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Pharmacodynamics and Pharmacokinetics of Aspirin in Pediatric Patients

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Abstract

Objective: To investigate pharmacodynamic and pharmacokinetic variability of aspirin in children and adolescents on antithrombotic prophylaxis for cardiac diseases.

Methods: Twenty-nine patients, aged between 6 months and 18 years, on stable Aspirin (ASA) treatment for at least one week were the study population. At 4, 5 and 6 hours after drug administration blood samples were collected to assay plasma concentrations of ASA and Salicylic Acid (SA), and arachidonic-induced platelet aggregation (Multiplate® analyzer). Residual platelet reactivity was measured by impedance change over 6 min and expressed as arbitrary units (U). The area under the concentration-time curves of ASA (AUC_{ASA}) and SA (AUC_{SA}) from 4 h to 6 h were calculated and correlated with the corresponding platelet reactivity measure (U).

Results: Platelet reactivity, AUC_{ASA}, AUC_{SA} and AUC_{SA}/AUC_{ASA} were highly variable among subjects. According to pre-set U cut-off values (<21, 21-28, >28), 54.2% of patients were full responders, 8.3% partial responders and 37.5% poor responders. Antiplatelet effect of ASA correlated inversely with AUC_{ASA} and directly with AUC_{SA}/AUC_{ASA} ratio (as a marker of ASA deacetylation rate). No thromboembolic or bleeding complications were recorded during follow-up.

Conclusion: We posit that our results might be explained acknowledging that platelet cyclooxygenase-1 is more influential than intestinal and liver carboxylesterases in pre-systemic ASA deacetylation.

Keywords: Pharmacodynamics; Pediatric patients; ASA

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Introduction

Despite a growing interest in 'aspirin resistance' in the medical literature, there is no consensus on its definition, prevalence and clinical burden [1]. Furthermore, while some studies support the prognostic relevance of aspirin resistance in adults [2,3], pediatric studies in this field are scarce [4]. The most recent position paper endorsed by the European Society of Cardiology (ESC) thrombosis working group defines two types of aspirin resistance [5]:

1. Clinical resistance: failure to prevent thrombosis;
2. Laboratory resistance, divided into:

- Pharmacokinetic resistance: Low serum levels of the drug and its metabolites due to low oral bioavailability or poor patient compliance;
- Pharmacodynamic resistance: Adequate serum levels of the drug fail to inhibit *in vivo* TxA2 production or ex vivo platelet aggregation.

The low incidence of thromboembolism in pediatric patients constitutes a limitation to conducting studies which can yield direct evidence. On the other hand, greater recourse to cardiac catheterization to treat congenital heart disease and technical advancement both in surgical and interventional procedures have increased the risk and the awareness of arterial thromboembolic complications [6].

According to the current guidelines for antithrombotic therapy in neonates and children, Aspirin (ASA) is still the most used drug for antithrombotic prophylaxis in pediatric patients [7].

Although optimal aspirin dosage has been empirically set at 1 mg/kg/day to 5 mg/kg/day, it is not supported by any pediatric clinical trial to date [7].

The aim of our study was to investigate pharmacodynamic and pharmacokinetic variability in children and adolescents on ASA antithrombotic prophylaxis by concomitantly measuring platelet reactivity and ASA systemic exposure.

Methods

Patients

Children aged between 6 months and 18 years, with congenital heart disease requiring antithrombotic prophylaxis with ASA, were the study population. All patients had to be on stable treatment with uncoated ASA according to local practice for at least one week. Exclusion criteria were: platelet count <100,000/mm^3, congenital or acquired coagulation disorders, or ongoing combination therapy with antithrombotic drugs other than ASA. The study was approved by the local Ethics Committee and the informed consent was obtained from children's parents in compliance with the Italian lows.

Study design

Our study was originally designed to determine the prevalence of laboratory resistance to ASA (primary outcome) and the incidence of treatment failures in term of thromboembolic and hemorrhagic events (secondary outcomes) in a pediatric population followed by the Cardiology Unit of the Pediatric Clinic, Padova University Hospital. Here we present an interim analysis based on the preliminary insights gleaned from 29 cases. Demographic characteristics (age, gender, weight, BSA, BMI) and clinical parameters (blood pressure, heart rate and oxygen saturation) were recorded. ASA doses ranged from 5 mg to 100 mg according to subject's age and body weight. The Hospital Pharmacy prepared doses <100 mg by crushing the 100 mg immediate-release product (Aspirinetta®, Bayer S.p.A., Milano, Italy).

During a scheduled visit, three blood samples were drawn into vacutainer tubes containing sodium citrate by peripheral venipuncture 4, 5 and 6 hours after oral drug administration. One aliquot was used for platelet aggregation tests and the remaining to assay plasma levels of ASA and Salicylic Acid (SA).

We followed-up all children with 3-monthly visits and recorded any thromboembolic or hemorrhagic complication. Echocardiographic exams were also performed to exclude intra-cardiac thrombi.

Platelet aggregation test

Arachidonic acid-induced aggregation was assessed by multiple electrode aggregometry (Multiplate® analyzer, Roche Diagnostics International Ltd, Rotkreutz, Swiss), which is based on changes in electrical impedance induced by platelet adhesion to the surface of two silver-coated electrodes.

Briefly, 0.3 mL of a 0.5 mM arachidonic acid solution in isotonic saline was added to 0.3 mL anticoagulated whole blood (ASPI test). Sodium citrate was used as anticoagulant instead of hirudin as both anticoagulants are known to have similar predicting value for high on-treatment platelet reactivity compared to the Light Transmission Aggregometry (LTA), which is currently considered the gold standard [8].

Measurements were made 0.5 h to 2 h after venipuncture. Aggregation (impedance) was continuously recorded in Arbitrary Units (AU) over 6 min and the area under the curve of AU *vs.* time (AUC-U) was taken as a measure of platelet residual reactivity. The manufacturer recommends to express the results in units (U), with 1U=10 AUC-U.

Drug assay

ASA and SA plasma concentrations were measured by an HPLC method modified after Venkata et al. [9]. Fifteen µL of the internal standard solution (m-toluic acid, 0.1 mg/mL) were added to 500 µL of plasma. Subsequently, 40 µL of distilled water and 3 mL of acetonitrile were added, followed by vortexing for 10 sec and centrifugation for 5 min at 3000 rpm. The supernatant was evaporated at 30°C under gentle nitrogen stream. The residue was reconstituted with 300 µL of mobile phase, transferred into 1.5 mL Eppendorf tubes and centrifuged at 13,000 rpm for 10 min to complete protein precipitation. Fifty microlitres were then injected into a chromatographic column (Zorbax Eclipse Plus-C18: 4.6 mm × 75 mm, 3.5 micron - Agilent) by means of a Waters 717 plus auto sampler. The mobile phase consisted of 848.5 mL of ultrapure water, 1.4 mL of orthophosphoric acid (85% w/v) and 150 mL of acetonitrile, with a flow rate of 1 mL/min (Water 1515 isocratic pump). The effluent was analyzed with an UV detector (mod. 2487, Waters) set at 237 nm, connected with the Empower software (Waters) to record and analyze the signal. The calibration curves for ASA and SA were linear up to 10 µL/mL and the coefficient of determination (r^2) was always >0.99. The coefficient of variations at 10 ng/mL and 500 ng/mL were 7.0% and 1.9% for ASA (n=10), 5.8% and 2.8% for SA(n=10), respectively.

The limits of detection, defined as a signal-to-noise ratio of 3:1, were 5 ng/mL for ASA and 3 nd/mL for SA.

Pharmacokinetic parameters

Based on the plasma concentrations of ASA and SA measured at the 4th, 5th and 6th hour, the area under the concentration-time curves (AUC) were calculated by means of the trapezoidal rule. The maximal concentrations (Cmax) and the time to Cmax (Tpeak) were also recorded by visual inspection. In addition, the extent of ASA deacetylation by intestinal and liver carboxyl-Esterases-1 and -2 (CES-1 and CES-2) [10] and platelet cyclooxygenase-1 (COX-1) was assessed through the ratio between the AUCs of SA and ASA (AUC_{SA}/AUC_{ASA}), (Figure 1).

Statistical analysis

Given the explorative nature of this study, sample size was not formally calculated. The results are described as means ± SDs (or SEs). Correlations between variables were first tested by means of linear regression. Then, the variables which reached a significance level (p) of at least 0.10 were included in a stepwise multiple regression analysis. A collinearity analysis was also performed to exclude redundancy among variables. The significance level was set at 0.05 for all tests.

Results

Patients' characteristics

The demographic and clinical characteristics of the 29 patients referred to our centre from July 2016 to August 2018 are summarized in Table 1.

Four patients suffered from chronic cyanosis (O_2 saturation 70% to 88%), though only 2 were polyglobulic. All patients had normal platelet count (above 100.000/mmc), and serum creatinine and albumin within the normal range.

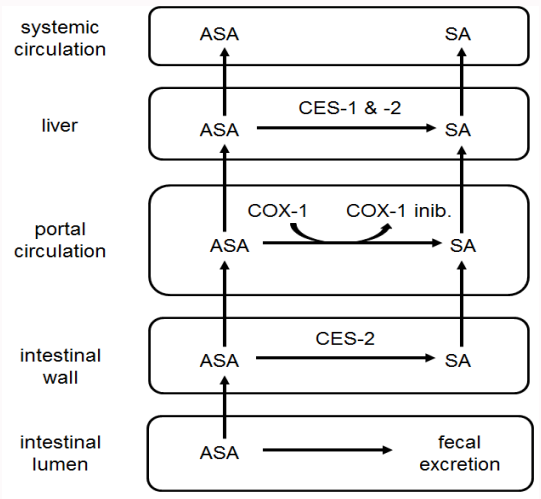

Figure 1: Aspirin (ASA) deacetylation pathways to Salicylic Acid (SA) by intestinal and liver Carboxylesterases 1 & 2 (CES-1 & -2) and platelet Cyclooxygenase-1 (COX-1).

The children suffered from the following heart diseases: Three dilated cardiomyopathies (2 post-infective and 1 post-actinic); sixteen isolated heart defects (14 atrial septal defects, 1 partial atrioventricular canal, 1 aortic coarctation); six complex heart defects (2 tetralogies of Fallot, 1 transposition of great arteries with aortic arch interruption, 1 truncus type 4, 1 levo-transposition of the great arteries with pulmonary atresia, 1 pulmonary atresia with intact ventricular septum); four univentricular hearts (2 hypoplastic left heart syndromes, 1 double outlet right ventricle with pulmonary stenosis and anomalous partial venous return, 1 unbalanced atrioventricular canal with pulmonary stenosis and heterotaxy). Two patients had a history of thrombosis prior to initiating aspirin therapy, none showed hemorrhagic diathesis.

All patients were on stable antiplatelet treatment with uncoated aspirin for at least one week (Table 2). Eleven patients were also taking one to five of the following drugs: furosemide (6), spironolactone (3), enalapril (3), lisinopril (3), lostartan (1), bisoprolol (2), carvedilol (1), metoprolol (1), bosentan (2), sildenafil (1), digoxin (1), ranitidine (1), oxybutinine (1), valproic acid (1), thyroxin (1), ampicillin (1), cefazolin (1). None of these drugs are known to interfere with aspirin serum concentration or effect.

Indication for antiplatelet therapy was antithrombotic prophylaxis for percutaneous device in ASD closure (15), percutaneous stent (4), percutaneous biological valve (2), valved conduit (1), left ventricular dysfunction with FE <30% (3), surgery on great vessels (2), aortic dissection (1).

ASA therapy was meant as a lifelong treatment in 12 patients and as a brief prophylaxis (3 to 6 months) in 17 patients.

Pharmacokinetic evaluation

The mean time course (± SE) of ASA and SA plasma concentrations are plotted in Figure 2 and the mean pharmacokinetic parameters of ASA and SA are shown in Table 3. These findings collectively indicate that, in the 4 h to 6 h interval post ASA administration, plasma levels of ASA and SA vary greatly among subjects and most of the administered ASA is deacetylated to SA by carboxylesterases (CES-1 and CES-2) and platelet COX-1 (Figure 1).

Table 1: Demographic and clinical characteristics of the study population.

	Mean	SD	Range
Sex (M/F)	15/14	-	-
Age (Years)	8.5	4.5	0.46-17.6
Weight (Kg)	29.1	16.7	5.2-71.0
Height (cm)	125	31	61-170
BSA (m^2)	0.99	0.4	0.30-1.80
BMI	16.6	3.5	12.2-26.1
Creatinine clearance [a] (mL/min)	113	17	75-145
Serum albumin (g/L)	45.2	4.9	39-66
Erythrocytes/mm^3 × 10^6	4,746.60	805.1	3,570-7,310
Haematocrit (%)	35.5	6.5	28.6-61.0
Mean cell volume (µL)	81.6	6.3	54.2-90.0
Haemoglobin (g/dL)	13.3	2.4	9.5-21.0
Leucocytes/mm^3 × 10^3	7,816.20	2,905.90	3,180-1,7450
Neutrophils/mm^3 × 10^3	3,976.80	2,446.00	1,580-13,510
Platelets/mm^3 × 10^3	262.7	61	157-396
Heart rate (bpm)	95	20	61 - 140
O_2 saturation (%)	96	7.9	70 - 100
Systolic BP (mmHg)	103	11	85 - 124
Diastolic BP (mmHg)	61	10	45 - 85

[a]Schwartz's formula

Table 2: Aspirin dose (mean, SD, and range).

	Mean	SD	Range
Dose (mg)	77.9	31	15 - 100
Dose/Kg (mg)	2.96	0.7	1.4 - 4.0
Dose/m^2	78	16	50.5 - 104.0

Table 3: Aspirin and salycilic acid pharmacokinetic parameters.

		Mean	SD	Range
Aspirin	AUC (ng/mLxh)	118.5	120.9	19.6 - 655.6
	Cmax (ng/mL)	82.5	96.7	7.5 - 512.3
	Tpeak (h)	5	0.78	4 - 6
	AUC_{SA}/AUC_{ASA}	60.4	78.1	3.31-387.6
Salycilic Acid	AUC (ng/mLxh)	4,009	4,240	91 - 20,170
	Cmax (ng/mL)	2,933.50	2,924.40	54.1 - 12,852
	Tpeak (h)	4.3	0.65	4 - 6

At univariate regression analysis none of the pharmacokinetic parameters listed in Table 3 correlated significantly with any demographic or clinical characteristic listed in Table 1.

Pharmacodynamic evaluation

ASPI test results were available only in 24 out of 29 patients for technical reasons. Residual platelet aggregation assessed as arbitrary units (U) varied between 9 and 50 in our population (mean ± SD: 23.0 ± 10.8). According to manufacturer's recommendations, when hirudin is used as anticoagulant U<40 warrants significant COX-1 inhibition by aspirin, while an U<30 reveals strong COX-1 inhibition [11]. However, no U cut-offs have been established concerning the use of sodium citrate as anticoagulant. To compare our U values with those reported with hirudin, we calculated a correction factor considering that: a) a linear correlation has been established between

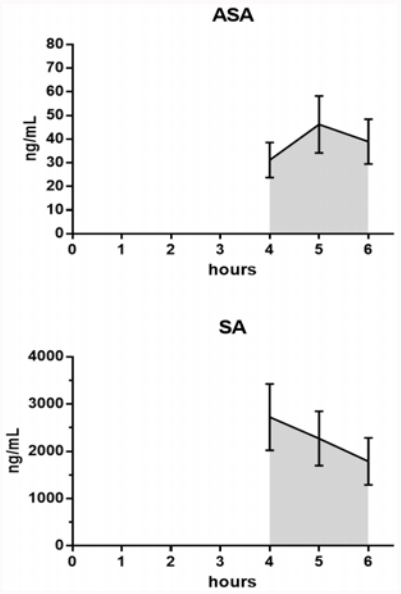

Figure 2: Mean time courses of aspirin (panel a) and salicylic acid (panel b) plasma concentrations. Bars represent standard errors and shaded areas are the mean areas under the concentration-time curves.

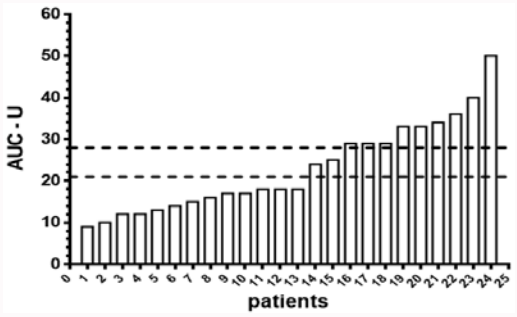

Figure 3: Distribution of the U values measured in 24 patients. The dotted lines indicate the upper and lower cut-off limits which identify poor responders (U>28), partial responders (U=21-28), and full responders (U<21).

platelet aggregation measured with the two anticoagulants (r=0.77) [8]; b) sodium citrate yields U values 24% to 36% lower than those obtained with hirudin [8,12,13]. Thus, we tentatively reduced the two hirudin cut-offs by 30% and obtained the cut-offs of 28 U and 21 U for sodium citrate, respectively. According to this new classification, 13 patients (54.2%) would be full responders, 9 poor responders (37.5%) and 2 partial responders (8.3%) (Figure 3).

Only two variables correlated with U at univariate regression analysis with a significance level p<0.10: AUC_{ASA} (p=0.059) and the ratio AUC_{SA}/AUC_{ASA} (p=0.062). These variables were included in a stepwise multiple regression analysis, which yielded the following equation:

$U= -18.6+0.0799 \times AUC_{ASA}-0.0708 \times AUC_{SA}/AUC_{ASA}$; adjusted $r^2=0.20$; p=0.035

No collinearity was found between the two variables (tolerance =0.97), which were independently albeit weakly-associated with U.

In clinical terms, the equation means that the higher the AUC_{ASA} and the lower the AUC_{SA}/AUC_{ASA} ratio, the lesser the antiplatelet effect of ASA.

Clinical outcomes

No thromboembolic (including intra-cardiac thrombi) or hemorrhagic events were documented during the follow-up (mean duration ± SD: 385 ± 225 days; range: 90-750 days). Although to date there is no evidence for adjusting aspirin treatment on the basis of laboratory tests, one of the two patients with the highest U value after cardiac surgery (U=50) was prudently switched to clopidogrel. The other (U=40), who was meant to continue prophylaxis for 3 months after atrial septal defect closure, maintained his ASA dose and underwent monthly echocardiographic exams for intra-cardiac thrombi detection until the discontinuation of therapy.

Discussion

Pharmacokinetic results

Our results indicate that systemic plasma levels of ASA and SA vary widely among individuals. Variability of AUC_{ASA} and AUC_{SA} could not explained by any of the demographic or clinical variable considered. Furthermore, neither ASA dose, dose/Kg nor dose/m^2 correlated with AUC_{ASA} and AUC_{SA}.

After oral administration ASA is deacetylated by various enzymes at different body sites (Figure 1). Even during absorption ASA interacts with the high affinity carboxylesterase CES-2 in the intestinal mucosa (Km=360 μM) and releases significant amounts of SA into portal blood. Here, residual ASA acetylates the serine residue of platelet COX-1, giving rise to additional SA. Finally, CES-2 and the low affinity carboxylesterase CES-1 (Km=2030 μM) expressed in liver cells further contribute to the pre-systemic ASA deacetylation and increase in systemic SA levels. Circulating SA is then eliminated partly unmodified by the kidney and partly metabolized to salicyuric acid, salicyl-phenol glucuronide, salicyl-acyl glucuronide and gentisic acid by the liver, with a final plasma half-life of about 2 h [14].

Because of low ASA dose, its short half-life (about 15 min) and extensive pre-systemic metabolism, SA plasma concentrations measured 4 h to 6 h after oral administration largely exceeded those of ASA, with a wide inter-individual variability of the AUC_{SA}/AUC_{ASA} ratio (3.3-388).

Each enzyme involved in ASA metabolism exhibits variable activity due to genetic and non-genetic factors. Specifically, three CES-2 variants have shown some decrease in aspirin hydrolysis – up to 40% for the variant A139T [10]. As to COX-1 genetics, haplotypes containing the mutant allele 842G in the promoter region have been associated with higher on-aspirin platelet reactivity and serum thromboxane B2 levels [15].

Among non genetic factors, Yang et al. [16] reported that mRNA and protein expression of CES-1 and CES-2 in human livers is age-dependent and microsomes from children aged 0 to 10 years have ~60% reduced hydrolytic activity on aspirin versus adults. In addition, they found a large inter-individual variability in the expression of these enzymes (particularly in the child group) and ascribed it to the presence of co-morbidities characterized by elevated cytokine levels, which are known to inhibit drug metabolism.

The literature data on ASA pharmacokinetics in children are very scanty, incomplete and relative to anti-inflammatory doses [14,17-19]. Nevertheless, a common feature with our data is high interindividual variability of pharmacokinetic parameters.

Pharmacodynamic results

Multivariate regression analysis indicated that ASA antiplatelet

effect correlated inversely with AUC_{ASA} and directly with AUC_{SA}/AUC_{ASA} ratio. At a first glance these results seem in contrast with the expected concentration-effect relationship. However, it bears reminding that ASA concentrations in the portal blood -rather than the systemic circulation- are solely responsible for the antiplatelet effect. It is, therefore, conceivable that high systemic ASA concentrations and low AUC_{SA}/AUC_{ASA} ratios may reflect decreased pre-systemic ASA deacetylation by COX-1 and reduced antiplatelet effect. This hypothesis has to be reconciled with two criticisms: 1) ASA is also metabolized by CES-1 and CES-2, which can contribute to determine AUC_{ASA} and AUC_{SA}/AUC_{ASA} ratio; 2) high plasma levels of SA have been shown to antagonize ASA antiplatelet effect [20,21], thus a lower AUC_{SA}/AUC_{ASA} ratio may be associated with greater antiplatelet effect.

Although the exact contribution of COX-1 and CES-2 to ASA deacetylation *in vivo* is unknown, *in vitro* studies on the hydrolysis of ASA by CES-1 and CES-2 have reported Km values which are quite higher (2.03 mM and 0.36 mM, respectively) than the ASA concentrations required to inhibit COX-1 in human platelets by 50% (IC_{50}=3.2 μM) [10,22], suggesting a dominant role of COX-1 in promoting pre-systemic ASA deacetylation. Moreover, liver microsomes from children aged 0 to 10 years have ~60% reduced CES-1/2 activities on ASA versus adults (see above) which may further strengthen the argument that COX-1 contributes to ASA deacetylation more than CES-1/2 in this age group [16].

With regard to the second criticism, there is evidence that SA can actually diminish ASA effect when tested in platelet-rich plasma [20,21]. By contrast, using whole blood (as we did) Gonzalez-Correa et al. [23] have shown that ASA effect is potentiated with SA concentrations of 50 to 125 μM, but antagonised with SA concentrations of 250 to 500 μM. Since SA plasma concentrations in our experimental conditions spanned between 0.33 to 73.0 μM, an antagonism with ASA seems to be excluded.

In conclusion, our postulate that pre-systemic ASA deacetylation is mainly catalyzed by platelet COX-1 is not contradicted by currently available experimental data and may explain why ASA antiplatelet effect inversely correlates with AUC_{ASA} and directly with AUC_{SA}/AUC_{ASA} ratio.

Clinical outcomes

None of our patients developed thrombosis or bleedings during the follow-up, though our study was not powered to assess clinical outcomes. So far, no tight correlation has been established between *ex-vivo* aggregation tests and clinical outcomes in adults, presumably owing to the several pathways involved in platelet activation and the diverse pathophysiology of thrombotic diseases. At this time, the official guidelines on antithrombotic therapy in neonates and children do not recommend the use of specific aggregation tests to individualize ASA therapy [7]. Nevertheless, in recent years several studies have been conducted in pediatric patients treated with ASA after cardiac surgery or catheterization with the aim to assess the prevalence of ASA resistance (or high on aspirin platelet reactivity) and its relation with clinical outcomes [4,24,25]. Aspirin "laboratory resistance" has been reported in 10% to 26% of patients, depending on the assay method and the pre-set cut-off values [26-30]. Only one study used the Multiplate System® to evaluate pharmacodynamic resistance to ASA in 14 children and found that 64% were responders after 3-6 month treatment [29], in line with our own findings (54.2%). A recent study retrospectively investigated *in vitro* and clinical ASA response in 430 pediatric patients undergoing cardiac surgery and reported that patients with suboptimal platelet response (aspirin reaction units ≥ 550 with the Verify Now® system) also carried a higher risk of thrombosis (OR: 30.1, p<0.001) [4]. Furthermore, none of the patients who underwent ASA dose escalation according to a recommended algorithm developed thromboses versus 38% of patients who did not. Similarly, a prospective observational study conducted on children treated with ASA found that urinary thromboxane B levels measured on the fifth post-operative day correlated with thrombosis risk [31]. It would therefore appear that point-of-care monitoring of ASA laboratory response may be useful to individualize dose and improve clinical response in children undergoing cardiac surgery [32]. What remains uncertain is which laboratory method and test cut-offs are most appropriate for clinical application. Larger prospective studies are needed to establish a relation between laboratory aspirin tests and clinical results.

Our study has several limitations. Firstly, we have so far enrolled a small number of pediatric patients which is insufficient to ascertain the incidence of unfavorable clinical events; however, clinical outcomes were not the primary end-point of our interim analysis. Secondly, the anticoagulant we used to test platelet function (sodium citrate) is not the one recommended by the manufacturer (hirudin); nevertheless, a significant correlation has been found between the results obtained with citrated and hirudinated blood and, more importantly, both methods are able to identify patients with high on-aspirin platelet reactivity compared to the gold standard method (light transmission aggregometry) [8]; we are therefore confident that the results of our regression analysis are sound. Thirdly, the limited number of ASA and SA concentrations prevented us from performing a thorough pharmacokinetic analysis. A detailed PK analysis was not among our aims. The interval between 4 h and 6 h after ASA administration was selected to allow complete drug absorption and onset of the effect, in order to analyze the PK-PD relationship in steady conditions.

Conclusion

Our preliminary results indicate that the wide inter-patient variability in ASA pharmacodynamics and pharmacokinetics documented in adults is also present in children. In addition, pharmacokinetic variability was not explained by the demographic and clinical variables considered. Conversely, the pharmacodynamic response was predicted by AUC_{ASA} and AUC_{SA}/AUC_{ASA} in a counterintuitive way, suggesting that presystemic ASA deacetylation in children is mainly catalyzed by platelet COX-1 rather than intestinal CES-1 and 2. This hypothesis needs to be confirmed in a larger trial.

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