WITHDRAWN:
Is Increased Platelet Aggregation Is a Risk Factor for Cardiovascular Disease in Women With Idiopathic Central Precocious Puberty

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Abstract

Background

Early menarche in girls is associated with an increased risk of cardiovascular events later in life, but the role of platelets in this risk has not been investigated during puberty. Here, we evaluated the effects of idiopathic central precocious puberty (ICPP) on platelet aggregation in platelet-rich plasma samples from female patients.

Methods

The study included 40 girls diagnosed with ICPP between February 2012 and June 2016, and a control group consisting of 30 healthy females. Adenosine diphosphate (ADP) and collagen-induced platelet aggregation were studied with photometric aggregometry.

Results

There was no difference in the platelet count or volume between girls with ICPP and the control group. In addition, the ADP-induced maximum aggregation time, value, and slope did not significantly differ between the study and control groups ($p > 0.05$). However, the collagen-induced maximum aggregation time, value, and slope were significantly higher in the study group ($p < 0.001$).

Conclusions

Increased collagen-induced platelet aggregation was detected in girls with ICPP. Thus, early treatment of ICPP may be important because of the increased risk of cardiovascular events later in life. Extensive studies with more patients are needed to determine the mechanisms of platelet dysfunction in girls with ICPP.

What Is Known

To our knowledge, no previous study has investigated the role of platelets in increased cardiovascular event risk has not been investigated in precocious puberty.

What Is New

We show the increased collagen-induced platelet aggregation in girls with idiopathic central precocious puberty (ICPP). This is significant because ICPP may cause increased cardiovascular event risk later in life.

Introduction

Precocious puberty (PP) is relatively common in girls, with an incidence of 29 per 100,000 per year (1). PP is divided into gonadotropin-dependent (idiopathic central PP) and gonadotropin-independent (peripheral PP) types. Idiopathic central precocious puberty (ICPP) occurs when sex steroids are released due to early activity of the hypothalamus pituitary-gonad (HPG) axis (2). Although it is unknown why the HPG axis becomes active early, complex interactions of genetic, stress, metabolic, nutritional and hormonal factors are thought to be involved (3). The plasma levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are increased in girls with ICPP, leading to an increase in circulating levels of 17β-estradiol (E2) (4). Breast development, pubic hair growth, accelerated somatic development, and vaginal bleeding (early menarche), which show periodicity over time, are important in girls with ICPP, and there may be variation in the order of development (5).

An earlier age at menarche has been associated with a higher risk of coronary heart disease, cardiovascular disease, and stroke in some women. The mechanisms underlying these associations remain unclear (6-10) but have been partially explained by excess adipose tissue in adulthood (6, 9) . Indeed, earlier pubertal timing is predictive of a higher adult body mass index (BMI) and greater risk of obesity in women (11). Childhood obesity has been consistently associated with early menarche (12).

Although there are various studies of children and adolescents regarding platelet number and coagulation factors, as well as various platelet aggregation studies regarding non-hematologic diseases, there are no studies on platelet count and platelet aggregation in girls with ICPP (13-18).

In this study, we investigated how platelet count and functions are affected by adenosine diphosphate (ADP) and collagen agonists in girls with ICPP.

Materials And Methods

The study population included 40 female patients diagnosed with ICPP between February 2012 and June 2016 (Group 1) at the Gülhane Training and Research Hospital pediatric outpatient clinic (Ankara, Turkey). The onset of secondary sex characters before the age of 8 years, a growth rate higher than expected for age, a bone age 2 standard deviations (SDs) higher than expected based on the calendar age according to the Greulich and Pyle method, and gonadotropin levels at the pubertal level were the diagnostic criteria for ICPP (4, 19). In addition, a uterine long axis ≥ 35 mm, loss of uterus tubular structure, corpus/cervix ratio ≥ 1, ovarian volume ≥ 2 mL, and the presence of follicles ≥ 10 mm in diameter in the ovaries were evaluated by ultrasonography at the onset of puberty (19, 20). Thirty healthy females were included as the control group (Group 2).

Written informed consent from the families of the patients, and approval of the local ethics committee, were obtained. The inclusion criteria for both groups of patients were as follows: no use of antiplatelet drugs in the last 30 days, and no hematological diseases, chronic heart, kidney, and/or liver diseases (excluding ICPP in the study group).

The whole blood count, ferritin level, prothrombin time (PTZ), activated partial thromboplastin time (aPTT), and fibrinogen and platelet aggregation were analyzed in both groups. Erythrocyte indices, and the platelet count and mean platelet volume (MPV), were obtained using an automatic device (Technicon H-1 System; Technicon Co, Tournai, Belgium)

Blood samples taken from the antecubital vein were collected into plastic syringes containing 1/10 volume 3.8% trisodium citrate. Platelet-rich and platelet-poor plasma were prepared by centrifugation (21). Platelet aggregation was assessed by photometric aggregometry using a whole blood aggregometer (Model 560; Chrono-Log Corporation, Havertown, PA, USA).

Collagen (5 µg/mL, Chrono Par No: 385; Chrono-Log Corporation) and ADP (10 µmol, Chrono Par No: 384; Chrono-Log Corporation) were used as agonists. The maximum aggregation time (s), value (%) and slope (%/min) were determined from the aggregation curves. The effects of ADP and collagen on aggregation were evaluated in both the control and study groups considering the effect of iron deficiency on aggregation (22, 23).

Statistical Analysis

Statistical analysis was performed with SPSS software (ver. 22; SPSS Inc., Chicago, IL, USA). Data are provided as mean, standard deviation, median, lowest, highest and percentage values. Normally distributed data were compared using the t-test for independent samples and Mann-Whitney U test in all other cases. Differences were considered statistically significant at $p < 0.05$.

Results

Forty female patients diagnosed with ICPP were included in the study group (Group 1) and thirty healthy females comprised the control group (Group 2). The demographic characteristics of Groups 1 and 2 are given in Table 1. There were no significant differences between the groups in age, height, weight, or BMI ($p > 0.05$).

The whole blood parameters of Groups 1 and 2 are given in Table 2. There were no significant differences between the groups in terms of the white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGB) level, hematocrit (HCT) level, mean erythrocyte volume (MCV), mean erythrocyte hemoglobin (MCH) level, mean erythrocyte hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet (PLT) count, or MPV ($p > 0.05$).

There was no significant difference between Groups 1 and 2 in the plasma ferritin level, PTZ, aPTT, or fibrinogen level ($p > 0.05$; Table 3).

The mean maximum aggregation time, value and slope induced by 10 µmol ADP and 5 µg/mL collagen in Groups 1 and 2 are shown in Table 4. In the study group, at 10 µmol ADP, the mean maximum aggregation time, value, and slope did not significantly differ from control group values ($p > 0.05$). However, in the study group, at a collagen concentration of 5 µg/mL, the mean maximum aggregation

time, value, and slope were significantly higher than in the control group (p = 0.001, 0.002 and 0.04, respectively).

Discussion

The onset of puberty before the age of 8 years in girls and 9 years in boys is considered to be PP, and is more common in girls. The vast majority of cases are ICPP (2). The loss of effectiveness of central systems suppressing neurons that secrete gonadotropin-releasing hormone (GnRH), and activity of systems that stimulate the release of LH and FSH in response to pulsatile release of GnRH, results in ICPP (24). Stimulation of the gonads by LH and FSH increases circulating levels of sex steroid hormones (especially E2 in girls) (4). Although sexual maturity has been shown to affect platelet aggregation in pigs, no published study has shown the effect of puberty and/or ICPP on platelet aggregation in humans (25).

In this study, platelet aggregation stimulated by ADP was not different between the study and control groups, whose demographic characteristics, whole blood parameters, ferritin levels, PTZ, aPTT and fibrinogen levels were not significantly different. However, the maximum aggregation time, value, and slope for collagen-induced platelet aggregation were significantly higher in the study group. Collagen is a strong agonist, but ADP is only a weak one (26). In our study, ADP was used at a concentration of 10 µmol, which is sufficient for platelet aggregation (26). We did not detect any change in aggregation at this level of ADP, but there was an increase in collagen, suggesting selectivity for collagen-induced platelet activation pathways. Similar to our findings, Leng *et al*. (27) achieved an increase in collagen-induced, but not thrombin-induced, platelet aggregation by giving different estrogen derivatives to ovariectomized mice, which was done to examine the effects of estrogens on arterial thrombosis. The authors suggested that the *in vivo* effects of estrogen on platelet function may be agonist-specific. In the same study, collagen induced glycoprotein-VI, a platelet surface glycoprotein, which initiated adhesion followed by aggregation. Collagen, unlike ADP, also stimulates the release of thromboxane A2, a strong aggregation agent (28). In our study, glycoprotein-VI and/or thromboxane A2 may explain why there was no change in platelet aggregation with ADP but an increase with collagen.

Early menarche has been associated with an increased risk of coronary heart disease in later life (6-9). Canoy *et al.* retrospectively examined over 1 million middle-aged women and compared girls with early menarche (aged ≤ 10 years) and late menarche (≥ 17 years) girls to girls with normal menarche (13 years) (10). They showed that early and late menarche increased the risk of coronary heart disease, whereas the risk of cerebrovascular disease, hypertension, and diabetes mellitus was significantly lower. In this study, impaired glucose homeostasis and hypertension may partly explain the associations among early menarche, coronary heart disease, and cerebrovascular disease. Although the increased risk of coronary heart disease in women with early menarche is partially explained by the excess adipose tissue present in these women in adulthood, Canoy *et al*. did not report an increased incidence of obesity in adult women with coronary heart disease after early puberty, unlike other studies (11, 29, 30). To explain this association, Hardy *et al*. investigated cardiovascular structure and function (carotid intima-media

thickness, pulse wave velocity, and left ventricular structure and function) in older women (60–64 years) with early menarche, but did not show any relationship with early menarche (31). It is thought that a process beginning with childhood obesity may ultimately lead to cardiovascular diseases in later life, in association with early puberty and adult obesity (29, 32-34). However, no study has investigated the role of platelet aggregation in the increased risk of cardiovascular disease in later life, either in girls or older women with early menarche.

Estrogens, especially E2, exert effects on tissues through their receptors. Platelets and their bone marrow precursors, megakaryocytes, also express estrogen receptors (35-37). Although many factors affect platelet function, the effects of hormones on platelet activity are poorly defined (35). Conflicting results have been reported regarding the effects of estrogen administration on platelet activation in mice and women (38). There have been few studies on the role of E2 in megakaryopoiesis and platelet production. In a study by Bord *et al*. (39), a higher number of medullary megakaryocytes was reported, without any change in the total bone marrow cell count, after long-term oral or transdermal estrogen hormone. In another study of post-menopausal women (40), a slight increase in platelet number and volume was found with estrogen treatment. In the study by Valéra *et al*. (41), the platelet count in mice was not significantly affected by chronic subcutaneous administration of E2 compared to ovariectomized mice. In a study by Miller *et al*. (42), in postmenopausal women, the number of platelets did not significantly differ from baseline after 48 months of E2 treatment. Similarly, Kaplan *et al.* (43) reported no change in the platelet count of a small group of women after 3 months of E2 treatment. However, another study reported a significant decrease in platelet count after 3 months of transdermal treatment of E2 (44). In our study, we found no difference between the study and control groups in terms of platelet number and volume.

The risk of thromboembolic events increases with oral E2 treatments in women (45). In an animal study by Rosenblum *et al*. (46), E2-treated rats showed more rapid aggregation after mesenteric endothelial damage compared to placebo. The reason for this has not been fully explained, but there are some putative mechanisms. Miller *et al*. (42) reported that estrogen increased the level of hepatic-derived coagulation factors. Thijs *et al*. (47) showed that E2 hormone therapy in postmenopausal women increased the levels of P-selectin and glycoprotein 53, which are associated with increased platelet activation and platelet degradation. Rank *et al.* (48) reported higher levels of platelet-derived microparticles in postmenopausal women receiving hormone replacement therapy, indicating platelet activation rather than endothelium-derived microparticles. Garcia-Martinez *et al*. (49) reported that E2 may cause an increase in P-selectin or calcium in platelets, leading to platelet activation and, ultimately, thromboembolism. Another proposed mechanism is increased activated protein C resistance in women taking E2 (50, 51).

By contrast, there are studies suggesting that E2 decreases platelet aggregation. Valéra *et al.* (52) reported that washed platelets isolated from E2-treated mice showed reduced aggregation after stimulation by thrombin and collagen. In the same study, the expression of various platelet proteins, including β1-tubulin, which is the main component of microtubules that modulate platelet production and

function, was reduced with E2 treatment. Geng et al. (53) found a decrease in collagen-induced platelet aggregation after E2 administration in ovariectomized mice, and attributed this to decreased platelet and megakaryocyte glycoprotein-VI expression after E2 administration. In platelet aggregation studies performed in both sexually matured and juvenile pigs, Jayachandran *et al*. (25) found that ADP and collagen-induced platelet aggregation decreased in mature female pigs and increased in male mature pigs, and that sexual maturity and platelet aggregation changed with maturity. In *in vitro* aggregation studies using different concentrations of E2 and thrombin and ADP agonists, Nakano *et al*. (54) found that these agonists reduced platelet aggregation, possibly due to the reduction of calcium associated with the increased production of cyclic guanosine monophosphate (which is dependent on NO). Similarly, Bar *et al*. (55) showed that, in postmenopausal women, a significant decrease in adrenaline-induced platelet aggregation and adenosine triphosphate release occurred due to E2 intake. These studies show that megakaryocytes and platelets are important targets of the prothrombotic or antithrombotic effects of estrogens.

Contrary to these studies, E2 was not found to have any effect on platelet aggregation in other studies performed using different agonists, in which various estrogen derivatives were given to postmenopausal women. (42, 43, 56, 57).

It is clear that there are many conflicting results regarding the relationship between E2 and platelet aggregation. In this study, we showed that collagen-induced platelet aggregation was increased in girls with ICPP, which may be due to increased E2 levels. As PP is associated with a higher risk of cardiovascular events later in life, early treatment of ICPP may be beneficial. However, our sample size was relatively small, and further studies with larger number of patients are needed.

Abbreviations

Precocious puberty (PP)

idiopathic central precocious puberty (ICPP)

Adenosine diphosphate (ADP)

hypothalamus pituitary-gonad (HPG)

follicle-stimulating hormone (FSH)

luteinizing hormone (LH)

estradiol (E2)

body mass index (BMI)

polycystic ovary syndrome (PCOS)

nitric oxide (NO)

standard deviation (SD)

prothrombin time (PTZ)

activated partial thromboplastin time (aPTT)

mean platelet volume (MPV)

white blood cell (WBC) count,

red blood cell (RBC)

hemoglobin (HGB)

hematocrit (HCT)

mean erythrocyte volume (MCV)

mean erythrocyte hemoglobin (MCH)

mean erythrocyte hemoglobin concentration (MCHC)

red cell distribution width (RDW)

platelet (PLT)

gonadotropin-releasing hormone (GnRH)

Declarations

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Availability of data and material: Data supply is available

Code availability: N/A

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Tables

Table 1. Demographic Characteristics of the Study and Control Groups

	Study Group	Control Group	
Patient characteristics	n = 40	N = 30	P value
Age (year)	7.38 ± 0.08 (7.21–7.54)	7.50 ± 0.09 (7.31–7.69)	0.321
Height (cm)	128.03 ± 0.82 (126.36–129.69)	127.97 ± 0.90 (126.13–129.81)	0.944
Weight (kg)	26.49 ± 0.45 (25.57–27.40)	27.10 ± 0.53 (26.02–28.18)	0.476
BMI (kg/m^2)	16.13 ± 0.18 (14.35–17.64)	16.52 ± 0.24 (14.35–19.84)	0.216

Data are given as mean ± SD (min-max).

Table 2. Whole Blood Parameters in the Study and Control Groups

	Study Group	Control Group	
Parameters	n=40	n=30	P value
WBC (/µL)	6370.27± 188.35	6433.33 ± 269.75	1.00
RBC (×10^6/µL)	4849.19 ± 51.36 13.07 ±0.10	4935.13 ± 62.58	0.117
HGB (g/dL)	38.84 ± 0.44	12.93 ± 0.13	0.376
HCT (%)	80.86 ± 0.49	39.19 ± 0.54	0.714
MCV (fL)	27.23 ± 0.17	80.88 ± 0.50	0.733
MCH (pg)	33.69 ± 0.21	27.02 ± 0.26	0.737
MCHC (g/dL)	13.64 ± 0.19	33.30 ± 0.19	0.13
RDW (%)	308621 ± 93	13.50 ± 0.14	0.81
PLT (/µL)	8.97 ± 0.86	282100 ± 13	0.58
MPV (fL)		9.21 ± 1.01	0.17

Data are given as mean ± SD.

Table 3. Ferritin Level, PTZ, aPTT, and Fibrinogen Level in the Study and Control Groups

	Study Group	Control Group	
Parameters (± SD)	n = 40	n = 30	P value
Ferritin (ng/mL)	29.18 ± 1.71	36.97 ± 4.73	0.61
PTZ (s)	13.66 ± 0.16	13.71 ± 0.22	0.808
aPTT (s)	30.00 ± 0.27	30.48 ± 0.29	0.222
Fibrinogen	290 ± 6.40	293 ± 7.88	0.980

Data are given as mean ± SD.

Table 4. Platelet Aggregation Parameters of the Study and Control Groups

	Study Group	Control Group	
Agonist	n = 40	n = 30	P value
ADP (10 µmol)			
Maximum aggregation time (s)	339.75 ± 12.89	342.50 ± 15.49	0.975
Maximum aggregation value (%)	70.63 ± 2.35	74.73 ± 2.07	0.16
Slope (%/min)	98.98 ± 3.98	114.30 ± 7.36	0.09
Collagen (5 µg/mL)			
Maximum aggregation time (s)	405.00 ± 14.64	337.83 ± 10.54	***0.001***
Maximum aggregation value (%)	74.95 ± 1.27	66.20 ± 2.33	***0.002***
Slope (%/min)	127.46 ± 4.69	111.30 ± 6.44	***0.04***

Data are given as mean ± SD.