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Increased Plasma Caspase-3 in Children with Down Syndrome is Associated with an Increasing Risk of Pulmonary Hypertension

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ABSTRACT

Background: Pulmonary hypertension (PH) is the one of the comorbidities in children with Down syndrome. The pathogenesis of this pulmonary hypertension remains to be investigated, although endothelial dysfunction and apoptotic activity are among the proposed mechanisms. Caspase-3 is a key regulator of apoptosis and appears to be an attractive predictor of pulmonary hypertension in children with Down syndrome.
Subjects and Method: A cross-sectional observational clinical study was performed in Dr. Moewardi General Hospital in Surakarta-Indonesia between January and March 2021 involving clinically diagnosed children with Down syndrome. Sampling method was using a consecutive sampling. The independent variable was plasma caspase-3 level and the dependent variable were the presence of pulmonary hypertension and congenital heart defects (CHD). Clinical data documentation, blood collection and echocardiography were performed on enrollment day. We first determined the plasma level of caspase-3 in 36 children with Down syndrome and CHD (n=18) or without CHD (n=18) and further determined the risk of having pulmonary hypertension using the plasma caspase-3 level. We also determined the biomarker performance of caspase-3 using a receiver-operating characteristic (ROC) analysis
Results: Children with Down syndrome with PH had a higher plasma caspase-3 compared to those without PH ($p<0.001$). In those with both CHD and PH, the plasma caspase-3 level was also high although not statistically significant ($p=0.145$). The highest plasma caspase-3 level was observed in subjects with PH without CHD ($p<0.01$). Relative risk and ROC analysis demonstrated that increased plasma caspase-3 level increased the risk to have PH 5 times (RR=5.00, 95% CI 1.74 to 14.34; $p<0.001$) and predicted the incidence of PH in children with Down syndrome (AUC 0.88, CI 0.76 to 0.99).
Conclusion: An elevation in plasma caspase-3 level of Down syndrome children is associated with the increasing risk of having PH regardless the presence of CHD.

Keywords: pulmonary hypertension; down syndrome; caspase-3; pathogenesis; apoptosis.

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BACKGROUND

Pulmonary hypertension (PH) is an increasing comorbidity risk of pediatric Down syndrome patients (Bush et al., 2020). The underlying mechanism of developing PH remains to be investigated, although the presence of chromosome 21 trisomy increases the incidence (Bush et al., 2018). Other comorbidities associated with the increased risk of PH in Down syndrome patients are congenital heart defects, chronic airway obstruction and recurrent infections (Bush et al., 2018; Espinola-Zavaleta et al., 2015; Verstegen et al., 2013). These comorbidities enhance the mortality risk of Down syndrome patients and to date the clinical intervention remains suboptimal (Hawkins et al., 2011).

Caspase-3 is an endoprotease which regulates the final phase of apoptosis (Cullen and Martin, 2009) and the role of caspase-3 in mediating endothelial apoptosis in PH has been previously described (White et al., 2014). In a real-time endothelial model, caspase-3 activity is associated with the increasing apoptotic rates of endothelial cell, likely due to hemodynamic stress and a high glucose level (Yu et al., 2013). In an in vitro endothelial model, Shioiri et al. (2009) further showed that LPS induces caspase-3 release into the culture supernatant (Shioiri et al., 2009) and inhibition of caspase-3 using α-1 antitrypsin (AAT) and RNA-inference further prevents endothelial cell apoptosis (Matsuda et al., 2007, Petrache et al., 2006). All together, these laboratory observations suggest that caspase-3 might play a key role in the pathogenesis of PH through endothelial apoptosis.

Increased plasma caspase-3 was previously demonstrated as a marker of the apoptosis in various conditions including atherosclerosis and sepsis patients (Lorente et al., 2016, Matulevicius et al., 2008). Moreover, increased activity of caspase-3 has been associated with the increasing risk of neuronal apoptosis and Alzheimer's disease in Down syndrome patients (Rueda et al., 2013; Stadelmann et al., 1999). In this study, we measure plasma caspase-3 level in children with Down syndrome patients with or without congenital heart defects (CHD) and in the presence or absence of PH.

SUBJECTS AND METHOD

1. Study Design

A cross-sectional observational clinical study was performed in pediatric outpatient department in Dr. Moewardi General Hospital in Surakarta Indonesia between January and March 2021.

2. Population and Sample

We included clinically diagnosed children with Down syndrome whom routinely followed up in Dr. Moewardi General Hospital. Subjects were enrolled using a consecutive sampling method. Children aged 2 months – 5 years old with physical characteristics of Down syndrome such as midfacial hypoplasia, hypertelorism, flat nasal bridges, oblique palpebral fissure, epicanthal folds, abnormal ear formation, macroglossia, mandibula hypoplasia, simian crease, fifth finger medial phalanx dysplasia, a longer distance between the foot toe and middle finger, muscle hypotonia and joint hyperflexibility were included. Patients with post shunt correction, right heart failure signs, Eisenmenger syndrome and critical congenital heart defects (e.g. truncus arteriosus, hypoplastic left ventricle syndrome and transposition of the great arteries) were excluded.

3. Study Variables

Caspase-3 was considered as the independent variable, whereas the presence of CHD and PH was considered as the dependent variables.

4. Operational Definition of Variables

Down syndrome is a genetic disorder due to triplication of Chromosome-21 and clinically diagnosed with physical characteristics such as midfacial hypoplasia, hypertelorism, flat nasal bridges, oblique palpebral fissure, epicanthal folds, abnormal ear formation, macroglossia, mandibula hypoplasia, simian crease, fifth finger medial phalanx dysplasia, a longer distance between the foot toe and middle finger, muscle hypotonia and joint hyper-flexibility.

Pulmonary hypertension (PH) is a chronically increased pulmonary artery vascular resistance and assessed using the echocardiography as determined with a peak velocity of tricuspid regurgitation >2.8 m/s in the absence of pulmonary blood flow as well as obstruction of right ventricular outflow.

Congenital heart defects (left to right shunt) was congenital disorders of cardiac development as well as impaired function as assessed using the echocardiography and comprised of ventricular septal defect (VSD), atrial septal defect (ASD), persistent ductus arteriosus (PDA) and persistent foramen ovale (PFO).

Caspase-3 is a final key effector enzyme in the apoptotic process and measured from the EDTA-anticoagulated blood plasma using Human Caspase-3 ELISA Kit with an end value designated in nanogram per milliliters (ng/mL).

Nutritional status is defined as the presence of short stature as assessed with body weight, height, head circumference and plotted to a standard curve with an end value of normal, undernourished and malnourished.

5. Study Instruments

Clinical data documentation, blood collection and echocardiography were performed on enrollment day. Clinical data were recorded using case record forms (CRFs), inputted and stored as a digital database form in a study computer. Plasma samples were obtained from EDTA-anticoagulated blood collected and centrifuged at 3000 RPM for 15 minutes. One mL of plasma was stored at -70°C freezer prior to a collective measurement. Caspase-3 was measured using Human Caspase-3 ELISA Kit (EIAab Science, Wuhan, China, Cat. No. E0626h) according to the manufacturer instruction. Echocardiography was performed in all patients to confirm the presence of congenital heart defects as well as type of shunt using GE Vivid S60 Echocardiography (GE Operations, Boston, Massachusetts, USA). Pulmonary hypertension was defined with the peak velocity of tricuspid regurgitation >2.8 m/s in the absence of pulmonary blood flow as well as obstruction of right ventricular outflow.

6. Data Analysis

Clinical characteristics of the patients were described in a descriptive analysis. Distribution normality of data was assessed using Shapiro-Wilk test. The difference between groups were analyzed using Mann-Whitney or Independent Student's t-test for numeric data and Fisher Exact test for categorical data depending on the distribution normality. Risk analysis was determined using Chi-Squared test and biomarker performance was analyzed using a receiver-operating characteristic (ROC) curve analysis. Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, NY, US) and p-value < 0.05 was considered to be statistically significant. Graphic visualizations were performed using GraphPad 9.0 (La Jolla, CA, USA).

7. Research Ethics

The study was approved by Medical Research Ethics Committees of Dr. Moewardi General Hospital, Surakarta (No.1.335/I/-HREC/2020). All procedures were perform-

ed according to the Declaration of Helsinki and written informed consents were obtained from all parents/ guardians.

RESULTS

1. Sample Characteristics

A total of 36 Down syndrome patients were enrolled. These subjects were grouped based on the presence (n=18) and absence (n=18) of PH. Most of patients in the PH group were male sex (72.2%) with a normal nutritional status (44.4%) or undernourished (44.8%). The most pronounced clinical symptom was a poor weight gain (88.9%). Half of these subjects had congenital heart defects (CHD) (50%) with the predominance of atrial septal defect (38.9%). Details of the characteristics were described in Table 1.

Table 1. Clinical Characteristics of Study Participants

Characteristics	Diagnosis		p
	Pulmonary Hypertension (n=18)	Non-Pulmonary Hypertension (n=18)	
Age (years)[a]	2.23 ± 1.33	2.31 ± 1.49	0.970
Sex [b] (n (%)			0.090
Male	13 (72.2)	8 (44.4)	
Female	5 (27.8)	10 (55.6)	
Nutritional Status[b]			0.490
Normal	8 (44.4)	9 (50)	
Undernourished	8 (44.4)	5 (27.8)	
Malnourished	2 (11.1)	4 (22.2)	
Symptoms[b]			0.010*
None	1 (5.6)	9 (50)	
Shortness of Breath	0 (0)	1 (5.6)	
Cyanosis	1 (5.6)	0 (0)	
Poor Weight Gain	16 (88.9)	8 (44.4)	
Congenital Heart Defects[b]	9 (50)	9 (50)	1.000
Shunt Type[b]			0.150
None	9 (50)	9 (50)	
VSD	1 (5.6)	3 (16.7)	
ASD	7 (38.9)	2 (11.1)	
PDA	0 (0)	3 (16.7)	
ASD and PDA	1 (5.6)	1 (5.6)	

Data were presented as mean with standard deviation (SD), number (n) or percentage (%). *Nutritional Status was determined using growth chart for Down Syndrome (Cronk et al., 1988). Differences between groups were analyzed using Mann-Whitney U or Chi-Squared/Fisher Exact Test. *= $p < 0.05$. Abbreviations: Ventricular Septal Defect (VSD), Atrial Septal Defect (PDA) and Persistent Ductus Arteriosus (PDA).

2. Differences of caspase-3 level in children with down syndrome according to the presence of pulmonary hypertension and congenital heart defects

Down syndrome subjects with PH had a higher plasma caspase-3 compared to those without PH (mean with standard deviation [Mean ± SD] of 12.63 ± 12.85 ng/mL vs 1.17 ± 1.68 ng/mL, $p<0.001$) (Figure 1A). In

subjects with CHD, caspase-3 level was also higher in those with PH compared to those without PH (3.71 ± 2.89 ng/mL vs 1.83 ± 2.21 ng/mL, p=0.145) although not statistically significant (Figure 1B). Interestingly, in the absence of CHD, caspase-3 level was also much higher in PH patients compared to those without (21.55 ± 12.79 ng/mL vs 0.50 ± 0.28 ng/mL, p<0.01) (Figure 1C). Furthermore, using a receiver-operating characteristic (ROC) analysis, we obtained that caspase-3 determined the presence of PH in all Down syndrome patients (AUC 0.88, CI 0.76 to 0.99), patients with CHD (AUC 0.71, CI 0.45 to 0.95) and patients without CHD (AUC 1.0, CI 1.0 to 1.0) (Figure 1A-C). Therefore, caspase-3 was best in predicting the presence of PH particularly in Down syndrome patients without CHD.

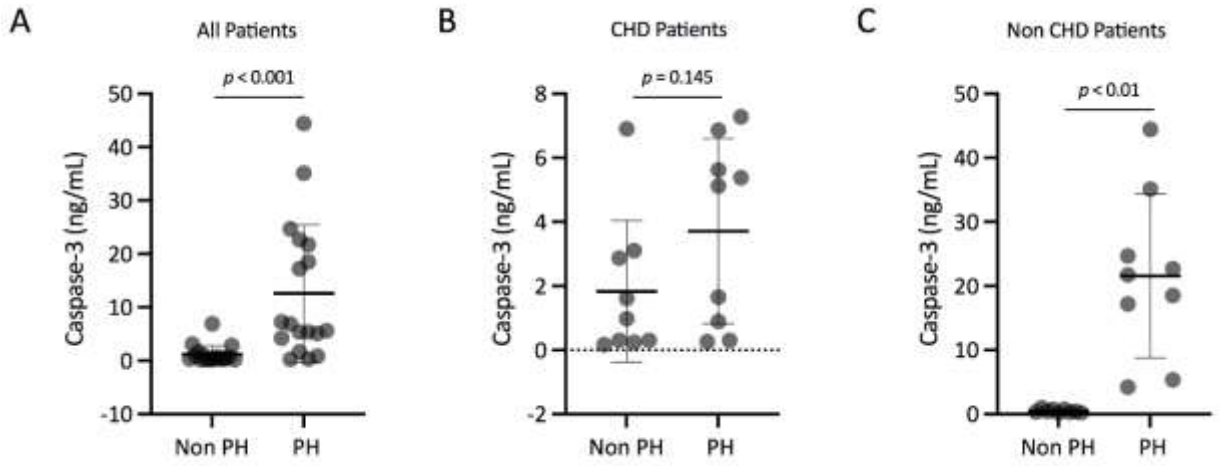

Figure 1. Circulating caspase-3 (ng/mL) in all Down syndrome patients (A), Down syndrome patients with congenital heart defects (B) and Down syndrome without congenital heart defects (C). Depicted are individual data together with lines showing mean with standard deviation range. Differences in between groups were analyzed using the Independent Student's t-test or Mann-Whitney U test. Abbreviations Congenital Heart Defects (CHD), Pulmonary Hypertension (PH).

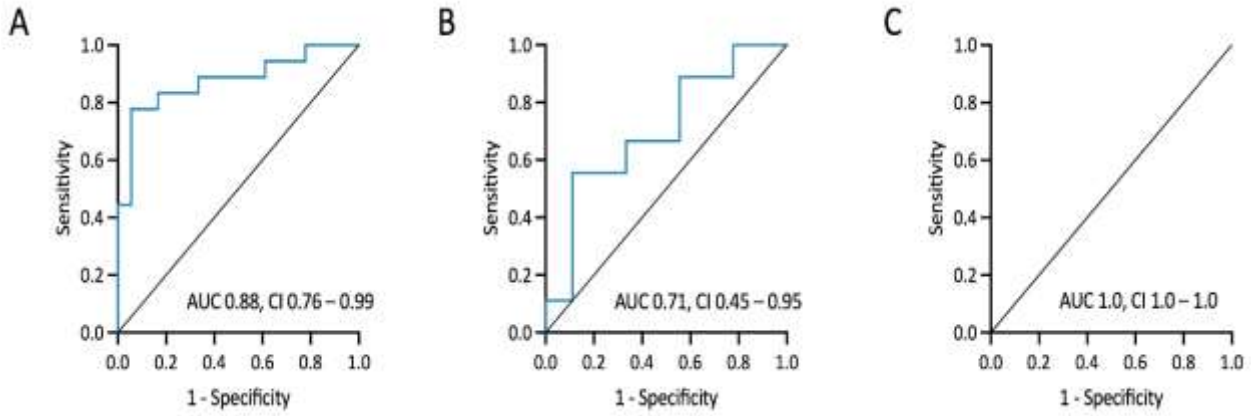

Figure 2. Receiver operating characteristic (ROC) analysis. ROC curves show the sensitivity and specificity of caspase-3 in the incidence of pulmonary hypertension in all Down syndrome patients (n=36) (**A**), Down syndrome patients with CHD (n=18) (**B**) and Down syndrome patients without CHD (n=18) (**C**). Abbreviation: Congenital Heart Defects (CHD).

3. Caspase-3 as a predictor of pulmonary hypertension incidence in children with Down syndrome

We further determined a cut-off value of plasma caspase-3 level of 1.64 ng/mL for Down syndrome patients in general or with CHD whereas 2.61 ng/mL for Down syndrome patients without CHD. Our risk analysis demonstrated that Down syndrome patients with plasma caspase-3 level ≥ 1.64ng/mL were at risk of having PH by 5 times (p<0.001) (Table 2). Down syndrome patients with CHD also have 2 times risk of having PH (p=0.35) although it was not statistically significant. All non-CHD Down syndrome patients and with plasma caspase-3 level ≥ 2.61 ng/mL were strongly associated with the incidence of PH.

Table 2. The association of increased plasma caspase-3 and risk of developing pulmonary hypertension in Down syndrome patients

Variable	PH	Non-PH	RR	95%CI		p
				Lower limit	Upper limit	
Caspase-3 **(Total)**						
≥ 1.63 ng/mL	15	3	5.00	1.74	14.34	<0.001**
< 1.64 ng/mL	3	15				
Caspase-3 **(CHD)**						
≥ 1.64 ng/mL	6	3	2.00	0.71	5.62	0.347
< 1.64 ng/mL	3	6				
Caspase-3 **(Non-CHD)**						
≥ 2.61 ng/mL	9	0	-	-	-	<0.001**
< 2.61 ng/mL	0	9				

Risk analysis was performed using Chi-Squared/Fisher exact test; ** = p < 0.001.
Abbreviations: Confidence Interval (CI), Pulmonary Hypertension (PH), Relative Risk (RR).

DISCUSSION

This current study demonstrates that Down syndrome children with pulmonary hypertension in general have a higher plasma caspase-3 level compared to those without pulmonary hypertension. Down syndrome children with CHD and PH also have a higher plasma caspase-3 level compared to those with CHD but without PH. Furthermore, plasma caspase-3 level is significantly highest in Down syndrome children with pulmonary hypertension in the absence of congenital heart defects.

Pulmonary hypertension is a significant comorbidity of children with Down syndrome (Bush et al., 2020, Bush et al., 2018, Saji, 2014). The underlying cause remains unknown, although chromosome 21 trisomy is one of the main contributors (Naumburg et al., 2017). Endothelial cell apoptosis is one of the speculated contributors involved in its pathogenesis (Sakao et al., 2009). Apoptosis of endothelial cells in PH is demonstrated to be mediated through programmed cell death-4 (PDCD4) axis and caspase-3 (White et al., 2014). This apoptotic process is closely related to the vascular remodeling process which contributes to the vascular wall thickening and PH (Happé et al., 2015). Plasma caspase-3 activity is previously demonstrated as an apoptosis marker in patients with atherosclerosis and sepsis (Lorente et al., 2016, Matulevicius et al., 2008). Caspase-3 activity is also increased in cerebral neurons (Stadelmann et al., 1999) as well as myocardial cells (Jiang et al.,

2010) of Down syndrome children. Therefore, in line with our current findings, it is speculated that the increased plasma caspase-3 level in children with Down syndrome and pulmonary hypertension may be due to the increased apoptotic activity of pulmonary vascular endothelial cells.

Congenital heart defect is also an important comorbidity which contributes to the development of pulmonary hypertension in children with Down syndrome (Bush et al., 2018; Espinola-Zavaleta et al., 2015). Intriguingly, although the plasma caspase-3 level of Down syndrome children with CHD and pulmonary hypertension is higher than those without pulmonary hypertension, it is not statistically significant. This precludes the possibility of caspase-3 involvement in this group and further can be explained by several alternative pathways. First, pulmonary hypertension in CHD patients is rather due to a mechanical compensation of the presence of a shunt, as most CHD patients in this study have atrial septal defect (ASD). Second, the mechanical pressure directly implicates to the endothelial cell proliferation and remodeling (Pascall and Tulloh, 2018; Van Der Feen et al., 2017) and followed by the imbalance of vasoactive mediators (Lau et al., 2017). In addition, it is generally acknowledged with the reversibility of pulmonary hypertension after shunt correction (Lévy et al., 2007) in which is known to be best performed prior to the development of plexiform lesion and intimal layer fibrosis (Van Der Feen et al., 2017). Third, apoptotic mechanism of endothelial cells is inhibited by hyper-proliferation response of endothelial cells which are resistant to apoptosis (Lévy et al., 2007, Sakao et al., 2005). Therefore, the plasma caspase-3 is not as high as compared in pulmonary hypertension patients without CHD.

Our finding revealed that plasma caspase-3 levels in children with Down syndrome and pulmonary hypertension is significantly the highest in the absence of CHD. This further corroborates with the notion that endothelial apoptosis mediates the pathogenesis of pulmonary hypertension in this group. In a mice model study, inhibition of caspase-3/8 activity prevents endothelial dysfunction due to endotoxemia shock (Matsuda et al., 2007). Caspase-3/8 RNA interference by α-1 antitrypsin (AAT) also regulates lung endothelial cell apoptosis (Matsuda et al., 2007; Petrache et al., 2006). Modulating PDCD4 micro-RNA (mRNA), a pro-apoptotic regulator of caspase-3 activity, is also proven to inhibit endothelial cell apoptosis (White et al., 2014). Our results further suggest that caspase-3 plays more role in the pathogenesis of pulmonary hypertension in Down syndrome patients without CHD. There are several possible explanations regarding this. First, in non CHD Down syndrome patients, other comorbidity such as chronic airway obstruction is observed (Mcdowell and Craven, 2011). This chronic hypoxia underlies a generalized pulmonary vasoconstriction as a response to hypoxia-inducible factor 1-alpha (HIF-1α) (Chan, 2013). Second, pulmonary hypoplasia in Down syndrome patients also contributes to this generalized pulmonary vasoconstriction and due to a reduced lung elasticity, so that it is prone to other pulmonary diseases (Bush et al., 2020). Third, Nitric Oxide (NO) is an important molecule which regulates vascular vasodilation, and NO deficiency is one of contributors in developing pulmonary hypertension (Lau et al., 2017). NO regulates endothelial cell survival as well (Dimmeler and Zeiher, 1999) and therefore the declining of NO production increasing the risk of endothelial cell apoptosis. Finally, chronic hyperinflammation is also more commonly observed in this group and potentially induces endothelial dysfunction in a longer period

(Bloemers et al., 2010).

Using a cut-off value ≥ 1.64ng/mL, plasma caspase-3 is also potentially used to predict pulmonary hypertension incidence in Down syndrome children, particularly in those without CHD. A cut-off value ≥ 2.61 ng/mL of caspase-3 is also significantly associated with the increasing risk of having pulmonary hypertension in non-CHD Down syndrome children. Another potential clinical implication is including the recommendation of antioxidant supplementation in Down syndrome children to minimize the intensification of pulmonary hypertension, as antioxidant is previously known to prevent and inhibit apoptotic activity (Aoki et al., 2001).

Our study provides possible link between the involvement of caspase-3 and endothelial apoptosis in Down syndrome children with pulmonary hypertension as well as a bridge for its use as a pulmonary hypertension biomarker in the clinic. Nevertheless, we acknowledge several limitations of our study. Firstly, we measured plasma caspase-3 and echocardiography in a single time point and therefore limit the use to assess progressivity of disease. Secondly, we also cannot rule out the possibility of other than endothelial cells as the source of caspase-3 in plasma of these patients.

In conclusion, our study suggests that increased plasma caspase-3 level in Down syndrome is associated with the increasing risk of having pulmonary hypertension regardless the presence of congenital heart defects.

AUTHOR CONTRIBUTION

Damayanti Ika Prasanti was the main author who conducted the study, processed data analysis, and wrote the manuscript. Sri Lilijanti Widjaja examined the background and discussion of the study. Evi Rokhayati formulated the framework study.

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The study was self-funded.

CONFLICT OF INTERESTS

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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