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Inflammatory biomarkers in very preterm infants during early intravenous paracetamol administration

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ABSTRACT

Background: Paracetamol promotes early closure of patent ductus arteriosus (PDA), and it may affect inflammation after preterm birth.
Objective: The aim of this study was to evaluate the association between paracetamol treatment and serum inflammatory biomarkers in very preterm infants with respiratory distress.
Study design: The infants were randomly assigned to intravenous paracetamol or placebo during the first 4 days of life, and others received a lower dose of paracetamol unblinded. Serum samples were used for the analysis of 10 cytokines, C-reactive protein (CRP) and malondialdehyde (MDA). The impact of paracetamol on the biomarkers was evaluated, based on the levels during the early (<60 h) and the later (60–120 h) postnatal age.
Results: Altogether, 296 serum samples from 31 paracetamol and 25 placebo group infants were analysed. Paracetamol had no effect on cytokine levels during the first 60 h when most induced PDA contractions took place. Later paracetamol treatment was associated with lower serum levels of several cytokines, including interleukin (IL-) 10, interferon gamma-induced protein (IP-) 10, and monocyte chemoattractant protein-1. CRP levels were lower in the paracetamol group during the early treatment. Amongst the infants who had severe morbidities, MDA was higher ($p = .045$), regardless of paracetamol treatment.
Conclusion: No significant differences in the cytokine levels were evident between the treatment and placebo groups. However, during early treatment, CRP levels were lower in the paracetamol group. To clarify whether this was due to a decrease in cardiopulmonary distress, or a distinct anti-inflammatory effect, requires further studies.

1. Introduction

Small preterm infants are susceptible to bronchopulmonary dysplasia (BPD), intraventricular haemorrhage (IVH), necrotising enterocolitis (NEC) and retinopathy of prematurity (ROP), which are associated with variable inflammatory responses and oxidative stress during early adaptation [1–4]. Soon after very preterm birth, the concentrations of specific cytokines and other inflammatory biomarkers have moderate power in the prediction of morbidities [5–7]. Unwanted consequences of prematurity have also been associated with early hyperoxic and hypoxaemic events; these cascades have been suggested as initiated by oxidative stress [8]. Malondialdehyde (MDA) is an oxidative stress biomarker, and increased indices of lipid peroxidation, including MDA levels, have been associated with neonatal morbidities in preterm infants [9–11].

Studies on the mechanisms of paracetamol (acetaminophen) action have focused on the decrease in prostaglandin synthesis as a result of the inhibition of the peroxidase subcomponent of the prostaglandin

Abbreviations: ANOVA, analysis of variance; BPD, bronchopulmonary dysplasia; CRP, C-reactive protein; IAM, index of acute morbidity; IL, interleukin; IP, interferon gamma-induced protein; IQR, interquartile range; ISM, index of serious morbidity; IVH, intraventricular haemorrhage; MCP, monocyte chemoattractant protein; MDA, malondialdehyde; MIG, monokine induced by gamma interferon; NEC, necrotising enterocolitis; NICU, neonatal intensive care unit; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity; SNAPPE-II, score for neonatal acute physiology with perinatal extension-II; TNF, tumour necrosis factor; VLGA, very low gestational age.

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synthase enzyme [12]. In a prophylactic setting, paracetamol constricted ductus arteriosus earlier than a placebo, and it has been suggested as a novel option in the treatment of patent ductus arteriosus (PDA) in preterm infants [13–15]. In the central nervous system, anti-inflammatory responses have been found after decreased prostaglandin synthesis by production of paracetamol metabolite N-arachidonoylphenolamine and after cannabinoid receptor antagonism [16,17]. In a cultured brain neuronal model, paracetamol showed anti-inflammatory properties by inhibiting the expression of inflammatory cytokines and chemokines and acted as an antioxidant by increasing the survival of cells exposed to the oxidant stressor menadione [18].

Although it has been proposed that paracetamol has no extracerebral anti-inflammatory properties, other possible target cells have been detected: studied in vitro, paracetamol prevented oxidative burst and delayed apoptosis in human neutrophils and increased the efficacy of mitochondrial oxidative phosphorylation [19,20]. Since paracetamol acutely constricts the ductus arteriosus soon after very preterm birth, it may have systemic anti-inflammatory properties amongst these high-risk patients [21]. We hypothesised that paracetamol may have an anti-inflammatory influence on preterm infants shortly after birth. Therefore, as part of a prospective trial of paracetamol treatment, we analysed inflammatory and oxidative stress biomarkers, including multiple cytokine, CRP and MDA levels in the sera of infants participating in prospective trials [22,23].

2. Methods

2.1. Participants

In a randomised placebo-controlled trial, infants with very low gestational age (VLGA; gestation <32 weeks) were assigned to intravenous paracetamol or placebo for 4 days (Premature infants' Paracetamol Study, PreParaS; NCT01938261; EudraCT 2013–008142-33) [15]. This is a follow-up to the original trial which has been described in detail [15,24,25]. The paracetamol loading dose was 20 mg/kg, followed by 7.5 mg/kg every 6 h for 4 days. Additionally, in a prospective paracetamol dosing study, eight preterm infants received lower trial doses (i.e. after the same loading dose, they received 7.5 mg/kg every 12 h for 4 days). Cardiac ultrasounds were performed before, daily during and 1 day after administration of the study drug, as previously described [15]. The ethical board of the hospital district approved both the original trial investigation plan and the prospective open paracetamol dosing study amendment using leftover blood specimens. All the parents gave their informed written consent.

2.2. Study samples

Serum samples were saved from all the infants during the study drug period. The leftover serum of all the routine laboratory blood samples was recovered, recorded and immediately stored at −70 °C. All the samples were analysed as duplicates. The mean values from two consecutive measurements were used. The following cytokine/chemokine serum levels were quantitated by an immunoassay (BD Biosciences, San Diego, CA, USA): interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, monokine induced by gamma interferon (MIG), RANTES and tumour necrosis factor (TNF). According to some previous studies, preterm infants' early cytokine levels are known to change rapidly during the first days of life [5,33,35]. Therefore, for further analysis of the paracetamol impact, the serum samples were divided, according to postnatal age at sampling during the study drug, into early (<60 h after birth) and late (60–120 h) samples. CRP values were taken as part of the clinical laboratory follow-up. The first reported CRP values were taken upon arrival to NICU, at next the morning after starting the study drug, and thereafter, CRP is reported daily during the study drug. It was measured by a photometric, immunoturbidimetric, accredited method (Nordlab, Northern Ostrobothnia Hospital District, Oulu, Finland) with a cut-off level of 10 mg/L. CRP levels <10 mg/L were marked as 0. MDA was measured using a chemical method (MDA assay kit, Abnova Corporation, Taipei City, Taiwan). The serum paracetamol levels were analysed using a paracetamol assay kit (Cambridge Life Sciences Ltd., Ely, UK). The sample blanks without colour reagent served as the reference. No change in paracetamol concentration was detected after prolonged storage. The inter- and intra-assay variation coefficients were calculated for the individual assays; they were less than 18.1% and 14.1%, respectively.

2.3. Evaluation of morbidities

For perinatal co-morbidities of the enrolled neonatal subjects, Score for Neonatal Acute Physiology with Perinatal Extension-II (SNAPPE-II) was used to characterize severity at early postnatal life [26]. Additionally, for the analysis of acute and chronic morbidity, the study infants were divided into two groups according to severe complications of prematurity diagnosed during hospitalisation and the need for intensive care treatments. Two composite indexes of severe morbidity were used: the index of acute morbidity (IAM) was considered positive (IAM+) if an infant had more than one dose of surfactant, inhaled nitric oxide therapy, ibuprofen and/or surgical PDA therapies. Infants who needed none of these treatments were IAM-negative (IAM-). If an infant presented any severe respiratory (BPD grades 2–3), neurological (IVH grades 3–4) or gastrointestinal (NEC stage 3) morbidities or had any ROP treatment during first hospitalisation, the index of serious morbidity (ISM) was considered positive (ISM+) [9]. ISM-negative (ISM-) infants had none of these morbidities or therapies. Severe BPD grades 2–3 was defined according to the need for supplemental oxygen and/or respiratory assistance at 36 weeks' postconceptional age [27]. Severe IVH grades 3–4 and NEC stage 3 were defined as previously described [28,29]. ROP treatments included intraocular injections of vascular endothelial growth factor antagonists and laser therapy [30].

2.4. Statistical analyses

For statistical analyses, IBM SPSS Statistics version 25 (IBM, Armonk, NY, USA) was used. Along with the original trial sample size calculation, the effect size of the proportions was estimated with the available sample size of this study. For instance, with the baseline change of CRP 13.7 (SD 16.0) mg/L during the study drug, present study had 80% power ($\alpha = 0.05$) to detect 12.27% change in the CRP values. This is likely clinically significant. The independent samples *t*-test, the chi-squared test, the non-parametrical Mann–Whitney *U* test, and the Pearson correlation were used as appropriate. Serial measurements were tested with the repeated measurements' analysis of variance (ANOVA). The significance was set at $p < .05$.

3. Results

Altogether, 296 serum samples were studied from 31 paracetamol-exposed infants and 25 placebo group infants. Their characteristics are presented in Table 1. Amongst the infants of the original trial, ductal closure tended to be more frequent in the paracetamol group ($p = .016$, Table 1) [15]. During administration of the study drug, no blood culture-positive septicaemia or viral antigen positivity in the respiratory track samples was detected. During their neonatal intensive care unit (NICU) stay, the study infants had 23 clinical infections altogether, and antibiotics were started, but the blood cultures were positive only in eight cases.

The medians (interquartile range [IQR]) of all the cytokine concentrations did not differ between the placebo ($n = 50$) and the treatment ($n = 61$) groups' samples (data not shown). The cytokine concentration changes from the early to the late samples were analysed by repeated measures ANOVA between the two study groups, and no significant

Table 1
Patient characteristics.

	Paracetamol group, $n = 31$		Placebo group, $n = 25$
	Preparas trial, $n = 23$	Open study, $n = 8$	
Gestational age at birth, wk., mean (SD)	28.4 (2.4)	27.8 (2.3)	28.3 (2.1)
Birth weight, kg, mean (SD)	1.22 (0.4)	1.15 (0.4)	1.12 (0.3)
Birth weight *Z*-score, mean (SD)	−0.97 (1.3)	−0.79 (1.3)	−1.33 (1.4)
Small for gestational age, n (%)	5 (22)	2 (25)	7 (28)
Preeclampsia, n (%)	6 (26)	2 (25)	7 (28)
Maternal antenatal infection, n (%)	9 (39)	1 (13)	6 (24)
Maternal antenatal steroids, n (%)	20 (87)	7 (88)	25 (100)
Cesarean section, n (%)	15 (65)	5 (63)	20 (80)
Singleton, n (%)	16 (70)	6 (75)	15 (60)
Male, n (%)	13 (57)	3 (38)	14 (56)
Apgar score at 1 min, median (range)	7 (2–9)	6 (3–8)	6 (1–9)
Apgar score at 5 min, median (range)	7 (4–10)	7.5 (3–9)	7 (2–9)
Respiratory distress syndrome, n (%)	17 (74)	4 (50)	22 (88)
Surfactant, n (%)	16 (70)	4 (50)	22 (88)
Intratracheal ventilation, n (%)	15 (65)	4 (50)	21 (84)
Assisted ventilation, days, median (range)	11.0 (0–56)	19.5 (1–199)	12.0 (1–51)
Supplemental oxygen >0.5, n (%)	7 (30)	1 (13)	11 (44)
Supplemental oxygen, days, mean (SD)	20.0 (24.5)	10.2 (23.5)	22.4 (25.0)
Bronchopulmonary dysplasia, grades 2–3, n (%)	0	2 (33)	1 (4)
Patent ductus arteriosus, n (%)	4 (17)	3 (38)	7 (28)
Postnatal age at ductal closure, h, mean (SD)*	177 (338)*	590 (238)	336 (517)*
Blood culture positive or viral infection, n (%)	8 (35)	3 (38)	8 (32)
Transfusions, n (%)	8 (35)	4 (50)	10 (40)
Parenteral nutrition, days, median (range)	7.0 (0–33)	7.5 (4–28)	7.0 (3–29)
Necrotising enterocolitis, stage 3, n (%)	0	0	1 (4)
Intraventricular haemorrhage, grades 3–4, n (%)	1 (4)	0	1 (4)
Transient diffuse periventricular leukomalacia, n (%)	2 (9)	0	4 (16)
SNAPPE-II, mean (SD)	27.0 (24.6)	14.4 (16.6)	23.0 (15.5)
Index of acute morbidity, positive, n (%)	8 (35)	4 (50)	10 (40)
Index of severe morbidity, positive, n (%)	1 (4)	2 (33)	3 (12)

Abbreviation: SNAPPE-II, score for neonatal acute physiology with perinatal extension-II.
* $p = .016$.

differences were revealed (Table 2). However, when the samples were divided by postnatal age into early and late samples, some trends were revealed. In both study groups, the postnatal decrease in IL-6 was significant (Table 2). IL-10 levels were mostly low. However, a significant decrease in IL-10 was found in the paracetamol group, while IL-10 increased significantly in the placebo group. The IP-10 and MCP-1 levels decreased in the paracetamol group (Table 2).

The serum concentrations of CRP were recorded upon arrival to NICU, and daily during the administration of the study drug, (Table 3). The CRP levels tended to be lower in the paracetamol group during the study drug administration; the difference was significant in the early (age < 60 h) samples (mean [standard deviation] 4.8 [7.2] vs. 11.1 [13.7] mg, $p = .026$) and during the first day (mean [SD] 4.2 [6.2] vs. 10.2 [11.8] mg/L), $p = .016$).

To evaluate oxidative stress during the first days of life, the MDA levels were measured from 42 infants' sera. There was no difference in the median (IQR) MDA levels between the paracetamol and the placebo groups: 9.2 (2.8) vs. 11.0 (6.1) nmol/mL, $p = .582$. The median (IQR) postnatal age of sampling did not differ between the treatment groups at 99.0 (103.0) vs. 129.5 (77.3) hours, respectively ($p = .068$). No associations between mean MDA levels (nmol/L) and SNAPPE-II scores were detected (Pearson correlation $r = -0.152$, $p = .344$). However, the IAM+ infants had significantly higher median (IQR) MDA levels when compared to the IAM- infants: 11.4 (5.5) vs. 9.0 (2.9) nmol/mL, $p = .045$. No significant difference between the ISM+ and the ISM- infants was found: median (IQR) MDA 9.9 (7.5) vs. 9.5 (4.3) nmol/mL, $p = .547$. The paracetamol subgroup analyses did not reveal any significant differences either.

4. Discussion

The present prospective study of inflammatory and oxidative stress biomarkers in preterm infants found a trend between early intravenous paracetamol use and decreased inflammatory biomarker levels. The following discussion deals with a possible significance of these findings.

According to previous studies on the cytokine levels during the early neonatal period in VLGA infants, these inflammatory mediators have wide range of blood concentrations that depend in part on the postnatal age [5]. In the paracetamol-treated infants, seven (70%) cytokines/chemokines decreased postnatally, and in five (IL-6, IL-8, IL-10, IP-10, MCP-1), the change from the early to late samples was significant (Table 2). In the placebo group, from the early to the late samples, IL-6 decreased significantly, IL-8 had a decreasing trend, and IL-10 increased significantly, while the others remained unchanged (Table 2). However, these differences between the two groups were not significant when evaluated with a repeated measures test. This may be explained on the basis of a rather small sample size and lack of prospectively defined age of recovering the specimens. In previous studies, the early cytokine storm during the first week of life, most notably the high levels of IL-8 and IL-10 had a modest association with the long term morbidities, particularly BPD [5,31–33]. Conversely, high cord blood cytokines have been associated with spontaneous preterm birth in chorioamnionitis [5,34]. Particularly after elective births without labour, high IL-8, IL-10 and matrix metalloproteinase-9 (MMP-9) levels during the first week associated with the risk of BPD [5,7,33]. A subsequent study confirmed that high inflammatory cytokines at birth due to chorioamnionitis were not associated with BPD [35]. Preterm infants shortly after birth have also been reported to have an anti-inflammatory shift in cytokine reaction to sepsis that may be associated with hypo-responsiveness, similar to sepsis-induced immunosuppression observed in adults [36,37]. Paracetamol's effect on cytokine expression was studied in a trial of adults requiring intensive care, and similar decreasing profiles of IL-6 and IL-10 were found in the paracetamol group [38]. Furthermore, studies using cells from the cerebral cortex, revealed that paracetamol inhibited expression of IL-1α, IL-1β, TNF, macrophage inhibitory protein (MIP)-1α and RANTES [39]. In the present study, the decreasing trend of the cytokines suggest an anti-inflammatory role for paracetamol. However, the fact that most of the present patients had an uneventful recovery complicates the evaluation of the systemic effect.

CRP has been considered an index of tissue inflammation, usually increasing in a delayed manner with the onset of inflammation and decreasing as inflammation resolves [40]. Increased levels have also been used as a sign of neonatal microbial infection, although the evidence from the early neonatal period does not support this association [7,41,42]. CRP, however, plays important roles in inflammatory processes and host responses to infection, including the complement pathway, apoptosis, phagocytosis, nitric oxide release and the production of cytokines, particularly IL-6 and TNF [40]. In terms of pro-inflammatory cytokine production, CRP increases IL-8 and MCP-1 production [42]. During the first week of life, elevated plasma levels of CRP predict the risk of BPD (odds ratio 3.4, $p = 2.9 \times 10^{-4}$) [7]. We found decreases in the early CRP levels during paracetamol treatment.

Table 2
Median (range) inflammatory biomarker levels (pg/mL) during the early (<60 h) and late (60-120 h) study drug periods.

	Paracetamol group patients, n = 31			Placebo group patients, n = 25			ANOVA**
	Early samples, *n* = 35	Late samples, *n* = 26	*p**	Early samples, n = 23	Late samples, *n* = 27	*p**	*p*
IL-1β	0 (18.1)	0 (8.0)	0.455	0 (32.3)	0 (4.5)	0.337	0.555 (12/16)
IL-6	42.0 (8470.6)	10.3 (29.5)	<0.001	56.6 (27,543.2)	13.0 (3552.6)	0.003	0.390 (12/16)
IL-8	142.8 (995.9)	109.8 (343.7)	0.028	174.0 (4623.8)	84.2 (2263.0)	0.082	0.217 (12/16)
IL-10	0 (40.4)	0 (1.7)	0.003	0 (35.4)	0 (272.3)	0.045	0.318 (12/16)
IL-12p70	0 (0)	0 (0)	1.000	0 (0)	0 (14.9)	0.234	0.232 (12/16)
TNF	0 (0)	0 (0)	1.000	0 (272.3)	0 (0)	0.404	0.379 (12/16)
IP-10	124.9 (383.4)	97.7 (266.3)	0.027	110.8 (1014.9)	111.3 (1476.5)	0.806	0.265 (13/16)
MCP-1	776.6 (15,388.5)	564.3 (72,060.1)	0.040	693.8 (12,267.3)	596.6 (24,923.1)	0.623	0.362 (13/16)
MIG	79.8 (240.7)	84.6 (127.8)	0.554	105.4 (1415.0)	94.8 (232.0)	0.311	0.222 (13/16)
RANTES	4551.4 (36,032.3)	4172.4 (36,050.6)	0.127	4485.8 (30,289.5)	4551.4 (33,774.4)	0.596	0.514 (13/16)

Abbreviations: IL, interleukin; IP-10, interferon gamma induced protein-10; MCP-1 monocyte chemoattractant protein-1; MIG, monokine induced by gamma interferon; TNF, tumour necrosis factor.
* Mann–Whitney *U* test.
** Repeated measures ANOVA. Patient numbers are included in the brackets (paracetamol group/placebo group).

Table 3
C-reactive protein mean (SD) plasma levels of the study patients. Baseline values were taken upon arrival to intensive care. Day 1 values were taken after starting the study drug.

C-reactive protein, mg/L	Paracetamol group n = 31	Placebo group n = 25	*t*-Test *p*	Repeated measures ANOVA *p*
Highest value during the study drug	7.0 (9.9)	13.7 (16.0)	0.058	-
Highest value during hospitalisation	25.2 (39.8)	32.2 (58.2)	0.594	-
Early samples (age < 60 h)	4.8 (7.2)	11.1 (13.7)	0.026	0.057
Late samples (age 60–120 h)	3.5 (6.3)	6.8 (11.4)	0.175	
Baseline	0.1 (0.6)	0.6 (2.8)	0.371	0.057
Day 1	4.2 (6.2)	10.2 (11.8)	0.016	
Day 2	5.4 (8.6)	11.9 (16.2)	0.058	
Day 3	4.3 (7.4)	8.2 (12.4)	0.146	
Day 4	2.7 (5.2)	5.4 (10.7)	0.228	

Previously, early paracetamol medication has been found to constrict the ductus arteriosus and improve the oxygenation of preterm infants [15,24]. Thus, we cannot exclude the possibility that besides the paracetamol-induced closure of PDA, the attenuated inflammatory biomarker profile may have contributed to the favourable effect of paracetamol on peripheral and cerebral oxygenation [24]. For the patients in the present study, two different and quite small study doses were used; whether the findings become dose-dependent at higher serum concentrations remains to be studied.

Oxidative stress has been associated with morbidities in preterm infants [8,10,11]. In practice, appropriate levels of supplemental oxygen are used to avoid oxidative stress factors amongst others [43]. MDA, a biomarker of oxidative stress, has been studied in preterm infants and associated with antenatal infection, respiratory distress syndrome and long-term complications of prematurity [11,44,45]. We found that preterm infants without major acute morbidities during the first week of life (IAM-) had lower MDA levels when compared to IAM+ infants. By contrast, in the ISM+ infants, no difference was detected. It is not known whether the low MDA levels in IAM- infants were due to diminished early oxidative stress or other confounding factors. Paracetamol did not impact the MDA levels. According to studies using isolated cells, paracetamol modulates the inflammatory process, for example by preventing neutrophil oxidative burst, increasing the survival of neurons and endothelial cells exposed to oxidative stress and decreasing mitochondrial free radical generation in vitro [18–20,39].

This study had some limitations. The number of patients was small, and the subgroup analyses may have been affected by unknown biases. In addition, the present paracetamol dosage was rather low, although the clinical consequences were detectable. The CRP laboratory method was not optimal for research purposes but merely a routine laboratory test with a high detection level. However, the trial patients were randomly assigned, and all the samples were prospectively collected for clinical assessment. Finally, only a few serious adverse events were observed in both the treatment and the control groups [15]. The acute and long-term outcomes are multifactorial, and the role of one medication may be limited. Our results do not rule out the possibility that paracetamol may actually have systemic anti-inflammatory properties that may prove to be beneficial.

5. Conclusion

In preterm infants, the levels of several cytokines decreased during the intravenous paracetamol treatment. However, there were no significant differences in the cytokine levels between paracetamol and placebo treatment groups. During the first 40 h of paracetamol treatment, the CRP levels were lower than in the placebo group. Paracetamol caused an acute contraction of ductus arteriosus, implying that it has a systemic effect. Further studies are required to elucidate whether paracetamol directly influences the systemic inflammatory mediators or whether this was the consequence of a decrease in circulatory stress.

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CRediT authorship contribution statement

Outi Aikio: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Antti Härmä:** Conceptualization, Formal analysis, Investigation, Visualization, Writing – review & editing. **Pia Härkin:** Conceptualization, Data curation, Investigation, Writing – review & editing. **Markku Leskinen:** Conceptualization, Validation,

Investigation, Supervision, Writing – review & editing. **Marita Valkama:** Conceptualization, Validation, Investigation, Writing – review & editing. **Timo Saarela:** Conceptualization, Resources, Supervision, Writing – review & editing. **Annamari Salminen:** Conceptualization, Data curation, Formal analysis, Validation, Writing – review & editing. **Mikko Hallman:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

None declared.

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