UNIVERSITÀ DEGLI STUDI DI MILANO

Dottorato in Scienze Biochimiche (XXX ciclo)

EVALUATION OF ASPIRIN RESPONSIVENESS IN ESSENTIAL THROMBOCYTHEMIA PATIENTS

Tesi di Dottorato di:

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Sommario

Introduzione. *La trombocitemia essenziale (ET) è una malattia mieloproliferativa caratterizzata da un aumento significativo del numero di piastrine, associato al rischio di complicanze sia emorragiche che trombotiche. I pazienti a rischio trombotico vengono trattati con acido acetilsalicilico (ASA) 100 mg al giorno. È stato osservato che buona parte dei pazienti con ET mostra una ridotta risposta farmacodinamica (PD) e l'aumento della produzione di piastrine viene identificato come possibile causa di questa inadeguata risposta perciò una doppia somministrazione giornaliera sembrerebbe essere preferibile. Lo scopo di questo studio è stato quello di indagare i potenziali meccanismi che causano la minor risposta al farmaco nei pazienti ET.*

Metodi*. Sono stati arruolati 17 pazienti ET in trattamento cronico con 100 mg al giorno di acido acetilsalicilico gastroresistente (EC)-ASA (formulazione comunemente prescritta) e 10 soggetti sani (HS). 7 pazienti ET sono stati identificati come "poor responder" (ET-PR) e 10 come "responder" (ET-R), in base ai livelli di TxB_2 sierico (valore limite = 10 ng / ml). I campioni di sangue sono stati prelevati al mattino, 24 ore dopo l'assunzione della compressa del giorno precedente e immediatamente dopo la somministrazione giornaliera del farmaco fino a 8 ore. Sono stati misurati i livelli di TxB_2 (ELISA) sierico, i livelli plasmatici di ASA e acido salicilico (SA) (ID-LC-MS / MS) e l'attività enzimatica delle esterasi, enzimi in grado di idrolizzare l'ASA in SA, presenti nel sangue e nel plasma. Inoltre è stata valutata la produzione di TxA_2 in seguito ad aggregazione piastrinica indotta da collageno (5 µg/ml) dopo aggiunta in vitro di ASA (10-1000 µM) sul sangue intero di HS e ET-PR. Infine sono state misurate le piastrine reticolate in 15 ET (8 R, 7 PR) e 8 HS mediante citofluorimetria.*

Risultati. *Lo studio in vitro sull'inibizione della sintesi di TxA_2 dopo incubazione con ASA ha mostrato curve dose-risposta comparabili sia nei HS che nei pazienti ET-PR. Non è stata osservata alcuna differente attività enzimatica delle esterasi nei tre gruppi studiati. La farmacocinetica (PK) dell'EC-ASA ha mostrato un andamento molto variabile sia nei pazienti ET che nei HS, mentre le PKs di ASA non rivestita sono risultate più uniformi in tutti e tre i gruppi. In tutti i pazienti ET-PR e in 3 ET-R i livelli di TxB_2 sierico superavano il valore limite (> 10 ng / mL) a 24 ore. In questi pazienti la differenza tra TxB_2 a 24 ore e il valore minimo di TxB_2 correlava (r = 0,6107; p = 0,0020) con il numero di piastrine reticolate e livelli di TxB_2 sierico venivano inibiti in seguito alla doppia somministrazione del farmaco.*

Conclusioni. *Le cause di una minor risposta all' ASA nei pazienti ET possono essere correlate da un lato a un ridotto assorbimento della formulazione gastroprotetta, dall'altro ad un aumento della produzione piastrinica.*

Abstract

Background. Essential thrombocythemia (ET) is characterized by an increased number of platelets, associated with risk for both bleeding and thrombotic complications. Prophylaxis with 100 mg acetylsalicylic acid (ASA) is indicated in patients at risk. A significant proportion of ET patients (ETs) display an inadequate pharmacodynamics (PD) response to ASA. It has been suggested that poor responsiveness is due to increased platelet production and a *bis in die* (*bid*) administration should be preferred. Aim of this study was to investigate further the potential mechanism(s) of poor responsiveness to ASA in ETs.

Methods. We enrolled 17 ETs on 100 mg enteric-coated (EC)-ASA (commonly used) treatment and 10 healthy subjects (HS). 7 ETs were identified as poor responders (ET-PR) and 10 as responders (ET-R), based on their levels of serum TxB_2 (cut-off=10 ng/ml). Blood samples were taken in the morning, 24 h after the previous intake, and immediately after taking the drug up to 8 h. We measured: serum TxB_2 (ELISA) and ASA and salicylic acid (SA) (ID-LC-MS/MS); the enzymatic activity in whole blood and plasma of esterase enzymes able to hydrolyse ASA to SA. Moreover, collagen (5 µg/ml)-induced platelet aggregation and TxA_2 production were tested by *in vitro* addition of ASA (10-1000 µM) to whole blood of HS and ET-PR. Reticulated platelets were also measured by flow-cytometry in 15 ET (8 R, 7 PR) and 8 HS.

Results. The in vitro dose-response curve of ASA for inhibition of TxA_2 generation was comparable in HS and ET-PR samples. Esterase activities were similar in all groups. The pharmacokinetics (PK) of EC-ASA was very variable within both ETs and HS, while PKs of plain ASA showed a uniform behaviour. All ET-PR and 3 ET-R showed an impaired TxB_2 (>10 ng/mL) at 24 h. In these ETs the difference between TxB_2 at 24 h and the min value of TxB_2

correlated (r=0.6107; p= 0.0020) with reticulated platelets and *bid* administration suppressed serum TxB_2 levels.

Conclusions. Causes of poor response may be related from one side to reduced absorption of EC-ASA, on the other to increased platelet turnover.

1 INTRODUCTION

1.1 Aspirin

1.1.1 Aspirin story

Aspirin (ASA, acetylsalicylic acid) (figure 1) story begins in ancient times when the bark from the willow tree was used to treat pain and fever. Unknown to the ancient Sumerians and Egyptians (as reported in the Edwin Smith Surgical Papyrus and in the Ebers Papyrus) who made use of it, the active agent within willow bark was Salicin (figure 1), which would later form the basis of the discovery of ASA. The use of willow bark for pain relief continued through ancient Greece, where it was recommended by Hippocrates to relieve the pain of childbirth, through to Roman times, when its use was recorded by Pliny the Elder.

In 1763 Reverend Edward Stone (1702-1768) [1] completed the first study about an extract from the bark and in 1828 the active ingredient in willow bark was discovered by Johann Buchner (1783–1852) who first refined willow bark into yellow crystals and named it Salicin (after Salix, the genus of the willow tree) (figure. 1). Over the years the process was further refined and in 1852, the French chemist, Charles Gerhardt (1816–1856), modified salicylic acid (SA) (figure 1) with the introduction of an acetyl group in place of a hydroxyl group, but the compound was not stable. In 1890, the German dye manufacturer, Bayer, set up a pharmaceutical division with research facilities for scientists and in 1897 Felix Hoffmann acetylated a phenol group of SA, producing ASA. Acetylation of SA later proved to be the key step in reducing its irritant properties [2].

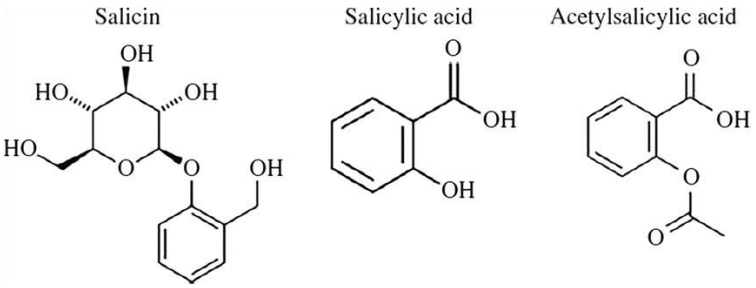


Figure 1. *Molecular structure of salicin, salicylic acid (SA) and acetylsalicylic acid (ASA)* [2].

1.1.2 Mechanism of action and targets.

ASA has been used therapeutically for over 100 years. It is a salicylate derivative drug and possesses the three properties of non-steroidal anti-inflammatory drugs (NSAIDs): analgesic, anti-pyretic and anti-inflammatory actions [3]–[5]. Until 1971 little was known about the mechanism of action of ASA and ASA-like drugs. It was Vane [6] to discover the mechanism by which ASA exerts its anti-inflammatory, analgesic and antipyretic actions. In fact he proved that ASA and other non-steroid anti-inflammatory drugs (NSAIDs) inhibit the activity of the enzyme involved in the synthesis of prostaglandins (PGs), lipid autacoids which cause inflammation, pain and fever.[7] This key enzyme is now called cyclooxygenase (COX), it has two distinct catalytic sites (a cyclooxygenase site and a peroxidase site) and catalyzes the conversion of arachidonic acid (AA) to prostaglandin H2 (PGH2), which is a precursor of other PGs, thromboxane and prostacyclin (figure 2).

Some PGs mediated pathogenic mechanisms (including inflammatory response) and some are involved in homeostatic function: protect the stomach mucosa from damage by hydrochloric acid, maintain kidney function and aggregate platelets when required [8] (figure 2).

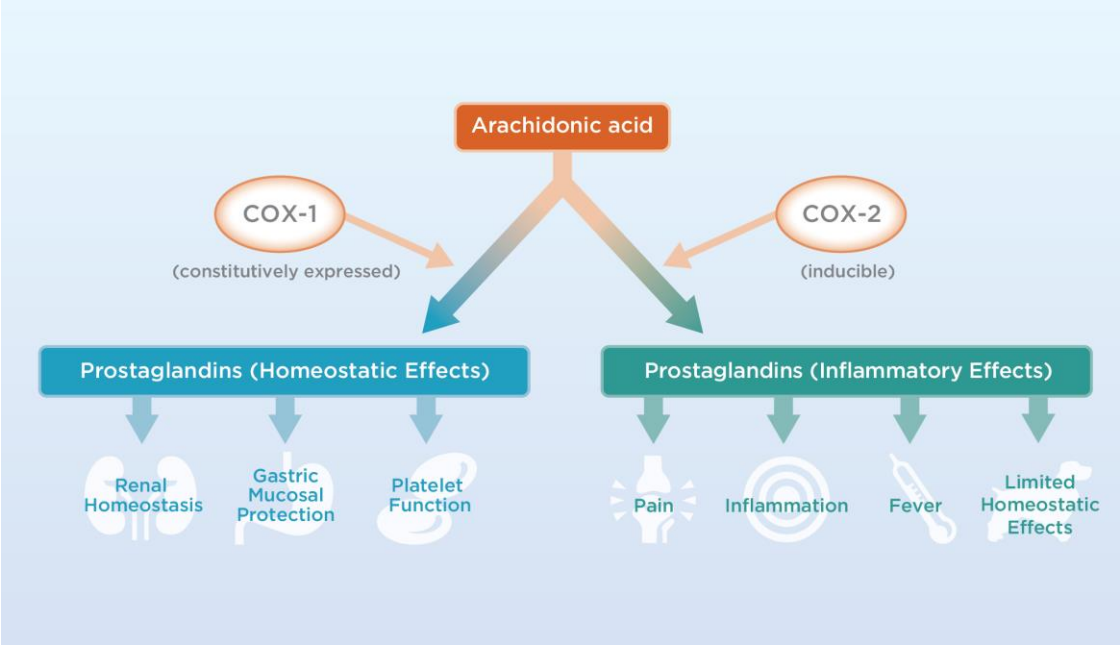


Figure 2. *Schematic representation of COX-1 and COX-2 pathway.*

By the late 1980s the discovery of a second COX gene clarified that there are two isoform of COX enzyme: the constitutive isoform (COX-1) and inducible isoform (COX-2). With this discovery the double function of PGs was better explained: COX-1 catalyzes the synthesis of PGs involved in homeostatic function; whereas COX-2 becomes upregulated by inflammatory mediators and catalyzes the synthesis of PGs involved in inflammation, pain and fever [7], [9]–[11].

Chandrasekharan et al. [12] describes a third COX (COX-3), which is selectively inhibited by paracetamol and also by low concentrations of some NSAIDs including ASA. COX-3 is a variant of COX-1 which has retained intron-1 during translation and which is found in human tissues in a polyadenylated form. Selective inhibition of COX-3 will discover potent and valuable new drugs for controlling pain and fever.

As previously mentioned the principal therapeutic effect of ASA is the ability to irreversibly inhibit PGs production by acetylating COX enzyme, in particular ASA is able to bind Ser-530 in the active site of COX-1 and Ser-516 in the active site of COX-2 (figure 3, thus preventing AA access to the COX catalytic site [13].

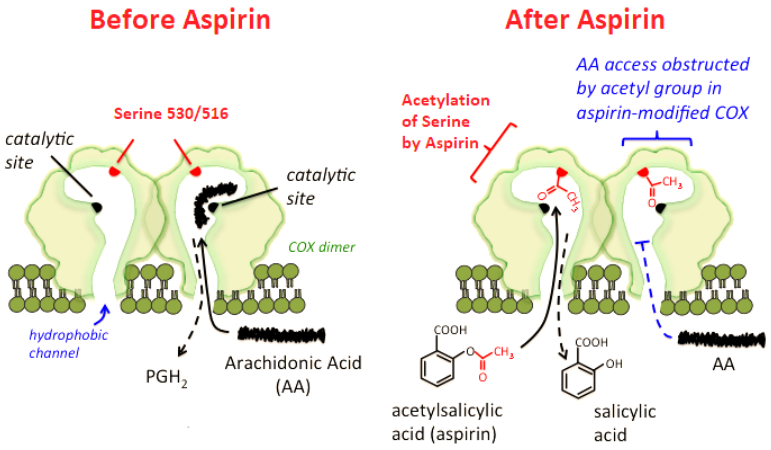


Figure 3. *Aspirin mechanism of action.*

1.2 Clinical use of Aspirin: doses, formulation and pharmacokinetics parameter

1.2.1 Aspirin used for pain fever and inflammation

At high dose (300-1000 mg) ASA is used to treat fever pain and inflammation. ASA inhibits both COX-1 and COX-2 enzymes. As mentioned above COX-1 is the constitutive isoform, which is linked to the mechanisms of cellular homeostasis. COX-2 is an inducible isoform whose production involves activated inflammatory cells. Furthermore, it also improves the synthesis of PGs that play an important role in inflammation, fever and pain. Thanks to the inhibitory action of COX-2, ASA makes the production of such PGs more difficult (figure 4).

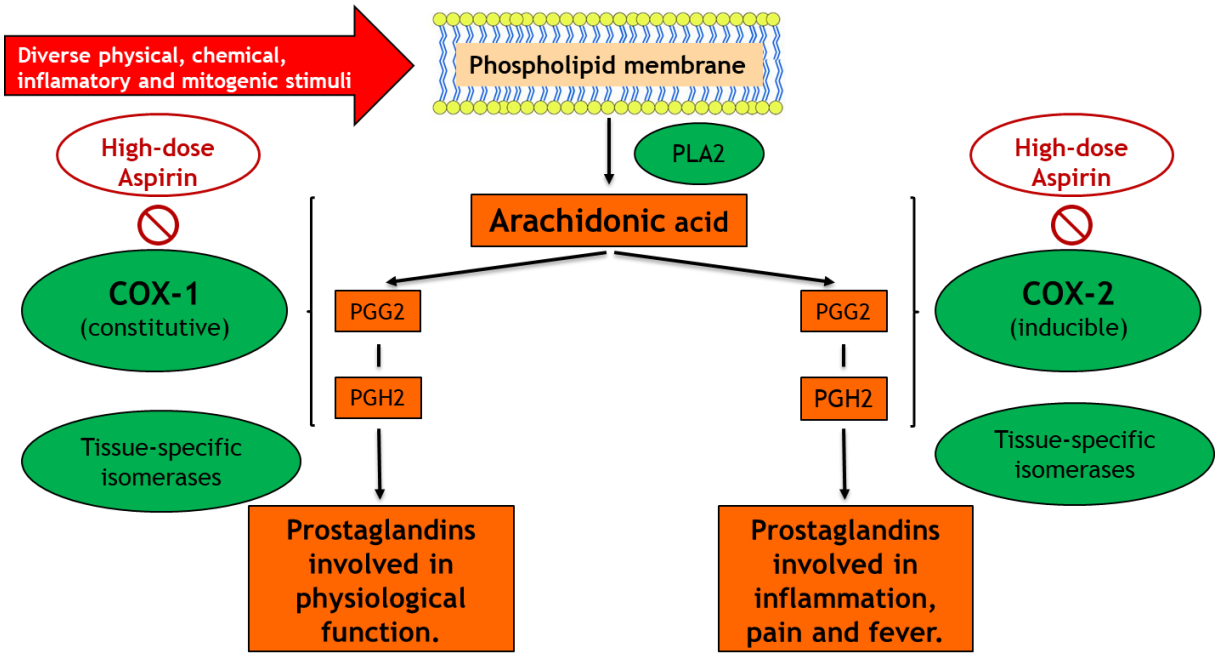


Figure 4. *Aspirin (ASA) mechanism of action and targets at high dose.*

ASA was synthesized in 1897 and sold for decades as tablet. After oral administration, the absorption in the gastrointestinal tract is rapid, complete and follows first order kinetics [3]. ASA in vivo acetylates COX enzyme and undergoes spontaneous hydrolyses

and enzymatic hydrolyses by esterase. Both way converted ASA into its main metabolite: SA. SA is mainly eliminated by metabolism in the liver; the metabolites include salicylic acid, salicyl-phenolic glucuronide, salicyl acyl glucuronide, gentisic acid and salyciluric acid. SA and its metabolites are excreted mainly through the kidney (figure 5) [14], [15]. The maximum plasma levels are reached at 10-20 minutes for ASA and at 0.3 to 2 hours for SA after intake.

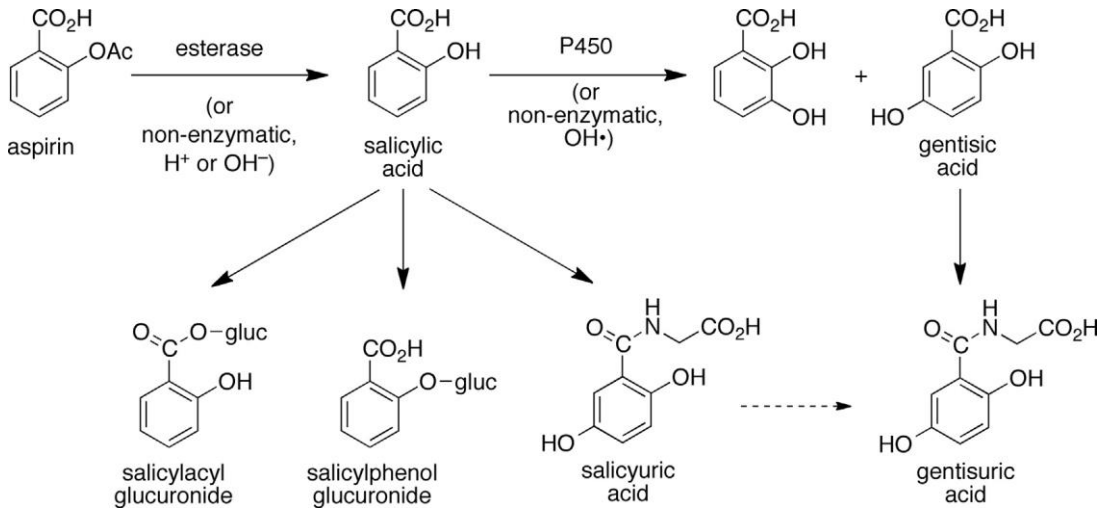


Figure 5. *Aspirin metabolism in vivo*[16].

Over the year different formulation have been developed for the treatment of fever, pain and inflammation in order to enhance pharmacokinetics (PK) and pharmacodynamics (PD) action of ASA: plain tablets, chewable tablets, effervescent tablets, extended-release tablets, granules and suspensions, fast release/disintegrating tablets, powders, creams, lotions, and intravenous solutions. For the treatment of pain and fever, these enhancements typically include bioavailability (total exposure of drug measured as area under the plasma-concentration time curve (AUC)), reduction in the time to reach maximum plasma concentration (t_{max}), increase in maximum plasma concentration (C_{max}), and extension of the release profile over time. A recent article [3] compares PK parameters of different ASA formulations at dose of 500 mg in healthy

subjects (HS). Data are extracted from Bayer's clinical trial database, grouped by formulation and analysed using descriptive and inferential statistics (mean, median and 95% CI) (figure 6).

Table 1. Summary pharmacokinetic data of acetylsalicylic acid for aspirin formulations.

Formulation	No. of studies	C_{max} (mg/L)	AUC [(mg × h)/L]	T_{max} (h)
Aspirin tablet	10	Mean: 5.43 ± 1.38 95% CI: 4.66–6.21 Median: 5.69 ± 1.38	Mean: 6.21 ± 1.24 95% CI: 5.51–6.91 Median: 6.22 ± 1.24	Median: 0.5 ± 0.16 95% CI: 0.40–0.60
Aspirin effervescent tablet	3	Mean: 10.45 ± 1.18 95% CI: 9.12–11.78 Median: 11.08 ± 1.18	Mean: 5.27 ± 0.51 95% CI: 4.69–5.85 Median: 5.31 ± 0.51	Median: 0.33 ± 0.02 95% CI: 0.30–0.36
Aspirin granules	6	Mean: 5.42 ± 1.03 95% CI: 4.59–6.25 Median: 5.48 ± 1.03	Mean: 6.18 ± 1.36 95% CI: 5.09–7.27 Median: 5.97 ± 1.36	Median: 0.46 ± 0.13 95% CI: 0.36–0.56
Aspirin granules in suspension	6	Mean: 12.77 ± 1.94 95% CI: 11.43–14.11 Median: 12.69 ± 1.94	Mean: 6.77 ± 1.63 95% CI: 5.64–7.90 Median: 6.02 ± 1.63	Median: 0.25 ± 0.04 95% CI: 0.22–0.28
Fast release aspirin tablet	6	Mean: 13.89 ± 1.08 95% CI: 13.03–14.77 Median: 13.75 ± 1.08	Mean: 6.95 ± 0.67 95% CI: 6.42–7.48 Median: 6.84 ± 0.66	Median: 0.30 ± 0.02 95% CI: 0.28–0.32
Aspirin chewable tablet	2	Mean: 6.25 ± 0.24 95% CI: 5.92–6.58 Median: 6.25 ± 0.24	Mean: 4.67 ± 0.03 95% CI: 4.63–4.71 Median: 4.67 ± 0.03	Median: 0.33 ± 0 95% CI: n/a

Table 2. Summary pharmacokinetic data of salicylic acid for aspirin formulations.

Formulation	No. of studies	C_{max} (mg/L)	AUC [(mg × h)/L]	T_{max} (h)
Aspirin tablet	10	Mean: 25.45 ± 3.64 95% CI: 23.19–27.71 Median: 25.56 ± 3.64	Mean: 145.67 ± 35.24 95% CI:123.82–167.50 Median: 125.76 ± 35.24	Median: 2.00 ± 0.54 95% CI: 1.66–2.34
Aspirin effervescent tablet	3	Mean: 27.54 ± 1.18 95% CI: 26.20–28.88 Median: 27.31 ± 1.18	Mean: 138.07 ± 8.89 95% CI: 128.00–148.13 Median: 141.90 ± 8.89	Median: 0.75 ± 0.05 95% CI: 0.70–0.80
Aspirin granules	6	Mean: 25.51 ± 4.59 95% CI: 21.84–29.18 Median: 25.50 ± 4.59	Mean: 158.4 ± 50.50 95% CI: 118.00–198.80 Median: 156.00 ± 50.50	Median: 2.00 ± 0.54 95% CI: 1.56–2.44
Aspirin granules in suspension	6	Mean: 29.08 ± 2.66 95% CI: 27.31–31.95 Median: 29.64 ± 2.66	Mean: 132.54 ± 16.22 95% CI: 121.30–143.78 Median: 133.51 ± 16.22	Median: 0.83 ± 0.15 95% CI: 0.72–0.94
Fast release aspirin tablet	6	Mean: 31.80 ± 1.81 95% CI: 30.35–33.25 Median: 31.00 ± 1.81	Mean: 179.07 ± 15.27 95% CI: 166.85–191.29 Median: 182.15 ± 15.27	Median: 0.75 ± 0.05 95% CI: 0.71–0.79
Aspirin chewable tablet	2	Mean: 23.24 ± 1.17 95% CI: 21.62–24.86 Median: 23.24 ± 1.17	Mean: 123.18 ± 0.24 95% CI: 122.85–123.51 Median: 123.18 ± 0.24	Median: 1.25 ± 0 95% CI: n/a

Figure 6. *Pharmacokinetics parameters (AUC, t_{max} and C_{max}) of acetylsalicylic acid and salicylic acid after different Aspirin (500 mg) formulations intake in healthy subjects* [3].

As we can see from figure 6 various formulations provide different C_{max} and t_{max}, in particular for tablet, chewable tablet and dry granule formulations, C_{max} levels are lower than C_{max} levels of effervescent tablet, granules in suspension and fast release

tablet formulation. Effervescent tablet and granules in suspension are highly bioavailable due to their liquid nature (before assumption are dissolved in water) and have a faster absorption time. The fast-release tablet combines a reduction in particle size (thereby increasing the available surface area for absorption) with the addition of sodium carbonate (which acts as both a disintegrate and local buffer). On the other hand, ASA dry granules, chewable tablets, and plain tablets have firstly to be dissolved in solution, after which they can be absorbed systemically, thereby increasing t_{max} and lowering C_{max}.

1.2.2 Aspirin used in prophylaxis as antiplatelet agent

At low-dose (75-100 mg/die) ASA is used as antiplatelet agent for preventing cardiovascular and cerebrovascular events. ASA inactivates platelet COX-1 and so blocking arachinodate-thromboxane A_2 (TxA_2) pathway.

TxA_2 is a powerful vasoconstrictor and platelet agonist (figure 7). TxA_2 is not stable and is immediately converted into thromboxane B_2 (TxB_2), which is biologically inactive.

The measurement ex-vivo of serum TxB_2 levels obtained after whole blood (WB) incubation at 37 °C for 1 hour is commonly used in order to evaluate the pharmacodynamic (PD) effect of ASA on platelets COX-1. Healthy subjects, not under ASA treatment, have TxB_2 between 200-300 ng/mL [17] aspirinated subjects have TxB_2 levels ≤ 10 ng/mL [18].

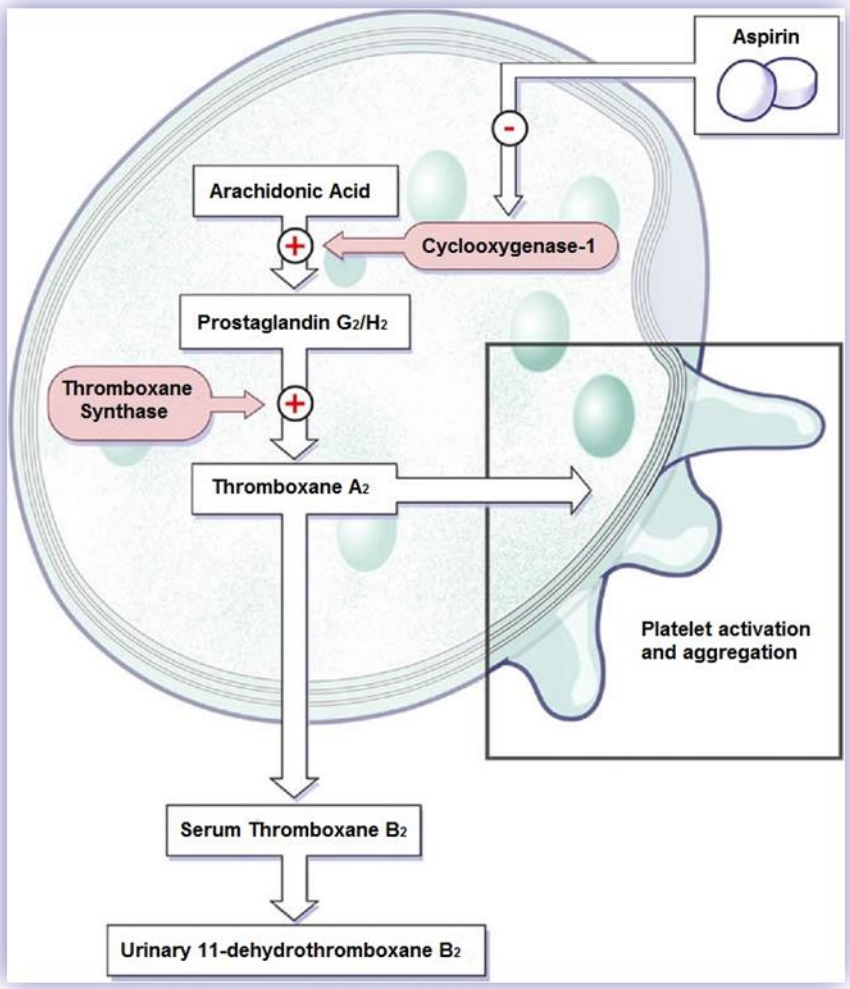


Figure 7. *Mechanism of action of low-dose ASA in platelets.*

Because platelets are anucleate cells (without transcriptional ability) and have a limited capacity of new protein synthesis [19] [20], platelets COX-1 inhibition is permanently and prolonged for their lifetime [21].

The mean life span of human platelets is approximately 8 to 10 days. Therefore, about 10 to 12% of circulating platelets are replaced every 24 h. ASA also acetylates the enzyme in bone marrow megakaryocytes and pro-platelets before new platelets are released into the circulation [22], [23]. Dose interval of 24 h is enough for maintaining TxA_2 levels inhibited because ASA inhibits COX-1 of both platelets and platelets progenitors, thus the new platelets progeny have non-functioning enzyme (figure 8) [24].

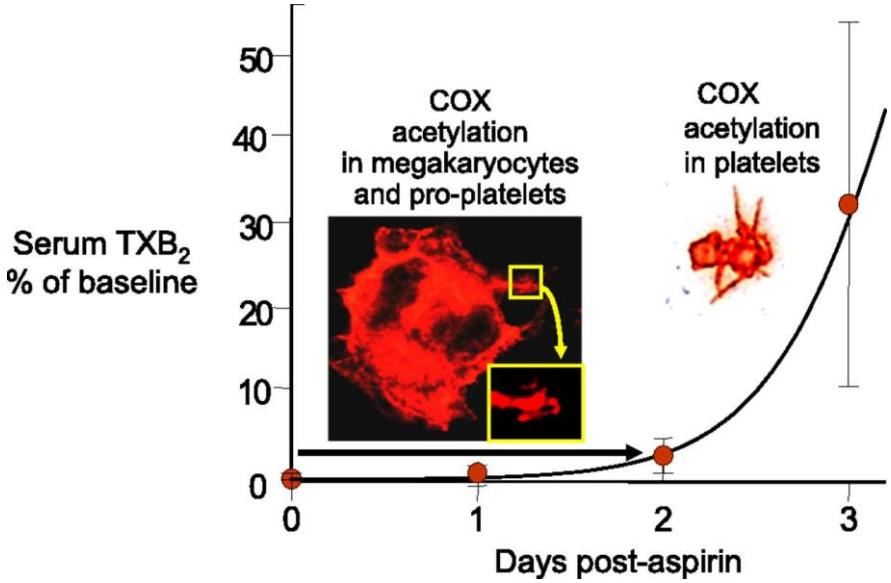


Figure 8. *Recovery of serum TxB_2 after ASA withdrawal in healthy subjects* [24].

For patients on chronic treatment (on daily ASA at dose of 75-100 mg) the enteric coated formulation (EC-ASA) is commonly prescribed instead of plain ASA for its lower side effect, in particular lower incidence of gastrointestinal bleeding or ulcers [25]: COX-1 is constitutively expressed in gastrointestinal mucosa where is involved in the synthesis of prostaglandin useful for the maintenance of mucosa integrity (for example prostacyclin) [26]–[28].

Plain ASA is rapid absorbed in the stomach and upper intestine and peak plasma levels occur between 30 to 40 min after intake, while the enteric-coating of EC-ASA is resistant to acid environment, so ASA is not released into the stomach but in the alkaline environment of the intestine and plasma peak levels occur between 2-6 h after intake. Both formulations have a bioavailability of 40-50%. Plasma concentration of ASA decay with an half-life of 15 to 20 min [29]–[31].

In literature there are few information published about PK of plain ASA and EC-ASA, at low dose, in HS. Nagelschmitz J et al. [32] compared the PK and PD of intravenous versus oral tablet formulations at different doses (250-500 mg for iv; 100, 300 and 500 for po) after single dose administration. Patrignani et al. [33] studied the extent of

COX-1 acetylation after the first and the seventh dose of daily 100 mg EC-ASA considering PK and PD parameters. Cox et al. [34] evaluated PD effect, before and after 14 days of treatment with ASA, of five different low-dose formulations: three 75 mg EC formulations, one 75 mg dispersible aspirin and asasantin (25-mg standard release aspirin plus 200-mg modified-release dipyridamole given twice daily).

1.3 Essential thrombocythemia

Essential thrombocythemia (ET) is a BCR-ABL1-negative myeloproliferative neoplasm (MPNs) characterized by an increased number of platelets (450 x 10^9/L) in the circulating blood. Other MPNs are polycythemia vera (PV), which main characteristic is increased red cells mass and primary myelofibrosis (PMF), characterized by anemia. ET, PV and PMF are hematopoietic disorders characterized by clonal proliferation of multilineage bone marrow progenitors.

No one knows what causes the onset of ET or other MPNs. ET is not a genetically inherited disorder, but there may be a familial predisposition to the disease in some patients.

However, researchers have discovered mutations that alter the activity of proteins that control signalling pathways (important regulators of cell growth and development) in many patients with ET: JAK2 (Janus kinase 2; located on chromosome 9p24), CALR (calreticulin; located on chromosome 19p13.2) and MPL (myeloproliferative leukemia virus oncogene; located on chromosome 1p34) mutations have a incidence in ET patients of 50–60%, 20-25% and 3-4% respectively [35]–[39].

Nowadays World Health Organization (WHO) diagnostic criteria for ET are:

1. platelets count > 450 x 10^9/L;
2. bone marrow biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei; no significant increase or left-shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers;

3. not meeting WHO criteria for BCR-ABL1+ chronic myeloid leukemia (CML), PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms;
4. presence of JAK2, CALR, or MPL mutation;
5. presence of a clonal marker or absence of evidence for reactive thrombocytosis.

Criteria 1-4 are classified as major, while the fifth is classified as minor criteria. Diagnosis requires meeting of all 4 major criteria or the first 3 major criteria and the minor criterion [40], [41].

Its prevalence is calculated to be around 20 per 100000 individuals and ET occurs in 50 to 60 years old patients and has a longer life expectancy and lower leukemic transformation rate as compared to other MPNs. 20% of ET sufferers are under the age of 40. Approximately 60% of patients with ET is characterized by a platelets proliferation higher than normal (platelet count > 450 X 10^9/L), with an incidence of 1-2 cases per 100000 inhabitants, had at least one thrombotic event in their lives, including transient ischemic attack, myocardial infarction or stroke. The female to male ratio is about 2:1 [42].

ET patients may develop both thrombotic and haemorrhagic complication, thus treatment is based on risk stratification with the clinical goal to reduce the frequency of thrombosis and haemorrhage. The classical factor in the stratification are age>60, previous thrombosis and platelet count>1000-1500 X10^9/L (for bleeding).

Low-dose ASA (100 mg), commonly the EC-ASA formulation, is usually given (for its lower risks of gastrointestinal bleeding) to reduce the risk of blood clotting and can be safely used if patients' platelet counts are < 1500 × 10^9 /L and there is no history of haemorrhage.

Hydroxyurea (HU) or hydroxycarbamide (HC) are often used to treat ET patients at high risk for clotting (over 60 years-old or patients with a prior blood clot) in order to reduce

platelet count. It is usually considered the first line agent for patients that require treatment. Anagrelide is another option for lowering platelet counts. It is frequently used after a patient has demonstrated intolerance or experienced complications with HU. Interferon (IFN) is sometimes prescribed for ET patients: women of childbearing age are often treated with INF because it hasn't been shown to cause birth defects [43]–[49].

1.4 Aspirin resistance

In literature there are different definitions of “ASA resistance”, for simplification it can consider “clinical resistance” and “laboratory resistance”. The first one can be diagnosed after the occurrence of an atherothrombotic ischaemic event in a patient taking a therapeutic dose of ASA [50]. So clinical diagnosis of “ASA resistance” is limited because it is retrospective and non-specific. It is more appropriate to classify these patients as having a failure of response to therapy, rather than “clinical resistance” to therapy. “Laboratory resistance" is referred to the failure of ASA to inhibit platelet TxA_2 production or the failure to inhibit TxA_2-dependent platelet function tests. In laboratory “ASA resistance” can be evaluated by measurement of platelet TxA_2 production. TxA_2 is not stable, thus the measurement is determined by considering TxB_2 levels in serum or and 11-dehydro thromboxane B_2 in the urine. Because serum TxB_2 production is largely dependent on platelet COX-1 (ASA’s therapeutic target), it’s commonly used as a measure of the inhibitory effects of low-dose ASA on platelets [51].

Moreover “laboratory resistance” can be evaluated by considering TxA_2-dependent platelet function [52]–[55], in particular agonist-induced platelet aggregation.

Tests of platelet function generally used are:

- light transmission aggregometry (LTA) measures the increase in light transmission through platelet rich plasma (PRP) when platelets are aggregated by an agonist (for example collagen, TxA_2, ADP ecc.);
- impedance aggregometry measures the change in electrical impedance between two electrodes when platelets are aggregated by an agonist. This method can be used also in WB;

- [PFA]-100 is an artificial vessel consisting of a sample reservoir, a capillarity and a biological active membrane with a central aperture, coated with collagen plus ADP or epinephrine. The application of a constant negative pressure aspirates the anticoagulated blood of the sample from the reservoir through the capillary and the aperture, while a platelet clot forms and occludes the aperture. The time taken to interrupt blood flow closure time is recorded;
- Ultegra rapid platelet function assay [RPFA] measures agglutination of fibrinogen-coated beads in response to propyl gallate or AA stimulation. If ASA produces the expected antiplatelet effect, fibrinogen-coated beads will not agglutinate, and light transmission will not increase. The result is expressed as ASA reaction units (ARU).

In figure 9 are reported advantages and limitations of each test.

	Advantages	Limitations
Thromboxane production		
Serum thromboxane B_2	Directly dependent on aspirin's therapeutic target, COX-1	May not be platelet specific Operator expertise required
Urinary 11-dehydro-thromboxane B_2	Dependent on aspirin's therapeutic target, COX-1 Correlated with clinical events	Not platelet specific Uncertain sensitivity Uncertain reproducibility Not widely evaluated
Thromboxane-dependent platelet function		
Light or optical aggregation	Traditional gold standard Widely available Correlated with clinical events	Not specific Uncertain sensitivity Limited reproducibility Labour intensive Operator and interpreter dependent
Impedance aggregation	Less sample preparation required	Not specific Uncertain sensitivity Operator and interpreter dependent
PFA-100	Simple Rapid Semi-automated Correlated with clinical events	Dependent on von Willebrand factor and haematocrit Not specific Uncertain sensitivity
Ultegra RPFA	Simple Rapid Semi-automated Point-of-care test Correlated with clinical events	Uncertain specificity Uncertain sensitivity

Adapted in part from Michelson.[11]

Table: **Laboratory tests commonly used to measure the antiplatelet effects of aspirin**

Figure 9. *Advantages and limitations of laboratory tests* [56].

Floyd et al [57], Hankey et al[56], Cattaneo [58] illustrated potential mechanisms for "ASA resistance" that could be summirize in:

1) poor adherence: it is probably the most frequent and plausible cause of ASA resistance;
2) decreased bioavailability of ASA. Some studies showed that at equivalent doses EC-ASA was less effective than plain ASA to ininhibit TxA_2 production [34], [59]–[61]. Some studies suggested that the standard low doses of daily aspirin (81–100 mg) may be inadequate in some subjects to inhibit completely COX-1 activity [62]–[64];
3) drug interaction: competition of ASA with other NSAIDs (for example ibuprofene, indometacine) which could block ASA access at binding site (Ser-530) in COX-1 [65];

   Proton pump inhibitors (PPIs) are routinely co-prescribed to patients on ASA chronic treatment at high risk of bleeding, and act to provide some gastric protection by reducing acid production, but the consequences of PPIs co-administration with ASA are not known;
4) esterase-mediated metabolism of ASA: *in vivo* ASA may undergo hydrolysis to salicylate by esterases in the gastrointestinal tract prior to absorption [66]. No-correlation between gastrointestinal esterase activity and ASA bioavailability has been studied. In contrast variations in blood-borne esterase activity have been documented in HS [67], but attempts to correlate activity with pathological states have yielded inconsistent results;
5) anion efflux pump: the oral bioavailability and intracellular concentration of certain drugs are modulated by unidirectional channels which pump them into

or out of cells. Massimi et al [68] demonstrated that platelet multidrug resistance protein-4 (MRP4) overexpression has a role in reducing ASA action in patients after by-pass surgery [69];

6) increased production of platelets by the bone marrow may introduce new platelets able to synthetized TxA_2, because new COX-1 is not inhibited by ASA [70];
7) circulating, aspirinated platelets may recover their ability to form TxA_2 through *de novo* synthesis of COX-1 [20], or through transcellular formation of TxA2 from prostaglandin H2 released by other blood cells or vascular cells [70];
8) Genetyc polymorfirsm: presence of COX-1 variants that may be less responsive to aspirin inhibition [71], [72];
9) other sources of TxA_2 production: biosynthesis of TxA_2 by pathways that are not blocked by ASA;
10) interventions of coronary revascularization with coronary artery bypass surgery or coronary angioplasty may induce temporary aspirin resistance [70];
11) loss of antiplatelet effect of ASA with prolonged administration: tachyphylaxis.

Considering results published [64], [73]–[76] the incidence of ASA "laboratory resistance" ranges from 1% to 61%.

It depends on the type of laboratory test used to monitor the pharmacological response and the cut-off values chosen to identify poor-responders. Nowadays there is not a standardized test that could identify "resistant patients" but it's clear that subjects on ASA therapy show a great variability in response to ASA.

1.4.1 Aspirin resistance in ET patients.

As previously reported ET patients at risk of thrombotic complication are treated with low-dose ASA (75-100 mg). ASA has been used for years in primary and secondary prevention of cardiovascular disease. It was shown that daily low doses of ASA are able to reduce the TxA_2 production by 97%-99% in HS [77], but some ET patients are less responder to ASA.

Causes of inadequate response to ASA in ET patients are not clear and have been investigated. Dragani et al [78] showed that the same ASA regimen is unable to fully inhibit platelet TxA_2 production in 80% of ET patients. The residual platelet COX activity (both COX-1 and COX-2) was fully suppressed to levels comparable to controls by adding ASA (50 μM) to WB *in vitro.* Moreover, they demonstrated that COX-2 expression was significantly increased in ET patients and correlated with thiazole orange (TO) positive platelets ($r=0.71$, $P>0.001$).

Pascale et al. [79] argues that the abnormal megakaryopoiesis that characterizes ET patients is responsible for shorter-lasting antiplatelet effects of low-dose ASA through faster renewal of platelet COX-1. The increase in platelet turnover in patients with ET leads to an increase in the amount of non-inhibited COX-1 levels, more than 10% expected in the 24h interval between doses of the drug. This abnormal biochemical and functional phenotype can be reverted to a normal pattern of platelet response by modulating the ASA dosing interval but not the dose. These results suggested that twice daily administration of ASA in ET patients may increase clinical ASA efficacy.

In a recent study [80] a correlation ($r=0.49$, $P<0.001$) was shown between residual AA-inducible platelet aggregation and the platelet count in patients with MPN (ET, PV or PMF).

2 AIM OF THE STUDY

Aim of the study is to elucidate the potential mechanism underlying the altered low-dose ASA response observed in some ET patients.

In particular, we explored three hypotheses:

1) lack PD of ASA
2) high plasma and/or whole blood esterase activity
3) low absorption of ASA

The first hypothesis was investigated by studying the *in vitro* effects of ASA on TxA_2 after platelet aggregation induced by agonist.

In order to be able to explore the second and the third hypotheses, it was necessary to develop a reliable, sensible, reproducible and accurate analytical method for the quantification of ASA and SA (the major ASA metabolite) in plasma samples. To this aim, we set up to develop and validate a method based on isotope dilution liquid chromatography mass spectrometry (ID-LC-MS/MS) for the simultaneous determination of the two analytes using the corresponding deuterated analogues.

Using this technique, we tested the *in vitro* activity of plasma and blood esterase as function of time and substrate concentrations and we evaluated the in vivo kinetics of ASA, SA and TxB_2 at different time points after ASA administration in healthy subjects, ET patients good ASA responders and ET patients poor ASA responders. We also compared the PK and PD of two clinically available ASA formulations: enteric-coated ASA and plain ASA.

3 MATERIALS AND METHOD

3.1 Chemicals and Materials

- ASA, SA, HPLC-grade acetonitrile, formic acid, potassium fluoride, dimethyl sulfoxide, indomethacin, thiazole orange (TO) and paraformaldehyde (PFA) were purchased from Sigma Aldrich (Milano, Italy).
- Acetylsalicylic acid-d4 (ASA-d4), salicylic acid-d4 (SA-d4) were purchased from Santa Cruz Biotecnology (CA, USA).
- Horm collagen from Mascia Brunelli (Milano, Italy).
- Inertsil ODS3, 150 × 3.0 mm i.d., 3 µm (GL Sciences, Tokio, Japan) was used for chromatography separation.
- Ultrapure water was obtained from MilliQ system.
- Thromboxane B_2 EIA kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA).
- CD42b APC antibody was purchased from Beckman (Cassina De' Pecchi (MI)).
- EDTA KE/2,7 mL, coagulation 9 NC/3 mL and serum Z/2,7 mL S-Monovette® were purchased from Sarstedt (Verona, Italy).
- Plain Aspirin and Cardioaspirin (enteric coated formulation) tablets at dose of 100 mg (Bayer, Germany).
- Revasc (INN-desirudin) injectable solution 15g/0,5 mL (Canyon Pharmaceuticals, London, United Kingdom).

3.2 Instrumentations

3.2.1 Liquid chromatography tandem mass spectrometry (LC-MS-MS)

ASA and SA were quantified from human plasma using HPLC coupled to a tandem mass spectrometer. The liquid chromatograph system is a Dionex 3000 UltiMate instrument with autosampler, binary pump, and column oven (Thermo Fisher Scientific, Rodano (MI), Italy). The tandem mass spectrometer is an AB Sciex 3200 QTRAP LC-MS/MS instrument with electrospray ionization (ESI) TurboIonSpray™ source (AB Sciex S.r.l., Milano, Italy). The analytical data were processed by Analyst software (version 1.4)

3.2.2 Other instruments

- Coulter hematology analyser (Beckman Coulter, Milano, Italy).
- Platelet Aggregation Profiler-8E (Biodata, Horsham, PA, USA).
- BD FACSVerse™ flow cytometer (Biosciences, USA).
- Ensight multimode Reader (Perkin Elmer, Milano, Italy).
- Multi-Block heater (Thermo-Fisher Scientific, Rodano (MI), Italy).
- Julabo TW8 water bath (Sigma Aldrich Milano, Italy).
- Centrifuges 5702R and 5415R (Eppendorf, Amburgo, Germany).
- Pipettes P10, P20, P200, P1000 (Eppendorf, Amburgo, Germany).

3.3 Study population and design

In a previous study with other aims, conducted in our laboratory, ET patients referring to "Divisione di Medicina 2, ASST Santi Paolo e Carlo, Dipartimento di Scienze della Salute - Università degli Studi di Milano" were classified considering serum TxB_2 levels at 24 h and 2 h after EC-ASA intake: as reported in literature [64] non responder patients are that with serum TxB_2 greater than 10 ng/mL (figure 10).

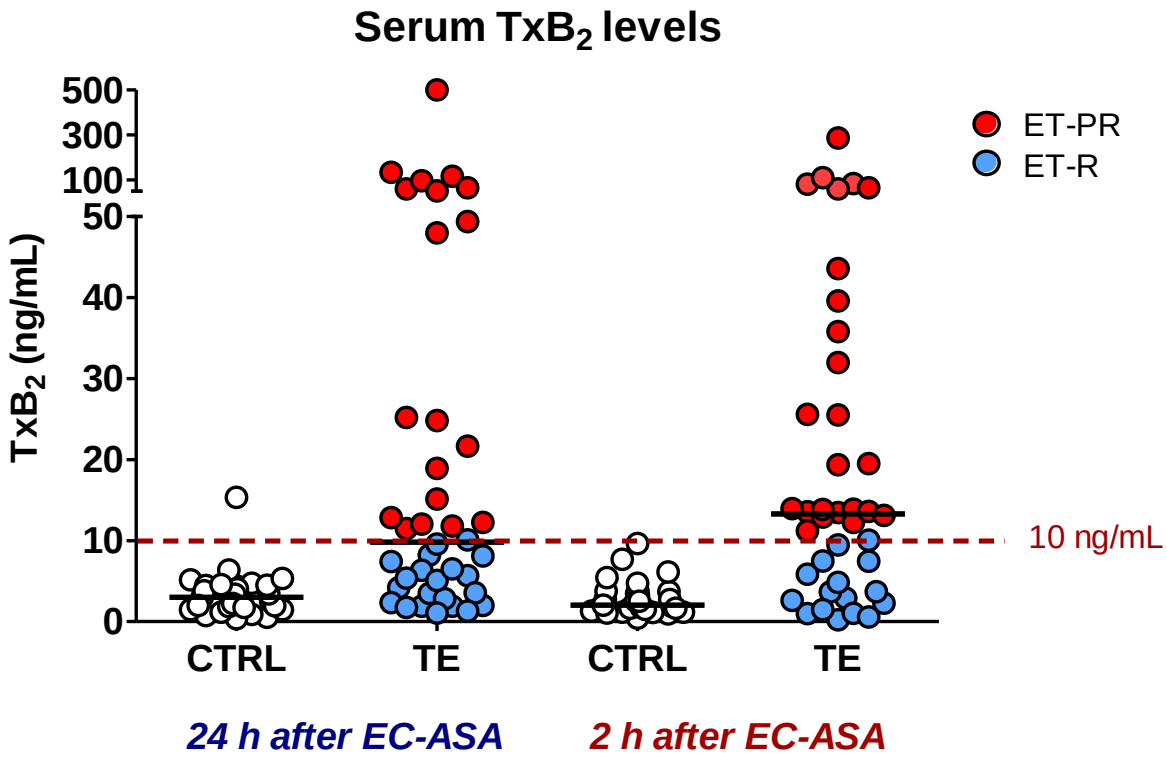


Figure 10. *Serum TxB_2 levels in ET patients and HS at 24 h and 2 h after EC-ASA intake.*

For this study ET patients were recalled and 10 ET responders (ET-R), 7 ET poor responder (ET-PR) and 10 healthy controls (HS) entered the study. All recruited subjects signed written informed consent. Subjects who took NSAIDs in the previous three days were excluded. For each enrolled subject a diary containing information about drugs assumption, weight, height, breakfast, age, withdrawal times was written off.

3.4 Blood collection

Blood sample were collected in the morning from antecubital vein.

For reticulated platelets measurement, the first 3 ml were collected into K-EDTA tubes and analysed by Coulter hematology analyser. For *in vitro* studies on TxA_2 production, 20 ml were collected in tubes containing 250 µg/ml of hirudin, gently mixed, left at room temperature for 15 min and analysed by Coulter haematology analyser. For esterase studies, 3 ml of blood were collected in commercial citrate tubes: blood was immediately used and plasma was obtained after centrifugation at 1400 g for 15 minutes. For PK studies 6 ml of blood were collected in two K-EDTA chilled tubes (3 mL) containing 20 µL of potassium fluoride 150 mg/mL (to minimize the hydrolysis of ASA to SA in human blood) and 3 ml were collected in tubes without anticoagulant (in order to obtain serum) at different time points after EC-ASA or plain ASA administration (figure 11). The chilled blood samples were centrifuged immediately at 14000g for 10 min at 4°C and the plasma samples were frozen at −20°C until LC/MS/MS analysis. Non-anticoagulated blood was allowed to clot in water bath at 37 °C for 1 hour, and then centrifuged at 1400g for 15 minutes and serum samples were frozen at -20 °C until ELISA.

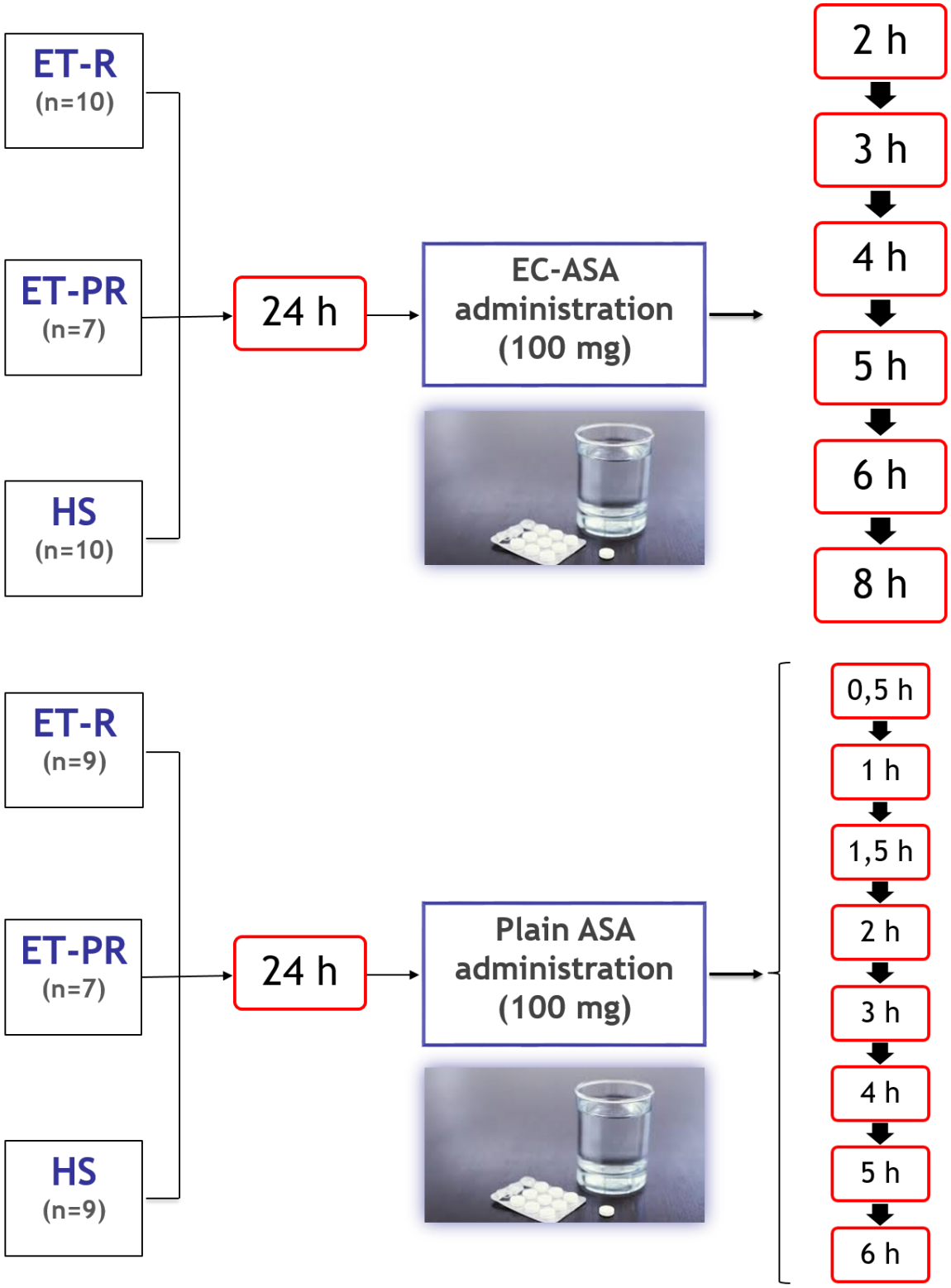


Figure 11. *Blood drawing scheme after EC-ASA (above) and plain ASA (below) intake.*

3.5 Evaluation of *in vitro* effects of Aspirin on TxA_2 production in whole blood and in platelet rich plasma

ASA activity on TxA_2 production was evaluated using Platelet Aggregation Profiler-8E.

The activity was measured in platelet rich plasma (PRP) and in (WB) diluted 1 to 1 with physiological solution. The experiments were carried out using different concentration of ASA from 10 µM to 1 mM.

PRP was obtained by centrifugation of hirudin WB samples at 200g for 15 minutes at room temperature, PRP was carefully removed into a stoppered plastic tube and platelet concentration was measured by coulter haematology. Platelet poor plasma (PPP) was obtained by further centrifugation at 1400g at room temperature for 15 minutes.

All tests were performed within 3 hours after blood collection. 300 µL of PRP or diluted WB was placed into a test tube containing a stir bar, 6 µL of ASA were added and incubated for 5 minutes at room temperature then 10 minutes at 37 °C. Aggregation was induced by collagen (5 µg/mL) at 37 °C under stirring (1000 r.p.m.) and recorded for 6 minutes. 2 µL of indomethacin 1 mM was used to stop platelet aggregation and after centrifugation (14000g, 2 minutes) supernatant was recovered and frozen at -20 °C until ELISA.

3.5.1 Quantification of Thromboxane B_2

TxB_2, the stable metabolite of TxA_2, was measured by selective, competitive enzyme immunoassay. Frozen samples were thawed at 37 °C and opportunely diluted (between 1:2 and 1:750) with phosphate buffer and tested in duplicate. Samples were assayed in parallel with standard calibration curve (detection limit= 1,6 pg/mL), prepared as

outlined in the manufacturer's instruction. The 96-well plate was red at 450 nm wavelength using Ensight multimode Reader. Results were expressed as ng/mL.

3.6 Development and validation of LC-MS/MS analytical method for ASA and SA determination in human plasma

3.6.1 Mass spectrometry and chromatographic conditions

The isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) technique in negative multiple reaction monitoring mode (MRM) was developed for determination of ASA and SA using the respective deuterated isotopomers (ASA-d4 and SA-d4) as internal standards (IS). The mass spectrometry operating conditions were optimized for each analyte by infusion of solution (10 µg/mL) into ESI source using the built-in syringe pump (rate 10 µL/min). ASA, ASA-d4, SA and SA-d4 were separated on reversed-phase column Inertsil ODS3, 150 × 3.0 mm i.d., 3 µm particle size (GL Sciences, Tokio, Japan) with an isocratic mobile phase consisting of acetonitrile and water containing 0.1% formic acid (80:20, v/v). The mobile phase was delivered at 0.3 mL/min, the autosampler and the column oven were kept at 5 °C and 20 °C, respectively.

3.6.2 Preparation of stocks and standard solutions

Stock solutions of ASA, ASA-d4, SA and SA-d4 were dissolved in 0.2% formic acid acetonitrile. ASA-d4 and SA-d4 standard working solutions were diluted 1 to 250 and 1 to 100 in order to obtain a final concentration of 4 µg/mL for ASA-d4 and 10 µg/mL for SA-d4. ASA and SA standard solutions were diluted with 0.2% formic acid in acetonitrile to prepare working solutions for calibration curves. Two independent calibration curves for ASA and SA were constructed in human plasma by spiking 250 µL of plasma (containing KF 1 mg/mL) with 50 µL of the appropriate working solution giving a final

concentration of 20, 50, 100, 200, 500, 1000, 2000 ng/mL for ASA and 20, 50, 100, 200, 500, 1000, 2000, 5000, 8000 ng/mL for SA.

Quality control (QC) samples were prepared by spiking control human plasma in bulk with ASA and SA at appropriate concentrations in the low, medium and high range: for ASA 20, 60, 500 and 1500 ng/mL; for SA 20, 100, 500, 5000 and 8000 ng/mL, then 250 µL of plasma aliquots were distributed in different tubes and stored at -20°C until extraction.

3.6.3 Sample preparation

ASA and SA were extracted from human plasma using protein precipitation: to 250 µL of human plasma were added 25 µL of ASA-d4 (4 µg/mL), 25 µL of SA-d4 (10 µg/mL) and 700 µL of 0.1% formic acid in acetonitrile. Samples were processed in ice-bath in order to prevent ASA hydrolysis by esterase. The mix was vortex for 1 minute, then centrifuged at 14000 g, 4°C for 10 minutes. The supernatant was transferred into an analytical vial and 10 µL were injected into LC/MS/MS system.

3.6.4 Method validation

The developed method was validated according to the US Food and Drug Administration guidelines [81] The parameters determined were selectivity, specificity, matrix effect, linearity, precision, accuracy, recovery and stability.

Selectivity and Specificity.

Selectivity was carried out by analysing six blank plasma samples, obtained from six different sources, spiked with analytes and internal standards at the respective LLOQ concentration.

Lower limit of quantification (LLOQ)

As reported in FDA guidelines the lowest standard on the calibration curve should be accepted as LLOQ if the analyte response is at least 5 times the response compared to blank response and analyte peak should be identifiable, discrete and reproducible with a precision of ±20% and accuracy of 80-120% [81].

Calibration curves

The linearity of the method was evaluated using analyte spiked plasma samples using the method of the least squares. Each calibration curves consisted of a blank sample, a zero sample (blank + ISs) and nonzero concentrations. Based on the concentration and peak area ratio two independent linear standard curves (20-2000 ng/mL for ASA and 20-8000 ng/mL for SA) were constructed and analysed by non-weighting linear regression. The calibration curves had to have a correlation coefficient (r^2) of 0.998. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20%.

Accuracy and precision

Intra-day accuracy and precision were evaluated by analysing five replicates containing ASA and SA at different QC levels prepared on the same day. The inter-day accuracy and precision were evaluated by analysing QC samples (n=5) in three different days. The accuracy was expressed as % bias: (observed concentration)/ (nominal concentration) x 100 and precision by coefficient of variation (CV). The acceptable criteria of the data included accuracy within ±15% deviation from the nominal value and precision within ±15% CV except for LLOQ, which was set at ±20%.

Recovery

The recovery of ASA and SA was determined at three different concentrations (low, medium and high) by comparing the analytical results for extracted samples with unextracted standards obtained by spiking extracted drug-free plasma samples with the same amount of ASA and SA. The unextracted standards represented 100% [81].

Stability

The stability experiments of ASA and SA in plasma samples were carried out by analysing QC samples at two different concentrations for ASA (60, 1250 ng/mL) and at three different concentrations for SA (60, 200 and 5000 ng/mL), under three different condition: after three freeze-thaw cycles (-20°C; 5 °C), after short term storage (6 h) in ice-bath, after long-term stability (2 months at -20 °C). Short term stability of post-extracted plasma was also evaluated in autosampler for 72 h.

3.7 Determination of esterase activity

Esterase activity was studied both as function of time and as function of substrate concentration. Plasma esterase activity as function of time was studied in plasma using the experimental condition described by Zhou et al. [82] which measure *in vitro* enzyme activity from the rate of SA formation during ASA incubation with plasma: 10 µL of plasma were added with 40 µL of ASA (4 mM) and incubated at 37 °C for 120, 180 and 240 minutes, before stopping the reaction with 150 µL of acetonitrile containing 0.1% of formic acid. Then samples were centrifuged at 14000 g for 10 minutes, diluted 1 to 10 with 0.1% formic acid in acetonitrile. At 100 µL of diluted samples were added 50 µL of SA-d4 (5 mM) and 2.5 µL were injected in LC-MS-MS system in order to measure the amount of SA formed after incubation (figure 12). Enzyme activity as function of substrate concentration was studied in fresh blood and plasma: 10 µL of plasma or blood were added with 40 µL of ASA at different concentration (from 0,4 µM to 10 mM) and incubated at 37 °C for 2 hours, then the reaction was stopped with 150 µL of acetonitrile containing 0,1 % of formic acid. Samples were centrifuged at 14000 g for 10 minutes and opportunely diluted (between 1:10 and 1:375), at 100 µL of diluted samples were added 50 µL of SA-d4 (5 mM) and 2.5 or 5 µL were injected in LC-MS-MS system (figure 13). We also evaluated non-enzymatic hydrolysis incubating ASA in phosphate buffer instead of plasma. The results were expressed by subtracting the unspecific hydrolysis. In order to quantified SA formation a specific SA calibration curve was constructed in phosphate buffer between 0.1 µM and 0.2 mM, calibrators were processed as described for samples.

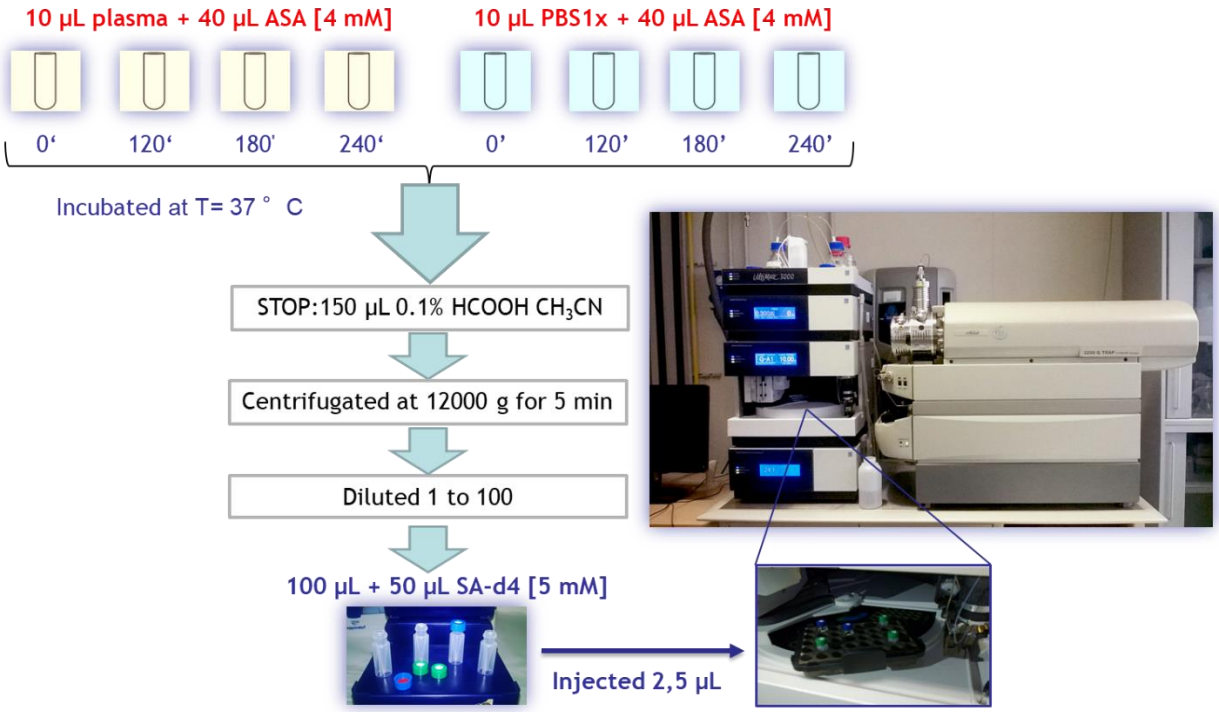


Figure 12. *Experimental scheme of esterase activity as function of time.*

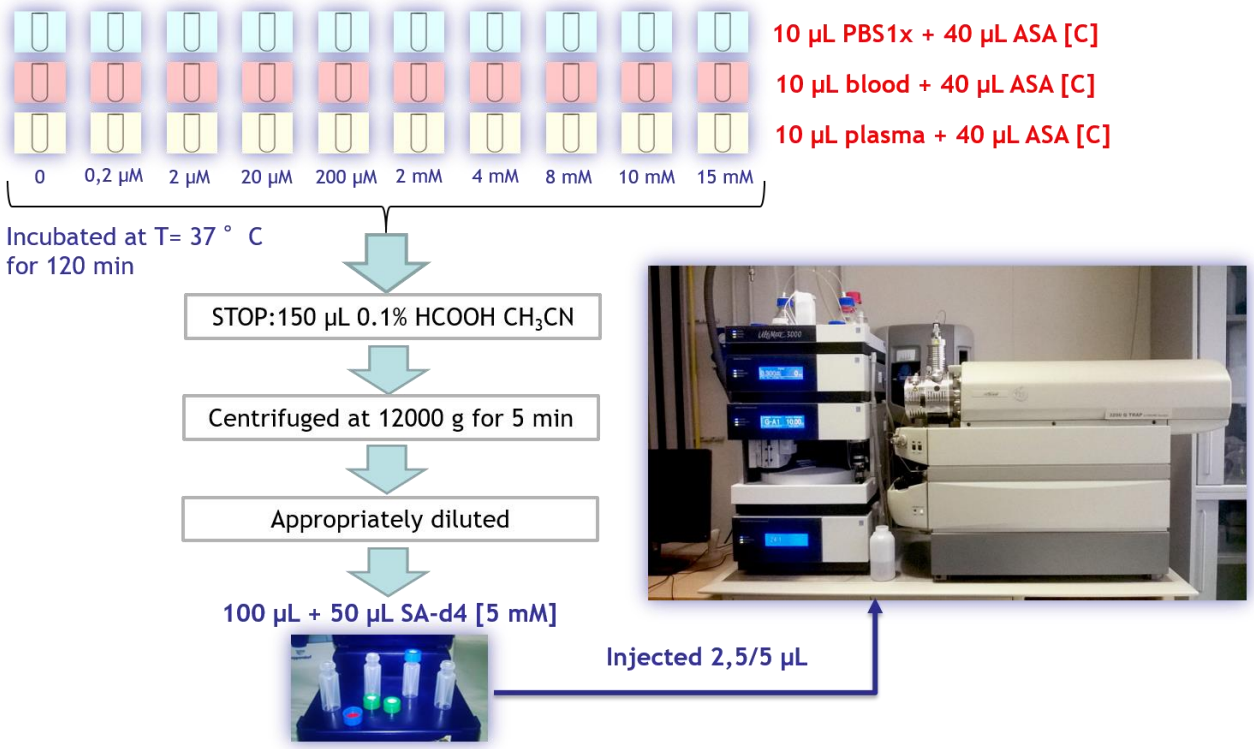


Figure 13. *Experimental scheme of esterase activity as function of substrate concentration.*

3.8 Reticulated platelets

Blood samples were prepared within one hour after sampling. A stock solution of TO was prepared by dissolving 1 mg of TO in 1 ml methanol and stored at -20 °C in the dark. TO working solution (0.05 µg/mL) was obtained by a 1 to 20000 dilution (in phosphate-buffered saline) immediately prior to use. 5 µl of WB were incubated for 15 min at room temperature with 2 µl of CD42b-APC (CD42b antibody recognize a platelet glycoprotein known as GPIbα) and with 40 µl of TO in the dark. Samples were then fixed by adding 1 ml of PFA (1%) and incubated for 30 min at room temperature in the dark. FS and SS were set to log-scale for the detection of platelets. An unstained sample was used to adjust the FS/SS PMT voltages. Samples were acquired by FACS verseTM BD 6 colours flow cytometry and analysed by BD FACSuite™ software. 5000 events in platelet gate (based on their size, forward-scattered light vs side-scattered light (FSC vs SSC)) were acquired with a medium flow rate. CD42b-positive cells were gated (SSC vs Allophycocyanin (APC)) to identify platelets and SSC vs TO settings were then used to identify TO-positive platelets.

3.9 Statistics

Statistical analyses were performed using GraphPad Prism v. 7 (GraphPad Software Inc, CA, USA). Peak integration and analytical method validation was performed using ABSciex Multiquant Software Version 2.0.

Population characteristics were reported as median (CI 25%-75%).

Results were expressed as mean ±SD or as mean± SEM. To assess significance among the three studied groups one-way analysis of variance (ANOVA) or Kruskal-Wallis test were performed. Statistical significance was assumed at $P<0.05$. Correlation was assessed by the Spearman rank test.

4 RESULTS AND DISCUSSION

4.1 Study population characteristics

Population characteristics are reported in tables 1 and 2. For each subject, information about weight, height, age and medication was recorded.

Table 1. Study population characteristics during EC-ASA PK study

	HS (n=10)	**ET-R (n=10)**	**ET-PR (n=7)**	***P* va*lue****
Sex (M/F)	5/5	5/5	4/3	-
Age (Y)[a]	53 (37-63)	69 (54-72)	52 (47-68)	0.211
BMI	22.7 (19.4-27.2)	25.3 (21.6-25.0)	26.0 (22.3-27.5)	0.783
WBC (x 10^9/L)[a]	6.1 (5.4-6.8)	5.4 (4.4-6.2)	8.2 (6.7-9.6)	0.373
RBC (x 10^{12}/L)	4.8 (4.3-5.0)	3.7 (3.3-4.5)	4.5 (3.4-4.9)	0.061
Hb (g/dL) [a]	14.3 (13.4-15.4)	12.5 (12.1-13.7)	13.7 (13.3-15.1)	0.054
Hematocrit (%)[a]	41.1 (39.2-43.0)	39.6 (38.4-44.1)	38.3 (35.3-40.8)	0.718
Platelets (x 10^9/L) [a]	223 (196-242)	373 (307-408)	583 (425-633)	<0.0001
MPV (fL) [a]	7.7 (7.4-7.9)	7.4 (7.0-7.7)	7.4 (7.1-8.5)	0.578
Medications				
Antihypertensive agents	*1/9*	3/10	-	
Antineoplastic agents	-	9/10	3/7	
alpha1-receptor antagonists	-	1/10	-	
Tapazole	-	1/10	-	
PPI	1/9	2/10	-	
Beta-blockers	*1/9*	2/10	-	
Diuretics	*1/9*	1/10	-	
Allopurinol	-	-	1/7	
Oral hypoglycemic agents	*1/9*	-	-	

[a]Results are expressed as median (CI 25%-75%); *P<0.05

Table 2. Study population characteristics during plain ASA PK study.

	HS (n=9)	ET-R (n=9)	ET-PR (n=7)	*P value**
Sex (M/F)	7/2	5/4	4/3	-
Age (Y)[a]	55 (31-66)	70 (52-74)	52 (48-70)	0.090
BMI	22.8 (22.3-25.4)	24.3 (21.6-25.0)	26.0 (22.3-27.5)	0.730
WBC (x 10^9/L)[a]	6.8 (5.3-7.1)	5.6 (5.1-7.6)	9.5 (6.3-11.1)	0.084
RBC (x 10^{12}/L)	5.0 (4.4-5.4)	3.8 (3.5-4.1)	4.4 (3.7-5.0)	0.012
Hb (g/dL) [a]	14.9 (13.9-15.4)	12.8 (12.7-13.5)	13.4 (12.8-14.5)	0.003
Hematocrit (%)[a]	43.5 (39.2-44)	38.3 (35.2-42.3)	37.3 (35.7-38.8)	0.009
Platelets (x 10^9/L) [a]	216 (192-224)	373 (307-408)	603 (344-645)	0.0003
MPV (fL) [a]	7.9 (7.7-8.1)	7.4 (6.9-8.2)	7.6 (7.2-8.6)	0.431
Medications				
Antihypertensive agents	*1/9*	3/9	1/7	
Antineoplastic agents	-	8/9	3/7	
alpha1-receptor antagonists	-	1/9	-	
Tapazole	-	1/9	1/7	
PPI	-	4/9	2/7	
Beta-blockers	*1/9*	4/9	-	
Diuretics	*1/9*	1/9	-	
Allopurinol	-	-	-	
Oral hypoglycemic agents	*1/9*	-	1/7	

[a]Results are expressed as median (CI 25%-75%); **P*<0.05

4.2 *In vitro* effect of ASA in ET-PR patients

In order to evaluate the PD of ASA, different concentration of ASA were added *in vitro* to hirudin-WB and PRP of ET-PR patients at 24 h after EC-ASA intake and in HS not under ASA. The levels of TxB_2 (stable metabolite of TxA_2) were measured by ELISA in the supernatant plasma after collagen (5 µg/mL) stimulation of PRP or WB (figure 14).

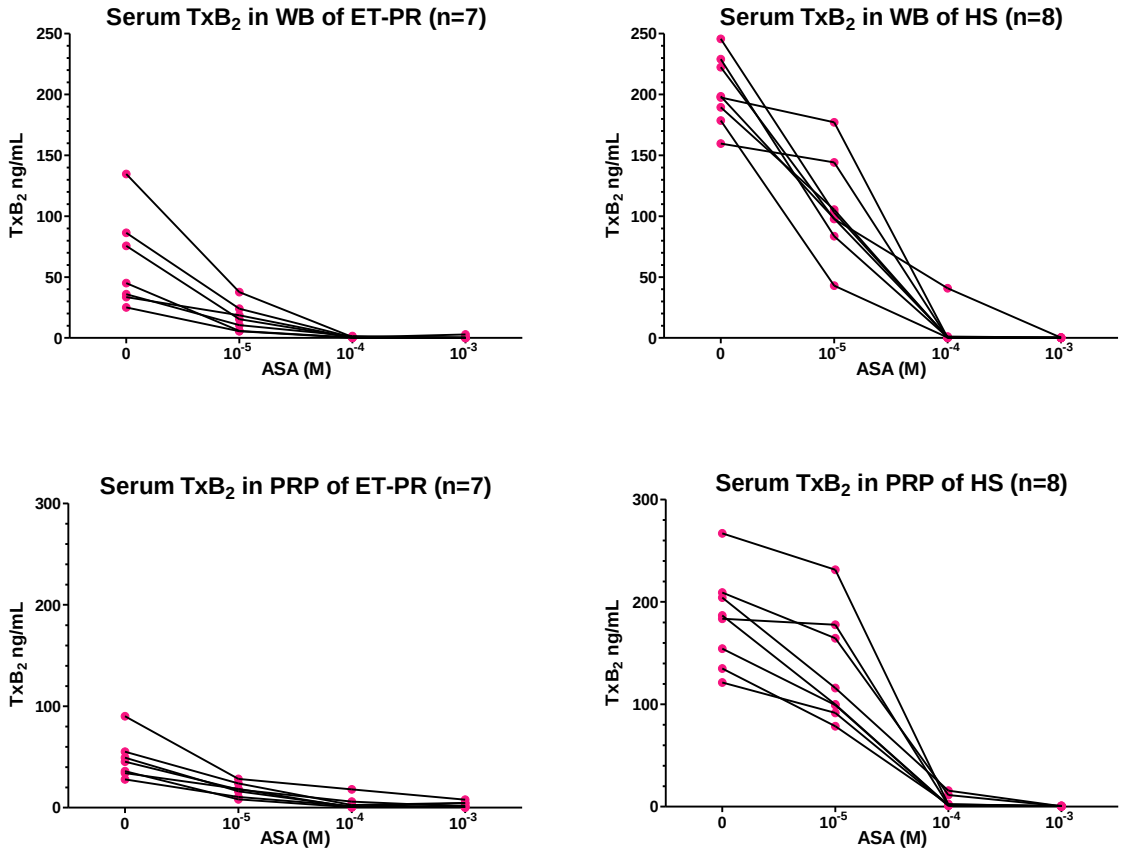


Figure 14. Effects of ASA, added in vitro at the indicated concentrations, on *collagen (5 µg/mL)-induced TxB_2 production in hirudin-WB (above)and hirudin-PRP (below) of ET-PR and HS not under ASA.*

Concentration-response curves in WB and PRP showed that TxB_2 production was inhibited when 100 µM of ASA were added both for ET-PR and HS. IC50 was 3.7 µM and 11 µM in WB of ET-PR and HS respectively. In PRP, IC50 was 4.9 µM for ET-PR and 21 µM for HS. This suggested that poor response to ASA in ET patients is not due to abnormal PD, because the addition of ASA *in vitro* inhibited TxB_2 production to the same extent in ET-PR and HS.

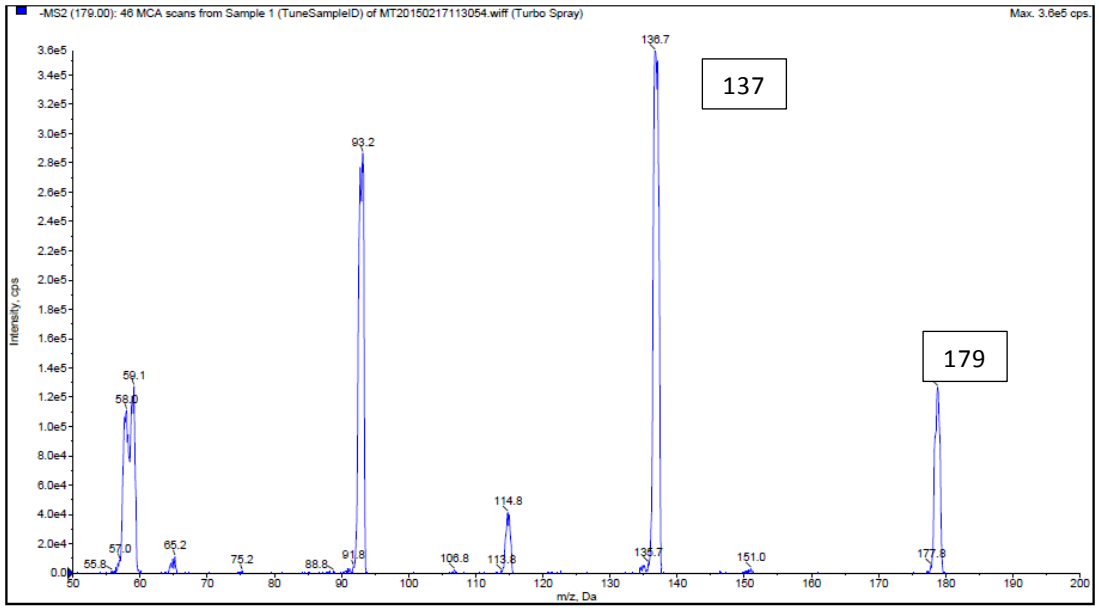
Our results are in agreement with those reported in literature: a recent study [78] showed that the residual TxB_2 production in ET patients was completely suppressed by adding ASA (50 µM) to WB *in vitro*. Another study [83] showed that ASA (100 µM) inhibited arachidonic acid (0.5-10 µM)-induced TxB_2 production of washed platelets.

4.3 Development and validation of LC-MS-MS for ASA and SA determination in plasma

In order to obtain the best analytical conditions for ASA and SA analysis, different chromatographic and mass spectrometric conditions were tested.

4.3.1 Optimization of Mass Spectrometry and liquid chromatography conditions

In order to optimize ESI conditions for ASA, ASA-d4, SA and SA-d4, quadrupole full scans were carried out in negative ion detection mode by infusing each analyte at concentration of 10 µg/mL. Carboxylic acid is present in ASA and SA structures, so they have a stronger mass response in the negative ionization mode than the positive mode. The most abundant precursor/product ions (figure 15) were chosen in the MRM acquisition in terms of better sensitivity: for ASA and SA at m/z 179.0 → 137.0 and m/z 137.0 → 93.0; for ASA-d4 and SA-d4 at m/z 183.0 → 141.0 and m/z 141.0 → 97.0, respectively (figure 16). During infusion experiment, source parameters were also optimized: temperature 500 °C, ion voltage -4500V, nebulizing gas -30 psi, heater gas -45 psi, curtain gas -45 psi and collision energy was set at medium. For each analyte and IS, compounds parameters, such as declustering potential (DP), collision energy (CE), entrance potential (EP), collision cell entrance potential (CEP) and collision cell exit potential (CXP) were optimized (table 3). Dwell time was set at 250 msec.



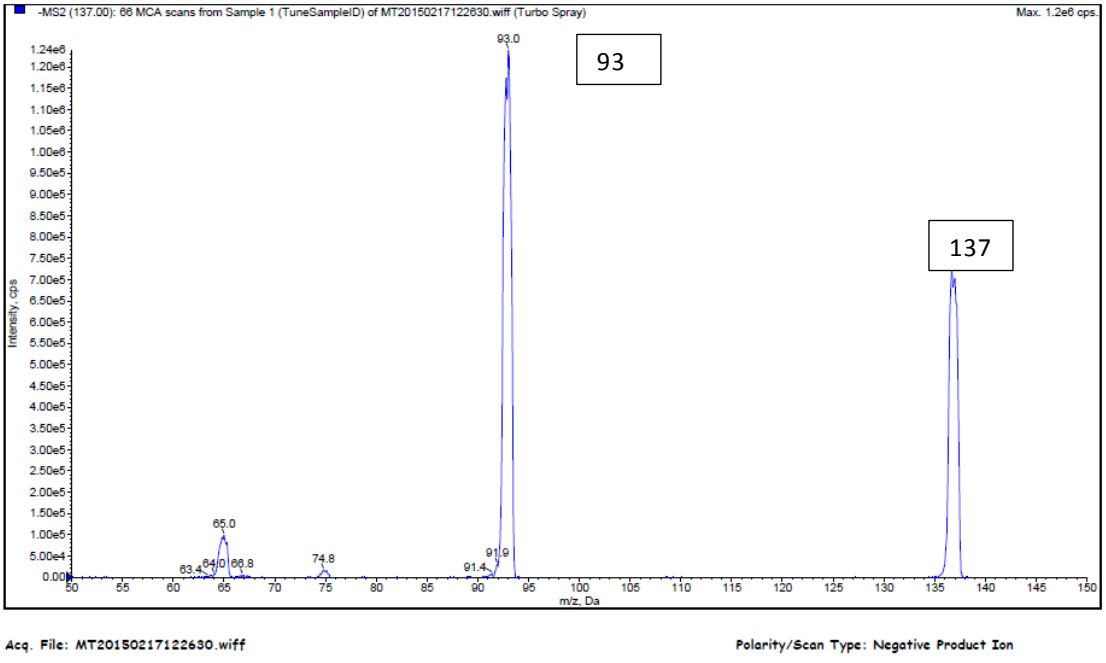


Figure 15. *Fragment ion spectrum of ASA (above) and SA (below).*

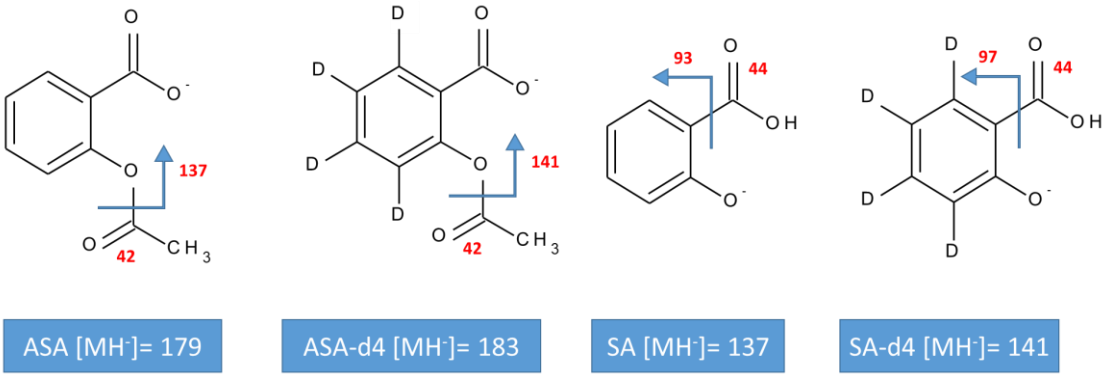


Figure 16 Chemical structure and fragmentation pattern of ASA, ASA-d4, SA and SA-d4 in negative MRM.

Table 3. *Compounds parameter for each analyte*.

Compound	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP(V)
ASA	179	137	-10	-10	-10	-14	-2
SA	137	93	-33	-10	-10	-25	-2
ASA-d4	183	141	-10	-10	-10	-14	-2
SA-d4	141	97	-33	-10	-10	-25	-2

It is worthy to note that more than 50% of ASA undergoes source fragmentation and forms SA, (this was also confirmed by ASA-d4 that forms SA-d4). Liquid chromatography plays an important role in the method development of ASA and SA: in fact, in PK studies, chromatographic separation is pivotal to distinguish between the SA fragment peak generated into the source, and the SA generated *in vivo* during ASA metabolism. Figure 17 reports an example of chromatogram of all components: retention time for ASA and ASA-d4 was 2.6 min; while for SA and SA-d4 was 3.5 min.

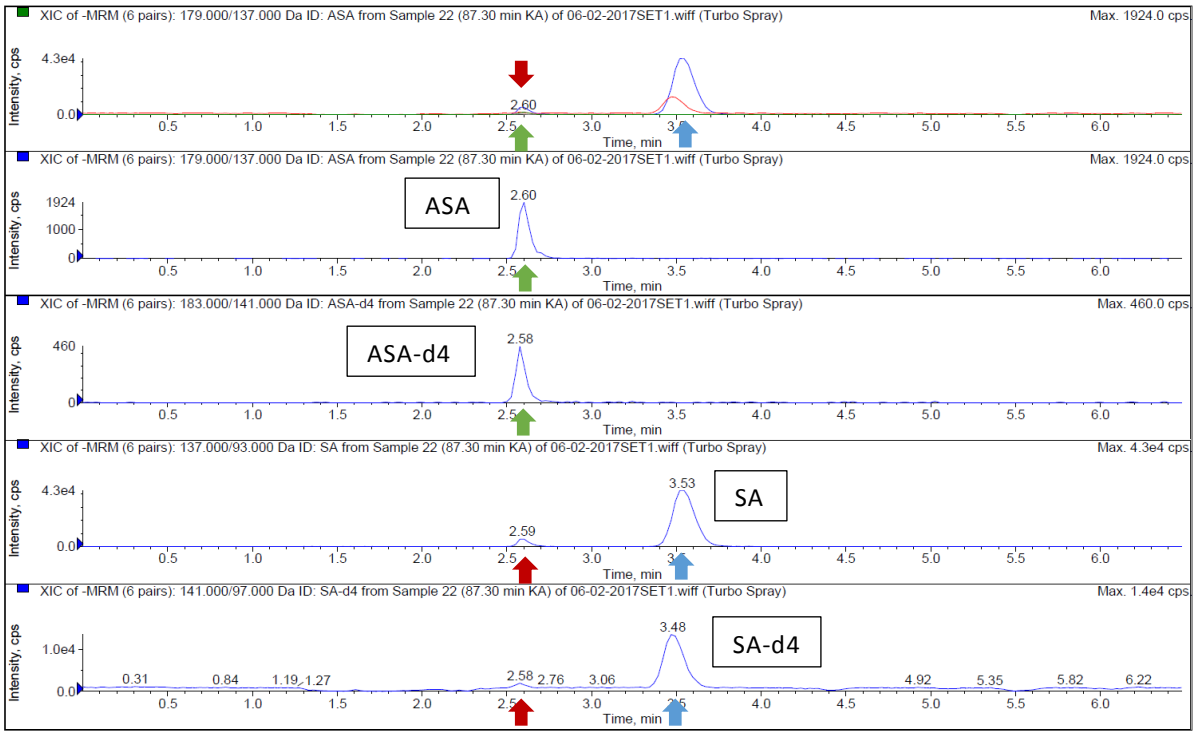


Figure 17. *Above the extracted ion (XIC) chromatograms of: all components. After from top to bottom the extracted ion chromatogram of ASA, ASA-d4, SA and SA-d4. Green arrows indicated chromatographic peaks of ASA and ASA-d4; blue arrows indicated chromatographic peaks of SA and SA-d4; red arrows indicated chromatographic peaks of SA generated in source from ASA and ASA-d4.*

4.3.2 Method validation

The developed method was validated according to the US Food and Drug Administration guideline [81] and performed using MultiQuant™ software 2.1 and GraphPad Prism v. 7

Selectivity and Specificity.

Selectivity was carried out by analysing six blank plasma samples, obtained from six different sources spiked with analytes at the respective LLOQ concentration (figure 18).

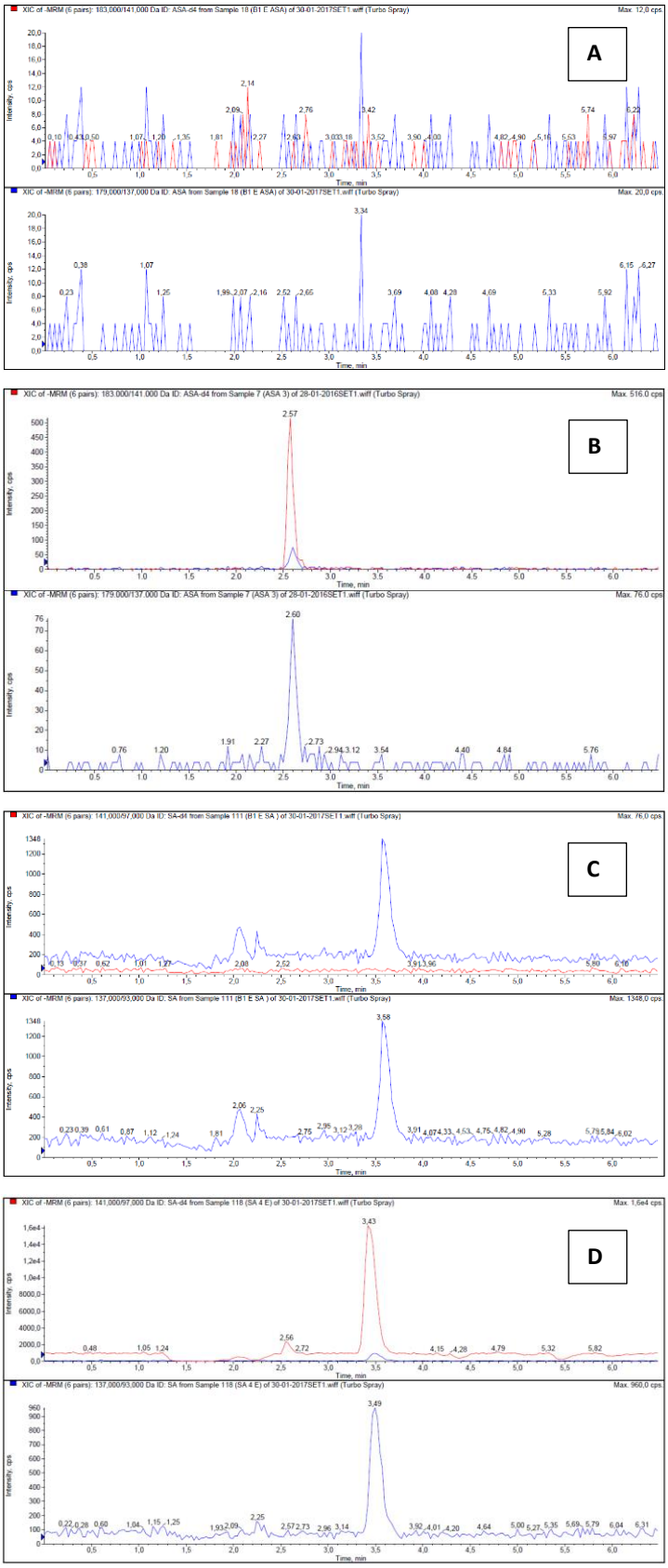


Figure 18. *Chromatogram of ASA and ASA-d4 in human blank plasma(A) and human plasma spiked with ASA (20 ng/mL) (C); chromatogram of SA and SA-d4 in human blank plasma (B) and human plasma spiked with SA (20 ng/mL)*

Lower limit of quantification (LLOQ)

Using this analytical method LLOQ for ASA and SA was 20 ng/mL.

Calibration curves

Calibration curves had a reliable reproducibility and linearity over the concentration range. The plasma concentration ranges were 20-2000 ng/mL for ASA and 20-8000 ng/mL for SA. The curves were linear with a mean correlation coefficient r^2 of 0,9991 for ASA and 0,9996 for SA. Each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was ±20% (figure 19).

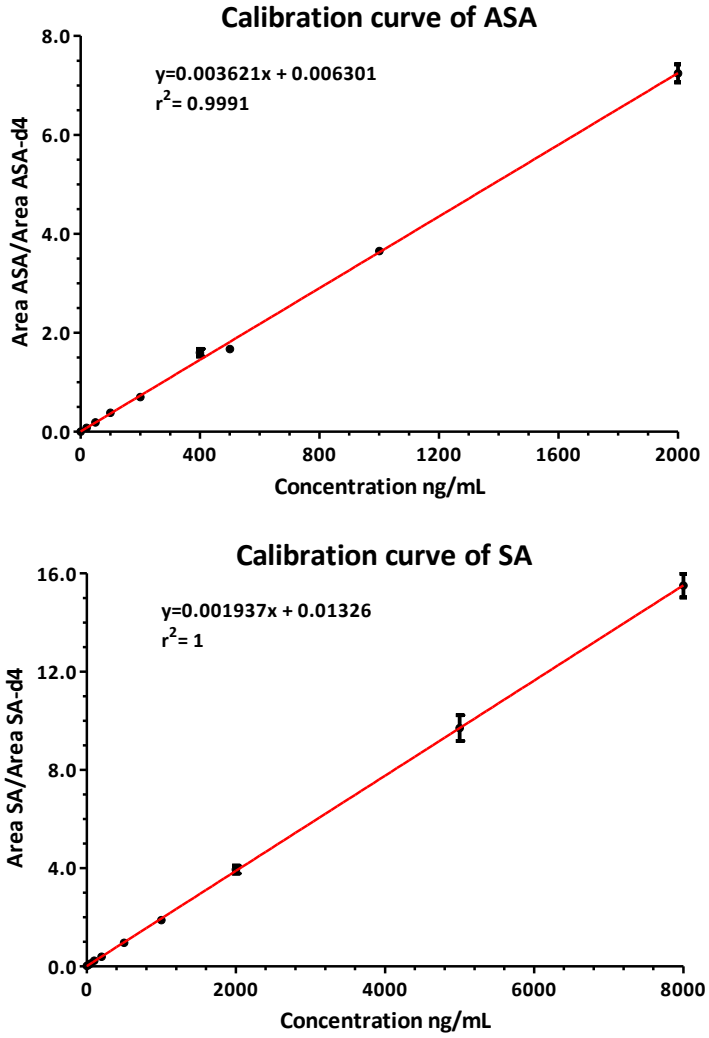


Figure 19. *Calibration curve in human plasma of ASA (above) and SA (below).*

Accuracy and precision

Results of intra-day and inter-day accuracy and precision are reported in tables 4 and 5. Intra-day and inter-day precision were expressed as % of CV and were within ±15% for each QC at low medium and high levels and within ±20% at LLOQ levels.

The intra-day and inter-day accuracies were all within 100 ±15% of the nominal value and were within 100±20% at LLOQ levels.

Table 4. *Intra-day precision and accuracy for ASA and SA QCs in human plasma (n=6).*

Analyte	Nominal concentration (ng/mL)	Concentration found[a] (ng/mL)	Accuracy (%)	Precision (% CV)
ASA	20	18.76±3.11	93.82	16.59
	60	52.23±3.63	87.04	6.94
	400	432.65±18.08	108.16	4.18
	1250	1230.48±28.63	98.44	2.33
SA	20	23.80±3.07	118.98	12.90
	60	66.00±4.03	110.01	6.10
	200	225.44±4.09	112.72	1.81
	500	561.14±21.94	112.23	3.91
	5000	5770.20±55.38	115.40	0.96

[a]Values are mean±SD

Table 5. Inter-day precision and accuracy for ASA and SA QCs in human plasma (n=6).

Analyte	Nominal concentration (ng/mL)	Concentration founda (ng/mL)	Accuracy (%)	Precision (% CV)
ASA	20	18.22±2.23	91.12	12.24
	60	52.22±3.75	87.04	7.17
	400	385.23±39.76	96.31	10.32
	1250	1214.62±72.45	97.17	5.96
SA	20	23.26±2.01	116.28	8.64
	60	67.29±3.62	112.14	5.38
	200	225.28±3.34	112.64	1.48
	500	557.94±15.88	111.59	2.85
	5000	5718.69±80.56	114.37	1.41

[a]Values are mean ± SD

Recovery of extraction

Liquid extraction with acetonitrile containing 0.1% of formic acid resulted to be robust and provided cleaned samples. The recoveries of analytes, reported in table 6, were good and reproducible.

Table 6. Extraction recoveries of the analytes in human plasma at different concentrations (n=6)

Analyte	Concentration (ng/mL)	Recovery[a] (%)	Precision (% CV)
ASA	20	85.60±4.74	5.60
	100	68.46±0.67	0.98
	500	57.12±4.15	7.20
	2000	56.02±5.05	9.01
SA	20	72.93±5.04	6.91
	100	70.71±0.80	1.13
	500	77.76±4.16	5.35
	5000	77.85±0.61	0.79
	8000	71.39±2.06	2.88

[a]Values are mean ± SD

Stability

The stability data of ASA and SA are reported in tables 7-8. No significant degradation of ASA and SA was observed under the conditions studied. Short-term stability of post-extracted plasma was also evaluated in autosampler for 72 h at 5 °C and no appreciable degradation was observed.

Table 7. Stability samples result for ASA in human plasma (n=6).

Stability test	QC nominal concentration (ng/mL)	Concentration found[a] (ng/mL)	Stability (%)	Precision (% CV)
Autosampler stability (at 5°C for 72 h)	60	51.01±2.35	85.02	4.62
	1250	1135.52±60.31	90.84	5.31
Short-term stability (in ice-bath for 6 h)	60	53.29±4.79	88.82	8.99
	1250	1247.33±106.80	99.79	8.56
Long-term stability (at -20°C for 60 day)	60	51.69±1.29	86.14	2.49
	1250	1258.46±94.44	100.68	7.50
Freeze-thaw stability (after 3 cycles)	60	50.12±0.67	83.53	1.35
	1250	1109.55±97.67	88.76	8.80

[a]Values are mean±SD

Table 8. Stability samples result for SA in human plasma (n=6).

Stability test	QC nominal concentration (ng/mL)	Concentration found[a] (ng/mL)	Stability (%)	Precision (% CV)
Autosampler stability (at 5°C for 72 h)	60	68.86±0.05	114.76	0.07
	200	225.23±0.29	112.62	0.13
	5000	5712.59±49.89	114.25	0.87
Short-term stability (in ice-bath for 6 h)	60	68.93±1.89	114.89	2.75
	200	198.50±6.95	99.25	3.50
	5000	5058.62±235.59	101.17	4.66
Long-term stability (at -20°C for 60 day)	60	68.51±3.16	114.18	4.61
	200	223.23±4.31	111.61	1.93
	5000	5747.83±46.21	114.96	0.80
Freeze-thaw stability (after 3 cycles)	60	68.88±2.81	114.81	4.09
	200	225.70±2.91	112.85	1.29
	5000	5743.87±35.66	114.88	0.62

[a]Values are mean±SD

4.4 Determination of esterase activity in ET-PR, ET-R and HS

In order to quantify SA formed after blood or plasma incubation, a specific SA calibration curve was constructed in PBS 1x buffer in the range of 0.1 µM-0.2 mM (corresponding to 0.004-1.6 nmol). The curve was linear over the concentration range of interest (R^2= 0.9992).

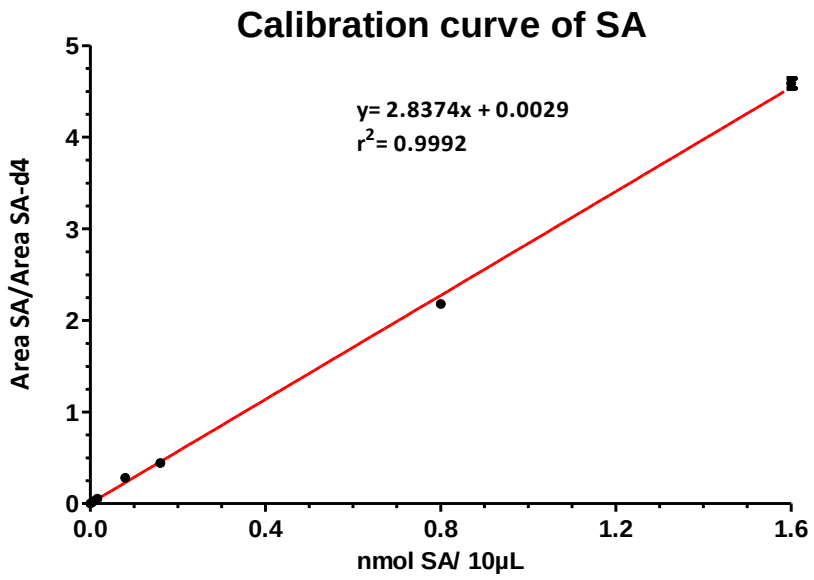


Figure 20. *Calibration curve of SA in PBS 1x.*

4.4.1 Plasma esterase activity *vs* time

Plasma esterase activity as function of time was studied in plasma samples, collected in this study and plasma samples collected in a previous study. Then we tested activity in 12 ET-PR, 29 ET-R and 31 HS. Maximal plasma esterase activity was observed after 120 min of incubation; the activity remained stable for additional 60 min, then declined. The results were expressed by subtracting the non-enzymatic contribution to ASA hydrolysis (spontaneous hydrolysis) observed in buffer samples to which the same amount of ASA had been added (figure 21).

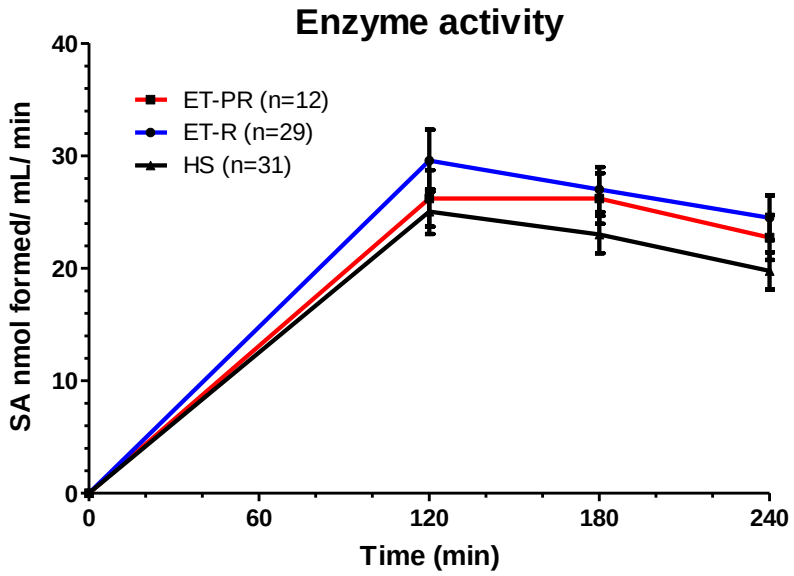


***Figure 21**. Plasma esterase activity as function of time.*

Using one-way analysis of variance, we compared activity among groups at each time of incubation. No significant difference was recorded among all groups (table 9).

***Table 9.** Esterase activity in HS, ET-R and ET-PR*

Enzyme activity (nmol/ mL/ min)[a]			
Subjects	t= 120 min	t= 180 min	t= 240 min
HS (n= 31)	25.04±2.00	23.00±1.66	19.77±1.65
ET-R (n= 29)	29.57±2.76	27.01±2.00	24.49±2.00
ET-PR (n= 12)	26.21±3.70	26.20±3.33	22.74±2.75
P value	*0.3987*	*0.3011*	*0.1863*

[a]Results are expressed as mean ± SEM

4.4.2 Esterase activity *vs* substrate concentration

Esterase activity as function of substrate concentration was studied both in whole blood and in plasma of 5 ET-PR, 6 ET-R and 6 HS. The results were expressed by subtracting the spontaneous hydrolysis (figure 22).

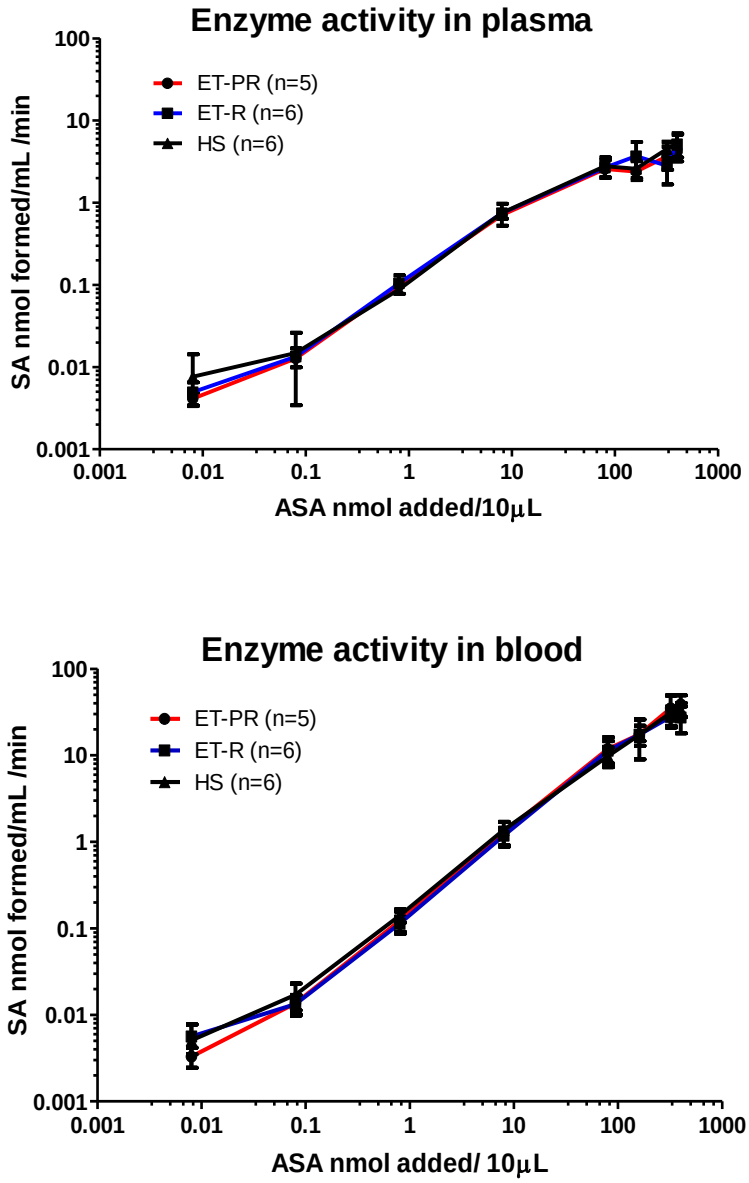


Figure 22. *Esterase activity as function of substrate concentration in plasma (above) and blood (below).*

Different substrate (ASA) concentrations (from 0.2 µM to 10 mM) were added (V= 40 µL) in plasma and in blood corresponding to 0.008 - 400 nmol added. Using Michaelis-Menten model V_{max} and K_M were calculated for each groups and means were compared. As we can see from tables 10 and 11 enzyme activity was similar in all study groups. Then our second hypothesis was rejected: ASA poor responsiveness in ET patients is not due to higher plasma and blood esterase activity.

Table 10. *V_{max} and K_M of plasma esterases.*

Subjects	**V_{max} [a] (nmol/ mL/ min)**	**K_M [a] (nmol/10 µL)**
HS (n= 6)	6.54±1.87	130.5±79.31
ET-R (n= 7)	6.02±1.39	163.0±108.7
ET-PR (n= 5)	5.80±2.19	147.5±64.45
P value	*0.9582*	*0.9695*

[a]Results are expressed as mean ±SEM

Table 11. *V_{max} and K_M of whole blood esterases.*

Subjects	**V_{max} [a] (nmol/ mL/ min)**	**K_M [a] (nmol/10 µL)**
HS (n= 6)	108.1±20.78	803.20±170.70
ET-R (n= 6)	80.88±17.91	620.30±185.10
ET-PR (n= 5)	76.02±14.05	425.30±141.10
P value	*0.4266*	*0.3340*

[a]Results are expressed as mean ±SEM

4.5 *In vivo* pharmacokinetics and pharmacodynamics of EC-ASA

In order to study PK and PD of EC–ASA, blood samples were collected in the morning after 24 h from the last EC-ASA dose and at 2, 3, 4, 5, 6 and 8 hours after the morning dose intake. We measured ASA and SA levels in plasma using the previously described validated method. At the same time points we measured serum TxB_2 levels (ELISA).

4.5.1 Pharmacokinetics and pharmacodynamics in HS

As we can see from figure 23, PK in HS showed a great variability. ASA reached maximal plasma concentration between 3 and 6 hours after intake with a median C_{max} (25%-75% CI) of 464.5 (412.1-678.9) ng/mL and a median AUC (25%-75% CI) of 701.8 (604.2-881.2) ng h/mL. As reported in literature, we found that ASA half-life is between 15-20 min, in fact ASA is rapidly converted to SA (figure 23). When ASA reaches the plasma compartment, the presence of SA is almost simultaneous. SA median C_{max} (25%-75% CI) and median AUC (25%-75% CI) were 2903 (1647-4326) ng/mL and 10547 (5410-14541) ng h/mL respectively. Two healthy subjects did not show any absorption within the 8 h observation period: in particular, in one subject (82K) we did not observe absorption at all within the 8 h observation period, and in one subject (57K) we observed a minimal ASA absorption confirmed by SA trend. PK of 82K subject was repeated (82K bis) and did not confirm this behaviour, showing in the second administration the appearance of ASA and SA plasma peak.

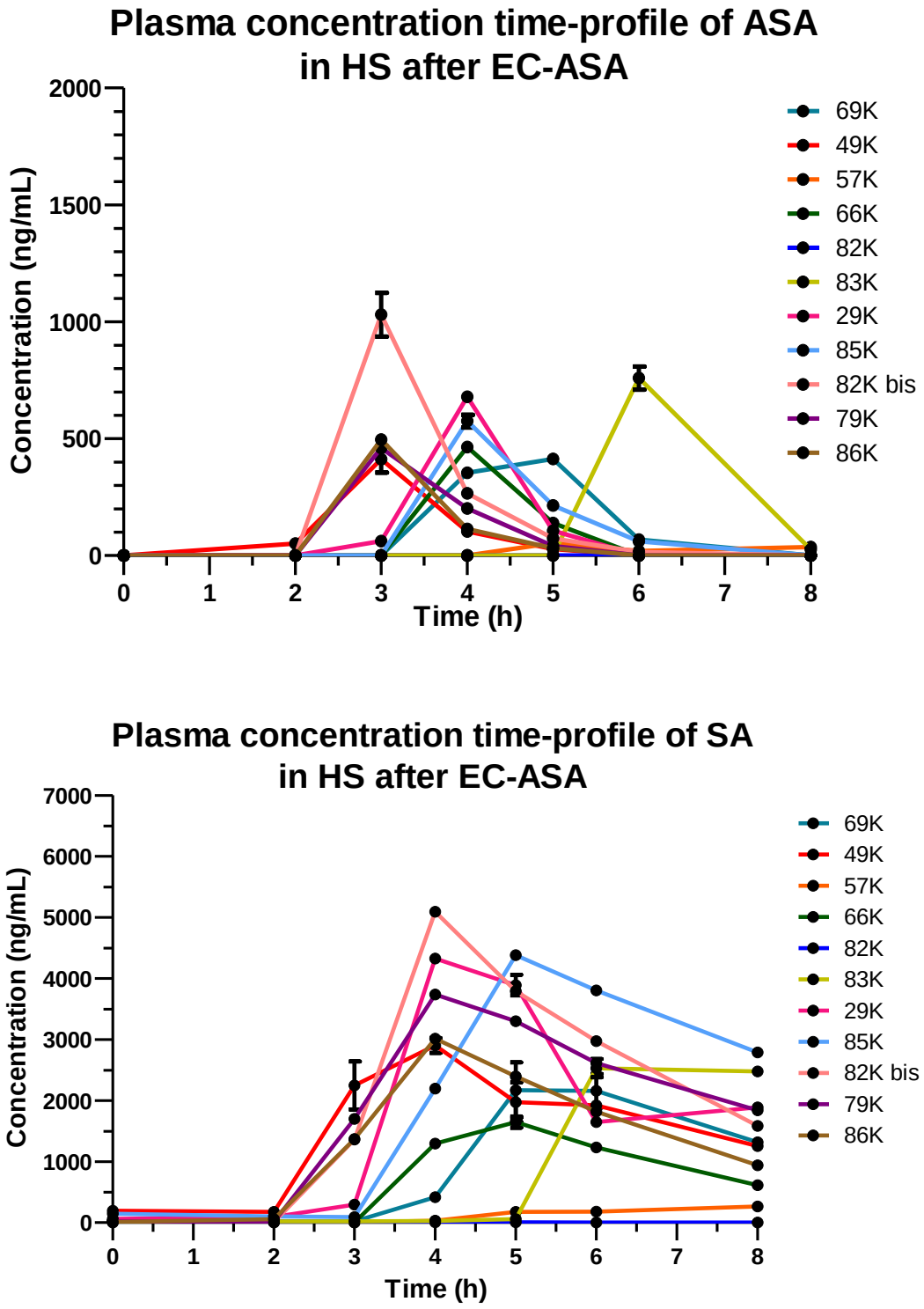


Figure 23. *Plasma concentration time-profiles of ASA (above) and SA (below) after EC-ASA in HS.*

Serum TxB_2 levels were low in all HS, also in the two subjects who showed impaired absorption (figure 24).

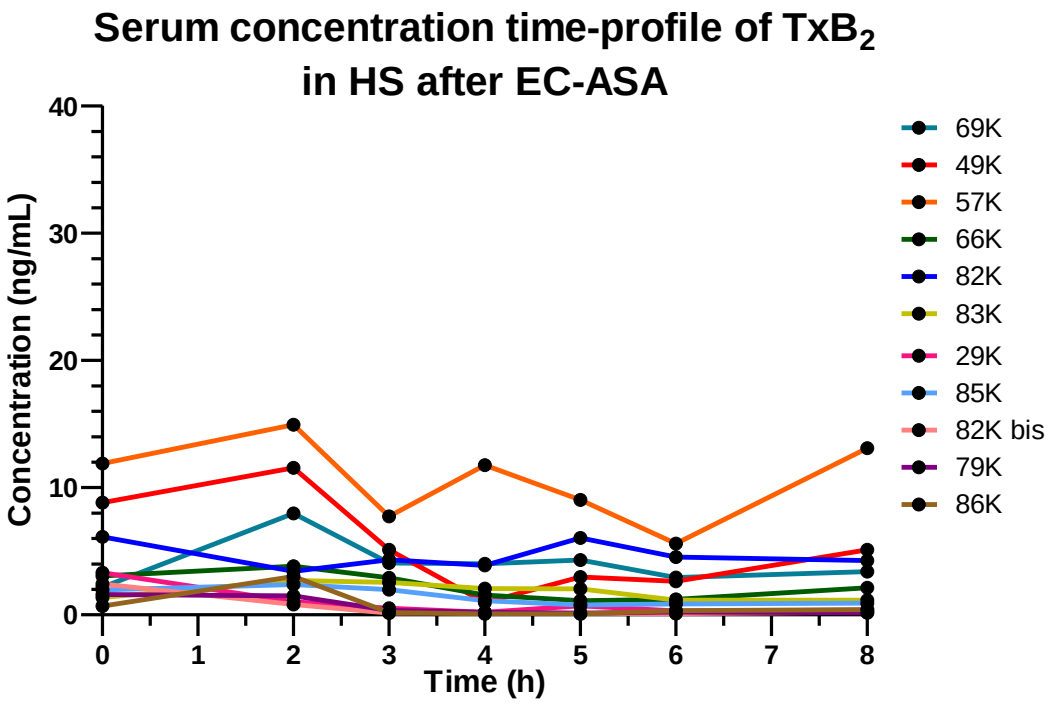


***Figure 24**. Serum concentration time-profiles of TxB_2 after EC-ASA in HS.*

4.5.2 Pharmacokinetics and pharmacodynamics in ET-R patients

All ET-R patients absorbed the drug between 2-8 hours after intake with ASA median C_{max} (25%-75% CI) of 429.2 (369.5-794.8) ng/mL and ASA median AUC of 856.2 (751.3-1162) ng h/mL. For one patient (3K) we missed the ASA plasma peak, which probably occurred at 7 h (time point no collected), but the presence of SA peak confirmed drug absorption (figure 25). SA median C_{max} (25%-75% CI) and SA median AUC (25%-75% CI) were 3472 (2927-3789) ng/mL and 8200 (5184-12544) ng h/mL respectively.

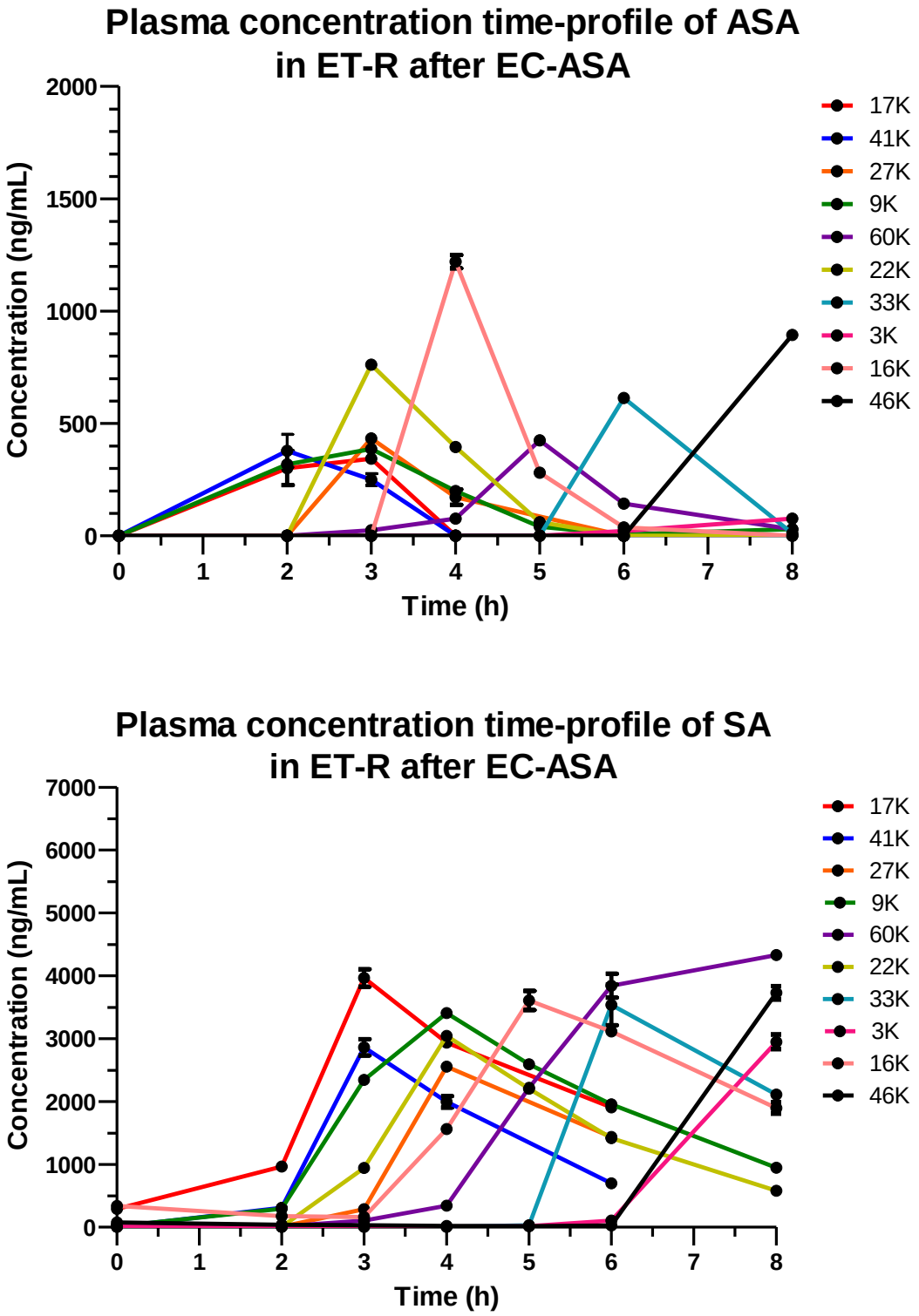


Figure 25. *Plasma concentration time-profiles of ASA (above) and SA (below) after EC-ASA in ET-R patients.*

Serum TxB_2 concentration at 24 h were lower than 10 ng/mL in all subjects but two (9K and 17K) (figure 26).

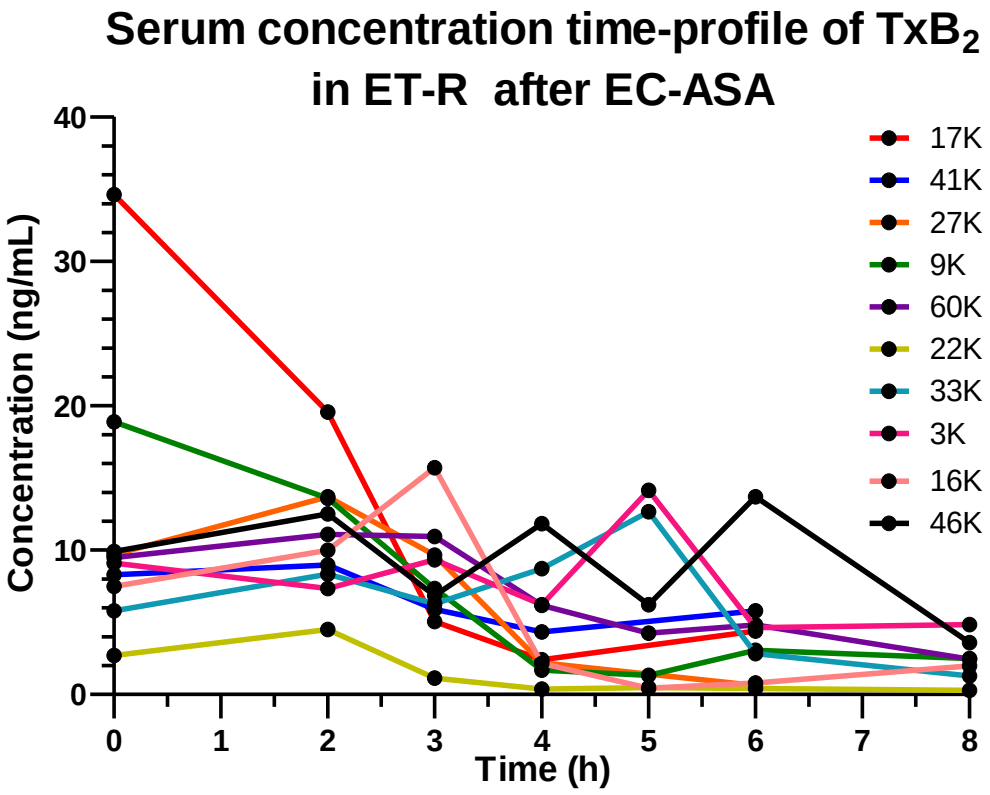


Figure 26. *Serum concentration time-profiles of TxB_2 after EC-ASA in ET-R patients.*

4.5.3 Pharmacokinetics and pharmacodynamics in ET-PR patients

Half of ET-PR patients absorbed between 3-8 hours after intake and half did not show any absorption within 8 hours, confirmed also by SA levels. Pharmacokinetics of 58K and 54K was repeated and 58K bis confirmed no absorption, while 54k bis showed ASA and SA plasma peak (figure 27). ASA median C_{max} (CI 25-75%) and ASA median AUC (CI 25%-75%) were 0 (0-414.3) ng/mL and 0 (0-644.1) ng h/mL; SA median C_{max} (CI 25-75%) and SA median AUC (CI 25%-75%) were 21.72 (0-2622) ng/mL and 89.56 (27.08-4054) ng h/mL.

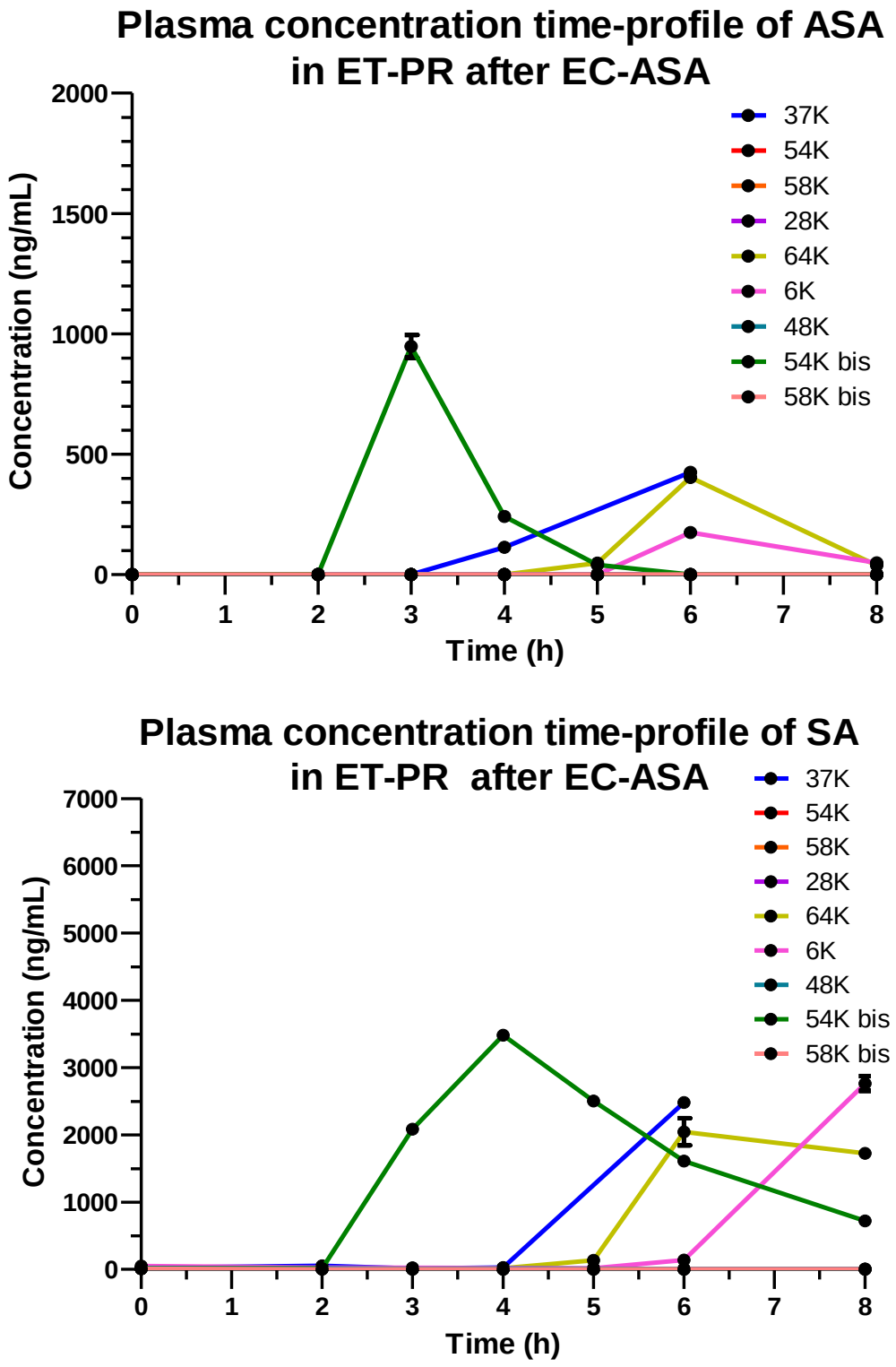


Figure 27 Plasma concentration time-profiles of ASA (above) and SA (below) after EC-ASA in ET-PR patients.

At 24 h, serum TxB_2 levels were >10 ng/mL in all patients: they declined in patients who absorbed ASA, but remained high in those who did not (54K, 58K, 28K, 48K and 58K bis) (figure 28).

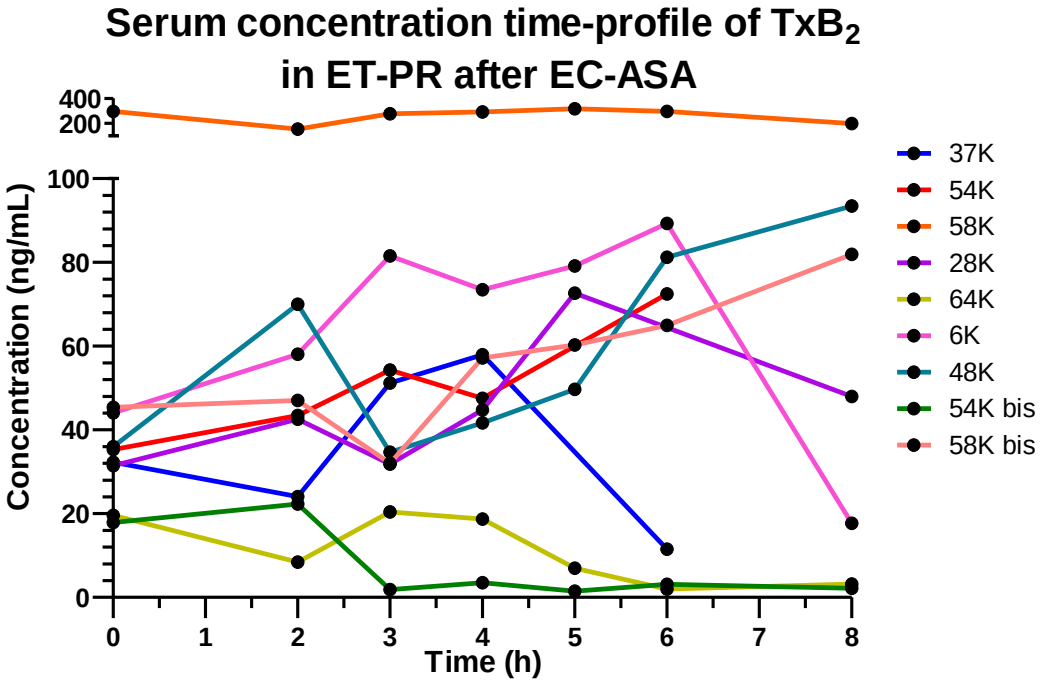


Figure 28. *Serum concentration time-profiles of TxB_2 after EC-ASA in ET-PR patients.*

4.6 In vivo pharmacokinetics and pharmacodynamics of plain ASA

In these same three groups, we studied also PK and PD of plain ASA, a non-gastro resistant formulation. In this case, blood samples were collected in the morning at 24h after the last plain ASA dose and at 0.5, 1, 1.5, 2, 3, 4, 5 and 6 hours after morning dose intake and at each time points we measured plasma levels of ASA and SA and serum TxB_2 (ELISA).

4.6.1 Pharmacokinetics and pharmacodynamics in HS

Drug absorption occurred in all HS (n=9) between 0.5-2 h after intake, also confirmed by SA plasma peak (figure 29). ASA median C_{max} (CI 25%-75%) and ASA median AUC (CI 25%-75%) were 575.8 (427.0-1125) ng/mL and 822.9 (635.4-1013) ng h/mL; SA median C_{max} (CI 25%-75%) and SA median AUC (CI 25%-75%) were 3055 (2074-4806) ng/mL and 9600 (7987-14808) ng h/mL. Serum TxB_2 levels were low in all HS (figure 30).

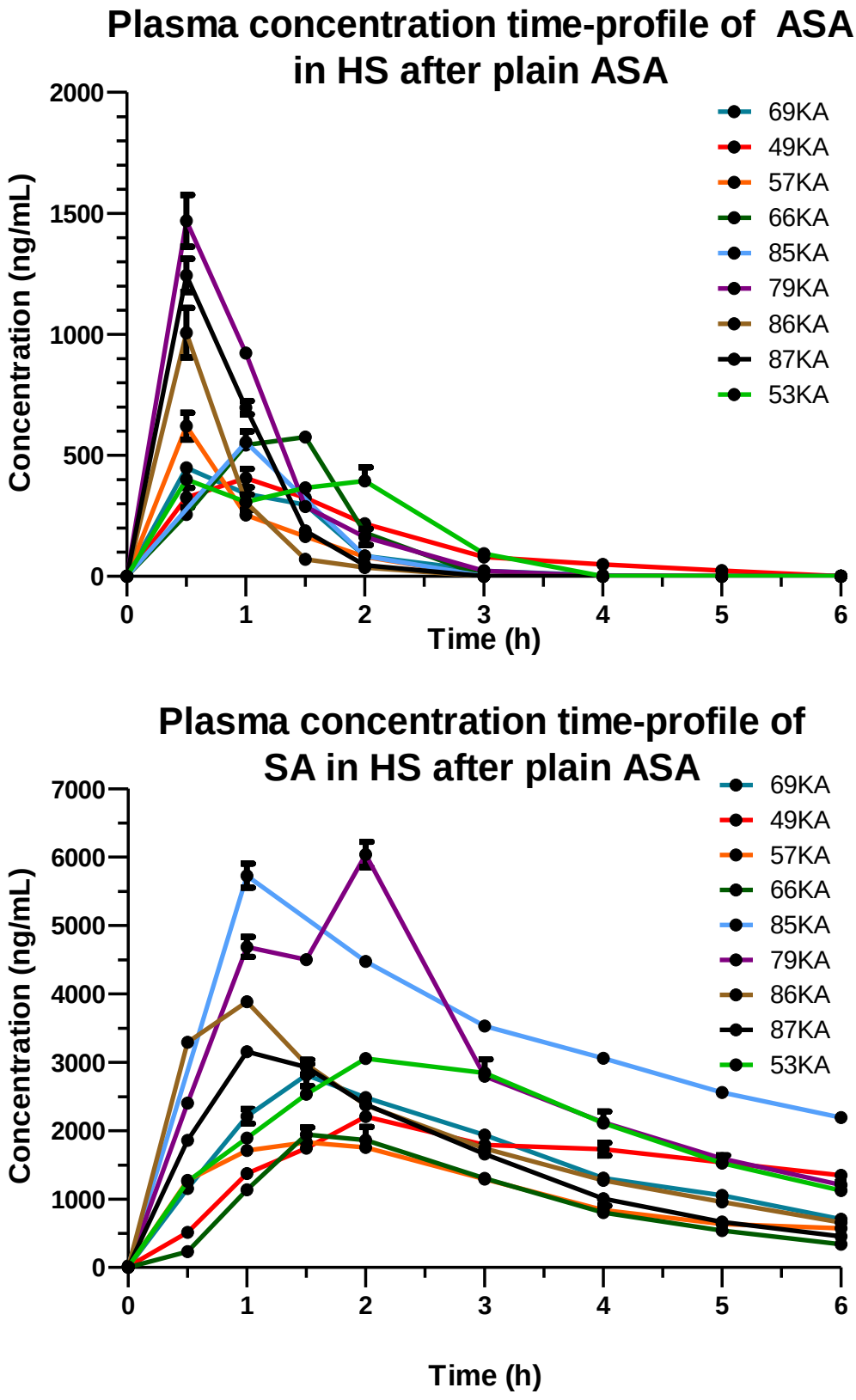


Figure 29. *Plasma concentration time-profiles of ASA (above) and SA (below) after plain ASA in HS.*

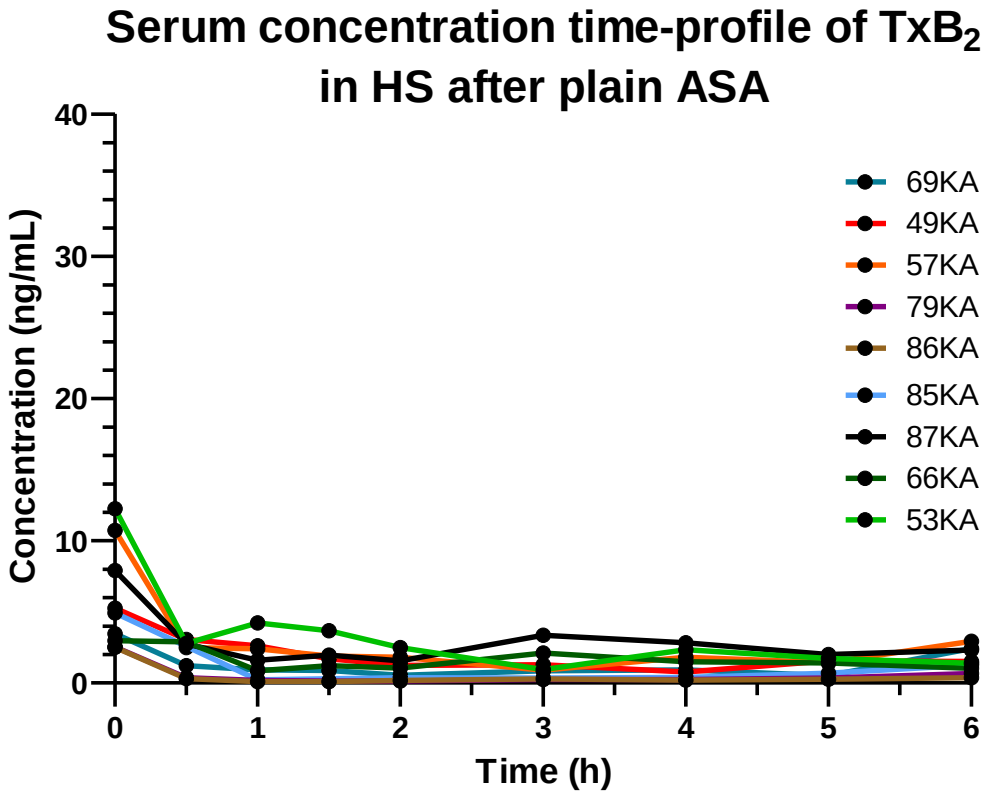


Figure 30. *Serum concentration time-profiles of TxB_2 after plain ASA in HS.*

4.6.2 Pharmacokinetics and pharmacodynamics in ET-R patients

As we can see from figure 31, drug absorption (t_{max}= 0.5-2 h) in ET-R patients was similar to HS (figure 29). ASA median C_{max} (CI 25%-75%) and ASA median AUC (CI 25%-75%) were 738.7 (598.1-1177) ng/mL and 836.5 (733.6-1292) ng h/mL; SA median C_{max} (CI 25%-75%) and SA median AUC (CI 25%-75%) were 2881 (2633-3704) ng/mL and 8903 (8057-12747) ng h/mL.

Serum TxB_2 levels were low in all ET-R patients after drug absorption, but in 4 patients (17KA, 41KA, 9KA and 16KA) at 24 h TxB_2 increased (figure 32).

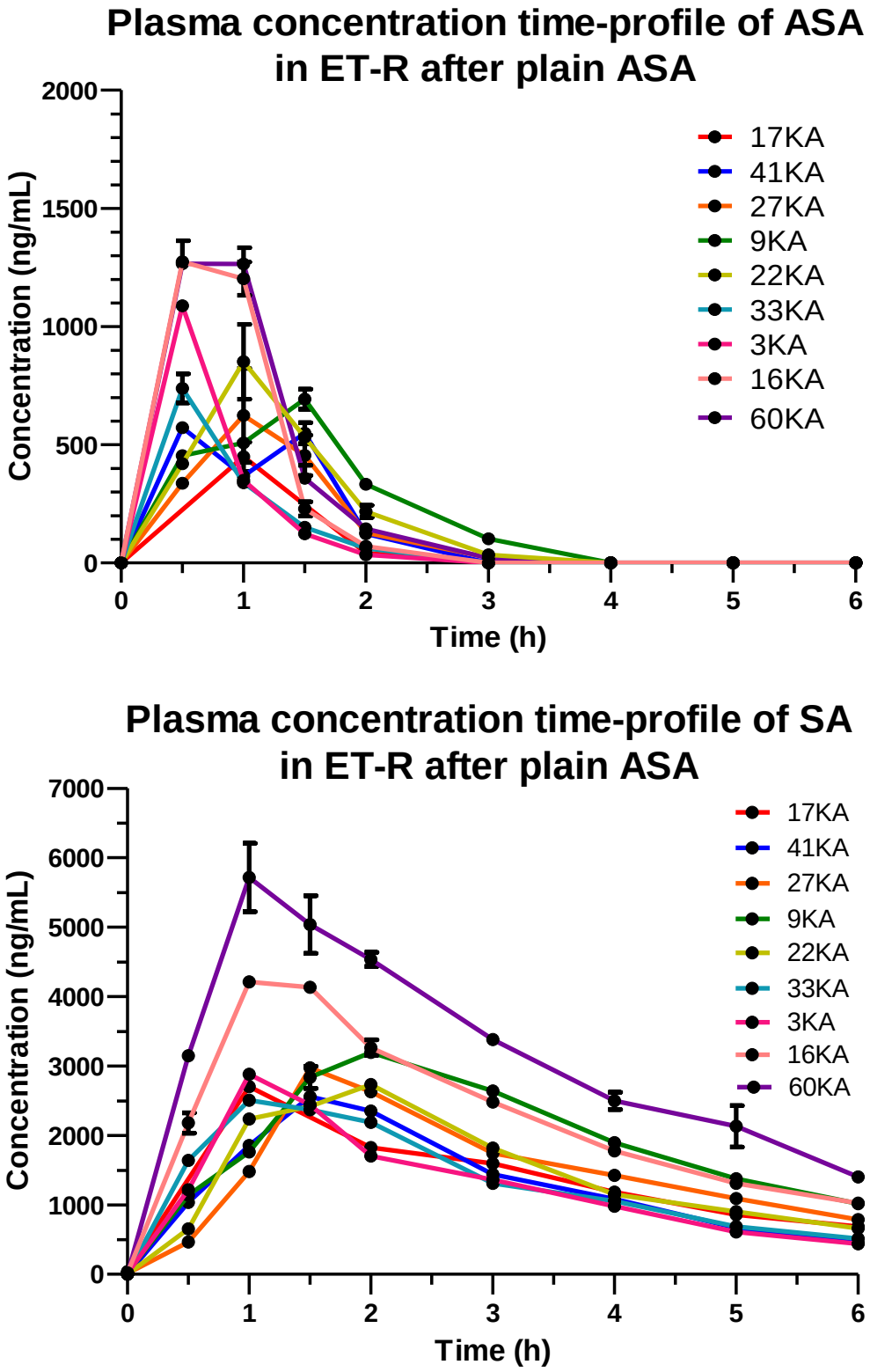


Figure 31. *Plasma concentration time-profiles of ASA (above) and SA (below) after plain ASA in ET-R patients.*

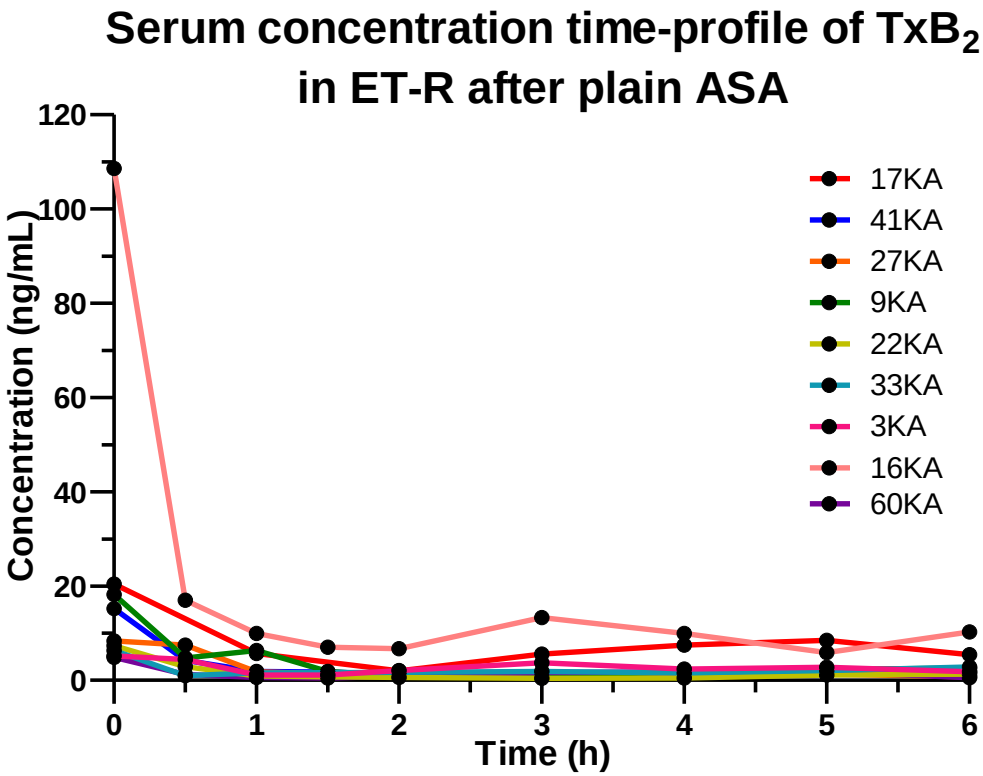


Figure 32. *Serum concentration time-profiles of TxB_2 after plain ASA in ET-R.*

4.6.3 Pharmacokinetics and pharmacodynamics in ET-PR patients

Also all ET-PR patients showed ASA and SA plasma peaks after plain ASA intake. Drug absorption occurred between 0.5 and 2 h after intake except in one subject (6KA) who reached plasma concentration peak at 5h (figure 33). ASA median C_{max} (CI 25%-75%) and ASA median AUC (CI 25%-75%) were 842.7 (306.4-1021) ng/mL and 872.8 (703.0-1103) ng h/mL; SA median C_{max} (CI 25%-75%) and SA median AUC (CI 25%-75%) were 3277 (2485-3626) ng/mL and 9432 (7429-10977) ng h/mL.

Serum TxB_2 levels were low in all ET-PR (n=7) patients after drug absorption, but at 24 h concentrations tended to increase in all patients (figure 34).

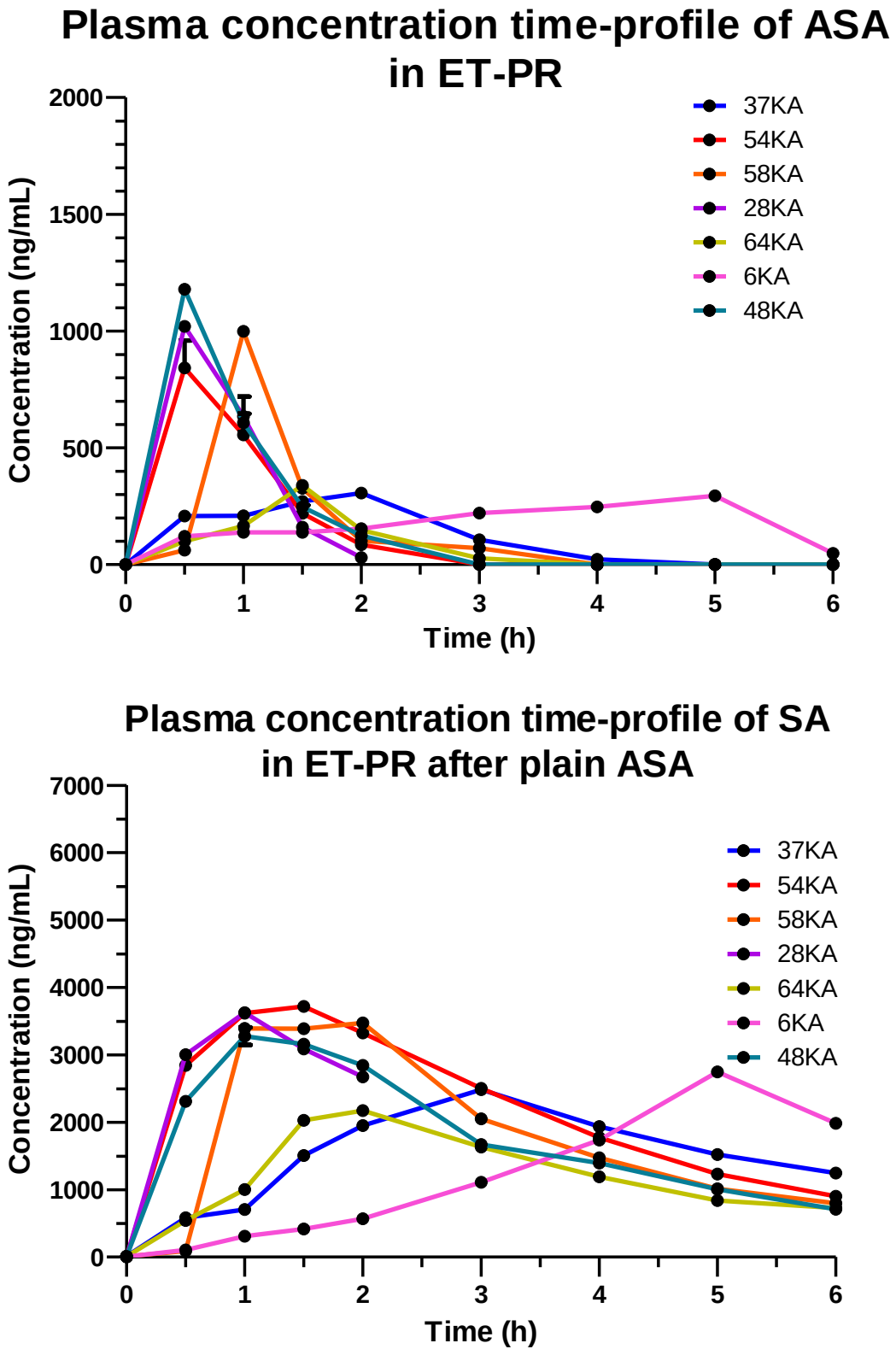


Figure 33. *Plasma concentration time-profiles of ASA (above) and SA (below) after plain ASA in ET-PR patients.*

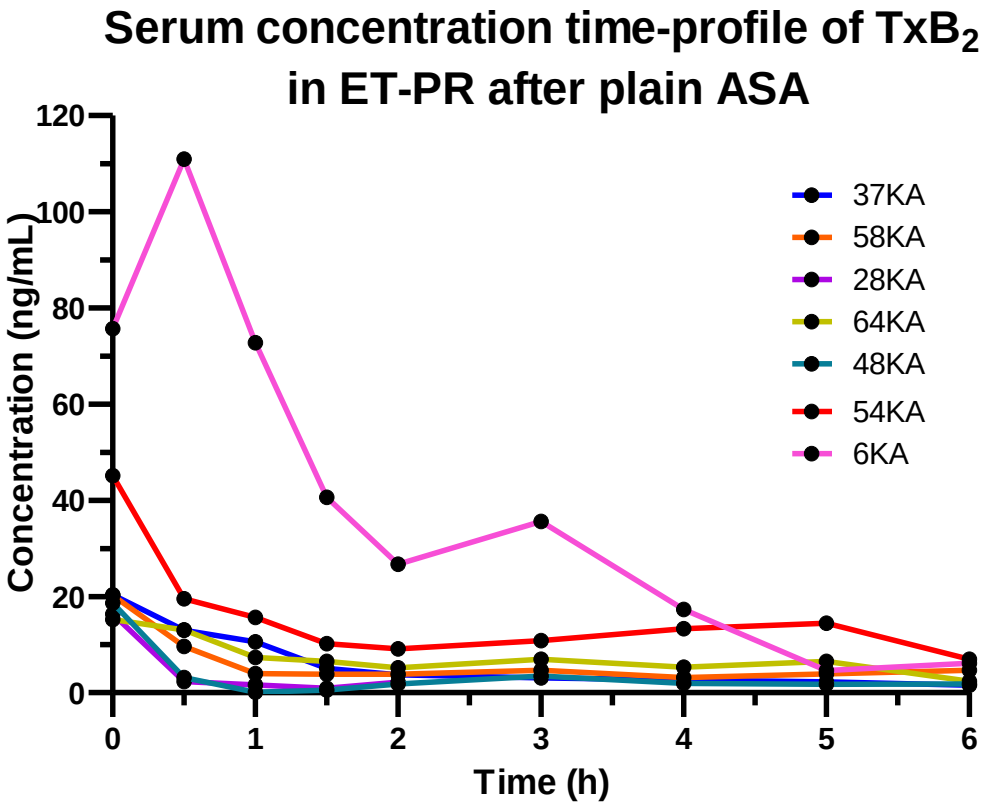


Figure 34. *Serum concentration time-profiles of TxB2 after plain ASA in ET-PR.*

4.7 Comparison between EC-ASA and plain ASA

As reported by the Italian Medicines Agency (AIFA): after oral administration, ASA is rapidly and completely absorbed from the gastro-intestinal tract. During and after absorption, ASA is converted to its main metabolite: SA. Plain ASA is rapidly absorbed in the stomach, while the enteric-coating of EC-ASA is resistant to acid environment, so ASA is not released into the stomach but in the alkaline environment of the intestine [30]. Observing our results, reported also considering the repeated pharmacokinetics (82K bis, 58K bis and 54K bis), it appears clear that in patients on treatment with EC-ASA, ASA and SA reached maximum plasma concentrations 3-8 hours after intake in all groups (figure 35). Plain ASA is rapidly absorbed in all subjects, with t_{max} between 0.5 and 2 h and SA t_{max} between 1 and 3 h after intake (1 ET-PR had ASA t_{max} at 5 h after intake, confirmed by SA trend, figure 33). By comparing ASA and SA C_{max} (figure 36), we noticed that if the drug is absorbed ASA and SA plasma levels were similar for all subjects independently of the drug formulation used. Then EC-ASA absorption problems seem to be related to enteric coating which probably didn't dissolve in the basic environment of intestine, consequently the active ingredient may not be released. This fact occurred more frequently in ET-PR patients but we observed it also in 2 HS.

In literature a recent study about PK of EC-ASA [33] in healthy subjects (n=24) treated for 7 days with 100 mg daily, showed ASA C_{max} (mean±SD) of 615±267 and t_{max} (mean±SD) of 4.33±1.23 and SA C_{max} (mean±SD) of 4706±2069 and t_{max} (mean±SD) of 5.50±1.32. Another study about plain ASA formulation [32] reported PK parameters after 100 mg of plain ASA single-dose: ASA C_{max} (geometric mean; %CV) of 1.01 (33.2)

mg/L (corresponding to 1001 ng/mL) and ASA t_{max} (median) of 0.5 h. Our results, obtained in healthy subjects, are comparable to those reported in literature.

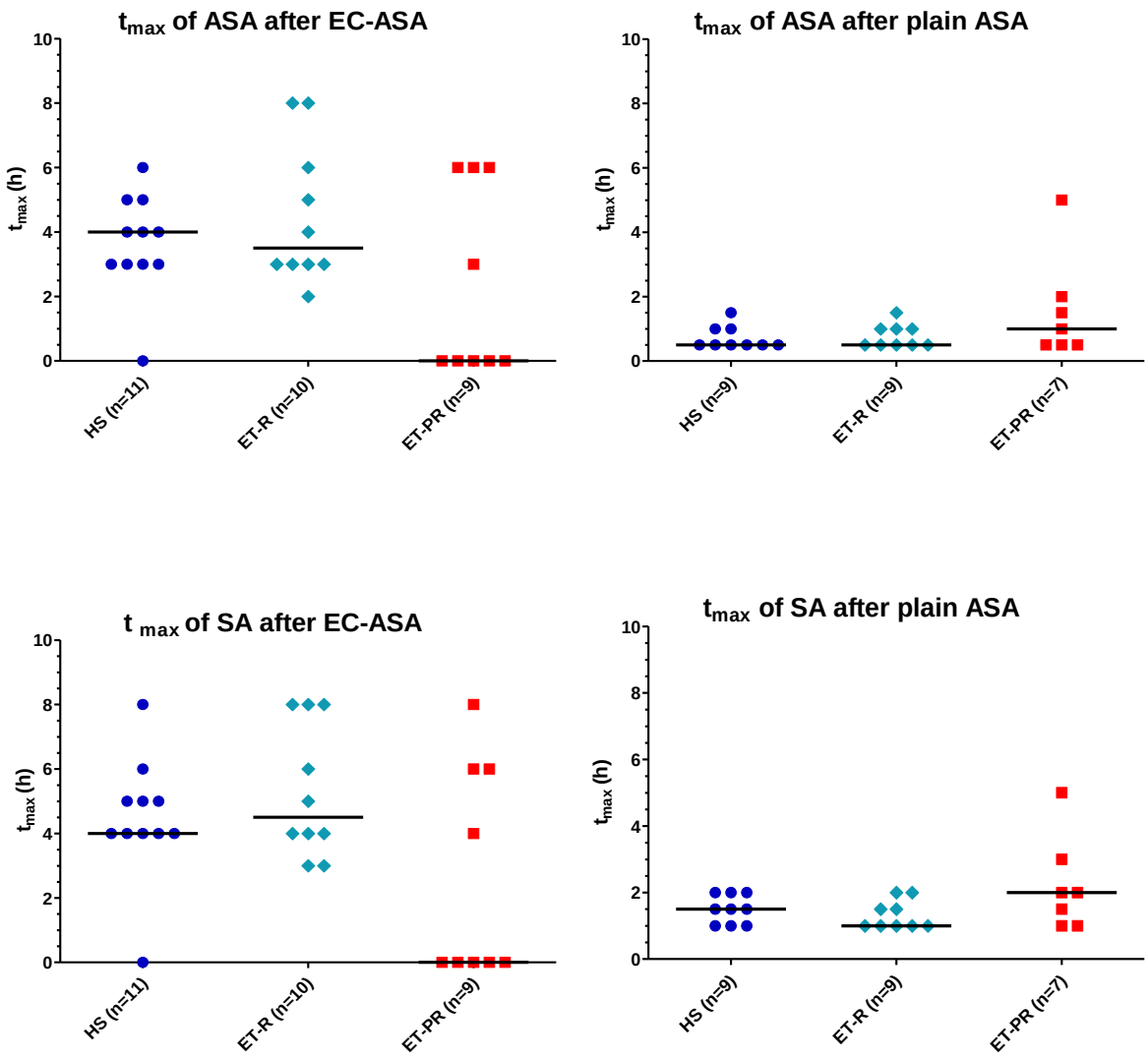


Figure 35. t_{max} of ASA (above) and SA (below) in HS, ET-R and ET-PR after EC-ASA (left) and plain ASA (right).

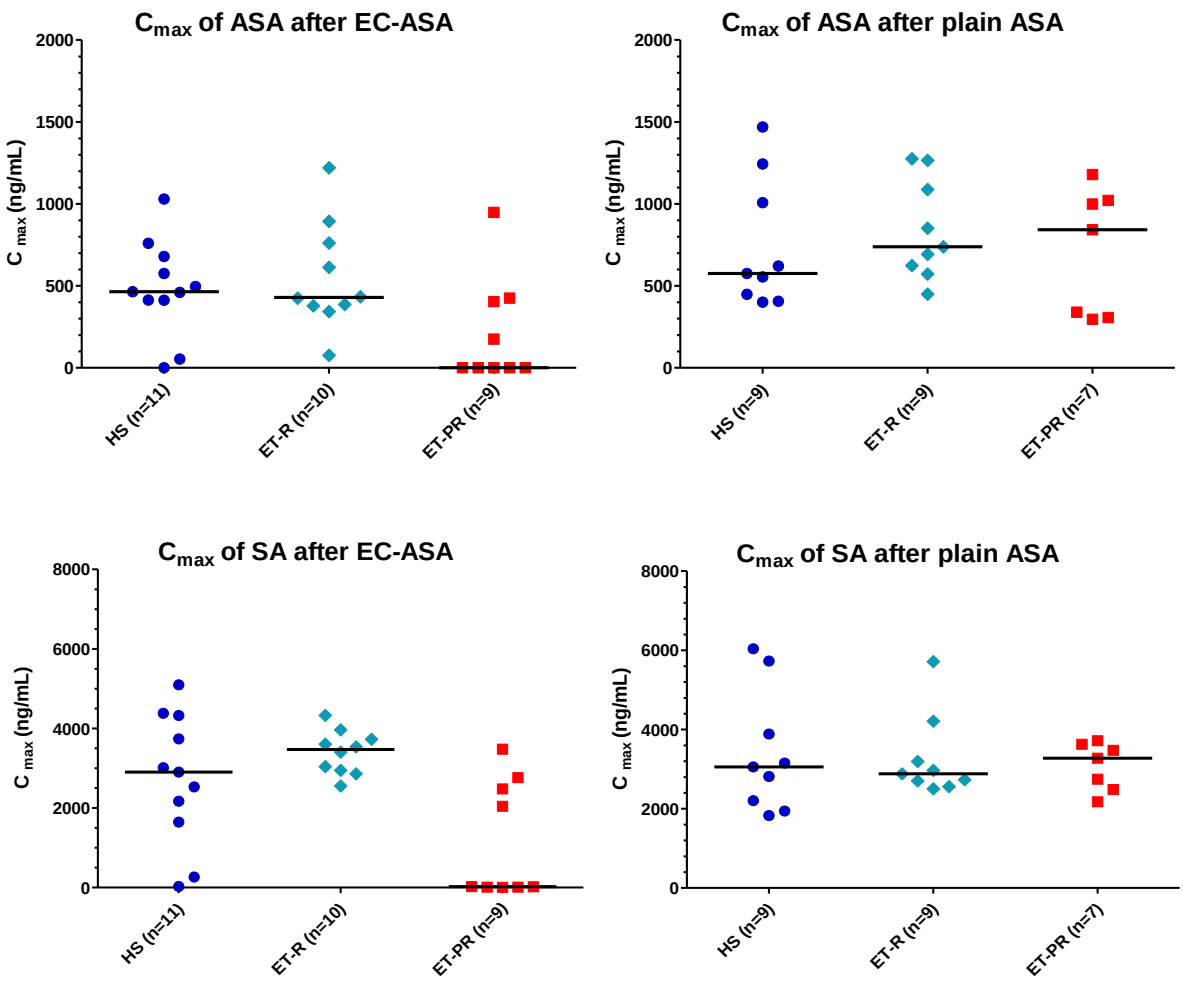


Figure 36. *C_{max} of ASA (above) and SA (below) in HS, ET-R and ET-PR after EC-ASA (left) and plain ASA (right).*

Serum TxB_2 at 24 h was higher in ET patients than in HS. As shown in table 12, PK of EC-ASA was statistically significant different between three groups: in particular, we compared mean of ASA AUC, SA AUC and serum TxB_2 levels (24h and 6h). Plain ASA pharmacokinetic didn't show any differences among groups (table 13).

Table 12. *AUC of ASA and SA in human plasma of HS (n=11), ET-R (n=10) and ET-PR (n=9) after EC-ASA intake. Serum TxB_2 levels at 24 and 6 h after EC-ASA intake.*

Subjects	AUC (ng h/mL)		Serum TxB_2 (ng/mL)	
	ASA[a]	SA[a]	24 h[a]	6h[a]
HS (n=11)	701.8 (604.2-881.2)	10550 (5410-14540)	2.42 (1.60-6.14)	1.16 (0.31-2.93)
ET-R (n=10)	856.2 (751.3-1162)	8200 (5184-12540)	9.28 (7.07-12.14)	3.73 (0.75-5.06)
ET-PR (n=9)	0 (0-644.1)	89.56 (27.08-4054)	35.29 (25.52-44.75)	68.71 (5.20-87.29)
P value*	0.0146	0.0053	0.0288	0.0048

[a]Results are expressed as median (CI 25-75%); *P<0.05

Table 13. *AUC of ASA and SA in human plasma of HS (n=9), ET-R (n=9) and ET-PR (n=7) after plain ASA intake. Serum TxB_2 levels at 24 and 6 h after plain ASA intake.*

Subjects	AUC (ng h/mL)		Serum TxB_2 (ng/mL)	
	ASA[a]	SA[a]	24 h[a]	6h[a]
HS (n=9)	822.9 (635.4-1013)	9600 (7987-14810)	4.93 (2.76-9.32)	1.38 (0.85-2.37)
ET-R (n=9)	836.5 (733.6-1292)	8903 (8057-12750)	8.37 (5.76-19.32)	1.84 (1.04-4.13)
ET-PR (n=7)	872.8 (703-1103)	9432 (7429-10980)	20.24 (16.38-45.11)	3.54 (1.75-6.31)
P value*	0.5987	0.6073	0.1239	0.1454

[a]Results are expressed as median (CI 25-75%); *P<0.05

4.8 Reticulated platelets

As mentioned before, TxB_2 at 24 h tended to be higher in ET patients even when plain ASA was used and drug absorption occurred (figures 32, 34). We hypothesized that this is due to the higher number of newly-formed reticulated platelets in ET patients. Our results showed that the percentage of reticulated platelets is similar in all groups while reticulated platelet count is higher in ET patients compared to healthy subjects due to elevated platelet count in ET patients (table 14). To test our hypothesis, we calculated the correlation between the number of reticulated platelets with the difference between serum TXB_2 at 24h and the lowest serum TxB_2 level after the morning dose of ASA. Indeed, we found a strong correlation between these two parameters (R= 0.6107; P=0.0020) (figure 37).

Table 14. *Percentage of reticulated platelets and reticulated platelets count in HS (n=8), ET-R (n=8) and ET-PR (n=7).*

Subjects	Percentage of reticulated platelet[a] (%)	Reticulated platelet count[a] (µL)
HS (n=8)	11.15±1.51	21083±1593
ET-R (n=8)	10.12±1.79	30991±5468
ET-PR (n=7)	9.32±0.92	47566±8103
P value*	0.8128	0.0105

[a]Mean ±SEM; *P<0,05

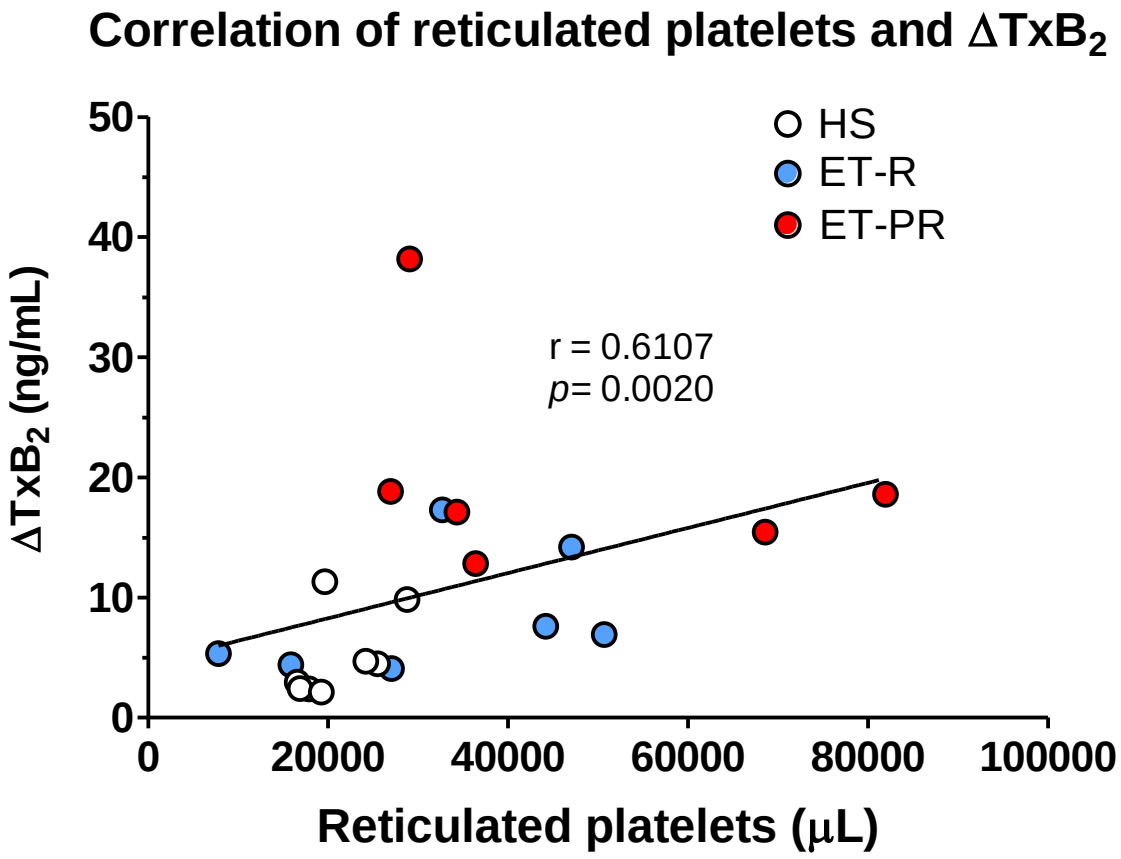


Figure 37. *Correlation between ΔTxB_2 and reticulated platelet count.*

4.9 Twice daily Aspirin

As shown before, serum TxB_2 level tend to be higher at 24h in ET patients regardless of the formulation administered. ET patients with serum TxB_2 level at 24h higher than 10 ng/mL were asked to take for one day a double dose of plain ASA at 12 h distance. Blood samples were collected at 12 h after the second dose and serum TxB_2 was measured as previously described.

As shown in figure 38, twice daily dose of plain ASA was able to reduced TxB_2 levels in those patients who showed high TxB_2 levels at 24 h.

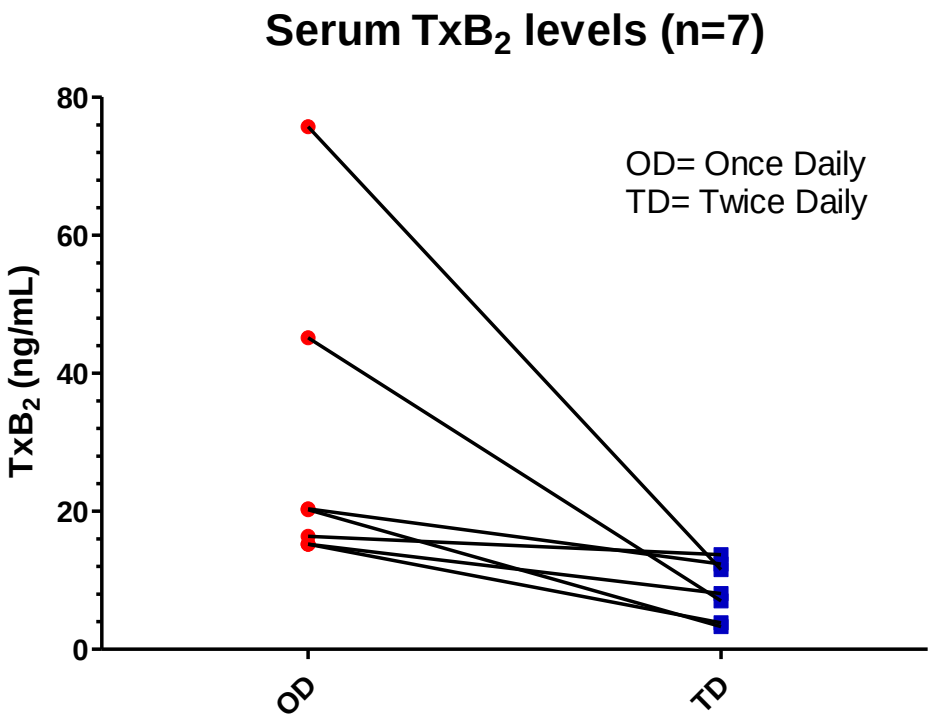


Figure 38. *Serum TxB_2 levels at 24h after once daily plain ASA and at 12h after twice daily plain ASA in ET patients.*

5 CONCLUSIONS

This study investigated the potential causes of poor response to ASA in some ET patients. First of all it was supposed a lack of PD effect in ET-PR patients previously classified on the basis of TxB_2 levels > 10 ng/mL [64]. This hypothesis was excluded because the *in vitro* addition of ASA (100 µM) to WB or PRP of ET-PR patients inhibits TxB_2-production induced by collagen (5 µg/mL) in the same manner as in HS.

A recent study [78] reported that the residual TxB_2 production in ET patients was completely suppressed by adding ASA (50 µM) to WB *in vitro*. Our first conclusion is that PD effect of ASA in ET-PR patients is not impaired and is not responsible for the lack of ASA activity.

The second hypothesis was that, in ET-PR patients, the presence of a stronger esterase activity could reduce the amount of ASA available to acetylate COX-1. This hypothesis was investigated by studying esterase activity as function of time and of substrate. We demonstrated that the activity of the enzyme was similar in all groups, thus excluding also this cause of poor response to ASA treatment in ET patients.

Finally, we studied the PK of EC-ASA (commonly prescribed) vs the PK of plain ASA.

EC-ASA absorption showed high variability in controls and patients. Two HS patients displayed no ASA absorption within the observation period, but their serum TxB_2 levels were low. We supposed a delayed absorption out of the observation period. Fifty percent of ET-PR patients displayed no absorption of ASA within 8 hours and their serum TxB_2 levels were high. This suggest that in these patients the cause of inadequate response to ASA treatment is an inadequate intestinal absorption of the drug. When plain ASA was given instead of EC-ASA, ASA and SA plasma peaks were

detectable and similar in all groups. Serum TxB_2 levels after plain ASA were similar in all groups but tended to be higher in all ET patients after 24 h from administration, irrespective of the ASA formulation used. The differences between TxB_2 at 24 h and the minimum TxB_2 serum concentration (after plain ASA absorption) correlated (R= 0.6107; P=0.0020) with reticulated platelets count in HS and ET patients. *Bid* administration (100 x 2 mg) in ET patients suppressed serum TxB_2 levels.

Concluding, ASA poor response in ET was observed only with EC-ASA and is likely associated with poor drug absorption. Increased platelet production is likely responsible for high TxB_2 in ET 24 post-dosing.

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7 LIST OF ABBREVIATIONS

AA (arachidonic acid)

APC (allophycocyanin)

ARU (Aspirin reaction units)

ASA (Aspirin, acetylsalicylic acid)

ASA-d4 (acetylsalicylic acid deuterated)

AUC (area under the curve)

CALR (calreticulin)

CE (collision energy)

CEP (cell entrance potential)

C_{max} (maximum plasma concentration)

CML (chronic myeloid leukemia)

COX (cyclooxygenase)

COX-1 (cyclooxygenase-1)

COX-2 (cyclooxygenase-2)

COX-3 (cyclooxygenase-3)

CXP (cell exit potential)

DP (declustering potential)

EC-ASA (enteric coated Aspirin)

EP (entrance potential)

ET (essential thrombocythemia)

ET-PR (essential thrombocythemia poor responder)

ET-R (essential thrombocythemia responder)

FITC (fluorescein isothiocyanate)

FSC (forward-scattered light)

HC (hydroxycarbamide)

HS (healthy subject)

HU (hydroxyurea)

ID-LC-MS/MS (isotope dilution liquid chromatography tandem mass spectrometry)

IFN (interferon)

IS (internal standard)

JAK2 (Janus kinase 2)

LLOQ (lower limit of quantification**)**

LTA (light transmission aggregometry)

MPNs (myeloproliferative neoplasm)

MRM (multiple reaction mode)

NSAIDs (non-steroidal anti-inflammatory drugs)

OD (once daily)

PD (pharmacodynamics)

PFA (paraformaldehyde)

PGH2 (prostaglandin H2)

PGs (prostaglandins)

PK (pharmacokinetics)

Plain ASA (plain Aspirin)

PMF (primary myelofibrosis)

PRP (platelet rich plasma)

PV (polycythemia vera)

SA (salicylic acid)

SA-d4 (salicylic acid deuterated)

SSC (side-scattered light)

TD (twice daily)

t_{max} (time to reach maximum plasma concentration)

TO (thiazole orange)

TxA_2 (thromboxane A_2)

TxB_2 (thromboxane B_2)

WB (whole blood)

WHO (world healthy organization)

XIC (extracted ion chromatogram)

8 LIST OF FIGURES

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10 SCIENTIFIC PRODUCTS

Oral presentation:

1. **Valutazione della risposta farmacologica all'Aspirina nei pazienti con Trombocitemia Essenziale.** XIX Riunione del Gruppo di Studio delle Piastrine Ostuni (BR), 7-9 ottobre 2018.

2. **Valutazione della risposta farmacologica all'Aspirina nei pazienti con Trombocitemia Essenziale.** XXV Congresso Nazionale Siset, Firenze 7-10 novembre 2018.

3. **Evaluation of aspirin responsiveness in healthy subjects and essential thrombocythemia patients.** Terza riunione biochimici dell'area Milanese, Gargnano (BS) 25-27 giugno 2017.

4. **Evaluation of aspirin responsiveness in healthy subjects and essential thrombocythemia patients**. 29a Riunione Nazionale "A. Castellani" dei Dottorandi di Ricerca in Discipline Biochimiche, Brallo di Pregola (PV) 5-9 giugno 2017.

5. **Evaluation of acetylsalicylic acid pharmacokinetics in patients with Essential Thrombocythemia.** 2° Congresso Diss, Milano 11 novembre 2016

Poster:

1. Jessica Rizzo, Michele Dei Cas, Federico Maria Rubino, Rita Paroni **Pharmacokinetics and bioavailability of different acetylsalicylic acid formulations in healthy subjects assessed by Liquid Chromatography-Tandem Mass Spectrometry.** IMSC-2018 – International Mass Spectrometry Conference – Firenze (I), August 26-31, 2018.

2. Jessica Rizzo, Eti Alessandra Femia, Rita Clara Paroni, Marco Cattaneo**. Evaluation of pharmacokynetics of acetylsalicylic acid using liquid chromatography-tandem mass spectrometry (LC/MS/MS). 1**° Congresso Diss, Milano 13 novembre 2015.

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