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ORIGINAL ARTICLE

Venous plasma serotonin is not a proper biomarker for pulmonary arterial hypertension

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Abstract

Objectives. Serotonin (5-HT) most likely plays an important role in the pathogenesis of pulmonary arterial hypertension (PAH). We aimed to test if venous plasma 5-HT is a potential biomarker of PAH. We also measured venous blood β-thromboglobulin (β-TG) in all participants to ensure that any increase in serotonin levels measured is due to platelet release. *Design.* Blood samples from patients ($n = 9$) with pulmonary arterial hypertension (Group 1 of the World Health Organization classification of pulmonary hypertension) as well as healthy volunteers ($n = 9$) were analyzed. We used enzyme-linked immunosorbent assay (ELISA) to measure venous platelet-poor plasma 5-HT and β-TG in patients with pulmonary arterial hypertension (PAH) and in age-matched normal controls. *Results.* Venous platelet-free plasma 5-HT and β-TG were almost similar in patients with PAH and healthy controls with only a slight trend toward increased 5-HT levels in patients with PAH. No correlation was found between venous platelet-poor plasma 5-HT and disease severity. There was no association between venous plasma 5-HT and the mean pulmonary artery pressure. *Conclusions.* Our data suggest that 5-HT is not significantly elevated in venous platelet-free plasma in patients with PAH and may accordingly not be a useful biomarker in this condition.

Key words: *platelet-poor plasma, pulmonary arterial hypertension, 5-HT,* β*-thromboglobulin, serotonin*

Introduction

Pulmonary arterial hypertension (PAH) is defined by a mean pulmonary artery pressure at or above 25 mmHg at rest, in the presence of a pulmonary capillary wedge pressure (PCWP) 15 mm Hg or less (1).

The amine serotonin (5-HT) most likely plays an important role in the pathogenesis of pulmonary hypertension (2). Platelet–vascular wall interactions are involved in the development of PAH (3) and platelets release 5-HT which is a major mediator of pulmonary vasoconstriction and leads to pulmonary artery smooth muscle cell proliferation (4). The original hypothesis of the role of 5-HT in the pathogenesis of PAH arose from the observation that patients using anorexigenic drugs such as aminorex and dexfenfluramine, developed PAH. Aminorex and dexfenfluramine are thought to increase free serotonin levels through interactions with the serotonin transporter (SERT) (5).

It is also recognized that 5-HT is synthesized in pulmonary endothelial cells by tryptophan hydroxylase 1 (Tph1). 5-HT acts at 5-HT receptors and SERT, and mediates constriction and proliferation of pulmonary artery smooth muscle cells (6). The 5-HT receptor antagonist tergurid has recently been shown to reduce experimentally induced pulmonary hypertension in rats (7). Therefore, the role of 5-HT in the development of PAH is not just by SERT interactions, but also via 5-HT receptors (8). Since pulmo-

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nary endothelial cells metabolize 5-HT, a reduction in this function could increase 5-HT concentration locally and thus play a role in the pathogenesis of PAH (9,10).

PAH can also occur in platelet storage pool disease and collagen vascular disease, conditions where there is an impaired storage of platelet serotonin and abnormal handling of 5-HT by platelets, causing increased free-plasma serotonin levels (3). Since 5-HT may be involved in the pathogenesis of PAH (2), it is of interest to evaluate whether venous plasma 5-HT may be a potential biomarker for the diagnosis and control of patients with PAH.

Free circulating 5-HT represents only a minor fraction of the total amount in blood as 5-HT is mainly sequestered within dense granules in platelets. Only free-plasma 5-HT has, however, biological activity on the vascular bed (9,10). Previous studies have given conflicting results concerning plasma 5-HT levels in patients with PAH (3,11,12). We have recently introduced a refined method for the determination of 5-HT in plasma based on concomitant determination of β-TG (13), and therefore we wanted to reexamine venous plasma 5-HT levels in patients with PAH. We also measured blood β-TG in all participants to ensure that any increase in 5-HT levels measured is due to platelet release.

Materials and methods

Study participants

The study followed the declaration of Helsinki guidelines for research involving human individuals and was approved by the regional ethical committee according to Norwegian law and regulations. Informed consent was obtained from all study participants. Nine patients with Group 1 (WHO classification of PAH, Dana Point 2008) and 9 age-matched healthy adult volunteers as controls were recruited at St. Olav's Hospital, Trondheim, Norway and enrolled in our study. Healthy controls were all women since the initial seven patients also were females; however, the last two patients were males. Healthy controls did not use acetylsalicylic acid (ASA), nor any other pharmaceutical drugs. All patients with PAH were under treatment with either sildenafil, ambrisentan or bosentan which are agents reducing pulmonary arterial resistance (14). Seven of nine patients were also treated with ASA. Patients' characteristics of sex, age, associated disease, N-terminal prohormone of brain natriuretic peptide (NT-proBNP), New York Heart association functional classification, mean pulmonary arterial pressure (MPAP), pulmonary vascular resistance (PVR), cardiac output (CO), PCWP, and mean right atrial pressure (MRAP) (Table I) were determined shortly (less than 1 month) before venous blood sampling

Table I. Patients' characteristics.

No. patient	Age years	Sex	PAH etiology	MPAP mmHg	PVR WU	MRAP mmHg	CO L/min	PCWP mmHg	NT-proBNP ng/L	Medications	NYHA	Serotonin ng/ml
1	76	F	Systemic sclerosis	*Missing value						Bosentan ASA	II	9.96
2	51	F	HIV	*Missing value						Bosentan Sildenafil	II	6.90
3	36	F	IPAH	64	18.4	3	3.2	5	3478	Ambrisentan Sildenafil ASA	II	4.18
4	79	F	Systemic sclerosis	50	8.8	3	4.9	7		Bosentan ASA	II	8.60
5	48	F	IPAH	39	7.5	4	3.6	12	170	Ambrisentan Sildenafil ASA	I	41.05
6	65	F	IPAH	57	12.2	4	4.1	7	169	Bosentan Sildenafil	II	11.61
7	44	M	IPAH	61	10.4	2	5.38	5	247	Bosentan Sildenafil ASA	III	7.21
8	64	F	IPAH	36	7.4	2	4.2	5	1202	Bosentan ASA	II	4.49
9	43	M	IPAH	50	9.8	2	4.6	5		Bosentan ASA	II	8.72

F, female; M, male; IPAH, idiopathic pulmonary arterial hypertension; PVR, pulmonary vascular resistance; CO, cardiac output; MRAP, mean right atrial pressure; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; ASA, acetylsalicylic acid; NYHA, New York Heart association functional classification; PCW, pulmonary capillary wedge pressure.

*SPAP, systolic pulmonary arterial pressure measured by echocardiography is 60 and 102 respectively.

for 5-HT determination. The diagnosis of PAH in seven of nine patients was based on an initial right heart catheterization which was done two or three years before blood sampling. Regarding the two patients without right heart catheterization the diagnosis of PAH was made after extensive echocardiographic evaluation demonstrating normal left ventricular function with normal EF and with no evidence of increased left ventricular filling pressure using the E/E' ratio. Neither was there any sign of pulmonary congestion at clinical nor X-ray examinations. Furthermore, one of these two patients had the diagnosis mixed connective tissue disorder that further makes it probable with PAH as a correct diagnosis.

Blood collection and analysis

After an overnight fast, blood was collected from an antecubital vein into Vacutainer (EDTA 9 mmol/L) tubes. The first tube was discarded. Rapid flow during sampling was emphasized. Blood was centrifuged within 5 min after blood sampling at 12,000 g for 2 min in an Eppendorf microcentrifuge at 22°C. The upper two-thirds of the platelet-poor plasma was transferred to a polystyrene tube and stored at −80° C until analyses.

The β-TG was measured using enzyme-linked immunosorbent assay (ELISA) kits (β-TG kit, E9037 HU) according to the manufacturer's instructions. Analysis of 5-HT was done using ELISA (IBL, Hamburg, Germany). A calibration curve of 5-HT was drawn based on the absorbance measured at 405 nm and 620 nm on the Microplate Reader and known concentrations of the standard solutions. Concentration values of samples were obtained by adjusting them to the calibration curve, using the five parameters non-linear regression curve fitting. The specificity of the assay was examined by testing the cross-reactivity of N-acyl-serotonin, 5-hydroxyindoleacetic acid (5-HIAA), melatonin, 5-methoxy-tryptamine, 3-indolacetic acid, 5-methoxytryptophol, and 5-OH-tryptophan and has been reported 0.12% or lesser. The threshold of detection was 0.01 nmol/L (13).

Statistics

Data are presented as mean ± standard deviation. The statistical significance of the difference between groups was tested using Wilcoxon rank-sum test. Correlation between data was evaluated using the nonparametric Spearman's rank order correlation test. A p value less than 0.05 is considered significant. All analyses were performed using SPSS 17.

Results

We enrolled nine PAH patients and nine controls. Two PAH patients had collagen vascular disease, one had HIV-associated PAH and six had idiopathic PAH. Mean age of controls and patients were 54 ± 8 and 56 ± 15 years, respectively. All participants in the control group were females, and seven of nine patients with PAH were females as well. All participants had normal platelet counts.

Seven patients underwent the initial right heart catheterization about two or three years before blood sampling. Two patients had not been examined by right heart catheterization due to technical issues; however, both had high systolic pulmonary arterial pressures estimated by echocardiography (60 and 102 mmHg, respectively). The average of mean pulmonary arterial pressure in patients with PAH was 51.00 mmHg.

5-HT and β-TG concentrations in healthy adult volunteers and patients with PAH are shown in Figure 1.

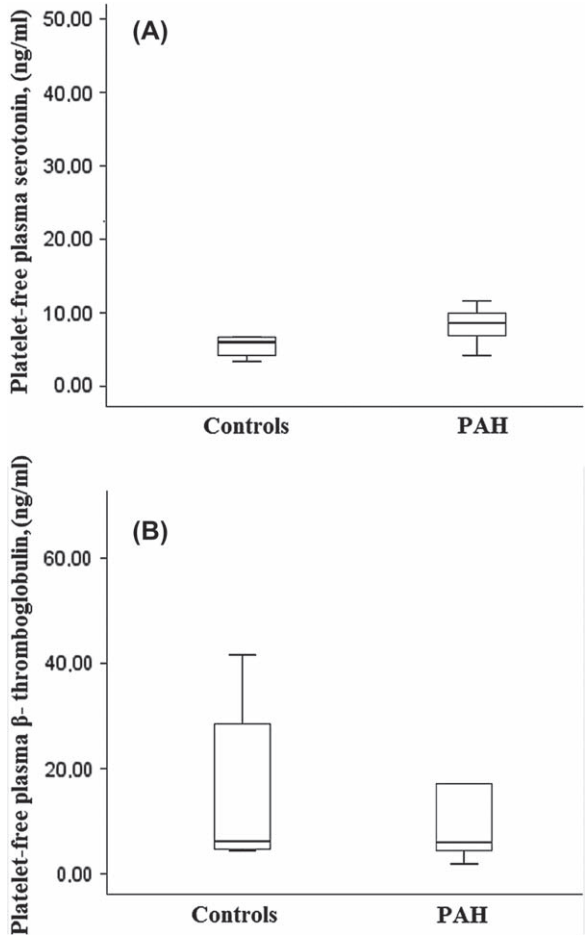

Figure 1. Serotonin (A) and β-thromboglobulin (B) concentrations measured in platelet-poor plasma from healthy controls and in patients with pulmonary arterial hypertension (PAH): median (*line*) and interquartile range (*box*).

Serotonin

The mean concentrations of venous platelet-poor plasma 5-HT in controls and patients with PAH were 9.2 ng/mL ($n = 9$) and 11.4 ng/mL ($n = 9$), respectively. Patients with PAH did not have significantly elevated venous plasma 5-HT levels compared to controls (Figure 1; $p = 0.13$).

No correlation was found between platelet-poor plasma 5-HT and disease severity according to PVR, MRAP, CO, and MPAP values ($p = 0.3$, 0.2, 0.9, and 0.4 respectively). The venous plasma 5-HT concentration in the control group who did not use any medication was not significantly different from patients who used ASA ($p = 0.20$).

β-*TG*

The mean concentrations of platelet-poor plasma β-TG in patients with PAH and controls were 17.4 ng/mL ($n = 9$) and 16.1 ng/mL ($n = 9$), respectively. Patients with PAH did not have significantly elevated venous plasma β-TG levels compared to controls (Figure 1; $p = 0.72$).

The plasma β-TG concentration in the control group who did not use medication was not significantly different from patients who used ASA ($p = 0.31$).

Discussion

Our study shows that the venous plasma 5-HT concentrations in patients with PAH were almost similar to those of healthy controls. A trend toward increased 5-HT levels in PAH patients was noted. It might be that inclusion of more patients would have shown a slight difference between groups. However, the measurement of venous plasma 5-HT concentrations is not suitable to distinguish between patients and healthy controls in a clinical setting.

Previous studies measuring free-plasma 5-HT levels in patients with PAH have given highly discrepant results with finding of both increased and normal 5-HT levels in patients with PAH (3,11,12,15). These variable results may be due to differences in the release of 5-HT from platelets during blood sampling and plasma preparation (16). We have recently introduced a refined methodology of measuring 5-HT in blood, based on extensive caution during sampling and preparation and concomitant determination of β-TG (13). We found that blood platelet numbers did not affect the level of serotonin in contrast to β-TG (13).

In this study, we also measured the platelet-specific substance, β-TG, which is stored in α- granules in platelets. Previous studies suggested that increased free 5-HT in plasma might result from impaired ability of the platelets to store 5-HT or abnormal platelet activation (3). Therefore measurement of this marker could help us to determine if platelets impairment or platelet activation is involved in PAH. In the present study plasma β-TG in patients with PAH did not differ from levels measured in normal subjects, which indicates that platelets are not activated in PAH. Our results are in agreement with the study by Schulman et al., who measured β-TG and platelet factor 4 (PF4) in patients with PAH and could not find any differences compared with healthy controls (17). The measurement of these two factors (5-HT and β-TG) was in peripheral blood. We have previously shown that there is no difference between these parameters in blood taken from peripheral artery and vein (13). However, the concentration in peripheral venous blood may not necessarily be the same as that in the pulmonary circulation, which is the concentration of interest with respect to the potential role of 5-HT in the pathogenesis of PAH. In the lungs, 5-HT may be released from pulmonary endothelial cells and from platelets resulting in higher local concentrations than those found in plasma and sufficient to induce a vasoconstrictor effect (18).

We know of one study where 5-HT has been determined in blood from the pulmonary circulation (12). They did not find any difference between the concentration of 5-HT in platelet-poor plasma prepared from blood taken from the pulmonary artery and peripheral veins or artery (12). On the other hand, they reported lower concentrations of 5-HT in platelet rich plasma taken from patients with PAH compared with healthy controls (12). Nevertheless, the study by Ulrich et al. suggests that free 5-HT concentrations are not different between blood taken from the pulmonary artery and a peripheral vein.

In the current study, we did not have sex-matched groups; however in a previous study, it was shown that gender did not affect venous plasma 5-HT (12).

In our study, patients were under treatment with sildenafil, ambrisentan, or bosentan and/or ASA. It could be argued that the lack of differences in venous plasma 5-HT levels between patients with PAH and controls were due to medical treatment. However, the finding of similar levels of β-TG between groups speaks against any platelet activation and thereby any platelet-derived serotonin released to the blood. It is highly unlikely that endothelin receptor antagonists and phosphodiesterase type-5 inhibitors that are used in the treatment of our patients could reduce/influence 5-HT release from the platelets. Prostacyclins which are potent inhibitors of platelet activation did not affect plasma serotonin levels in the study by Ulrich et al. as well (12). However, we

did not treat the patients with prostacyclin compounds at the time of blood sampling. ASA affects platelet functions, but in our previous study we did not find any effect of ASA on 5-HT concentration when determined by our method (13).

The MPAP of all patients determined shortly before blood sampling was above 25 mmHg, and there was no association between severity of disease and the level of plasma 5-HT. The information about patient's catheterization is from the right-side heart catheterization closest to our blood sampling. We did not measure plasma serotonin at the time of diagnosis and do not know whether thrombocyte function and serotonin concentration vary over time. We know of one study where plasma serotonin has been determined at the time of diagnosis/initial right heart catheterization, and it did not differ between patients and controls (12). Lederer et al. found in their study that higher levels of 5-HT were associated with milder PAH. However, findings have differed between studies (3,11,15).

We included patients with PAH due to different etiologies. It remains to be determined whether plasma serotonin differs depending on the etiology of PAH. In our study, most of the patients enrolled had idiopathic PAH and hence the results of this study mainly apply for this group. However, in the few patients there were similar findings with PAH due to systemic sclerosis and HIV.

In conclusion, venous free-plasma 5-HT levels were not significantly increased in PAH patients and hence may not be used as a biomarker in the diagnosis and control of PAH. However, serotonin concentration in the pulmonary vascular bed may be higher, and thereby serotonin may play a role in the pathogenesis of PAH. In our study serotonin levels were found almost similar in PAH patients and healthy individuals and is thereby not suitable as a biomarker in the assessment of PAH patients.

***Declaration of interest*:** The authors report no declarartions of interest. The authors alone are responsible of the content and writing of the paper.

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