activation[48]. Functional analysis by Yasui et al.[44], aiming to explain the mechanisms of mtDNA in triggering ART showed that mtDNA could activate innate immunity cells (e.g. neutrophils, monocytes) but the found amounts in the ATR group are not able to induce white blood cells activation. They hypothesized that mtDNA would act in concert with synergetic factors.

Our findings could explain these results since the most abundant protein identified in the up-regulated ones in the platelet pellet fraction was the Eukaryotic Translation Initiation Factor 3 subunit G (EIF3G), 86498 times higher in the ATR group. EIF3G is a subunit of the eukaryotic translation initiation factor 3 (eIF3) complex, which is required for initiation of protein translation[49]. Kim et al.[50] found that the subunit G of the eIF3 complex activates caspase-3, and also has a strong DNase activity.

In our study, we did not investigate the mtDNA, but we identidied another DAMP which is the HMGB1 (30 times higher in the ATR group in the platelet pellet fraction) and that could be a good candidate for answering the hypothesis of Yasui et al.[44] concerning the synergistic factors. Ultimately to HMGB1, two other molecules with evident roles in inflammation were identified in the ATR group: the macrophage migration inhibitory factor MIF (47 times higher in the ATR group) and IL27 known also as the myeloid-derived growth factor (2268 times higher in the ATR group). The identification of the IL27 confirms a previous study by our team by Cognasse-Hamzeh et al.[8]. In addition, the supernatant study revealed many actors of the complement to be higher in the ATR group. Altogether and with the previous identified BRMS involved in the pathological effect of PCs in ATRs, PCs are revealed to be extremely inflammatory.

Limitations of the study

As it was the first proteome and transcriptome study on PCs involved in ATR, it is warranted to confirm our results in a larger cohort and in other blood banks. In addition, in this study, although we identified new inflammatory BRMs, we failed to identify the previously identified ones (i.e. CD40L, OX40L, IL13, RANTES, MIP-1α). This could be explained by the fact that our approach and the other used to investigate the whole proteome are not as sensitive as the ELISA and the Luminex technologies.

Conclusion

The present study describes, for the first time, the proteomic and transcriptomic footprint of PC involved in ATRs. Differentially expressed proteins and genes between PCs involved in ATRs and controls PCs were characterized by an enrichment of proteins that have primarily a role in platelet activation, blood coagulation, apoptotic process, platelet degranulation and also many proteins involved in different signaling pathways. Previous studies carried on PCs involved in ATRs identified many BRMs in higher amounts in the ATR groups. We hypothesize that platelets in PCs involved in ATRs inherit higher amount of proteins and mRNAs involved in platelet activation and apoptosis process. During PCs processing and storage, platelets are subsequently more easily activated and engage the apoptotic process via the intrinsic pathway. Thus, they release a large amount of proinflammatory BRMs and DAMPS (acting also as BRMs) which could induce an ATR after transfusion.

In addition, we identified new inflammatory markers with cytokine activity highly expressed in the ATR group, namely the HMGB, the MIF, eIF3G and STIM1. These markers need to be

more investigated in vitro and in vivo and whether their roles are confirmed, they could be targets for prevention strategies to avoid inflammatory transfusion reactions.

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Author contributions:

SL, OG, FC and CA conceived and initiated the study. SL and CA designed the study and interpreted the results. HB, CM and JF collected the samples. SC performed the proteome study. CA, JF, SL and FS performed the RNA-seq experiment. CA, IL and SC performed statistical and bioinformatics analysis of the data. CA, SL and OG wrote the manuscript. All authors read the manuscript and discussed the interpretation of results.

Conflict of interest:

The authors declare that they have no conflict of interest.

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Supplemental Table S1 : Most enriched biological process (with a minimum of 10 protein per pathway) of the up-regulated proteins in the platelet pellet fraction.

Items	Items_Details	Support	Reference Support	P-value	FDR correction	Proteins
GO:0007596	blood coagulation (BP)	31	457	4.03E-19	4.38E-16	GRB2, PDE5A, VAV1, SYK, PROS1, ATP2A2, PTK2, PTPN11, HBB, FGG, PRKCD, KIF2A, RAP1B, CD36, APOB, VWF, PRKACB, CAP1, CAPZB, PTPN1, FN1, EGF, FLNA, CDK5, LRRC16A, GUCY1A3, RAC1, F5, TIMP1, GNAI2, FGB
GO:0007165	signal transduction (BP)	27	1176	2.48E-06	6.56E-05	EEF1D, NCKIPSD, PDE5A, ARHGAP4, MAPRE2, PHB, ROCK1, FGG, PRKCD, RAP1B, CORO1C, RAN, PRKACB, CAP1, EGF, COPS2, HINT1, GDI2, FYB, PEX11B, GNAI2, STAT3, FGB, RSU1, MPP1, CLIC1, IMPA1
GO:0006915	apoptotic process (BP)	25	594	4.93E-11	5.36E-09	PSMA2, ACTC1, ARHGAP4, VAV1, CASP6, PPM1F, PSMC2, TJP2, PSMD10, PSMB3, PTK2, PSMD13, ROCK1, PSMB10, PRKCD, PSMB7, LMNA, PSMB4, PSMA3, RAC1, BAX, VDAC1, PSMA4, ARF6, HMGB1
GO:0006810	transport (BP)	22	604	1.04E-08	5.12E-07	NDUFB9, SLC25A1, ARCN1, DYNC1H1, ATP5B, AGFG1, ATP2A2, UQCRC2, AP3S1, NDUFAB1, CPT1A, UQCRC1, HBB, SLC25A15, CD36, APOB, GOT2, SLC25A11, ORM2, NDUFB5, SDHA, CLIC1
GO:0030168	platelet activation (BP)	21	234	9.97E-16	5.42E-13	PDE5A, VAV1, SYK, PROS1, ATP2A2, PTPN11, FGG, PRKCD, CD36, APOB, VWF, CAP1, FN1, EGF, FLNA, GUCY1A3, RAC1, F5, TIMP1, GNAI2, FGB
GO:0016070	RNA metabolic process (BP)	18	257	7.92E-12	2.15E-09	RPS16, PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, EIF4G2, PSMB10, PRKCD, RPS4X, EIF4A1, PSMB7, PSMB4, LSM1, EIF4E, PSMA3, PSMA4, DDX6
GO:0007264	small GTPase mediated signal transduction (BP)	17	312	1.48E-09	8.46E-08	ARHGAP4, VAV1, ARL8B, YWHAQ, RAB2A, RAB21, RAP1B, RAN, RAB30, RAC2, GDI2, RAB38, SRPRB, RAC1, RAB6B, IFT27, ARF6
GO:0016071	mRNA metabolic process (BP)	17	223	7.95E-12	1.73E-09	RPS16, PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PRKCD, RPS4X, EIF4A1, PSMB7, PSMB4, LSM1, EIF4E, PSMA3, PSMA4, DDX6
GO:0006508	proteolysis (BP)	16	543	1.60E-05	0.00037074	CTSC, BLMH, CASP6, PSMC2, PROS1, UQCRC2, COPS5, USP47, UQCRC1, ERAP1, ABHD10, AFG3L2, SCPEP1, CTSW, NPEPPS, OTUB1
GO:0044419	interspecies interaction between organisms (BP)	16	328	2.15E-08	9.36E-07	GRB2, SND1, PSMC2, SYK, PSMB3, PSMB10, RAN, TSG101, PSMB7, HTATIP2, PSMB4, EIF4E, PSMA3, STAT3, VDAC1, PSMA4
GO:0042981	regulation of apoptotic process (BP)	16	205	2.32E-11	3.16E-09	PSMA2, PSMC2, TPT1, PSMD10, PHB, PSMB3, PSMD13, PSMB10, PSMB7, HTATIP2, CDK5, LMNA, PSMB4, PSMA3, BAX, PSMA4
GO:0034641	cellular nitrogen compound metabolic process (BP)	16	200	1.60E-11	2.48E-09	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, SLC25A15, GOT2, PSMB7, DLST, QDPR, PSMB4, PSMA3, HSD17B10, PSMA4, ALDH4A1

GO:0044267	cellular protein metabolic process (BP)	15	284	2.10E-08	9.50E-07	EEF1D, RPS16, PROS1, CCT6A, EEF1A1, RPS4X, CCT5, EIF3G, EIF4A1, FUT8, GANAB, EIF2B1, EIF4E, HEXB, SEC31A
GO:0000278	mitotic cell cycle (BP)	15	304	5.15E-08	2.00E-06	PSMA2, MAPRE1, DYNC1H1, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, KIF2A, CKAP5, PSMB7, PSMB4, PSMA3, NUDC, PSMA4
GO:0016032	viral reproduction (BP)	15	329	1.44E-07	4.88E-06	RPS16, PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, RPS4X, RAN, AP1G1, PSMB7, PSMB4, PSMA3, RAC1, PSMA4
GO:0005975	carbohydrate metabolic process (BP)	14	290	1.88E-07	5.85E-06	SLC25A1, AKR7A2, DCXR, PGD, SLC2A3, GOT2, PRKACB, SLC25A11, GANAB, CS, SLC25A12, PGAM1, HEXB, RPIA
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I (BP)	14	94	5.68E-14	2.06E-11	PSMA2, BLMH, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, ERAP1, PSMB7, PSMB4, NPEPPS, PSMA3, PSMA4, SEC31A
GO:0016192	vesicle-mediated transport (BP)	12	192	8.99E-08	3.37E-06	ARCN1, NSF, RAB2A, AP3S1, COPA, SAR1A, AP1G1, COPB2, VAMP7, RAB6B, ARF6, SEC31A
GO:0000209	protein polyubiquitination (BP)	12	107	1.13E-10	1.03E-08	PSMA2, BLMH, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, NPEPPS, PSMA3, PSMA4
GO:0055114	oxidation-reduction process (BP)	12	233	7.21E-07	2.18E-05	ACADM, ACOX1, PGD, PDIA5, UQCRC1, NNT, HADHB, ACADVL, HTATIP2, DHRS4, SDHA, TBXAS1
GO:0006886	intracellular protein transport (BP)	12	196	1.13E-07	4.08E-06	ARCN1, SYTL4, RAB3GAP2, AP3S1, YWHAH, COPA, SAR1A, STON2, RAN, AP1G1, CDK5, COPB2
GO:0006184	GTP catabolic process (BP)	12	151	6.18E-09	3.20E-07	ARL8B, RAB2A, RAB21, SAR1A, RAP1B, RAN, RAB30, RAB38, RAC1, GNAI2, RAB6B, ARF6
GO:0010467	gene expression (BP)	11	408	0.00070898	0.00928502	EEF1D, RPS16, GARS, DARS, EEF1A1, PTBP1, RPS4X, EIF3G, EIF4A1, EIF2B1, EIF4E
GO:0015031	protein transport (BP)	11	413	0.00078293	0.00989585	NSF, RAB2A, RAB21, TSG101, RAB30, GDI2, RAB38, VAMP7, RAB6B, ARF6, SEC31A
GO:0006412	translation (BP)	11	241	6.68E-06	0.00016882	EEF1D, RPS16, DARS, COPS5, EIF4G2, EEF1A1, RPS4X, EIF3G, EIF4A1, EIF2B1, EIF4E
GO:0000082	G1/S transition of mitotic cell cycle (BP)	11	145	4.33E-08	1.74E-06	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, EIF4E, PSMA3, PSMA4
GO:0044255	cellular lipid metabolic process (BP)	11	128	1.18E-08	5.57E-07	SLC25A1, ACLY, ACADM, ACOX1, CPT1A, AGPAT1, HSD17B12, HADHB, GPD2, ACADVL, HSD17B4
GO:0002576	platelet degranulation (BP)	11	79	6.29E-11	6.21E-09	PROS1, FGG, CD36, VWF, CAP1, FN1, EGF, FLNA, F5, TIMP1, FGB
GO:0000084	S phase of mitotic cell cycle (BP)	10	112	3.81E-08	1.59E-06	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4
GO:0051436	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle (BP)	10	65	1.70E-10	1.42E-08	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4
GO:0051437	positive regulation of ubiquitin-protein	10	71	4.20E-10	3.04E-08	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4

	ligase activity involved in mitotic cell cycle (BP)					
GO:0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process (BP)	10	80	1.40E-09	8.47E-08	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4
GO:0051439	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle (BP)	10	75	7.32E-10	4.98E-08	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4
GO:0006977	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest (BP)	10	66	1.99E-10	1.55E-08	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4
GO:0000216	M/G1 transition of mitotic cell cycle (BP)	10	78	1.09E-09	6.95E-08	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4
GO:0006521	regulation of cellular amino acid metabolic process (BP)	10	51	1.35E-11	2.44E-09	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4
GO:0000075	cell cycle checkpoint (BP)	10	131	1.69E-07	5.40E-06	PSMA2, PSMC2, PSMD10, PSMB3, PSMD13, PSMB10, PSMB7, PSMB4, PSMA3, PSMA4

Supplemental Table S2: Most enriched biological process of the up-regulated proteins in the supernatant fraction.

Items	Items_Details	Support	Reference Support	P-value	FDR correction	Genes
GO:0007596	blood coagulation (BP)	20	457	7.11452e-24	1.01026e-21	TUBA4A, GP1BB, HRG, PLG, YWHAZ, RAP1B, ALDOA, VWF, CAP1, ITGA2B, VCL, ACTG1, FLNA, PFN1, ACTN1, PROC, F13A1, FGB, TLN1, ITGB1
GO:0030168	platelet activation (BP)	16	234	3.21109e-22	3.03983e-20	TUBA4A, GP1BB, HRG, PLG, YWHAZ, ALDOA, VWF, CAP1, ITGA2B, VCL, FLNA, PFN1, ACTN1, F13A1, FGB, TLN1
GO:0002576	platelet degranulation (BP)	14	79	1.46517e-25	4.16107e-23	TUBA4A, HRG, PLG, ALDOA, VWF, CAP1, ITGA2B, VCL, FLNA, PFN1, ACTN1, F13A1, FGB, TLN1
GO:0007155	cell adhesion (BP)	9	556	2.42786e-07	8.61889e-06	AMBP, GP1BB, VWF, ITGA2B, VCL, PARVB, TNXB, TLN1, ITGB1
GO:0007165	signal transduction (BP)	8	1176	0.000552467	0.00682177	ECM1, PDIA3, YWHAZ, RAP1B, CAP1, TNXB, FGB, RSU1
GO:0006928	cellular component movement (BP)	8	95	2.86352e-12	2.0331e-10	ACTR2, TUBB, MYH9, TPM4, VCL, ACTG1, TLN1, MACF1
GO:0034329	cell junction assembly (BP)	7	90	1.25962e-10	7.15466e-09	ACTG1, FLNA, PARVB, ACTN1, RSU1, VASP, ITGB1

GO:0007411	axon guidance (BP)	7	307	6.14505e-07	1.9391e-05	MYH9, CAP1, ITGA2B, ACTG1, VASP, TLN1, ITGB1
GO:0006096	glycolysis (BP)	5	45	1.03731e-08	4.90993e-07	LDHB, ENO1, ALDOA, TPI1, GAPDH
GO:0005975	carbohydrate metabolic process (BP)	4	290	0.00122824	0.0120283	ENO1, ALDOA, TPI1, GAPDH
GO:0006006	glucose metabolic process (BP)	4	98	1.93071e-05	0.000498473	ENO1, ALDOA, TPI1, GAPDH
GO:0044267	cellular protein metabolic process (BP)	4	284	0.00113726	0.0119623	TUBB1, TUBA4A, PDIA3, PROC
GO:0030036	actin cytoskeleton organization (BP)	4	128	5.50189e-05	0.00130211	CAP1, PFN1, TNXB, VASP
GO:0051258	protein polymerization (BP)	4	29	1.37825e-07	5.59176e-06	TUBB1, TUBB, TUBA4A, FGB
GO:0006094	gluconeogenesis (BP)	4	42	6.39528e-07	1.81626e-05	ENO1, ALDOA, TPI1, GAPDH
GO:0007264	small GTPase mediated signal transduction (BP)	3	312	0.0139004	0.040698	RAP1B, RAB8A, RAB11B
GO:0007018	microtubule-based movement (BP)	3	90	0.000418452	0.00594202	TUBB1, TUBB, TUBA4A
GO:0000278	mitotic cell cycle (BP)	3	304	0.0129708	0.0387759	TUBB, TUBA4A, UBC
GO:0007229	integrin-mediated signaling pathway (BP)	3	70	0.00019946	0.00377643	MYH9, ITGA2B, ITGB1
GO:0050900	leukocyte migration (BP)	3	106	0.000674795	0.00737084	MYH9, PROC, ITGB1
GO:0007160	cell-matrix adhesion (BP)	3	75	0.000244651	0.00434255	ITGA2B, VCL, ITGB1
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade (BP)	3	140	0.00150636	0.0142602	ECM1, UBC, FLNA
GO:0006457	protein folding (BP)	3	182	0.00317176	0.0225195	TUBB1, TUBA4A, PDIA3
GO:0006936	muscle contraction (BP)	3	96	0.000505469	0.00652515	TPM4, VCL, TLN1
GO:0008360	regulation of cell shape (BP)	2	90	0.00928549	0.0361244	MYH9, ALDOA

Cellular Component (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0070062	extracellular exosome	8	0.000251
GO:0030863	cortical cytoskeleton	3	0.00083
GO:0005925	focal adhesion	4	0.00156
GO:0005576	extracellular region	8	0.00227
GO:0030054	cell junction	5	0.0035
GO:0005938	cell cortex	3	0.00734
GO:0031093	platelet alpha granule lumen	2	0.0175
GO:0005884	actin filament	2	0.0233
GO:0031091	platelet alpha granule	2	0.0246
GO:0005829	cytosol	6	0.0401
			(less ...)

Supplemental Table S3: 9 common elements in "Supernatants" and "platelet pellets": ACTR2, CAP1, RSU1, RAP1B, TPM4, PKM, FLNA, VWF, FGB.

Discussion générale et perspectives

Les plaquettes sont des médiateurs clés de l'hémostase mais également de la réponse immunitaire[90]. Ainsi, les concentrés plaquettaires (CP) sont une composante importante des soins de soutien pour les patients thrombopéniques. Malgré des décennies d'améliorations, la préparation des PC représente encore l'un des principaux défis pour les banques de sang, à la lumière des limitations de stockage standard de plaquettes. Il a été montré que des niveaux élevés de médiateurs inflammatoires plaquettaires libérés (BRM) pourrait être à l'origine, au moins en partie, des effets indésirables chez les receveurs (EIR) malgré la leucoréduction.

Bien que 97,9% des transfusions de concentrés de globules rouges (CGR), de concentrés plaquettaires et de plasma à usage thérapeutique ne donnent pas lieu à la survenue d'EIR immédiat ou différé, les 2.1% restant peuvent donner lieu à une manifestation modérée (EIR) ou sévère. On notera cependant que ce faible pourcentage n'est pas également réparti selon la nature du produit sanguin transfusé. En effet, bien qu'elle ne représente que 10% des produits sanguins transfusés, la transfusion de plaquettes est à l'origine de près de 25% des effets indésirables enregistrés et de 50% des effets indésirables graves. Les plaquettes sont impliquées dans plusieurs types d'EIR dont les plus fréquents sont de type inflammatoire notamment les réactions de type allergie et les réactions fébriles non-hémolytiques (RFNH)[138].

Comme mentionné dans la problématique, notre équipe de recherche s'intéresse au rôle inflammatoire des plaquettes dans les RFNH. A ce propos, plusieurs études « cas-témoin » ont montré la présence de BRM plaquettaires avec des taux plus élevés dans les CP qui étaient associés à la survenue d'EIR[132–136]. Toutefois, la sur-expression de ces molécules n'induit pas systématiquement un EIR. Notre première reflexion a été de vérifier au niveau du gène *CD40LG* l'existence de polymorphismes génétiques qui pourraient modifier l'affinitité du couple récepteur/ligand : CD40L et son récepteur principal CD40. Afin de vérifier cette hypothèse, nous avons débuté nos explorations par l'investigation des partie codantes des gènes *CD40* et *CD40LG* ainsi que des région régulatrices (i.e. jonctions exon-intron, 3'UTR et 5'UTR) en utilisant une stratégie de criblage de mutation de haute résolution, la dHPLC et le séquençage de Sanger. Nos résultats ont montré l'absence de différences significatives dans le profil génétique du gène *CD40LG* chez les donneurs dont les CP ont induit ou non un EIR dans la population française[285]. Les résultats étaient similaires pour le gène *CD40* (résultats non publiés). Bien que notre population étudiée fût de faible effectif (n = 30 versus 211 contrôles), les résultats statistiques nous ont permis de conclure à l'absence d'association génétique entre *CD40LG* et les EIR. Malgré ce résultat inattendu, notre étude était originale et

a fourni des informations importantes sur le gène *CD40LG* dans les populations française et tunisienne qui pourraient être utiles pour d'autres études d'association où *CD40LG* pourrait être un gène candidat.

En plus des 12 polymorphismes investigués dans cette première étude, *CD40LG* contenait d'autres polymorphismes introniques qui étaient décrit comme associés à des pathologies inflammatoires présentant des manifestations cliniques proches de celles des EIR. Ceci nous a incités à continuer l'investigation de ce gène en ciblant 6 polymorphismes introniques. Les informations obtenues dans la première étude concernant les fréquences alléliques des SNP et la répartition des blocs haplotypiques nous ont permis de choisir judicieusement ces 6 polymorphismes selon la stratégie de « tagSNP ». Dans le but d'étudier ces 6 polymorphismes supplémentaires, nous avons développé une technique multiplexe de génotypage, la « quadruplex tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) »[150]. Nos résultats non publiés ont confirmé, encore une fois, l'absence d'association entre le gène *CD40LG* et les EIR.

Le sCD40L circulant provient essentiellement des plaquettes après leur activation. La libération de sCD40L augmente progressivement dans les CP au cours du stockage et des taux élevés sont associés à la survenue d'EIR. D'après la littérature, deux polymorphismes de *CD40LG* seraient impliqués dans la régulation de *CD40LG*, à savoir un SNP dans la région 5'UTR (rs3092952)[284] associé à l'augmentation de sCD40L dans le sérum et un microsatellite $(CA)_n$ dans la région 3'UTR qui affecte la stabilité de l'ARNm[282,283]. En outre, il a été rapporté que le taux de sCD40L peut aussi être associé à des marqueurs génétiques indépendants tels que rs1883832 dans la région promotrice de son récepteur *CD40* et le polymorphisme C807T (rs1126643) dans la région codante du récepteur plaquettaire au collagène (*ITGA2*)[286–288]. Dans le but d'identifier un ou plusieurs marqueurs génétiques de surexpression de sCD40L et par conséquence d'EIR, nous avons réalisé la troisième étude[289]. Afin d'identifier une association génétique entre les polymorphismes régulateur et la libération de sCD40L dans les CP au cours du stockage, nous avons mesuré la sécrétion de sCD40L dans 142 CP destinés à la transfusion le jour de préparation (J0) et le jour de délivrance. Parallèlement, chez les 142 donneurs correspondant aux CPU investigués, nous avons génotypé 10 polymorphismes de *CD40LG* en se servant de la technique optimisée, la T-ARMS-PCR[150] en plus des deux polymorphismes de *CD40* et d'*ITGA2*. L'analyse d'association des SNP a montré que seul le polymorphisme rs1126643 du gène *ITGA2* est associé avec une modification significative de la sécrétion de sCD40L. Par contre, l'analyse des haplotypes de *CD40LG* a montré que

l'haplotype « TGGC » des rs975379 (C / T), rs3092952 (A / G), rs3092933 (A / G) et rs3092929 (A / C) est aussi associé à des niveaux élevés de sCD40L. L'analyse d'haplotypes inter-chromosomiques a également montré une association positive entre l'haplotype « ATC » des polymorphismes rs3092952 (A / G), rs1883832 (C / T) et rs1126643 (C / T), porté respectivement par les gènes *CD40LG*, *CD40* et *ITGA2*, et des taux élevés de sCD40L dans les CP. Vue ces résultats positifs d'association, nous avons testé ces haplotypes dans notre cohorte de donneurs dont les CP ont induit ou non un EIR. Toutefois, les résultats se sont avérés être négatifs (data non publiées).

Toujours dans le but d'identifier des marqueurs génétiques de taux élevés de BRM dans les CP, nous avons effectué une étude dans laquelle nous avons évalué les lésions de stockage des CP et testé plusieurs polymorphismes génétiques connus dans la littérature comme étant régulateurs ou associés à des concentrations élevées de molécules solubles. Dans cette étude (Aloui et al. 2016. *Blood transfusion*, accepté), 158 échantillons de CPU non déleucocytés ont été obtenus à partir de donneurs de sang tunisiens. Nous avons caractérisé deux BRM principalement d'origine leucocytaire (IL-1ß, IL-8), deux principalement d'origine plaquettaire (sCD62P, sCD40L) et deux communs aux deux types de cellules (TNF-α, RANTES) ; ceci en présence ou en absence de stimulation par la thrombine et à différents jours de stockage (jour 0 à 5). Huit polymorphismes génétiques régulateurs ont été testés pour une éventuelle association avec des niveaux de sécrétion de BRM : (CD62P : rs6127 [A/G] and rs6136 [A/C][290–292]; CD40L: rs3092952 [A/G][284]; IL-8: rs1126647 [A/T][293] and rs4073 [T/A][294,295]; RANTES: rs2280788 [C/G][296]; TNF-α: rs1799964 [T/C][297,298]; and IL-1β: rs1143627 [C/T][299,300]). Les résultats ont montré que les BRM d'origine leucocytaire ou plaquettaire ont des profils de sécrétion bien distincts durant la période de stockage. Les BRM leucocytaires dominent largement ceux dérivées des plaquettes et influencent davantage le programme de sécrétion des BRM plaquettaires. Cette étude a contribué à l'obtention de nouvelles informations substantielles sur les interactions leucocytes/plaquettes et leur rôle probable dans la transfusion lorsque la déleucocytation ne peut pas être réalisée ou même partiellement réalisée. L'analyse génétique a montré l'absence d'association entre les polymorphismes étudiés et les taux de BRM dans les CPU (data non publiés). Une étude similaire de Addas-Cavalho et al., 2004 a identifié l'association entre un polymorphisme de l'IL-1β et l'accumulation de la protéine correspondante dans 30 échantillons de CP non leucoréduits[301].

A notre connaissance, aucune autre étude génétique en relation avec les EIR n'a été réalisée excepté une autre étude de Addas-Cavalho et al., en 2006[302]. Ils ont identifié une association significative entre le gène *IL1RN* et les EIR dans une étude cas-témoins chez des receveurs de concentrés de globules rouges. Toutefois, cette étude n'a pas été confirmée.
L'absence de marqueurs génétiques en association avec les quantités de BRM pourrait être expliquée par la nature anucléée des plaquettes. Au regard de nos résultats, il s'avère que la sécrétion des BRM dans les CP est un processus plus complexe que dans les cellules nucléées.

Outre les BRM classiques (i.e. Cytokines, chémokines, facteurs de croissances et molécules apparentées), Lee et al.[303] furent les premiers à identifier un autre type de BRM dans les produits sanguins transfusés (i.e. CGR, plasma frais congelé et CP). Il s'agit de l'ADN mitochondrial (ADNmt) soluble qui a été classé comme un ensemble de signaux de dangers endogènes (Damaged-associated molecular patterns, DAMP). Les DAMP, aussi connu comme alarmines, sont des molécules libérées par les cellules dans des conditions de stress et agissent comme des signaux de danger endogènes afin de promouvoir et exacerber la réponse inflammatoire[304]. Les DAMP les plus connus sont les protéines S100, le high-mobility group box-1 (HMGB1), les protéines du choc thermique (HSP) et les amyloïde sérique A. Des niveaux élevés de ces DAMP dans le sérum ont été associés à de nombreuses maladies inflammatoires, y compris le sepsis, l'arthrite, l'athérosclérose, le lupus, et la maladie de Crohn[305–313]. Des études ont montré que l'ADNmt libéré par les cellules en cas de traumatisme peut activer les neutrophiles humains et alors induire une inflammation. Par conséquent, les mitochondries sont maintenant reconnues comme source importante de DAMP. Les auteurs ont également rapporté que les DAMP mitochondriaux (i.e. l'ADNmt et les N-formyl peptides) peuvent induire l'adhérence des PMN aux cellules endothéliales et activer les deux types de cellules par l'intermédiaire de multiples voies, y compris celle des TLR-9. Considérant ces modèles inflammatoires, Bouderau et al.[314], furent les premiers à identifier des taux élevés d'ADNmt soluble dans des CP ayant induit des EIR en comparaison avec un groupe contrôle. Notre étude, (Cognasse et al.[142]) a confirmé les résultats de Boudreau et al. [314]. Récemment, Yasui et al.[315] ont pu confirmer nos résultats et ceux de Boudreau et al., concernant la présence de taux plus élevés des ADNmt dans les CP impliqués dans des RFNH. Toutefois les essais étendus aux CGR et au plasma frais congelé se sont révélés négatifs. Leurs analyses fonctionnelles ont montré que les quantités d'ADNmt nécessaire à activer des leucocytes et notamment les monocytes, les basophiles et les PMN sont plus élevées à celles identifiées dans le groupe d'EIR. Les ADNmt semblent induire des

EIR qu'en présence d'autres agonistes comme les N-formyl-$_L$-methionyl-$_L$-leucyl-$_L$-phenylalanine ou les anticorp HLA[315].

Notre équipe de recherche et d'autres avons largement évalué l'évolution des BRM dérivés des plaquettes dans les CP leucoréduits, et avons montré l'implication potentielle de ces derniers sur la physiopathologie de la transfusion plaquettaire[133–136,140,141]. Cependant, les mécanismes moléculaires sous-jacents de la libération des BRM plaquettaire sont encore inconnus. Par conséquent, nous avons utilisé des techniques de protéomes et de transcriptome de « dernière génération » (i.e. LC-MS/MS et RNA-seq) afin de mieux comprendre le mécanisme physiopathologique de libération des BRM. Notre étude « omique » (manuscrit en préparation) a décrit, pour la première fois, l'empreinte protéomique et transcriptomique des CP impliqués dans des EIR. L'enrichissement fonctionnel des protéines et des gènes différentiellement exprimés entre les CP impliqués dans des EIR et les CP contrôles a montré un enrichissement de protéines qui ont principalement un rôle dans l'activation plaquettaire, la coagulation, le processus apoptotique, la dégranulation des plaquettes. De nombreuses protéines ont aussi été retrouvées comme impliquées dans différentes voies de signalisation dont la principale était celle des intégrines. L'activation plaquettaire, la coagulation et l'apoptose sont des processus fortement liés[316–318]. Au cours du stockage, bien que les plaquettes soient stockées dans les conditions conventionnelles optimales en termes de température, d'échanges gazeux et d'absence de stimulations exogènes tel que des pathogènes entiers ou des fragments de pathogène, des marqueurs d'apoptose surviennent et augmentent au cours du stockage[319–321]. Ce phénomène constitue apparemment une des lésions cruciales de stockage important qui réduit la viabilité et le nombre des plaquettes dans les CP après une conservation prolongée. Potentiellement, l'apoptose plaquettaire pourrait avoir des impacts négatifs chez les receveurs lors de la transfusion comme la diminution de la fonction des plaquettes transfusées et du CCI (Corrected Count Increment)[319] et pourrait tout à fait être à l'origine des réactions indésirables dues aux propriétés proinflammatoires et pro-thrombotique des microparticules générées au cours de l'apoptose[52,322]. Notre étude a mis en évidence l'enrichissement de la voie intrinsèque (dite aussi voie mitochondriale) d'apoptose plaquettaire. Ceci pourrait expliquer les résultats d'abondance d'ADNmt solubles dans les CP impliqués dans les EIR. De plus, l'état d'activation et de dégranulation des plaquettes expliquent bien la présence des BRM classiquement identifié (e.g. sCD40L, RANTES, sCD62P, le MIP-1α, OX40L, IL13) étant donné que la plupart des BRM identifiés en relation avec les EIR sont d'origine granulaire (principalement les granules α) et sont fréquemment

corrélés entre eux[132,135,136]. Le protéome des surnageants et celui des culots plaquettaires se sont révélés cohérents et ont été confirmés par l'étude transcriptomique qui a mis en évidence une dérégulation du métabolisme calcique lui-même associé à l'activation plaquettaire et à l'apoptose via la voie mitochondriale.

Au cours de la conservation des PC, ce programme de sécrétion pourrait être dépendant des stimuli endogènes comme les DAMP en cas de stress ainsi que les lésions de stockage [105,323]. Les données de l'hémovigilance (non publiées) montrent que les donneurs dont les CP sont associés à des EIR sont des donneurs réguliers et leurs produits n'induisent pas systématiquement des EIR. Ceci pourrait nous orienter à nouveau vers l'hypothèse des polymorphismes génétiques. Toutefois, les résultats de l'étude « omique » mettrait plus en cause le processus de préparation et de stockage des CP. Nous ne pouvons pas également écarter une autre hypothèse concernant l'état de santé du donneur le jour du don de sang total ou de plaquettes d'aphérèse. L'état de santé du receveur au moment de la transfusion pourrait également tolérer des produits plus « inflammatoires » comme elle pourrait compliquer son état et ceci nécessiterait d'être investigué. Une étude cas-témoins du protéome du sérum des receveurs pourrait répondre à cette question.

En perspective, concernant l'étude génétique, vu les résultats négatifs de l'étude *CD40LG* et celle d'autre gènes (data non publiées), il serait intéressant de s'orienter vers les approches globales telles que l'approche *GWAS* chez les donneurs. Toutefois, cette approche nécessite une large population d'étude (au moins 1000 échantillons pour la phase de découverte) et vu la rareté des EIR, il nous faudra plusieurs années pour les collecter. Il faudra aussi établir des collaborations avec d'autres établissements de transfusion afin de pouvoir répliquer et confirmer les résultats. Une deuxième approche pourrait être envisagée, plus réalisable que le *GWAS*. Il s'agit du « *whole exome sequencing* » qui nous permettrait de regarder minutieusement les variations génétiques aux niveaux des protéines codantes et de pouvoir étudier les interactions récepteurs-ligands d'une large gamme de BRM. L'idée serait de comparer l'exome de plusieurs donneurs qui ont donné un CP à un receveur polytransfusé.

Notre étude « omique » a révélé des plaquettes plus activées et plus apoptotiques dans les CP ayant induit des EIR. A notre connaissance, cette étude est unique et mérite d'être confirmée sur un échantillonnage plus large et dans d'autres établissements de transfusion. Un nombre plus important d'échantillons nous permettrait de comparer plusieurs paramètres tels que

l'influence des méthodes de préparations, du sexe des donneurs, des techniques d'inactivation de pathogènes, des solutions additives de plaquettes et des jours de stockage.
En outre, nous avons identifié de nouveaux marqueurs inflammatoires fortement exprimés dans le groupe EIR, à savoir le HMGB1, le MIF, eIF3G et STIM1. Ces marqueurs méritent des études complémentaires *in vitro* et *in vivo* et si leurs rôles sont confirmés, ils pourraient représenter des cibles pour les stratégies de prévention des EIR. Notre étude a également mis en évidence l'enrichissement de la voie mitochondriale d'apoptose plaquettaires, permettant ainsi d'orienter le développement de nouvelles stratégies pour améliorer la viabilité et la fonction des plaquettes pendant le stockage.

Etant donné la grande importance du rôle des miARN dans la régulation de la traduction et leur utilisation comme des biomarqueurs stables, ce serait très intéressant de les investiguer dans une étude cas-témoins.

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Despite the implementation of systematic leucoreduction, platelet transfusions are still generating transfusion reactions (also called "Adverse Events or AEs"). We know that platelets release proinflammatory molecules during preparation and storage of platelet components (PCs) notably the soluble CD40 ligand or sCD40L and high levels are associated with AEs. In this thesis, we aimed to understand the mechanisms of occurrence of inflammatory platelet transfusion reactions.

In the first part, we investigated the genetic polymorphisms of *CD40LG*. We failed to identify a significant association with AEs but we identified genetic markers of sCD40L overexpression: a *CD40LG* haplotype and also an interchromosomal haplotype (*CD40LG-CD40-ITGA2*). However, these haplotypes have not been found to be associated with AEs.

In the second part, we showed that, during storage of non leucoreduced PCs, leukocyte-derived "biological response modifiers" (BRMs) dominate and influence the platelet-derived BRMs. Then, we found increased levels of another individualized BRM in PCs involved in AEs: mitochondrial DNA, classified as an endogenous danger-associated molecular pattern (DAMP).

In the last part, we performed an integrated proteomic and transcriptomic study by LC-MS/MS and RNA-seq, respectively, to better understand the pathophysiology of AEs in a case-control approach. The biological enrichment of differentially expressed genes revealed a significant association with platelet activation, apoptosis and inflammatory mechanisms which may be involved in platelet transfusion reactions. This study provides novel insights into the molecular mechanisms underlying the occurrence of AEs and could give paths to prevent them.