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DOTTORATO DI RICERCA IN METODOLOGIA CLINICA

XXVIII CICLO

Effects of cysteinyl leukotrienes on platelet activation

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List of abbreviation

5-HT = Serotonin

AA = Arachidonic Acid

ADP = Adenosine diphosphate

cAMP = cyclic AMP (Adenosine monophosphate)

COX = Cyclooxygenase

cPLA = cytosolic phospholipase A2

CysLTs = cystenyl leukotrienes

DAG = diacyl-glycerol

ECs = Endothelial stem cell

HUVECs = Human umbilical vein endothelial cells

IP_3 = Inositol trisphosphate

LTs = Leukotrienes

$P2Y_{12}$ R= $P2Y_{12}$ receptor

PAR-1 = Protease-activated receptors 1

PAR-4 = Protease-activated receptors 4

PGE_1 = Prostaglandin E_1

PKC = Protein kinase C

PLC = Phospholipase C

TF = Tissue factor

Summary

Platelet are key players in haemostasis and represent a pivotal link between inflammation, immunity and atherogenesis. Cysteinyl leukotrienes (CysLTs) such as $LTC_{4,}$ LTD_4, and LTE_4 are potent lipid inflammatory mediators which interact with G protein-coupled receptors, $CysLT_1R$, $CysLT_2R$. However, LTE_4, the most stable cystenyl leukotriene, is not putative substrate for these two receptor. Recently have been hypothesized that a third cystenyl leukotreine receptor exists, and a computer modeling suggests $P2Y_{12}$, receptor for ADP present on platelet surface, as the putative receptor. But GPR99 is also hypothesized as LTE_4 putative receptor. It is known that LTE_4 needs $P2Y_{12}$ on platelet to mediate inflammation in sensitized mice, and LTC_4 can induce release of P-selectin in sensitized mice. In human platelet LTE_4 cannot increase level of cAMP, or of p-selectin.

Aim of this study was to test whether cystenyl leukotrienes elicit platelet functional responses, by interacting with the platelet $P2Y_{12,}$ receptor for ADP.

We measure the platelet aggregation induced by CysLTs alone or in combination with epinephrine or ADP; the cAMP level in presence of PGE_1, ADP and CysLTs, and finally the p-selectin expression on platelet surface after stimulation with ADP and CysLTs and in presence/absence of cangrelor.

Ours results shows that CysLTs cannot affect platelet aggregation alone or in combination with other agonist, independently of presence of physiological level of calcium.

CysLTs failed to show an effect also on cAMP level. Also when platelet activation was tested by measuring the expression of p-selectin on the platelet membrane induced by ADP, CysLTs failed to show any effect.

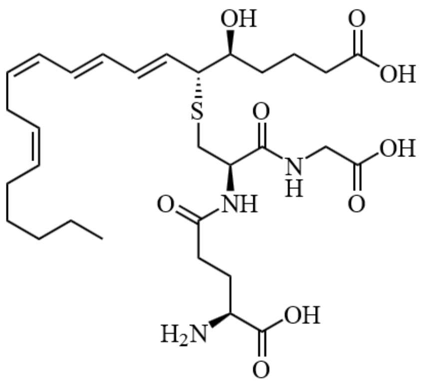
The negative results of our studies are not due to alterations of the CysLTs that we used, as they were identified correctly at mass spectrometry and induced normal cellular response of HUVEC, as previously shown. The inflammatory effects of CysLTs mediated by the platelet $P2Y_{12}$ receptor, which have been demonstrated in vivo experiments, were most likely indirect, rather than induced by a direct interaction of CysLTs with platelets.

1. INTRODUCTION

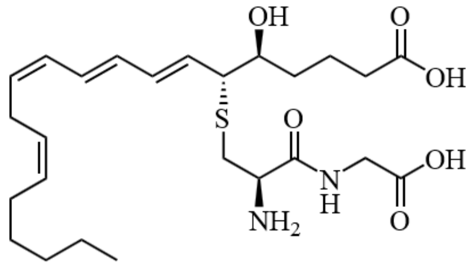
1.1 CYSTENYL LEUKOTRIENES

1.1.1 Leukotrienes : structure and biosynthesis

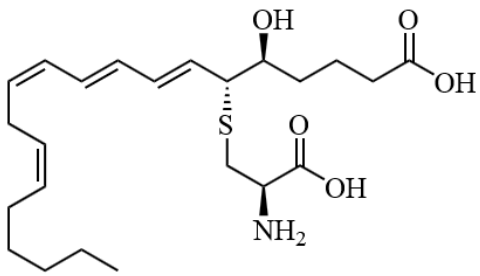
Leukotrienes are a family members of eicosanoid inflammatory mediators produced by immunocompetent cells starting from arachidonic acid [1]. The name leukotriene, introduced by the swedish biochemist Bengt Samuelsson in 1979, comes from the word *leukocyte*, where they were first discovered, and *triene* (indicating the compound's three conjugated double bonds). Leukotriene family consists of five molecules: LTA_4, LTB_4, LTC_4, LTD_4, LTE_4. The latter three are collectively known as “cysteinyl leukotrienes (cysLTs)” due to the presence of cysteine amino acid in their structures.[2]



LTC_4



LTD_4



LTE_4

Figure 1.1 *cysteinyl leukotrienes LTC_4, LTD_4, LTE_4.*

The leukotriene synthesis pathway starts from the release of arachidonic acid from the perinuclear phospholipid membrane, endoplasmic reticulum, and/or

Golgi apparatus by activation of cytosolic phospholipase (cPL) A2α [3]. Arachidonic acid is then converted into LTA_4 through the enzyme of 5-lipoxygenase (5-LO). The lipoxygenase pathway is active in leukocytes (eosinophils, neutrophils, monocytes, and basophils) and other immunocompetent cells (mast cells, macrophages, myeloid dendritic cells). LTA_4 can be converted either into LTB_4 by the LTA_4 hydrolase (LTA_4H) or into LTC_4 by LTC_4 synthase (LTC_4S) (Figure2).

Once synthesized, LTB_4 and LTC_4 are exported from the cell by two members of the multidrug resistance-associated protein (MRP) transporter family, MRP4 and MRP1, respectively [4]. All members of this proteins family mediates the efflux transport of amphipathic anions from the cell (e.g. signaling molecules, uric acid, eicosanoids) and also have an affinity for several drugs, including anticancer and antiviral agents as well as nucleoside analogues [5]. LTC_4 is then converted into LTD_4 by the actions of γ-glutamyl transferase, which removes the glutamate residue from glutathione [6]. LTD_4 is in turn converted to LTE_4 by dipeptidase, which removes the glycine residue [2].

Very few cells express the three key enzymes for the whole leukotriene synthesis (5-LO, LTA_4H, LTC_4S). This indicates that in vivo leukotriene biosynthesis involves cooperation between multiple cell types. Neutrophils, the primary source of LTA_4 generation, do not express LTC_4S [7, 8], which is expressed in eosinophils, mast cells, monocytes, and macrophages. Red blood cells which do not express 5-LO and cannot produce $LTA_{4,}$ are capable to convert exogenous LTA_4 into LTB_4, as shown by in vitro experiments[9, 10], as well as platelets[11] [7] which express LTC_4S and release $LTC_{4.}$ [7, 12].

LTC_4 and LTD_4 have been considered the only active cysLTs, exert their effects locally following secretion. As their short half-lives, LTE_4 is the most stable LTs, but to date data about its biological effects are contradictory [13, 14].

The biological action of cysLT are mediated by two receptor discusses in next paragraph.

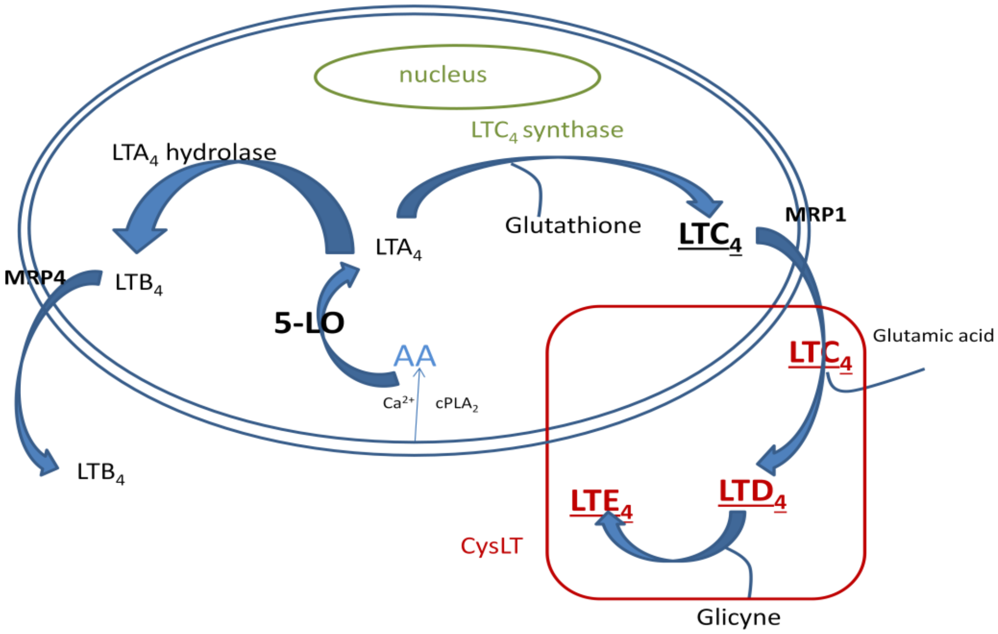


Figure 1.2 : *Schematic figure of leukotriene biosynthesis. Increases in intracellular calcium concentrations promotes the translocation of $cPLA_2\alpha$ to the nuclear membrane, this process liberates arachidonic acid (AA) from the phospholipid bilayer and presents it to 5-LO, which converts it to leukotriene A_4 (LTA_4). LTA_4 is then converted into either leukotriene B_4 (LTB_4) by LTA_4 hydrolase (LTA_4H) present in the cytosol or to leukotriene C_4 by LTC_4 synthase(LTC_4S), at the nuclear membrane. LTB_4 and LTC_4 are exported from the cell by multidrug-resistance proteins (MRPs) 4 and 1, respectively. LTC_4 can also be converted to leukotriene D_4 (LTD_4), which also binds to $CysLT_1R$ and $CysLT_2R$, by γ-glutamyl transferase (GGT). Finally, LTD_4 is converted to the stable metabolite leukotriene E_4 (LTE_4).*

1.1.2 Leuokotrienes activity

Cysteinyl leukotrienes are very potent lipid inflammatory mediators, they induce smooth muscle constriction that is very well studied, and as a result, anti-leukotriene pharmacological agents have become a popular asthma treatment option [15, 16] CysLTs also affect vascular smooth muscle, and can elicit vasoconstriction in both arteries and veins [17, 18]. Interestingly, CysLTs have been shown to induce vasoconstriction in atherosclerotic coronary arteries, but not in healthy people [19]- indicating that pathogenesis may increase the scale and severity of leukotriene-mediated responses. CysLTs also exert effects on circulating cells and on endothelial cells. CysLT stimulation induces chemotaxis of monocytes [20] [21], eosinophils [22], dendritic cells [23] and CD34+ progenitor cells [24, 25]. CysLT stimulation also upregulates Mac-1 in eosinophils [22], promotes proliferation in CD34+ progenitor cells [26], and induces release of the chemokine RANTES from platelets [27]. LTC_4 and LTD_4 stimulation of endothelial cells in vitro results in production of PAF, promotion of neutrophil adhesion [28], secretion of von Willebrand factor, and surface expression of p-selectin [29]. In addition, CysLT stimulation triggers vascular hyperpermeability in multiple vascular beds [2, 30, 31]. CysLTs have also been implicated with an important role in host defense, as attenuated leukotriene synthesis has been linked to increased susceptibility to infectious disease in both humans and animal models [32].

While LTE_4 received its fair share of attention following its discovery, its poor affinity for the classical CysLT receptors resulted in its fading from the spotlight. However, early studies noted that LTE_4 potency was 10-fold greater than that of LTC_4 or LTD_4 in guinea pig trachea [33]. LTE_4 was also distinct in its ability to augment contractile response to histamine, something that LTC_4 or LTD_4 stimulation could not do [34]. LTE_4 was also found to induce vascular permeability with equal potency as LTC_4 or LTD_4 when injected intradermally in humans [35]. These findings suggest that LTE_4 either uniquely mediates pathways that LTC_4 or LTD_4 do not, or that there are receptors that favor LTE_4 as a ligand over LTC_4 and LTD_4.

1.1.3 Cysteinyl leukotrienes receptors

Cysteinyl leukotrienes bind two G-protein coupled receptors, named cysteinyl leukotriene receptor 1 ($CysLT_1R$) and cysteinyl leukotriene receptor 2 ($CysLT_2R$). Further investigation yielded a receptor favouring leukotriene E_4 rather than C_4 and/or D_4[36]. However, until now the two most investigated receptors remains $CysLT_1R$ and $CysLT_2R$.

➢ *$CysLT_1R$*

The human $CysLT_1R$ was first characterized in 1999 by two separate groups. It is a 337 amino acid G-protein coupled receptor that signals predominantly through $G_{q/11}$ class G proteins, although it does show limited activation of $G_{i/0}$ pathways [37, 38]. The human $CysLT_1R$ shares only 38% homology with the human $CysLT_2R$, and is actually more similar to the purinergic receptor $P2Y_1$ (32% homology)[37-39]

Activation of $CysLT_1R$ results in mobilization of intracellular Ca^{2+} via both $G_{q/11}$ and $G_{i/o}$ pathway activation [37, 40]. Induction of proliferation has been observed in numerous cell line following $CysLT_1R$ activation, including human hematopoietic cells [26] epithelial cells [41, 42], smooth muscle cells [43, 44], and astrocytes [45, 46]. In addition, $CysLT_1R$ activation upregulates pro-inflammatory mediators, including β-integrins [25, 47], IL-4[48] , IL-5[27, 49, 50] , IL-8[51] , IL-11[52], TGF-β1[53] , as well as MIP-1α and MIP-1β [54], Finally, CysLTR pharmacological blockade results in decreased of IL-4, IL-5, IL-8, IL-11, IL-13, TNFα, RANTES, and TGF-β [52, 55-57]

➢ *$CysLT_2R$*

The human $CysLT_2R$ was first characterized in 2000 by three independent groups [39, 58, 59] It is a 346 aa protein that shares 33% homology with the orphan receptor GPR17 (and as mentioned before, only 38% homology with $CysLT_1R$)[39]. The human $CysLT_2R$ expression profile is distinct from that of $CysLT_1R$, being found predominantly in the heart (expressed in atria, ventricles, and Purkinje fibers - but not the aorta), spleen, brain, lymphatic system, placenta, and adrenal gland [39, 58, 59]; Indeed, it is the dominant cysLT receptor in heart and brain[58] and unlike $CysLT_1R$, is not found high abundance in the lung [39]. $CysLT_2R$ is also expressed by numerous circulating cells, including eosinophils [60], monocytes, macrophages[39] , mast cells [61] and platelets [27] , but not neutrophils [62]. It is also expressed in HUVECs [63]and coronary artery SMCs [64] .

$CysLT_2R$ activation results in altered endothelial cell function, as well as cytokine secretion. Activation of $CysLT_2R$ in HUVECs leads to increased intracellular calcium concentration, myosin light chain kinase activation [63, 65], P-selectin surface expression [66], and the up regulation of a myriad of pro-inflammatory genes including CXCL2 (which encodes the MIP-2α protein), SELE (E-selectin), IL-8, EGR1, and PTGS2 [67]. In human mast cells, $CysLT_2R$ activation facilitates IL-8 secretion, but through a pertussis toxin-sensitive pathway [61]

➢ *Other CysLT receptors*

In addition to $CysLT_1R$ and $CysLT_2R$, data has implicated CysLT activation of other GPCRs. A possible third cysLT receptor was first proposed after [68] noted that IL-4 treatment in mast cells upregulated two receptors: $CysLT_1R$ and a receptor that could not be $CysLT_2R$ based on pharmacological antagonism experiments. Moreover, these cells responded to both cysLTs and UDP, and as $CysLT_1R$ activation by UDP was later ruled out[69, 70]. Further investigation revealed that GPR17, a receptor structrually and phylogenetically related to other P2Y receptors and CysLT receptors[71], could be activated in a specific and dose-dependent manner by both cysLTs and uracil nucleotides [71, 72]. Nonaka et al. [73] used a computer model to determine that LTE_4 is a potential ligand for $P2Y_{12}$

Of the cysteinyl leukotrienes (LTs; LTC_4, LTD_4, and LTE_4), only LTE_4 is stable and abundant in vivo and it's shows negligible activity at $CysLT_1R$ and $CysLT_2R$ [14]shown that the adenosine diphosphate (ADP)–reactive purinergic ($P2Y_{12}$) receptor is required for LTE_4-mediated pulmonary inflammation. This effect, in mice, is mediated by $P2Y_{12}$ receptor on platelets[14]. There is no evidence of a direct binding between LTs and $P2Y_{12}$.

Another receptor has been recently identified as GPR99 [36]. The LTE_4-mediated ear edema observed in $CysLT_1R$/$CysLT_2R$ double knockout mice was almost completely abolished in GPR99/$CysLT_1R$/$CysLT_2R$ triple knockout mice. Stimulation with LTC_4 or LTD_4 did elicit a mild response in $CysLT_1R$/$CysLT_2R$ double knockout mice, indicating that GPR99 can bind $LTC4_4$ or LTD_4 in the absence of the classical cysLT receptors[36]

1.2 PLATELETS

1.2.1 Platelet biology

Platelets are small anucleate blood cells, with a discoid shape ranging between 1 to 3 µm in diameter. These cell fragments originate from the cytoplasm of megakaryocytes (MKs) in the bone marrow and circulate in the human bloodstream for about 10 days. Platelets lack genomic DNA [74] but contain megakaryocyte-derived messenger RNA (mRNA) and the translational machinery needed for protein synthesis including ribosomes, and initiation and termination factors [75]. Furthermore, platelets contain three types of secretory organelles known as α-granules, δ-granules (dense) and lysosomes, which are generated by the budding of small vesicles containing granule cargo from the *trans*-Golgi zone of the Golgi complex in MKs [76]. The number of α-granules per platelet depends on cell size but and may range between 40 and 80. They contain many proteins, such as coagulation factor V, thrombospondin, P-selectin, von Willebrand Factor (vWF) and fibrinogen. The δ-granules, compared with α-granules, are smaller, fewer, and have high morphological variability. They are rich in ATP and ADP, serotonin, pyrophosphate, calcium, and magnesium. Human platelets also contain few lysosomes (no more than 3), which contain at least 13 acid hydrolases. Other organelles present in the platelet cytoplasm include a small number of simple mitochondria involved in energy metabolism, glycosomes [77], electron dense chains and clusters [78], and tubular inclusions [79].

1.2.2 *Platelets in primary haemostasis*

The main role of blood platelets is to ensure primary haemostasis, which means the rapid cessation of bleeding after tissue trauma and the maintenance of the integrity of the endothelium, in part through the release of proangiogenic cytokines and growth factors. The balance between blood fluidity and rapid thrombus formation in response to injury is regulated by endothelial cells, which synthesize either inhibitors or activators of platelet aggregation and blood clotting [80, 81]. Under normal physiological conditions, platelets circulate close to the endothelium without establishing/ forming stable adhesion contacts. The anti-adhesive phenotype of vascular endothelium cells towards platelet is maintained by at least 4 intrinsic pathways. The arachidonic acid-prostacyclin (PGI_2) and the L-arginine-nitric oxide (NO) pathways inhibit platelet activation by the stimulation of cAMP and cGMP production respectively, whereas endothelial ecto-adenosine diphosphatase (ecto-ADPase/CD39) is involved in ADP metabolism, which is necessary to prevent premature platelet activation at the vessel wall. Furthermore, thrombomodulin rapidly inhibits the prothrombotic effect of α thrombin, reducing platelet activation and fibrin generation (Figure 2).

At sites of vascular injury, platelets interact with the damaged vessel, to form a platelet aggregate. The initial platelet tethering at the surface and subsequent platelet-platelet cohesion are typically differentiated into the following steps: adhesion, activation, secretion and aggregation of platelets [82].

- ### *Platelet Adhesion*

After vascular injury, such as rupture or erosion of the vessel wall, subendothelial matrix proteins such as collagen, von Willebrand factor (vWF), fibronectin and laminin become exposed to the circulating blood. These proteins support platelet adhesion via the engagement of specific receptors, thus rapidly recruiting individual platelets at the site of subendothelial damage. The initial tethering of platelets occurs via the interaction between glycoprotein Ib (GPIb), a component of GPIb-V-IX platelet complex, and exposed collagen-bound vWF. This bond has a rapid dissociation rate and is therefore unable to support stable adhesion, resulting in platelet translocation along the vessel wall. Translocating platelets engage with collagen in the vessel wall through their adhesion receptors glycoprotein VI (GPVI) and GPIa. GPVI is the major collagen receptor, whose stimulation induces the intracellular calcium flux necessary for stable platelet adhesion, cytoskeletal reorganization, integrin glycoprotein IIb/IIIa (αIIbβ3) activation and the release of soluble agonists.

➢ *Platelet Activation and Secretion*

After the initial adhesion, platelets undergo the repair process that requires a quick response to autocrine and paracrine mediators. Platelets experience a complex series of morphological and biochemical changes that lead to the release of platelet granular content such as ADP and serotonin (5-HT), as well as to the synthesis of TxA_2. These endogenous agonists act to enhance platelet activation by interacting with specific G-protein coupled receptors expressed on the platelet membrane. Briefly, ADP and 5-HT are released from platelet dense granules and bind their specific receptors. Activation of the 5-HT_{2A} receptor by 5-HT and the P2Y1 receptor by ADP (both coupled to a Gq protein) induces an increase in intracellular Ca^{2+} levels, whereas activation of $P2Y_{12}$ (couple to Gi protein) by ADP inhibits adenylate cyclase, blocking cyclic adenosine monophosphate (cAMP) production, a potent endogenous platelet inhibitor, and activates PI_3kinase signallling leading to the integrins activation. TxA_2 is synthesized in activated platelets starting from arachidonic acid (AA) by cyclooxygenase (COX). Once formed, TxA_2 diffuses across the platelet membrane and activates other platelets through the interaction with two surface membrane TxA_2 receptors, TPα and TPβ, coupled to the proteins G_q and G_{12} or G_{13}, which activate phospholipase C (PLC). This enzyme degrades membrane phospholipids, thus releasing secondary messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG activates intracellular protein kinase C (PKC), which causes protein phosphorylation, whereas IP_3 increases cytosolic Ca^{2+} levels from the endoplasmic reticulum. In addition platelets provide a catalytic surface necessary for local production of thrombin thus enhancing platelet activation. Indeed, at the site of injury prothrombin is proteolytically cleaved to form thrombin, a serine protease that converts soluble fibrinogen into insoluble strands of fibrin. Subsequently, thrombin mediates cleavage of the N-terminal extradomain of protease-activated receptors (PAR)-1 and (PAR)-4, that increases intracellular calcium (Ca^{2+}). The generation of thrombin is contingent upon the expression of tissue factor (TF) on the surface of fibroblasts, smooth muscle cells, endothelial cells and leukocytes. Thrombin is among the most potent stimulators of platelets.

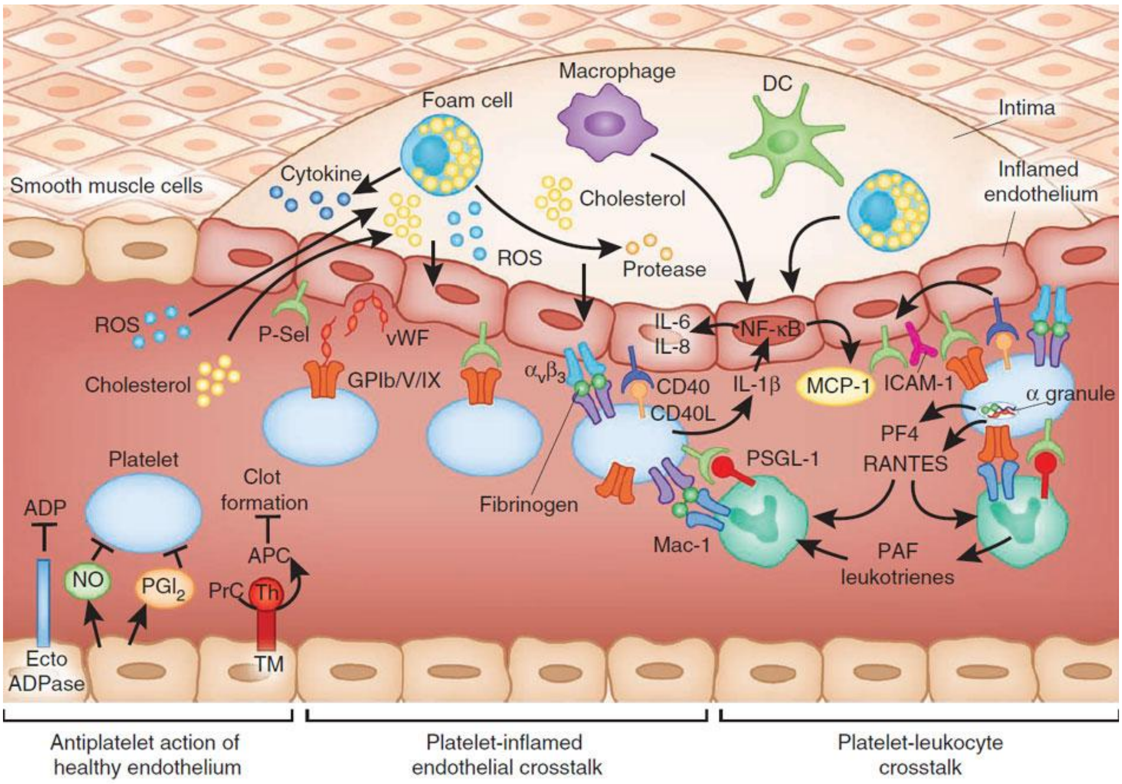


Figure 1.3. *Picture modified from* [83] *The antiadhesive phenotype of endothelial cells is maintained through four intrinsic pathways: ecto-ADPase, prostaglandin I2 (PGI$_2$), nitric oxide (NO) and the thrombomodulin (TM)-activated protein C (APC) pathways.*

➢ *Platelet Aggregation*

Aggregation is the amplification step that involves accumulation of platelets into the hemostatic thrombus through release of soluble agonists that enhance recruitment of further platelets. The stimulation of Gq and Gi signaling pathways leads to activation of the glycoprotein complex GPIIb/IIIa. Activated GPIIb/IIIa binds multiple ligands, including vWF [84] [85], fibrinogen [86], fibrin and fibronectin [87], able to form stable platelet aggregates [88]. The primary hemostatic plug is consolidated by fibrin generation at the site of injury. Platelet activation is under tight negative control to limit and contain thrombus formation within the boundaries of the lesion in the vessel wall.

1.2.3 *The platelet P2Y12 receptor for adenosine diphosphate*

- *ADP signalling in platelets*

Adenosine diphosphate (ADP), the first known low molecular weight platelet aggregating agent, plays an important role in platelet function despite being a weak platelet agonist. As such, it only induces platelet shape change and reversible aggregation in humans. Platelet secretion and secondary aggregation observed after stimulation with ADP of normal, human citrated platelet-rich plasma are due to the aggregation-dependent formation of TxA_2. ADP is released in high concentration from platelet dense granules where it is stored and amplifies platelet responses induced by other agonists [89, 90] and stabilizes platelet aggregate [91, 92]. As previously described, ADP interacts with two different G protein coupled P2 receptors on the platelet surface: $P2Y_1$ and $P2Y_{12}$.The signal transduction involves a transient rise in free cytoplasmic calcium trough the Gq-linked $P2Y_1$ receptor, and the inhibition of adenylyl cyclase, mediated by the Gi-linked $P2Y_{12}$ receptor [93]. The activation of $P2Y_1$ receptor by ADP mediates platelet shape change and initiates platelet aggregation, while $P2Y_{12}$ amplifies platelet aggregation response[94]. Concomitant activation of both G protein-coupled receptors is essential to elicit normal platelet aggregation [93, 95] (Figure 3).

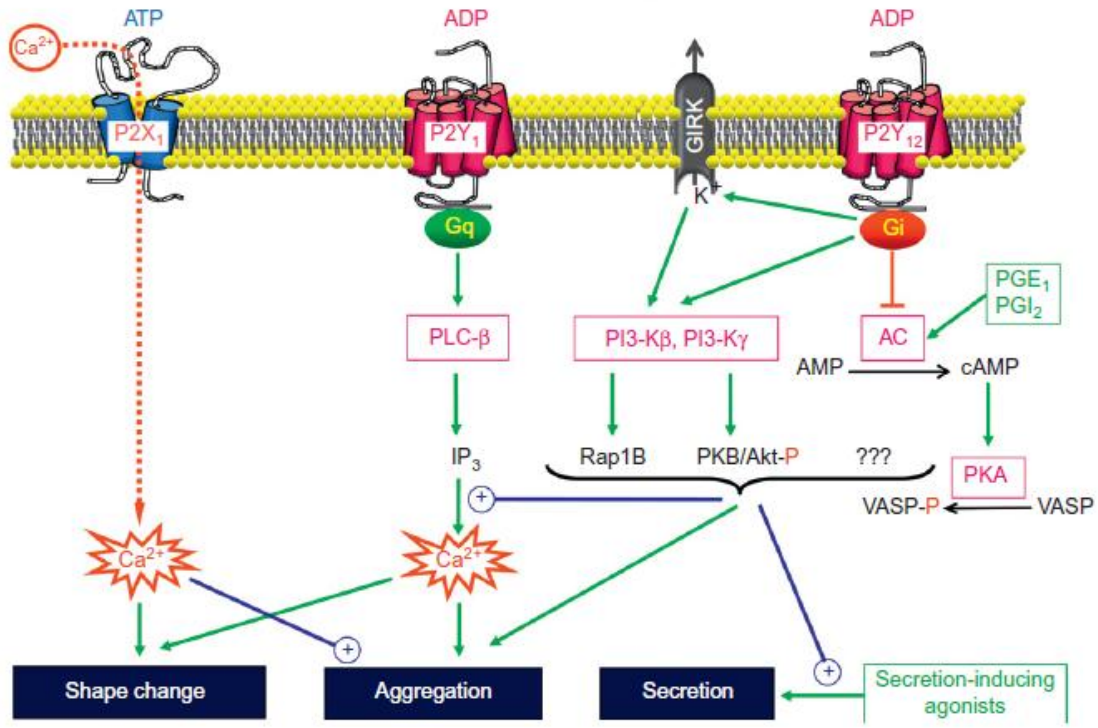


Figure 1.4. *Figure modified from [96]. Role of $P2Y_{12}$ in platelet aggregation. ADP interacts with $P2Y_{12}$, a seven-transmembrane receptor that is coupled to Gi protein. This bond induces platelet aggregation and amplifies the aggregation response that is induced by other agonists or by ADP itself, by interacting with its other platelet receptor, $P2Y_1$. $P2Y_{12}$ stabilizes platelet aggregates and amplifies the secretion of platelet dense granules stimulated by secretion-inducing agonists (coupled to Gq). $P2Y_{12}$ stimulation inhibits adenylyl cyclase (AC) through Gi, this function does not appear to be directly related to $P2Y_{12}$ -mediated platelet activation. However, it could have important implications in vivo, where platelets are exposed to the inhibitory prostaglandin PGI_2 (prostacyclin), which inhibits platelet aggregation by increasing platelet cyclic adenosine monophosphate (cAMP) through activation of AC mediated by Gs: inhibition of AC by $P2Y_{12}$ counteracts the inhibitory effect of prostacyclin, thereby favoring the formation of platelet aggregates in vivo.*

➢ *Platelet $P2Y_{12}$ receptor*

The P2 receptors, which interact with purine and pyrimidine nucleotides, are divided into two groups: G protein-linked or metabotropic, termed P2Y, and ligand-gated ion channels or ionotropic, termed P2X [97]. The P2Y receptors are seven-membrane-spanning proteins with a molecular mass of 41 to 53 kD after glycolysation [97]. The carboxyl terminal domain is on the cytoplasmatic side, whereas the amino terminal domain is exposed to extracellular environment. The mechanisms of signal transduction are shared by most seven-membrane-spanning receptors, and include activation of phospholipase C and regulation of adenylyl cyclase activity. The Gq coupled receptor $P2Y_1$ leads to activation of β-isoforms of phospholipase C (PLC) and triggers the mobilization of Ca^{2+} into the cytoplasm. The Gi coupled receptor $P2Y_{12}$ leads to inhibition of adenyl cyclase (AC) with a decrease of intraplatelet cAMP. Co-interaction of $P2Y_1$ and $P2Y_{12}$ is necessary for normal ADP-induced platelet aggregation, in fact separate inhibition of either of them with selective antagonists results in a dramatic decrease in aggregation [95, 98, 99]. The stimulation of the ADP receptors, predominately the $P2Y_{12}$ receptor, assists to activation of integrin GP IIb/IIIa (fibrinogen receptor)[100, 101]. $P2Y_{12}$ is important for both normal hemostasis and pathological thrombosis, this explains why $P2Y_{12}$ receptor is one of the main target for antiplatelet drug.

1.2.4 *Platelet and inflammation*

In addition to their well characterized and established role in hemostasis and thrombosis, platelets have also inflammatory functions and influence innate and adaptive immune responses [102-104]. Basically, platelet increase endothelial permeability, leading to extravascular fluid accumulation, and when pathologically in disease.[105-107]

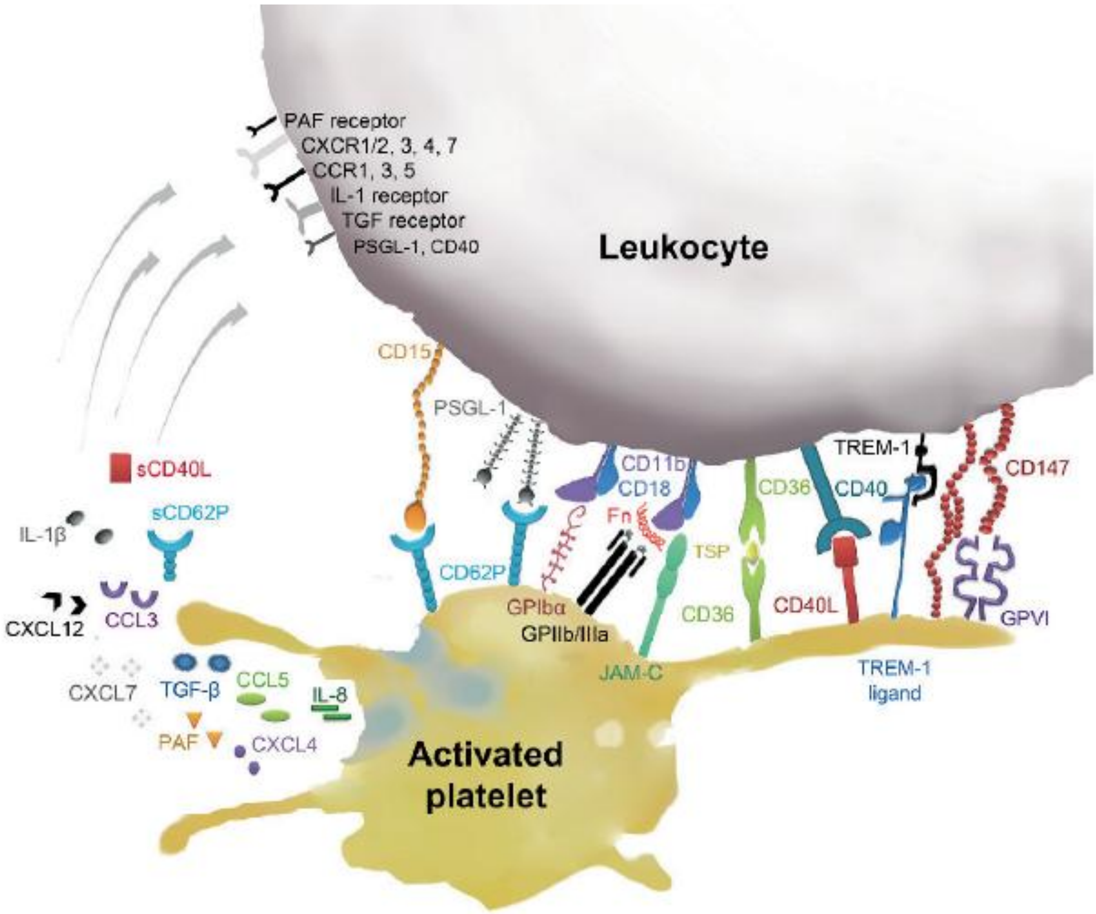


Figure 1.5: *modified from [108] Direct and indirect platelet-leukocyte interactions: platelet and leukocyte surface receptors and the most important platelet α-granule derived cytokines that modulate leukocyte responses are depicted*

Platelet activation triggers exocytosis of platelet granules, which comprise a plethora of immune-modulatory factors[109] (Figure 1.5). Platelets express toll-like receptors, which initiate the innate immune response; interact with activated endothelium, undergo chemotaxis, “prime” leukocytes for efficient tissue recruitment, and activate other inflammatory cells. [102-104].

Platelet dense granules content, including ADP and ATP, are important for activation and recruitment of further platelets. Platelet α-granules contain platelet factor 4 (PF4, CXCL4), macrophage inflammatory protein 1 (MIP-1, CCL3), regulated on activation, normal T cell expressed and secreted (RANTES, CCL5), neutrophil activating protein 2 (NAP-2, CXCL7), interleukin 8

(IL-8) and IL-1, CD40 ligand (CD40L) and P-selectin (CD62P), which are involved in recruitment and/or activation of leukocytes. [110-112]

In particular, CD62P will bind to P-selectin glycoprotein ligand-1 on leukocytes and create platelet-leukocyte aggregates and trigger leukocyte activation. CD40 interaction with CD40L on the platelet surface or soluble CD40L is an important mediator of platelet-induced adaptive immune responses. CD40 is expressed on mature B-cells, some T-helper (Th) cells and cytotoxic T-lymphocytes as well as platelets. Via CD40L platelets can directly induce B-cell antibody production and support Th1-cell-mediated germinal centre formation. [108]

Recently, microRNAs, small non-coding RNA molecules such as miRNA-223, -126, -21, -24, and -197, were found in platelets, secreted in exosomes and/or microvesicles upon platelet activation and once taken up by cells, can further trigger inflammatory processes and increase atherosclerosis and angiogenesis [113, 114].

As result of leukocytes and EC activation, at the site of inflammation release of pro-inflammatory cytokines and adhesion receptors expression increase that further amplify the inflammatory process.[115]

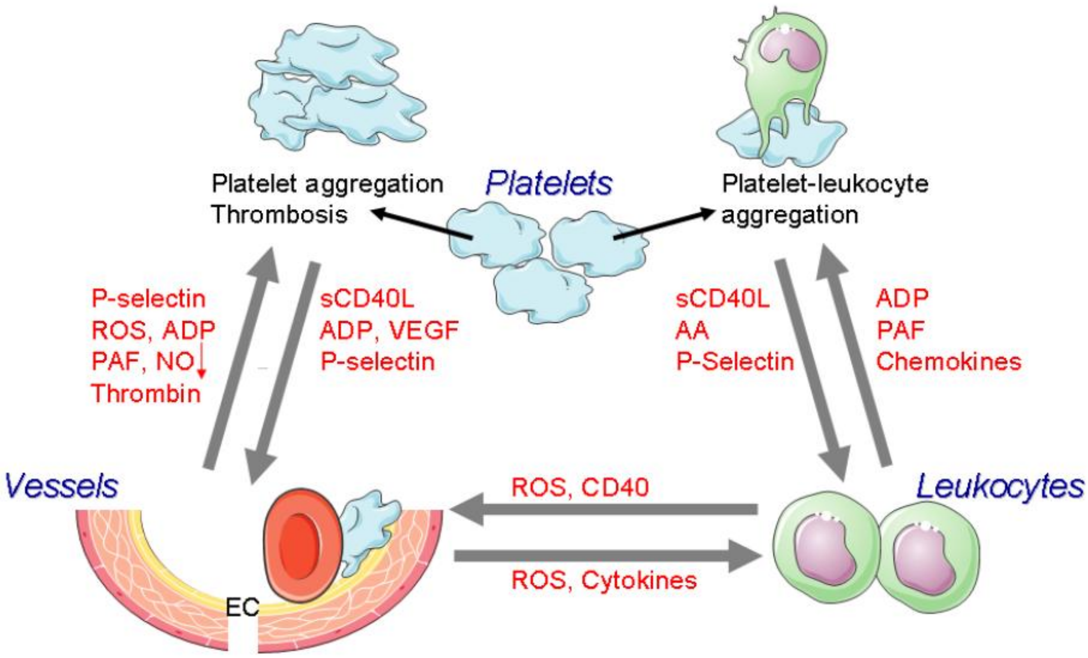


Figure 1.6*: modified by [115] Illustration of the role of platelets in amplification of inflammation via interactions with leukocytes and endothelial cells and the release of cytokines from all cells involved. Platelets store pro-inflammatory cytokines that are released when platelets are activated. In addition activated platelets translocate adhesion receptors e.g. CD62P (P-selectin) and CD40L from their alpha granules to the plasma membrane. CD62P will bind to P-selectin glycoprotein ligand-1 on leukocytes and create platelet-leukocyte aggregates and trigger leukocyte activation. CD40L will bind to CD40 on a number of cells including endothelial cells (EC) and trigger their activation.*

➢ *The platelet $P2Y_{12}$ receptor in inflammation*

Several studies indicated that inflammatory disease conditions are associated with the extracellular release of nucleotides and highlighted fundamental roles for P2Y receptors during inflammatory and infectious diseases. The two P2Y receptor expressed on platelets have been shown to potentially play a role in inflammation. The platelet $P2Y_1$ receptor contributes to P-selectin exposure, platelet-leukocyte aggregates formation and tissue factor exposure when platelets are stimulated with ADP, collagen or low concentrations of thrombin receptor agonist peptides [116, 117].

Some studies [118, 119] have documented decreased exposure of P-selectin, diminished formation of platelet-leukocyte aggregates, and less subsequent tissue factor exposure also in patients on treatment with thienopyridines, inhibitors of $P2Y_{12}$ receptor such as ticlopidine and clopidogrel, demonstrating a potential role of the platelet $P2Y_{12}$receptor in inflammation in vivo.

In addition, it has been reported that the inhibition of CD40L exposure and release [120] and the reduction of circulating levels of C-reactive protein in response to $P2Y_{12}$ antagonists [121-123], supporting the role of $P2Y_{12}$ in inflammatory processes. Clopidogrel withdrawal was associated with an increase in platelet and inflammatory biomarkers in diabetic patients[124]

The critical role of platelets in vascular inflammation and its inhibition by $P2Y_{12}$ antagonists has been demonstrated also in vitro both in humans and experimental animals. Indeed in a murine model of abdominal aortic aneurism, treatment with clopidogrel significantly suppressed aneurysm formation, inflammatory cytokine expression, infiltration of macrophages and production of matrix metalloproteinases[125]. Inhibition of $P2Y_{12}$ receptors in vitro by clopidogrel's active metabolite reduced P-selectin expression, platelet-polymorphonuclear leukocytes adhesion and production of reactive oxygen species by polymorphonuclear leukocytes [126].

However, the inflammation-reducing effects of blocking $P2Y_{12}$ have not been a persistent finding in all trials and the results of different studies in this field are sometimes contradictory [127-131].

Another inhibitor of $P2Y_{12}$ receptor, the active metabolite of prasugrel yielded in vitro a concentration-dependent inhibition of platelet aggregation, soluble CD40L release, and platelet-leukocyte aggregates formation in healthy volunteers [132]. The latter finding was confirmed by others [133] in human blood samples and extended to the inhibition of agonist-stimulated platelet-monocyte adhesion in blood samples from mice [134].

Prasugrel reduced tumour necrosis factor (TNF)-alpha synthesis and increased nitric oxide (NO) metabolites in endotoxin-treated mice in vivo [134]. On the other hand, the anti-inflammatory actions of the active metabolite of prasugrel was likely derived from direct targeting of neutrophils isolated from human blood and was $P2Y_{12}$ receptor independent [135]. In a randomized, placebo controlled, cross-over study it was recently shown that treatment with the thienopyridine $P2Y_{12}$ antagonist prasugrel of patients with allergic asthma for 15 days tended to reduce bronchial hyper-reactivity to mannitol. [136] This effect of prasugrel likely reflects a reduction in airway inflammation, because the mannitol test, like other forms of indirect airway challenge, more closely reflects active airway inflammation than the direct challenges, such as the metacholine test[137] The greater specificity of the mannitol test for detecting changes in airway hyper-responsiveness in asthma patients is likely explained by the fact that it mimics the normal pathophysiology of bronchial asthma, causing the release of various mediators of bronchoconstriction[138] Although the results of this study cannot clarify whether the effect of prasugrel was mediated by its interaction with $P2Y_{12}$ on platelets or other cells, the former hypothesis is supported by the results of experimental studies that demonstrated the important role of platelet $P2Y_{12}$ in the recruitment of inflammatory cells in lungs of sensitized mice challenged with cysteinyl leukotrienes [14, 139]

1.3 CYSTENYL LEUKOTRIENES AND PLATELET IN INFLAMMATION

Allergic bronchial asthma is a chronic inflammatory disease that impairs the quality of life and is associated with significant mortality rate. In 1981, it was shown that platelets are activated during antigen-induced bronchocostriction [140] As mentioned before Cysteinyl-Leukotrienes interact with G protein-coupled receptors and play a role in allergic asthma. Platelets adhere to leukocytes and amplify the production of Cysteinyl-LTs [141], express $CysLT_1R$ and $CysLT_2R$ and, when exposed to LTD_4 or LTE_4, release RANTES, a powerful eosinophil chemoattractant [27]. More recently, it was shown that platelets accumulate in lungs of asthmatic patients, are required for airway wall remodelling and recruitment of inflammatory cells in murine allergic lung inflammation [142, 143] and migrate into the lungs of ovalbumin sensitized and -challenged mice by an IgE dependent mechanism [144]. Recently, it was shown that LTE_4 enhances inflammatory cell recruitment in lungs of sensitized mice, which is abrogated by platelet depletion, by treatment with the anti-$P2Y_{12}$ thienopyridine drug clopidogrel[145], and in mice lacking $P2Y_{12}$, but not in mice lacking $CysLT_1R$ and $CysLT_2R$ [14] Moreover, intranasal administration of LTC_4 in sensitized mice before ovalbumin challenges potentiated the recruitment of eosinophils in the bronchoalveolar lavage, which was dependent on $CysLT_2R$, but also on $P2Y_{12}$ and platelets [139]. The mechanism by which the platelet $P2Y_{12}$ contributes to the effects of cysteinyl- leukotrienes is uncertain. Because LTE_4 shows negligible activity at $CysLT_1R$ and $CysLT_2R$, its biological effects are likely mediated by a third, elusive receptor, which was tentatively identified with $P2Y_{12}$, based on computer modelling and the demonstration that LTE_4 signals through $P2Y_{12}$ in transfected cells [73] However, more recently GPR99 was identified as the elusive receptor for LTE_4[36]. Moreover, studies that demonstrated the important role played by platelet $P2Y_{12}$ in LTE_4 or LTC_4-induced enhanced recruitment of inflammatory cells in the lungs of sensitized mice failed to show that the CysLT interact directly with the platelet $P2Y_{12}$, which might therefore play an indirect, albeit important role in the process [14, 139, 141] CysLTs induced concentration dependent calcium mobilisation in cells overexpressing $CysLT_1R$ and $CysLT_2R$ but failed to induce any calcium response in cells expressing $P2Y_{12}$. Similarly, specific response to 2-MeS-ADP, but not to cysLTs was also observed in cells expressing $P2Y_{12}$ when intracellular cAMP and beta-arrestin signalling. [146] Also in human platelets LTE_4 cannot affect the externalization of P-selectin or the production of cAMP. [146]. So far, the role of cystenyl leukotrienes in platelet aggregation is not well described. In 1986 Metha et al.[147] indicate that leukotrienes alone had no direct effect on platelet aggregation, but potentiated the effects of subthreshold concentrations of epinephrine and thrombin, indendenty by prence of Calcium. Cystenyl leukotrienes potentiate epinephrine-induced platelet aggregation by modulating TXA_2 synthetase activity. Independently of whether it plays a direct or an indirect role in the inflammatory process, these data clearly suggest that the platelet $P2Y_{12}$ represents an ideal pharmacological target for the treatment of allergic asthma. The demonstration that $P2Y_{12}$ variants are associated with lung function in a large family-based asthma cohort and that house dust mite modulates these associations through gene-by-environment effects provided the first human evidence supporting a role for $P2Y_{12}$ in this disorder [148].The role of platelets in inflammatory processes, in particular, has gained particular attention in the last three decades. Among the many platelet receptors and molecules that are involved in inflammation, the platelet $P2Y_{12}$ receptor for ADP has recently been implicated in the pathogenesis of allergic asthma, through its direct or indirect interactions with cysteinyl-leukotrienes. Both an observational, epidemiologic study and, more recently, a small, proof-of-concept randomized clinical trial supported the hypothesis that $P2Y_{12}$ may represent an important pharmacological target for the treatment of patients with allergic bronchial asthma.

2. AIM OF THE STUDY

Aim of this study was to test whether cysteinyl leukotrienes elicit platelet functional responses, by interacting with the platelet $P2Y_{12,}$ receptor for ADP.

3. MATERIALS AND METHODS

3.1 REAGENTS

Epinephrine and ADP were supplied by Sigma Aldrich (Milano, IT). Cangrelor (Can) was from The Medicines Company, USA. LTC_4, LTD_4 and LTE_4 purity ⩾ 97%), were supplied by Cayman Chemical Company, Ann Arbor, MI, USA.

The fixative solvent PamFIX were supplied by Platelet Solution (Nottingham, UK).

Hirudin ReVasc powder was supplied by Boehringer Ingelheim RCV GmbH (Vienna, Austria) Each vial contains 15 mg desirudin corresponding to approximately 270,000 antithrombin units (ATU) or 18,000 ATU per mg of desirudin with reference to the WHO Second International Standard for alpha-thrombin.

Antibody for P-selectin is a PE Mouse Anti-Human CD62P, clone AK-4. It's matching isotype control is a Mouse IgG1, κ, as indicated in data sheet.

Antibody used to identify platelet is an APC-Mouse. Anti-Human CD42b, clone HIP1.

All antibodies were supplied by Bekton Dickinson Italia S.p.A.

Cell culture supplies (media, serum, supplements and antibiotics) and TRIzol were from Gibco Life Technologies (LifeTechnologies Italia, Monza, MB, Italy). Laboratory disposable products (Petri, multi-well plates, etc.) were from Euroclone (Pero,MI, Italy).

Montelukast was kindly provided by Merck (Merck & Co., West Point, PA).

AP100984-2A was a kind gift from Dr. J. Evans, Amira Pharmaceuticals (San Diego, CA).

3.2 HEALTHY SUBJECTS

Twenty-four apparently healthy subjects were studied, who were recruited among the laboratory personnel and medical students of our institution. All subjects abstained from any drug known to affect platelet function for at least 10 days before blood sampling.

3.3 BLOOD SAMPLING

Patients had to refrain from smoking for at least 2h before blood sampling; a light breakfast was allowed in the morning of the study. Blood samples were collected from an antecubital vein, using a 21-gauge butterfly needle and a tourniquet, released soon after needle insertion. The first 3 mL of blood were collected into K-EDTA and analyzed by coulter hematology analyzer (Beckman Coulter, Milano, IT); the following blood was collected into plastic PP tubes containing trisodium citrate (109 mM, 1:9, v/v) or Hirudin ReVasc (5 mg/mL; 18,000 ATU per mg of desirudin) gently mixed, allowed " to rest" at room temperature for 15 min, and used for platelet aggregation studies.

For P-selectin experiments (flow cytometry) blood samples were collected in commercial tubes containing sodium citrate (109 mM, 1:9, v/v; Sarsted) and a cocktail, simultaneously added, containing Na_2EDTA (50mM), N-ethylmaleimide (60mM), and aprotinin (2000 KIU/mL) to inhibit the activity of protease. [149]

3.4 PLATELET AGGREGATION

Platelet aggregation was measured by light transmission aggregometry (model PAP-8E, Biodata, Horsham, PA, USA) in platelet rich plasma (PRP), obtained by centrifugation of citrate or hirudin whole blood at 200 x g for 10 min at room temperature [150]. Autologous platelet-poor plasma (PPP) was obtained by centrifugation of blood samples at 1.400 x g for 15 min at room temperature. Autologous PPP was used to set the instrument's 100% light transmission, while unstimulated PRP was used to set 0% light transmission. The individual platelet count of PRP samples was not adjusted to a pre-determined range, because this procedure may induce artefacts [151]. We tested the effect of leukotrienes on platelet aggregation in two different experimental situations: in citrate-PRP (low concentration of ionized calcium) and in hirudin-PRP (physiological concentration of ionized calcium). All aggregation tests were performed within 3 hours after blood collection.

Briefly, PRP was placed into a test tube containing a stir bar, test compound (LTC_4 0.8 µM , LTD_4 1 µM, or LTE_4 1.1 µM) or vehicle (ethanol 0.25%) was added without stirring and incubated at 37 °C for three different times: 0, 3, and 6 minutes, then the aggregation was induced by ADP or Epinephrine at two different concentrations (0.1 µM and 1 µM) and recorded for 6 minutes. The maximal aggregation response, expressed as percent increase in light transmission, was measured.

3.5 MEASUREMENT OF PLATELET cAMP

Platelet cAMP was measured by a radioisotopic assay, using a commercially available kit (Cyclic AMP [3H] assay system; Amersham International, UK). Duplicate samples of 1 mL citrated PRP were incubated at 37°C for 2 minutes with a mixture containing theophylline (1 mM), PGE_1 (2 µM), and Tyrode's buffer or ADP (0.1 µM) in presence and absence of LTC_4 (0.5 µM) or LTE_4 (0.5 µM). A set of these experiments with LTC_4 was carried out in presence and absence of cangrelor (1 µM). After incubation, 1 mL of 5% trichloroacetic acid was added, and the samples were snap-frozen in dry ice and methanol, thawed at ambient temperature, and then shaken at 4°C for 45 minutes. After centrifugation at 4°C for 30 minutes, the supernatant was extracted 3 times with 5 mL of water-saturated ether, dried under a stream of nitrogen at 60°C, and stored at −20°C. Before assay, the samples were reconstituted with 0.05 mol/L Tris buffer containing 4 mmol/L EDTA. [152]

3.6 FLOW CYTOMETRY

3.6.1 Stimulation of blood samples

Blood samples were diluted 1:2 with sterile saline solution [153, 154] and then stimulated with LTC_4 (0.8 µM) or LTD_4 (1 µM) or LTE_4 (1.1 µM) or vehicle (Ethanol, compounds used for dissolving leukotrienes) in presence or not of cangrelor 1µM, at room temperature, without stirring, for 25 minutes. Then saline, ADP 0,1 µM or ADP 1 µM was added, the samples were gently mixed and left for 5 minutes at room temperature without stirring.

The reaction was stopped by addition of fixative (PAMFix) at a ratio of 2 volumes of PAMFix to 1 volume of the sample. Fixed samples were stored at 4°C and then analysed between 24 hours and 9 days [153]

3.6.2 Labelling and analyses at flow cytometer

Fixed samples were labelled with a cocktail of the following antibodies: anti-human APC labelled CD42b (used to identify platelets), - PE labelled anti-CD62P (used to identify P-selectin) or isotype-matched control antibodies in a ratio of 9 volumes of stimulated blood and 1 volume of each antibody. After one hour of incubation in dark, the samples were analyse by using FACSverse™ BD FACS verse 6 color flow cytometry.

Calibration beads automate the characterization of cytometer fluorescence detectors and the entire optical configuration by creating baselines with performance values, which have to be targeted prior to each measurement to ensure standardized performance.

BD FACSuite™ software controls the connection between the flow cytometer and instruments being the platform to perform calibration and acquisition.

All samples were acquired with a slow flow rate, and for each samples 5000 events were recorded.

3.6.3 Titration curve

Immediately after the puncture the blood was stimulated at room temperature, without stirring for 5 minutes with TRAP 10 µM. The same amount of blood was incubated with increasing amount of antibody CD42b-APC and CD62P-PE:0.25; 0.5; 1, 2 and 5 µL of each antibody with 20 µL of blood.

The sample were acquired with same instrument and program of sample described above.

3.6.4 Gating strategy

We evaluated P-selectin expression in a whole blood sample, but we were interested only in platelet, then we acquired with threshold in APC labelled to CD42b (identifier of the platelets). The cloud of platelet appeared clear in a dot-plot graph with FSC parameter on *x* axis and SSC parameter on *y* axis, in a logarithmic scale. In this graph we traced the cloud named platelet. (Fig 3.1)

By using a dot plot CD42b-APC *vs* CD62P-PE, we plotted cells gated in "platelet" and we defined the positive cells basing on isotype control. (Fig 3.1)

We fixed the isotype control at a maximum of 4% of positive to CD62P cells.

The analytic parameter used to evaluate the level of P-selectin expression was always the % of cells positive to the antibody CD62P.

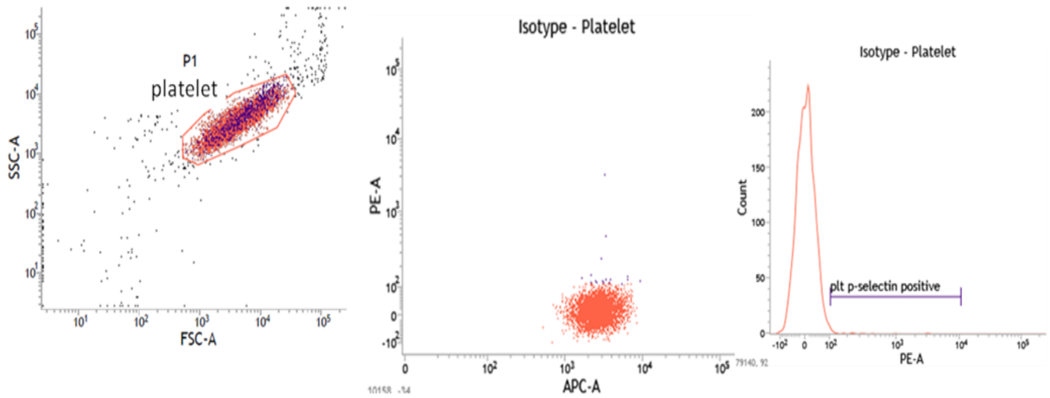


Figure 3.1: *Measurement of P-selectin by flowcytometer: isotype control.*

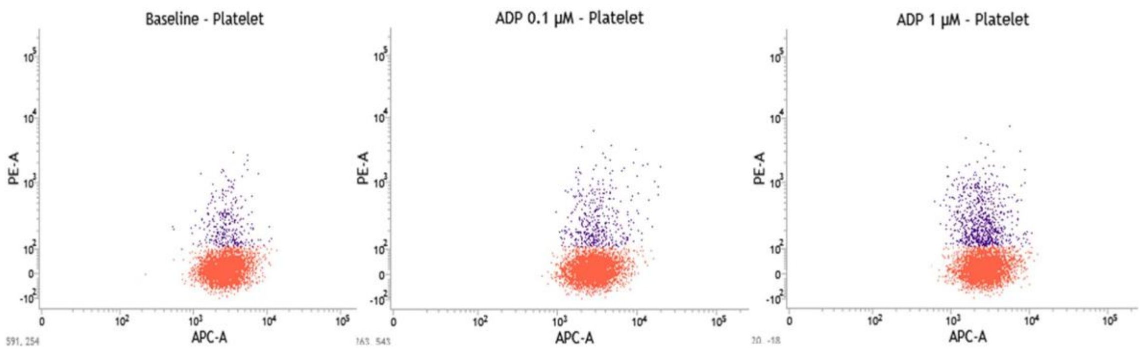


Figure 3.2: *Measurement of P-selectin by flowcytometer: expression of p-selectin after stimulation with increasing concentration of ADP*

3.7 MASS SPECTROMETRY

Mass spectrometry was performed using an ABSciex 3200 QTrap mass spectrometer equipped with an ESI ion source and combined with HPLC (Ultimate 3000 Dionex) . Data acquisition and elaboration were carried out by Analyst software Ver. 1.6 (ABSciex).
Operating conditions for the ESI source, used in the negative ionization mode, were optimized by adding standard stock solutions containing LTC_4, LTD_4, and LTE_4 in ethanol 10 ng/μL) to the HPLC flow. Mobile phase consisted of acetonitrile–water–formic acid (20:80:0.1, v/v/v) and the flow rate was 0.3 mL/min. The injection volume was 10 μL for each LT . The parameter settings were the followings: source temperature of 600°C, spray voltage of 4000 V, curtain gas of 25 psi, ion source gas 1 of 55 psi, ion source gas 2 of 70 psi, collision gas of 6 arbitrary units and dwell time of 65 ms per ion. [155]
The resulting mass spectra were acquired in the full-scan mode (scan range: m/z 150 – m/z 650) and the product ions (624 m/z for LTC_4 , 495 for LTD_4, 438 for LTE_4) were extracted and analysed.

3.8 RESPONSES OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS TO CysLTs

3.8.1 Preparation of human umbilical vein endothelial cells

ECs were isolated from human umbilical cord veins (HUVECs) obtained after informed consent at the G. Salvini Hospital (Garbagnate Milanese, MI, Italy) and at the Fatebenefratelli Hospital (Erba, CO, Italy). The veins were cannulated and perfused with sterile physiological solution (0.9% NaCl); after incubation with Collagenase A (0.015–0.035% in Dulbecco's Phosphate Buffered Saline – D-PBS) at 37◦C for 15 min, ECs were centrifuged (15 min, 280 g), resuspended in M199 supplemented with 20%fetal calf serum (FCS), 0.1 mg/ml heparin, 0.1 mg/mL ECGF, 1% penicillin/streptomycin/fungizone solution, and then cultured in gelatin-precoated flasks (25 cm^2). After reaching confluence, cells were treated with trypsin and seeded into the appropriate Petri dishes, trans well filters or glass covers lips and allowed to expand to the desired confluence for subsequent use. Cells were used up to the third passage. [[156]]

3.8.2 Real-time impedance analysis of HUVECs response to cysteinyl-LTs

HUVECs were seeded onto E-Plate L8 (ACEA Bioscience, SanDiego, CA) precoated with gelatin 0.1% and used at 80% confluence. $CysLT_2$cpd or vehicle were added 15 min before CysLTs (LTC_4 or LTD_4 or LTE_4 0.1µM) and changes in electrical impedance were measured by i-Celligence Real Time Cell Analyzer (ACEA Bioscience) at 37◦C and 5% CO_2 for 15 minutes. [[156]]

3.8.3 *Determination of cytosolic Ca^{2+} levels*

HUVECs cells were seeded onto 12-mm diameter glass covers lips and used when 80–90% confluence was reached. Cells were incubated for 60 min at 37°C in the dark with 2 µmol/L fura2/AM in saline solution (NaCl 145 mmol/L, KCl 5 mmol/L, $MgCl_2$1 mmol/L, $CaCl_2$1.8 mmol/L, HEPES 10 mmol/L, glucose 10 mmol/L; pH 7.4) plus 0.03% pluronic F-127. After loading, the cells were washed twice with a saline solution. The covers lips were transferred to a spectrofluorimeter (Perkin Elmer LS50) cuvette and fluorescence was monitored at 37◦C (505 nm emission, 340 and380 nm excitation). In order to evaluate the concentration of cytosolic-free Ca^{2+} ion ($[Ca^{2+}]_i$) from fluorescence recording, calibration was performed as follows: F_{max}(maximal fluorescence of the system) was obtained by adding 2 µmol/L ionomycin and 100 µmol/L digitonin, F_{min} was obtained by adding 5 mmol/L EGTA and 60 mmol/L Tris base. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz [157] with a K_d= 224 nmol/L. Values were expressed as fold increase over basal. [156]

3.9 STATISTICAL ANALYSIS

All statistical analyses were performed using GraphPad Prism Ver.5 (GraphPad Software Inc, CA, USA). Normal distribution was evaluated by D'Agostino-Pearson test. Parametric or non-parametric tests were used as appropriated. For comparison among multiple groups one-way ANOVA or Friedman test was used followed by Bonferroni or Dunn’ post hoc test, respectively.

Differences were considered statistically significant with p-value<0.05.
Results were expressed as mean and standard deviations.

4. RESULTS

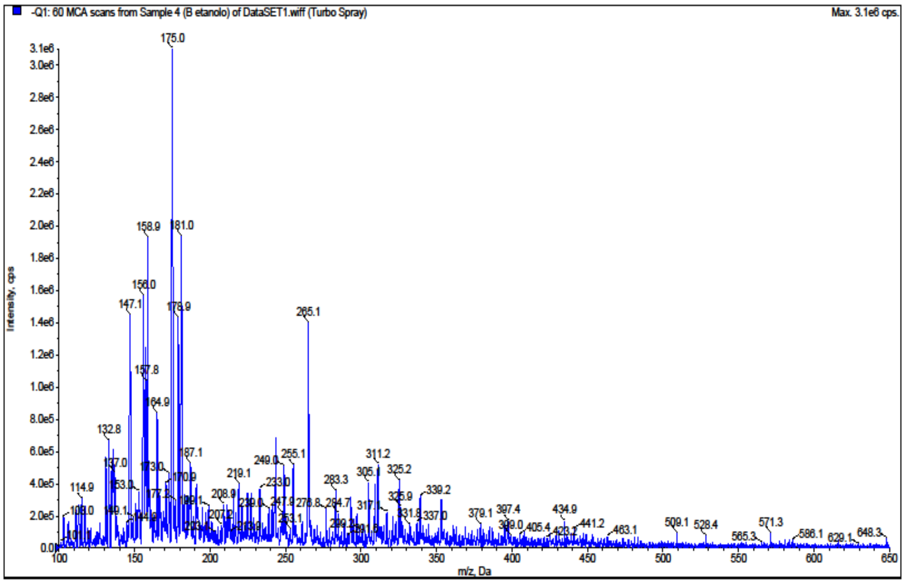
4.1 CHARACTERIZATION OF CYSTENYL LEUKOTRIENES

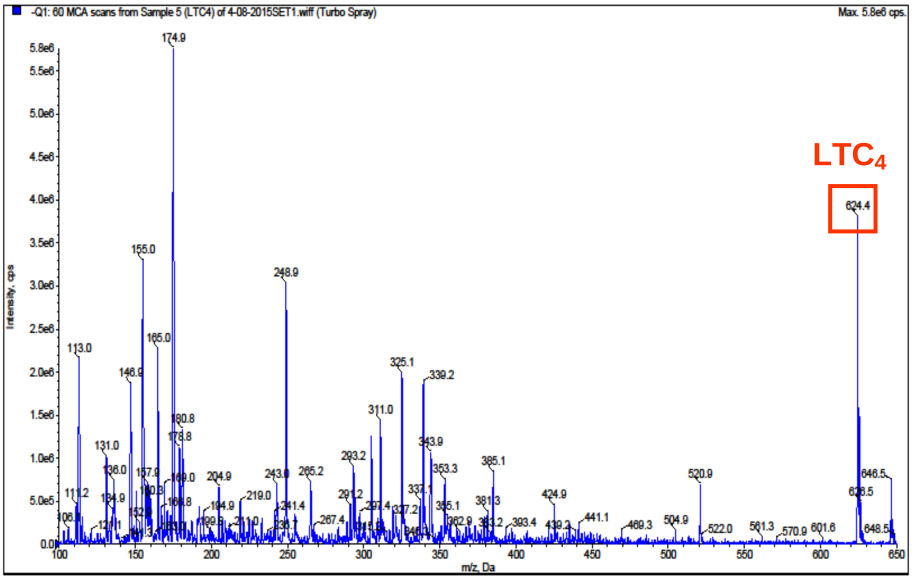
4.1.1 Identification of leukotrienes by mass spectrometry

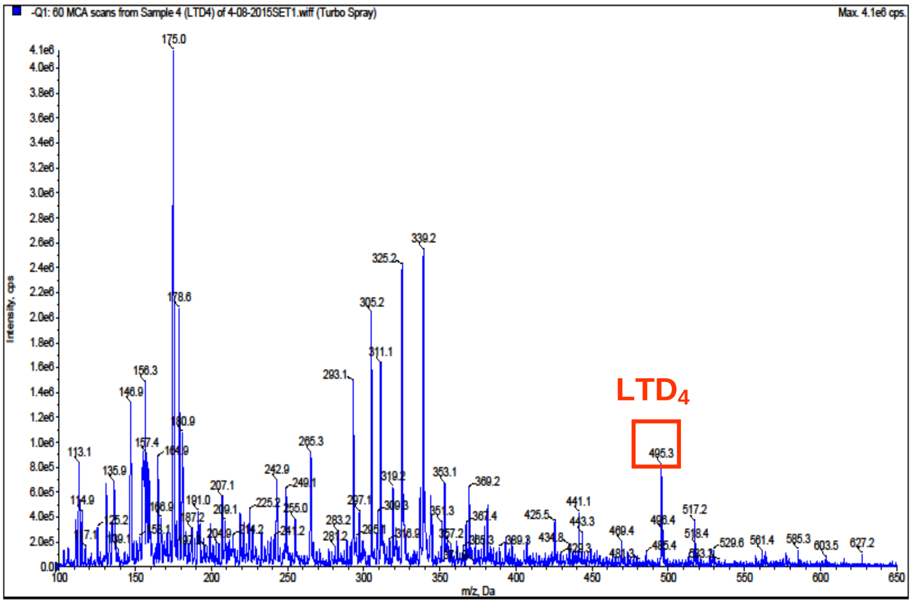
The mass spectra acquisition was performed in negative ESI, because all tested compounds have a carboxyl group which confers a negative charge. Negative electrospray full-scan mass spectra of CysLTs indicated the presence of the proper deprotonated molecules $[M-H]^-$ as the predominant ion for each compound, confirming the presence of the stable CysLTs in the solutions used in all experiments reported in this work . The product ion mass spectra of the analytes $[M-H]^-$ are reported in table 4.1 and Figure 4.1.

Compound	**Molecular Formula**	**Molecular Weight**	**Monomer $[M-H]^-$ m/z**
LTC_4	$C_{30}H_{47}N_3O_9S$	625	624
LTD_4	$C_{25}H_{40}N_2O_6S$	496	495
LTE_4	$C_{23}H_{37}NO_5S$	439	438

Table 4.*1 Pseudomolecular and adduct ions in the ESI negative mass spectra of CysLTs*


-Q1: 60 MCA scans from Sample 4 (B etanolo) of DataSET1.wiff (Turbo Spray)
Max. 3.1e6 cps.
Intensity, cps
m/z, Da


-Q1: 60 MCA scans from Sample 5 (LTC4) of 4-08-2015SET1.wiff (Turbo Spray)
Max. 5.8e6 cps.
LTC4
624.4
Intensity, cps
m/z, Da



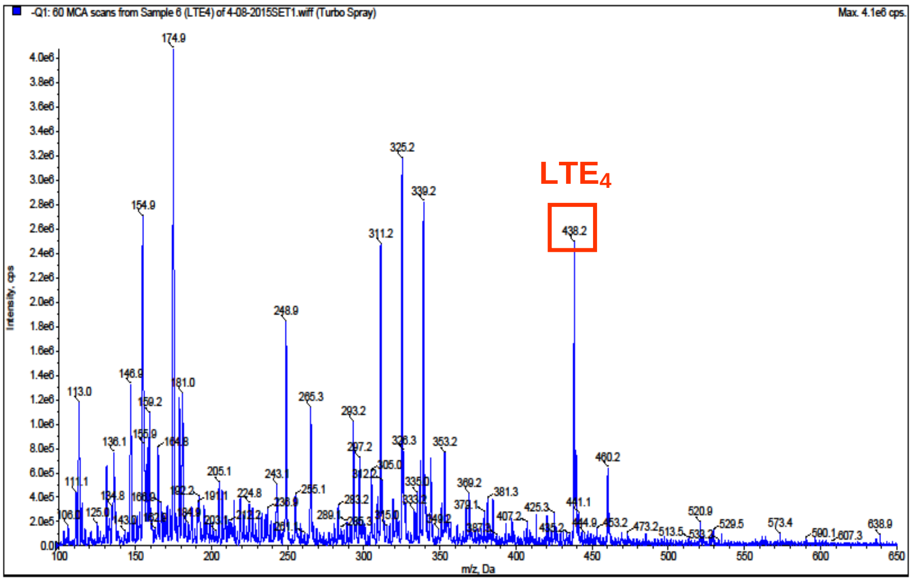


Figure 4.1 *Full scan spectra of Vehicle (ethanol), LTC_4, LTD_4, and LTE_4*

4.1.2 Effects of CysLTs on cytosolic free Ca^{2+} levels

All tested CysLTs induced an increase in Ca^{2+} levels in HUVEC: the highest increase was observed with LTC_4, the lowest with LTE_4. AP100984-2A (1 µM), a selective inhibitor of $CysLT_2R$, inhibited the increase in $[Ca^{2+}]$ activated by LTC_4 (Fig 4.2)

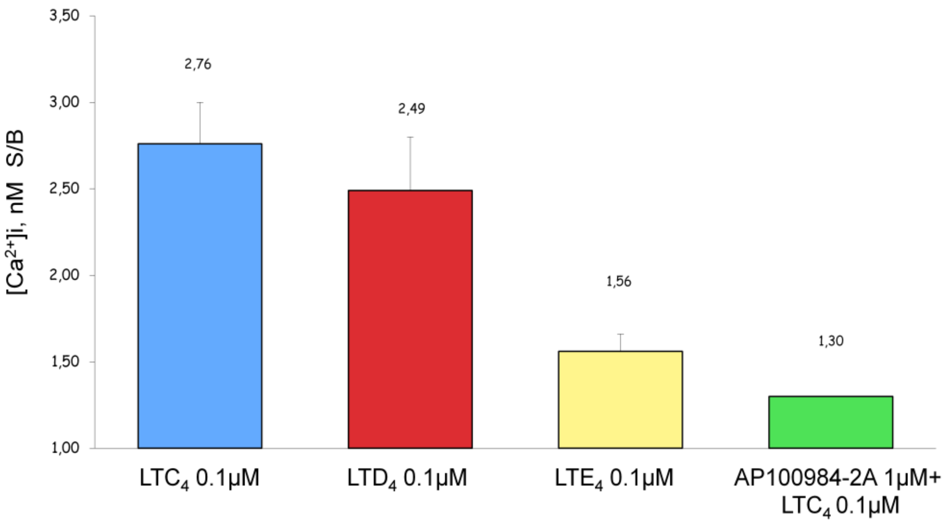


Figure 4.2: *Effects of LTC_4, LTD_4, and LTE_4 at the indicated concentrations on $[Ca^{2+}]_i$ transients in HUVEC. The effects of LTC_4 were measured also in the presence of the $CYsLT_2$ receptor antagonist (AP100984-2A).*

4.1.3 Effects of CysLTs on impedance of HUVEC cells

LTC_4 decreased the impedance of HUVEC (Fig4.3); its effect was inhibited by AP100984-2A (1µM), but not by Montelukast (1 µM), a selective inhibitor of $CysLT_1R$. (FIG 4.3). Similar results, albeit of lower intensity, were observed with LTD_4 (Fig 4.4) and LTE_4 (Fig4.5), confirming previously published results by Capra et al. [156].

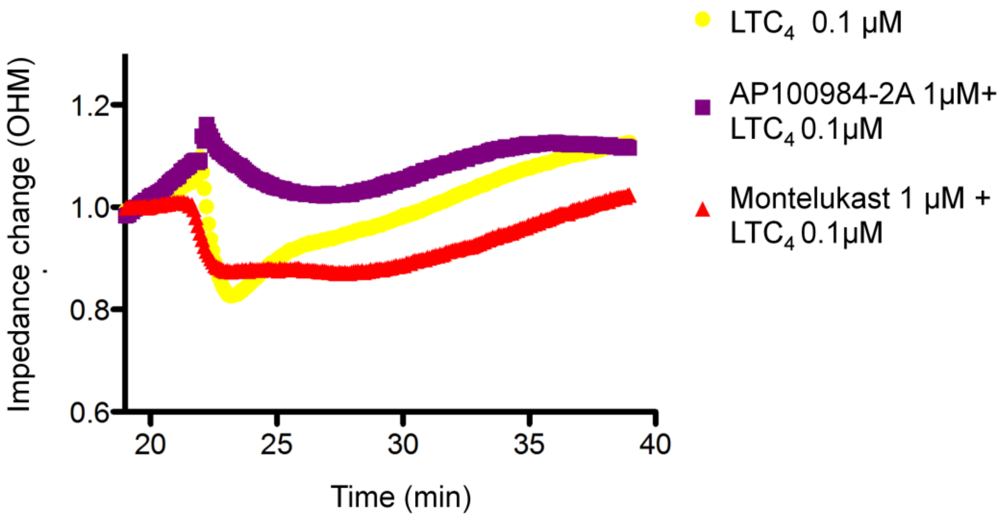


Figure 4.3: *Effects of LTC_4 on impedance of HUVEC, in the presence and absence of AP100984-2A (1μM), a selective inhibitor of $CysLT_2R$ or Montelukast (1 μM), a selective inhibitor of $CysLT_1R$*

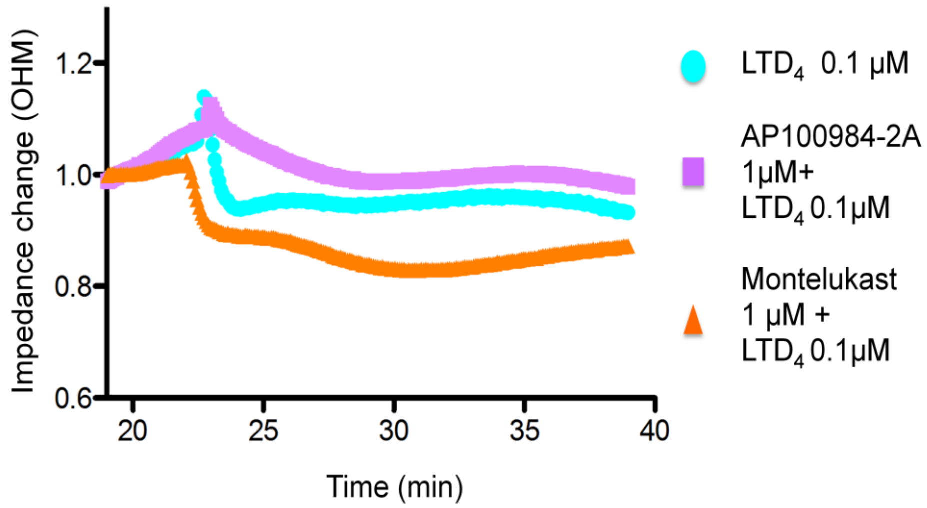


Figure 4.4: *Effects of LTD_4 on impedance of HUVEC, in the presence and absence of AP100984-2A (1μM), a selective inhibitor of $CysLT_2R$ or Montelukast (1 μM), a selective inhibitor of $CysLT_1R$*

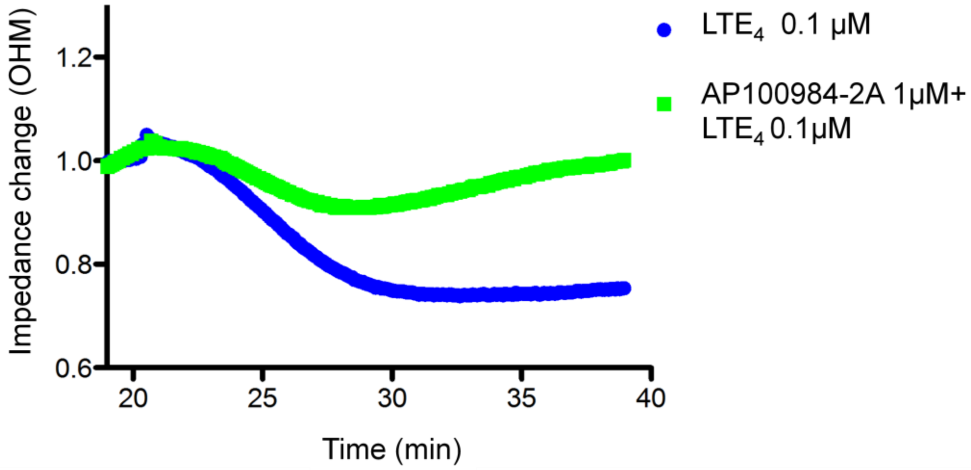


Figure 4.5: *Effects of LTE_4 on impedance of HUVEC, in the presence and absence of AP100984-2A (1µM), a selective inhibitor of $CysLT_2R$ or Montelukast (1 µM), a selective inhibitor of $CysLT_1$.*

4.2 Platelet aggregation

Incubation of LTC_4 (0.8 µM), LTD_4 (1 µM), and LTE_4 (1.1 µM) with normal human PRP for up to 6 min did not cause platelet aggregation, independently of the type of anticoagulant used to collect blood samples (citrate or hirudin) (Table 4.2)

	Max aggregation (%)					
	CITRATE			**HIRUDIN**		
	0 min	*3 min*	*6 min*	*0 min*	*3 min*	*6 min*
Vehicle	3,3 (1,8)	2,0 (1,7)	1,6 (2,1)	1,6 (0,9)	0,8 (0,4)	1,2 (0,4)
LTC_4 (0.8 µM)	2,8 (1,3)	2,3 (1,5)	1,6 (1,3)	1,2 (0,4)	1,2 (0,4)	1,2 (0,8)
Vehicle	2,0 (0,9)	1,8 (1,2)	1,7 (1,4)	1,8 (0,8)	0,8 (0,4)	1,0 (0,6)
LTD_4 (1 µM)	2,0 (0,9)	1,2 (0,8)	1,5 (0,5)	1,5 (1,0)	1,0 (0,6)	0,8 (0,8)
Vehicle	2,7 (1,6)	2,3 (1,7)	2,1 (2,3)	1,5 (0,7)	0,6 (0,5)	0,8 (0,6)
LTE_4 (1.1 µM)	3,1 (2,1)	2,5 (1,6)	2,4 (2,4)	1,8 (1,9)	0,7 (0,7)	0,9 (0,7)

Table 4.2: *Platelet aggregation in human PRP, measured as % increase of light transmission. Date are reported as means and standard deviations: n=6 for LTC_4 and LTD_4, and n=11 for LTE_4.*

When added to citrate or hirudin PRP in combination with different concentrations of the platelet agonists ADP or epinephrine (0.1 – 1.0 µM) LTC_4 (Tab. 4.3 and Tab 4.6), LTD_4 (Tab. 4.4 and Tab 4.6), and LTE_4 (Tab. 4.5 and Tab 4.7) did not enhance agonist-induced platelet aggregation, independently of the length of incubation of CysLTs with PRP.

	% of aggregation			
	Agonist 0.1 µM		*Agonist 1 µM*	
	Vehicle	**LTC_4 0,8µM**	**Vehicle**	**LTC_4 0,8µM**
	0 min incubation			
Vehicle	3.7 (2.1)	**3.0 (1.7)**	3 (1.7)	**2,7 (1.2)**
Epinephrine	8.0 (4.4)	**7.0 (4.6)**	73,7 (8.0)	**71,7 (4.7)**
ADP	4.0 (4.4)	**4.0 (3.5)**	11,3 (3.2)	**10,7 (2.1)**
	3 min incubation			
Vehicle	2,3 (2.3)	**2,7 (2.1)**	1,7 (1.2)	**2 (1.0)**
Epinephrine	5,7 (4.0)	**5,3 (2.5)**	66.3 (8.5)	**71,0 (14.4)**
ADP	2,7 (2.9)	**4,7 (5.5)**	9.3 (1.5)	**9,7 (2.5)**
	6 min incubation			
Vehicle	2,0 (2.6)	**2,0 (1.7)**	1 (1.4)	**1 (0.0)**
Epinephrine	4,3 (2.3)	**5,7 (4.0)**	71,3 (17.0)	**70 (16.6)**
ADP	3,0 (4.4)	**3,0 (2.6)**	8,7 (2.9)	**8,3 (3.2)**

Table 4.3: *Plateletaggregation in citrate-PRP induced by ADP or Epinephrine in presence/absence of LTC4, which had been incubated with PRP for 0 min (added together with the platelet agonist), 3 or 6 min before the addition of the platelet agonist. Data are reported as means and standard deviations (n=3)*

	% of aggregation			
	Agonist 0.1 µM		*Agonist 1 µM*	
	Vehicle	**LTD_4 0,8µM**	**Vehicle**	**LTD_4 0,8µM**
	0 min incubation			
Vehicle	2.0 (1.0)	**2.0 (1.0)**	2.0 (1.0)	**2.0 (1.0)**
Epinephrine	15 (20.1)	**18.0 (23.5)**	30.3 (23.5)	**26.7 (27.1)**
ADP	2.3 (1.5)	**1.7 (0.6)**	13.0 (4.4)	**13.3 (2.3)**
	3 min incubation			
Vehicle	1.3 (1.5)	**1.0 (1.0)**	2.3 (0.6)	**1.3 (0.6)**
Epinephrine	14.7 (21.1)	**18,3 (25.7)**	26.0 (25.0)	**25.7 (34.1)**
ADP	1.0 (1.0)	**1,7 (1.2)**	11.7 (3.1)	**11.0 (3.6)**
	6 min incubation			
Vehicle	1.7 (2.1)	**1.3 (0.6)**	1.7 (0.6)	**1.7 (0.6)**
Epinephrine	3.0 (2.6)	**3.0 (2.6)**	24.7 (31.5)	**20.3 (27.5)**
ADP	1.7 (1.2)	**2.0 (0.0)**	12.3 (2.5)	**11.7 (2.5)**

Table 4.4: *Platelet aggregation in citrate-PRP induced by ADP or Epinephrine in presence/absence of LTD_4, which had been incubated with PRP for 0 min (added together with the platelet agonist), 3 or 6 min before the addition of the platelet agonist. Data are reported as means and standard deviations (n=3)*

	% of aggregation			
	Agonist 0.1 µM		*Agonist 1 µM*	
	Veh	**LTE_4 1.1 µM**	**veh**	**LTE_4 1.1 µM**
	0 min			
Veh	4.0 (1.0)	5.0 (2.2)	1,7 (0.6)	2,3 (1.2)
ADP	4.3 (1.5)	4.0 (1.0)	9,3 (6.1)	8,7 (6.7)
	3 min			
Veh	3.3 (0.6)	3.3 (0.6)	1.3 (1.5)	1.7 (0.6)
ADP	3.7 (2.1)	3.3 (2.1)	7.0 (6.1)	8.3 (5.1)
	6 min			
Veh	2,7 (2.5)	3,3 (1.5)	1.0 (1.0)	1.0 (1.0)
ADP	3,3 (1.5)	3,7 (2.1)	7.0 (5.3)	12 (13.9)

A

	% of aggregation			
	Agonist 0.1 µM		*Agonist 1 µM*	
	veh	**LTE_4 1.1 µM**	**veh**	**LTE_4 1.1 µM**
	0 min			
Veh	3.1 (1.7)	3.4 (2.3)	2.3 (1.6)	2,4 (1.7)
EPI	8.4 (5.4)	9.3 (7.0)	30.3 (27.6)	32.1 (25.8)
	3 min			
Veh	2.6 (1.8)	2.8 (1.8)	1.9 (1.9)	2,1 (1.8)
EPI	12,8 (19.1)	6,9 (5.6)	30,4 (25.2)	28,5 (21.7)
	6 min			
Veh	2,6 (2.6)	3.0 (2.6)	1,9 (2.3)	2 (2.7)
EPI	10,1 (11.4)	13,3 (19.0)	25,4 (28.6)	25,4 (25.1)

B

Table 4.5: *Platelet aggregation in citrate-PRP induced by ADP (Panel A,) or Epinephrine (Panel B;) in presence/absence of LTE_4, which had been incubated with PRP for 0 min (added together with the platelet agonist), 3 or 6 min before the addition of the platelet agonist. The results are reported as mean and standard deviation of 3 experiments for aggregation induced by ADP, and as mean and standard deviation of 8 experiments for aggregation induced by epinephrine*

	% of aggregation					% of aggregation			
	Agonist 0.1 µM		*Agonist 1 µM*			*Agonist 0.1 µM*		*Agonist 1 µM*	
	Veh	**LTC_4 0,8µM**	**Veh**	**LTC_4 0,8µM**		**Vehi**	**LTD_4 1 µM**	**Vehi**	**LTD_4 1 µM**
	0 min					0 min			
Vehicle	2.0 (1.0)	1,3 (0.6)	1.0 (0.0)	1.0 (0.0)	**Vehicle**	2.0 (1.0)	2.0 (1.0)	1.7 (0.6)	1.0 (1.0)
EPI	1.7 (0.6)	2.0 (1.0)	1.7 (0.6)	1.7 (0.6)	**EPI**	1.7 (1.2)	1.3 (0.6)	2.3 (1.5)	1.3 (0.6)
ADP	0.7 (1.2)	0.7 (0.6)	15.0 (2.0)	11.7 (4.9)	**ADP**	0.3 (0.6)	0.7 (0.6)	19.0 (7.2)	17.0 (1.7)
	3 min					3 min			
Vehicle	1.0 (0.0)	1.3 (0.6)	0.7 (0.6)	1 (0.0)	**Vehicle**	0.7 (0.6)	1.3 (0.6)	1.0 (0.0)	0.7 (0.6)
EPI	1.7 (1.2)	1.3 (0.6)	0.3 (0.6)	0.3 (0.6)	**EPI**	0.7 (0.6)	0.3 (0.6)	1.0 (0.0)	0.7 (0.6)
ADP	0.0 (0.0)	0,7 (0.6)	12.0 (0.0)	11.3 (1.2)	**ADP**	0.3 (0.6)	0.0 (0.0)	15.0 (4.6)	14.0 (3.5)
	6 min					6 min			
Vehicle	1.3 (0.6)	1.0 (1.0)	1.0 (0.0)	1.5 (0.7)	**Vehicle**	1.0 (1.0)	1.0 (1.0)	1.0 (0.0)	0.7 (0.0)
EPI	1.0 (1.0)	0.7 (0.6)	0.7 (0.6)	1.0 (0.0)	**EPI**	0.7 (1.2)	1.0 (1.0)	1.0 (0.0)	1.0 (0.0)
ADP	0.0 (0.0)	1.0 (1.0)	13.0 (1.0)	9.7 (4.2)	**ADP**	1.0 (1.0)	0.0 (0.0)	16.3 (5.0)	14.3 (1.2)

A *B*

Table 4.6: *Platelet aggregation in hirudin-PRP induced by ADP or Epinephrine in presence/absence of LTC_4 (Panel A) or LTD_4 (Panel B), which had been incubated with PRP for 0 min (added together with the platelet agonist), 3 or 6 min before the addition of the platelet agonist. Data are reported as mean and standard deviation (n=3)*

	Hirudine (% of aggregation)			
	Agonist 0.1 µM		*Agonist 1 µM*	
	Vehi	**LTE_4 1.1µM**	**Vehi**	**LTE_4 1.1µM**
	0 min			
Vehicle	1.7 (1.2)	3.3 (3.2)	1.3 (0.6)	1.0 (0.0)
ADP	0.3 (0.6)	0.3 (0.6)	9.7 (6.0)	10.0 (6.0)
	3 min			
Vehicle	1.0 (0.0)	1.0 (1.0)	0.3 (0.6)	0.7 (0.6)
ADP	0.0 (0.0)	0.0 (0.0)	7.7 (4.2)	9.0 (4.6)
	6 min			
Vehicle	1.3 (0.6)	1.3 (0.6)	0.3 (1.0)	1.0 0.6)
ADP	0.3 (0.6)	0.0 (0.0)	10.0 (6.2)	7.3 (5.2)

A

	Hirudine (% of aggregation)			
	Agonist 0.1 µM		*Agonist 1 µM*	
	Vehi	**LTE_4 1.1µM**	**Vehi**	**LTE_4 1.1µM**
	0 min			
Vehicle	1.6 (0.8)	2.1 (2.2)	1.4 (0.5)	1.1 (0.4)
EPI	1.3 (1.0)	2.0 (1.1)	1.3 (1.2)	1.6 (1.1)
	3 min			
Vehicle	0.7 (0.5)	0.7 (0.8)	0.4 (0.5)	0.6 (0.5)
EPI	0.8 (0.7)	1.0 (0.8)	0.9 (0.4)	0.8 (0.5)
	6 min			
Vehicle	1.0 (0.6)	0.9 (0.7)	0.6 (0.5)	0.7 (0.8)
EPI	0.9 (0.8)	1.1 (1.1)	0.6 (0.7)	1.4 (1.1)

B

Table 4.7: *Platelet aggregation in hirudin-PRP induced by ADP (Panel A) or Epinephrine (Panel B) in presence/absence of LTE_4, which had been incubated with PRP for 0 min (added together with the platelet agonist), 3 or 6 min before the addition of the platelet agonist. The results are reported as mean and standard deviation of 3 experiments for aggregation induced by ADP, and as mean and standard deviation of 8 experiments for aggregation induced by epinephrine.*

4.3 EFFECTS OF CYSTENYL LEUKOTRIENES ON THE INHIBITION BY ADP OF PGE_1-INDUCED PLATELET PRODUCTION OF CYCLIC-AMP

PGE_1 (2µM) increased platelet cAMP levels in citrate PRP, which were partially decreased by treatment with ADP. CysLTs had no significant effect on cAMP accumulation, both in the presence and absence of ADP. (Fig 4.5)

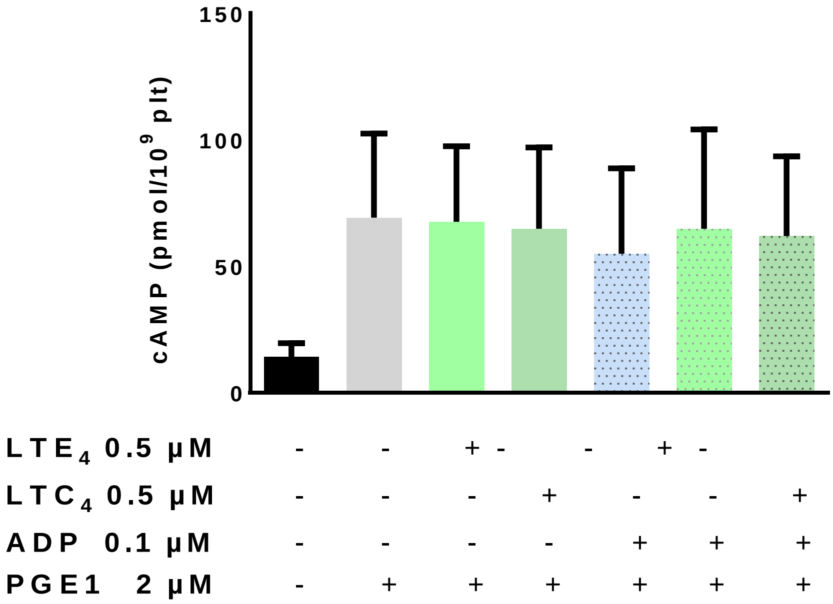


Figure 4.5. *Effects of ADP, LTE_4 and LTC_4 in various combinations on the increase in platelet cAMP induced by PGE_1.*

4.4 EFFECTS OF CYSTENYL LEUKOTRIENES ON P-SELECTIN EXPRESSION

ADP caused an increase in platelet p-selectin expression in a dose-dependent manner [158, 159], which was inhibited by cangrelor (1 µM) (FIG 4.6)

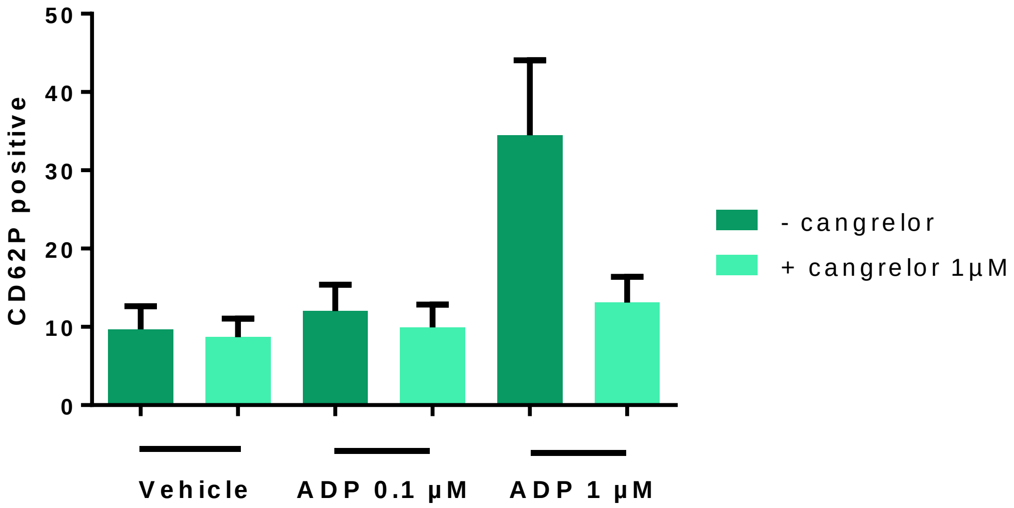


Figure 4.6: *Expression of platelet P-selectin after stimulation of whole blood by ADP in the presence and absence of cangrelor. Values were normally distributed. $p<0.0001$ (ANOVA for repeated measures). Internal contrasts (Bonferroni's test): vehicle vs ADP 0.1 µM $p<0.01$, vehicle vs ADP 1 µM $p<0.0001$.*

Incubation of LTC_4 (0.8 µM), LTD_4 (1 µM), or LTE_4 (1.1 µM) with whole blood for 30 min, in the presence/absence of cangrelor, did not increase the expression of P-selectin on platelets. ANOVA: p>ns (FIG 4.7).

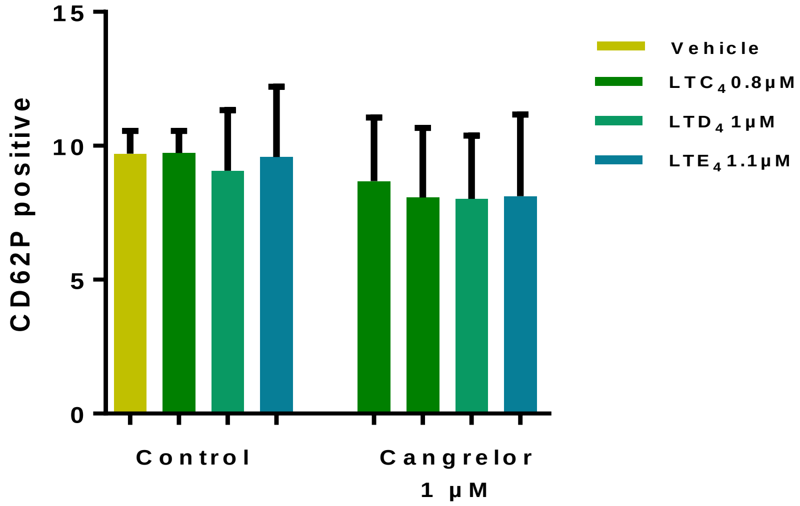


Figure 4.7: *Expression of P-selectin after treatment with CysLTs: none of them increased the level of P-selectin expression (ANOVA p=ns), in presence or absence of cangrelor*

Incubation of whole blood with LTC_4 (0.8 µM), LTD_4 (1 µM), or LTE_4 (1.1 µM) in combination with ADP 0.1 µM for 30 min did not significantly increase the expression of p-selectin on platelets, in the presence/absence of cangrelor (FIG 4.8).

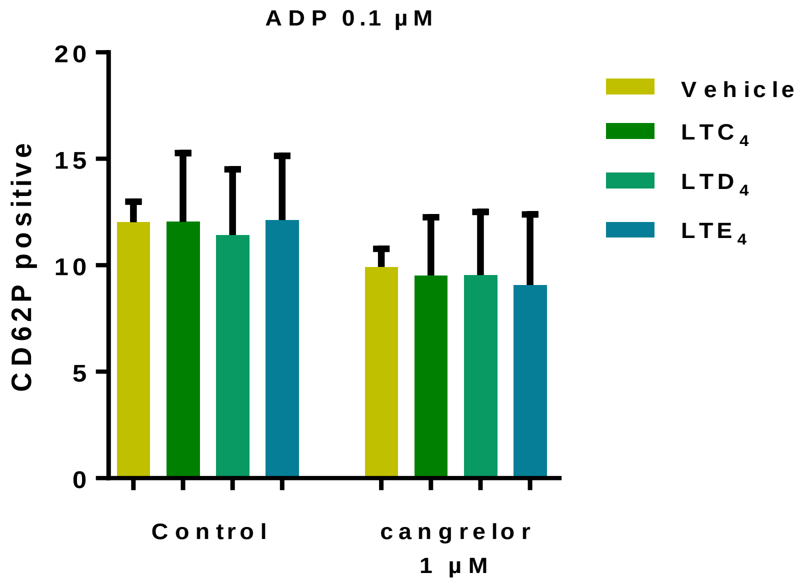


Figure 4.8: *Expression of P-selectin in whole blood after treatment with CysLTs and stimulation by ADP 0.1 µM in presence/absence of cangrelor. p=ns (Friedman test)*

When whole blood was stimulated by an higher concentration of ADP (1 µM) with LTC_4 (0.8 µM), LTD_4 (1 µM), or LTE_4 (1.1 µM,) CysLTs had a modulatory effect on p-selectin expression, which disappeared in the presence of the $P2Y_{12}$ inhibitor cangrelor (FIG 4.9) . However, none of the CysLTs significantly affected p-selectin expression induced by ADP.

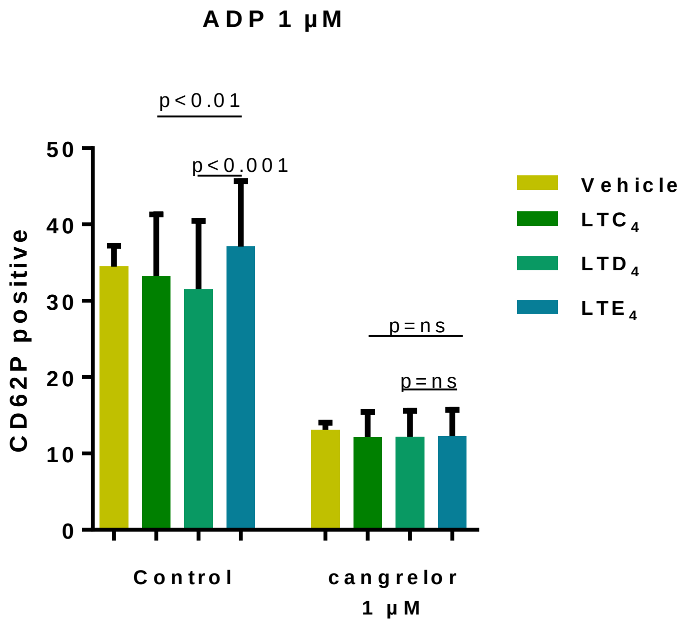


Fig 4.9: *Expression of P-selectin in whole blood after treatment with CysLTs and stimulation induced by ADP 1 µM in presence/absence of cangrelor. Data were normally distributed; $p<0.001$ (ANOVA., Bonferroni's test for internal contrast LTC_4 vs LTE_4 $p<0.01$ and LTD_4 vs LTE_4 $p<0.001$.)*

5. DISCUSSION AND CONCLUSION

Platelets play a role in inflammation and CysLTs are important pro-inflammatory molecules. It has been hypothesized that some CysLTs may interact with the platelet $P2Y_{12}$ receptor for ADP. However, contrasting results have been reported[14, 36, 139, 146]. In our study we tested the effects of CysLTs on several tests of platelet function that are dependent on $P2Y_{12}$ activity.

Our results indicated that CysLTs alone have no direct effect on platelet aggregation in citrate PRP with CysLTs, confirming the findings by Metha et al. [147].

In order to evaluate whether LTC_4, LTD_4, and LTE_4 were able to affect platelet aggregation induced by physiological agonists, we tested two concentrations of epinephrine and ADP. In contrast with Mehta et al, we were unable to demonstrate that CysLTs potentiate the aggregatory effects of these agonists. at both tested concentrations (0.1 and 1.0 µM).

At variance with Mehta et al, we studied the effects of CysLTs on platelet aggregation also in hirudin PRP, in which the concentration of plasma Ca^{2+} is maintained at physiological levels. Also under these experimental conditions, we were unable to demonstrate any effect of CysLTs.

Also when platelet activation was tested by measuring the expression of p-selectin on the platelet membrane induced by ADP, CysLTs failed to show any effect.

We then focused our studies on the inhibition by ADP of PGE_1 induced increase in platelet cAMP, a function that is dependent of $P2Y_{12}$ only. CysLTs had no effects also in this platelet function

Overall, therefore, the results of our studies failed to show any significant effects of CysLTs on $P2Y_{12}$-mediated platelet function, in agreement with some previous studies.[146] The negative results of our studies are not due to alterations of the CysLTs that we used, as they were identified correctly at mass spectrometry and induced normal cellular response of HUVEC, as previously shown.

The inflammatory effects of CysLTs mediated by the platelet $P2Y_{12}$ receptor, which have been demonstrated in *in vivo* experiments[14, 36, 139], were most likely indirect, rather than induced by a direct interaction of CysLTs with platelets. Whether or not this indirect effect is active only in subjects with an inflammatory state [8], such as in sensitized mice [3-5] or patients with asthma [6] should be studied in properly designed experiments.

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