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Original Article

Etiology and clinical profile of neonates with pathological unconjugated hyperbilirubinemia with special reference to Rhesus (Rh) D, C, and E incompatibilities: A tertiary care center experience

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ABSTRACT

Objective: To assess etiology, clinical profile, and outcome of pathological neonatal unconjugated hyperbilirubinemia, including various Rhesus (Rh) blood group incompatibilities.

Methods: This was a prospective observational study of 200 neonates, with unconjugated hyperbilirubinemia requiring phototherapy and/or exchange transfusion, presented at tertiary care neonatal unit during August 2011 to July 2012. Detailed history, physical examination, and laboratory work-up were performed. In case, if major blood group incompatibility was found negative, the blood was further processed for G-6PD status, hereditary spherocytosis, and for non-Rh D incompatibility by testing non-Rh D antigens status in mother–infant pair.

Result: ABO incompatibility (64 cases, 32%), Rh D incompatibility (28 cases, 14%), and sepsis (42 cases, 21%) were major causes of neonatal unconjugated hyperbilirubinemia, while the etiology could not be ascertained in 31 (15.5%) cases. Rh C and Rh E incompatibilities were observed in 28 (14%) and 6 (3%) cases, respectively among those neonates who did not exhibit major blood group incompatibility (ABO and Rh D). Rh C incompatibility was less severe than Rh D, and Rh E was least.

Conclusions: Blood group incompatibilities (ABO and Rh D) and sepsis are important causes of unconjugated hyperbilirubinemia in neonates requiring treatment. Though less common, non-Rh D incompatibility can contribute to severe neonatal hyperbilirubinemia, and needs to be investigated.

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1. Introduction

Neonatal hyperbilirubinemia is a common condition requiring inpatient treatment, as well as an important reason for readmission to hospital.^{1–3} It occurs in 70–80% of the neonates, more commonly in preterms.^{4,5} Although, only 5–10% of the newborns need to be treated due to pathological hyperbilirubinemia, the threat of neurologic damage always remains, especially with very high bilirubin level, in presence of certain risk factors and in cases where management remains inappropriate.^{6,7} Etiology of hyperbilirubinemia is not only crucial for optimal management of the patient but also it may have implications for subsequent pregnancies. However, the etiology of neonatal hyperbilirubinemia may remain obscured in more than half of the cases.^{8–10} Hemolytic disease of the newborn (HDN) is one of the common pathologic cause of hyperbilirubinemia during the early neonatal period, mostly due to Rhesus (Rh) incompatibility, ABO incompatibility, G6PD deficiency, and rarely induced by other alloimmune antibodies.^{9,10} Despite available guideline to perform predischarge hour specific serum bilirubin in infants and their follow-up to predict severe hyperbilirubinemia,^{7,11} wide practice of anti-D prophylaxis to prevent Rh D disease, and wide availability of effective phototherapy, exchange transfusion, and intravenous immunoglobulin for treatment of hyperbilirubinemia, neonates presenting at tertiary hospital with severe hyperbilirubinemia and bilirubin encephalopathy are not a rarity. Limited reports are available on Rh incompatibility other than Rh-D in causation of neonatal hyperbilirubinemia.^{12–15} The present study aimed to delineate the etiology of pathological unconjugated hyperbilirubinemia (requiring treatment) in neonates, study its clinical profile, and to explore the select group of patients not detected to have ABO or Rh D incompatibility, for other non-Rh D incompatibility.

2. Methods

This was a prospective observational study done at a tertiary care neonatal unit between August 2011 and July 2012. The study was approved by the Institutional Ethics Committee. The subjects were neonates with pathological unconjugated hyperbilirubinemia requiring phototherapy and/or exchange transfusion. Patients having major anomalies, weight <600 g, gestational maturity <26 weeks, and perinatal asphyxia with HIE stage 3, were excluded. History, including mother's age, parity, use of oxytocin injection during labor, period of gestation, past history of blood transfusion/abortion, place of delivery, mode of delivery, mother's blood group (ABO and Rh), predominant feed (mother's milk/other feed) before admission, and age at onset of jaundice, was recorded. Complete physical examination of the neonate was performed carefully and the data, including weight on admission, maturity, cephalhematoma, and presence of acute bilirubin encephalopathy (ABE), were recorded. Only moderate to severe grade of ABE was noted, based on mental status, muscle tone, and cry.¹⁶

All neonates were investigated for major blood group status (ABO and Rh D), total serum bilirubin (TSB), as well as direct

fraction, complete blood count, reticulocyte count, and peripheral blood smear. Serum bilirubin (total and direct) was measured by modified Malloy-Evelyn colorimetric/end point method. Sepsis screen and blood culture were performed when indicated. A diagnosis of sepsis was made when blood culture grew organism or sepsis screen was found positive (any two of the five screen parameters, namely, total leukocyte count <5000/mm³, reduced absolute neutrophil count, immature to total neutrophil ratio ≥20%, CRP ≥10 mg/L, and raised micro-ESR of ≥15 mm in first hour).^{17–19} Direct antiglobulin test (Coomb's test) by using antihuman globulin reagent was performed in cases having blood group incompatibility and/or evidence of hemolysis. A diagnosis of ABO incompatibility was made when the mother had blood group 'O' and the baby's blood group was either 'A' or 'B'. Similarly, diagnosis of Rh D incompatibility was made when the mother had Rh D negative blood group and the baby was found to be Rh D positive. When both the ABO and Rh D incompatibilities were detected in any patient, it was decided to categorize the patient under Rh D incompatibility group. In case if more than one etiology was identified in a patient, the predominant cause leading to hyperbilirubinemia was ascertained by consensus. When no ABO and/or Rh D incompatibility scenario was detected (N = 108), we performed quantitative test for G-6-PD deficiency (by G-Six test) and osmotic fragility test in the infant, and agglutination test in both the infant and the mother to detect Rh C and Rh E antigens on the RBCs. The agglutination test was performed in the Department of Transfusion Medicine using ready-to-use reagents, Eryclone (anti-C and anti-E antibody) from Tulip Diagnostics. Red blood cells possessing the C or E antigens would agglutinate in the presence of the antibodies directed toward the antigens, which are readily detected by naked eye/microscopy. Rh C and Rh E incompatibilities were regarded as a cause of neonatal hyperbilirubinemia when an infant was detected to have Rh C or Rh E antigen, while the mother was found to be negative for the same, in absence of any other explainable reason.

The decision to initiate phototherapy and to perform exchange transfusion was uniform and in accordance with unit protocol, based on American Academy of Pediatrics, clinical practice guideline – 2004,²⁰ and National Neonatology Forum, India – 2010 guidelines.²¹ The TSB level was plotted against postnatal age in hours, gestation, and risk category for starting phototherapy and doing exchange transfusion in infants >35 weeks of gestation. We considered early exchange transfusion in cases of hydrops; in Rh incompatibility, when there was history of previous sibling receiving exchange transfusion, the following were noted: cord blood hemoglobin <11 gm/dl, cord TSB >5 mg/dl, rate of rise of TSB >1 mg/dl/h despite phototherapy or rise >0.5 mg/dl/h despite phototherapy if hemoglobin is between 11 and 13 gm/dl, any TSB >12 mg/dl in first 24 h of life, and TSB >20 mg/dl in neonatal period. The phototherapy and exchange transfusion decision for preterm infants <35 weeks of gestation were in accordance with TSB, weight category, and whether the infant is healthy or sick (National Neonatology Forum, India – 2010 guidelines). Presence of ABE in any infant was also considered for doing exchange transfusion. Phototherapy was stopped when TSB level fell below 2 mg/dl lower than the phototherapy threshold for that postnatal age. Standard treatment was

Table 1 – Causes of neonatal unconjugated hyperbilirubinemia.

Causes	No. of neonates	
	N = 200	%
ABO incompatibility	64	32
Rh D incompatibility	28	14
Rh C incompatibility	28	14
Rh E incompatibility	6	3
Sepsis	42	21
G-6PD deficiency	1	0.5
Unknown	31	15.5

provided to all the subjects and intensive phototherapy was ensured for all. Repeat serum bilirubin measurements and complete hemogram were performed as indicated. Other parameters recorded were evidence of hemolysis (Hb <13 gm% or PCV <40 along with reticulocyte count >6%), peak TSB level, age (days) at peak TSB, duration of phototherapy provided (days), and need of exchange transfusion and its number. It was recorded as 'a day' when age or duration of phototherapy exceeded 12 h. For the statistical analysis of results, SPSS 16.0 software was used. Data were summarized using descriptive statistics. Continuous variable was analyzed using independent Student's t-test and chi-square test/Fisher's exact test was used to test associations between groups.

3. Results

During the study period, 1208 patients were admitted in the neonatal unit and 233 presented with hyperbilirubinemia. Of these, 200 fulfilled the inclusion criteria and were investigated. ABO incompatibility was the commonest cause (64 cases, 32%) of pathological hyperbilirubinemia, followed by sepsis (42 cases, 21%), while the cause could not be ascertained in 31 (15.5%) of the cases (Table 1). Three of the patients had both ABO and Rh D incompatibilities, and were grouped under Rh D

incompatibility. Baseline maternal and neonatal parameters of the cases having pathological hyperbilirubinemia due to various etiologies are displayed in Table 2. There was male preponderance in all the groups of causes. Rh blood group incompatibilities (Rh D, Rh C, and Rh E groups), as a cause of hyperbilirubinemia, were more commonly observed in babies born to multipara mother. Table 3 shows neonatal characteristics, lab parameters, and treatment details of the patients. Majority of the infants in Rh C incompatibility (21, 75%) and sepsis (28, 66.6%) groups were preterm. The proportion of babies presenting with hyperbilirubinemia within first 48 h of life was maximum in Rh D incompatibility group (22, 78.6%) followed by Rh C (20, 71.4%), Rh E (4, 66.7%), unknown etiology (20, 62.5%), ABO incompatibility (39, 61.9%), and sepsis (22, 53.7%) groups. Blood culture was positive in only three patients; one in each of the group belonged to sepsis, Rh D incompatibility, and G-6PD deficiency. Evidence of hemolysis was found in 10 patients (35.7%) among Rh D incompatibility group, 5 patients (17.9%) of Rh C incompatibility, and 7 patients (10.9%) of ABO incompatibility group, while none in Rh E incompatibility group. The mean peak TSB (mg/dl) was maximal in Rh D incompatibility patients (22.7 ± 8.9). The number of patients requiring exchange transfusion was maximum in Rh D incompatibility group (16, 57.1%) followed by ABO incompatibility (16, 25%), Rh C group (4, 14.3%), and one each in sepsis and unknown groups, while none in the Rh E incompatibility group.

The data were compared for various parameters between Rh D and ABO incompatibility groups and it was observed that babies born to multipara women (67.9% vs 42.2%; $p = 0.023$), evidence of hemolysis (35.7% vs 10.9%; $p = 0.005$), and higher peak TSB level (mg/dl) [mean difference 4.31, 0.82–7.80 (95%CI); $p = 0.006$] were significantly more common in Rh D group. Also, the duration of phototherapy requirement (days) [mean difference 1.08, 0.32–1.83 (95%CI); $p = 0.016$] was longer in the Rh D group and more babies of Rh D group underwent exchange transfusion (57.1% vs 25%; $p = 0.003$). Two patients in Rh D incompatibility group required exchange transfusion

Table 2 – Baseline maternal and neonatal parameters.

Parameters	ABO Incom. N = 64 (%)	Rh D Incom. N = 28 (%)	Rh C Incom. N = 28 (%)	Rh E Incom. N = 6 (%)	Sepsis N = 42 (%)	G-6PD def. N = 1 (%)	Unknown N = 31 (%)
Maternal age (yrs), mean ± SD	25.3 ± 2.9	26.2 ± 2.4	25.3 ± 2.4	24.8 ± 1.2	25.7 ± 2.8	34	25.9 ± 2.1
Parity							
Primi	37 (57.8)	9 (32.1)	15 (53.6)	4 (66.7)	21 (50)	0 (0)	14 (45.2)
Multi	27 (42.2)	19 (67.9)	13 (46.4)	2 (33.3)	21 (50)	0 (0)	17 (54.8)
Blood transfusion/abortion	7 (10.9)	1 (3.6)	3 (10.7)	2 (33.3)	7 (16.7)	0 (0)	4 (12.9)
H/o Inj oxytocin	35 (54.7)	13 (46.4)	16 (57.1)	2 (33.3)	18 (43.9)	0 (0)	17 (53.1)
Hospital born	56 (87.5)	24 (85.7)	27 (96.4)	5 (83.3)	27 (64.3)	1 (100)	26 (83.9)
Mode of delivery							
Vaginal	55 (85.9)	24 (85.7)	20 (71.4)	4 (66.7)	36 (85.7)	1 (100)	26 (83.9)
Cesarean	9 (14.1)	4 (14.7)	8 (28.6)	2 (33.3)	6 (14.3)	0 (0)	5 (16.1)
Weight (g) at admission, mean ± SD	2379 ± 726	2300 ± 703	1639 ± 573	1820 ± 620	1860 ± 600	2000	2131 ± 732
Male:female	47:17	20:8	21:7	6:0	26:16	1:0	25:6
Term	43 (67.2)	18 (64.3)	7 (25)	3 (50)	14 (33.3)	0 (0)	18 (58.1)
Preterm	21 (32.8)	10 (35.7)	21 (75)	3 (50)	28 (66.6)	1 (100)	13 (41.9)
Predominant mother's milk feeding	39 (60.9)	18 (64.3)	14 (50)	5 (83.3)	23 (56.1)	0 (0)	27 (84.4)

Table 3 – Neonatal characteristics, lab investigations, and treatment.

Parameters	ABO Incom. N = 64 (%)	Rh (D) Incom. N = 28 (%)	Rh (C) Incom. N = 28 (%)	Rh (E) Incom. N = 6 (%)	Sepsis N = 42 (%)	G-6-PD def. N = 1 (%)	Unknown N = 31 (%)
Age at admission (days), mean $\pm$ SD	2.5 $\pm$ 1.2	1.8 $\pm$ 1.3	2.8 $\pm$ 1.4	2.8 $\pm$ 0.9	3.1 $\pm$ 1.5	2.0	2.9 $\pm$ 1.1
Cephalhematoma	4 (6.3)	0	0	0	0	0	2 (6.6)
ABE (moderate/severe)	6 (9.4)	4 (14.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TSB at admission (mg/dl), mean $\pm$ SD	15.5 $\pm$ 7.0	15.7 $\pm$ 11.38	13.1 $\pm$ 7.1	13.3 $\pm$ 2.8	14.3 $\pm$ 5.31	21.3	14.9 $\pm$ 4.9
Peak TSB (mg%), mean $\pm$ SD	18.4 $\pm$ 7.2	22.7 $\pm$ 8.97	15.2 $\pm$ 6.5	15.4 $\pm$ 3.3	15.4 $\pm$ 4.9	21.3	16.1 $\pm$ 4.3
Age at peak TSB (days), mean $\pm$ SD	3.8 $\pm$ 1.3	3.4 $\pm$ 1.3	4.1 $\pm$ 1.5	4.2 $\pm$ 0.4	4.2 $\pm$ 1.7	2	3.8 $\pm$ 1.7
TSB > 20 mg/dl (no.)	12 (18.7)	6 (19.8)	5 (17.8)	0 (0)	4 (9.5)	1 (100)	5 (16.1)
Blood culture + (N = 199)	0 (0)	1 (3.6)	0 (0)	0 (0)	1 (4.8)	1 (100)	0 (0)
Sepsis screen + (N = 199)	7 (10.9)	5 (17.9)	3 (10.7)	1 (16.7)	41 (97.6)	1 (100)	0 (0)
Coomb's test							
+ve	2 (3.1)	3 (10.7)	5 (17.9)	0 (0)	0 (0)	0 (0)	0 (0)
–ve	4 (6.2)	7 (25)	11 (39.3)	3 (50)	6 (14.3)	1 (0)	10 (32.3)
Not done	58 (90.6)	18 (64.3)	12 (42.9)	3 (50)	36 (85.7)	0 (0)	21 (67.7)
Hemolysis	7 (10.9)	10 (35.7)	5 (17.9)	0 (0)	1 (2.4)	0 (0)	0 (0)
Duration of phototherapy (day), mean $\pm$ SD	7.17 $\pm$ 1.76	8.25 $\pm$ 1.45	8.06 $\pm$ 2.29	7.17 $\pm$ 2.04	7.02 $\pm$ 1.56	7.00	6.00 $\pm$ 1.46
Exchange transfusion	16 (25)	16 (57.1)	4 (14.3)	0 (0)	1 (2.4)	0 (0)	1 (3.2)

ABE, acute bilirubin encephalopathy; TSB, total serum bilirubin.

twice, while none in ABO incompatibility group required repeat exchange transfusion. On comparison of data of patients having Rh D and Rh C incompatibilities, we found that number of patients having ABE (p -value = 0.005) and number of patients needing exchange transfusion (57.1% vs 10.7%; p -value = 0.001) were significantly higher in Rh (D) incompatibility group. Similarly, higher mean peak TSB level (mg/dl) [mean difference 7.47, 3.27–11.67 (95%CI); p = 0.001] and peak TSB level at earlier age (days) [mean difference –0.75, –1.50 to 0.00 (95%CI); p = 0.05] were found in Rh D incompatibility group compared to Rh C group. On comparing patient's data between Rh C and Rh E incompatibilities, no significant differences were observed. We also evaluated the patients having ABO incompatibility to look at any difference between O-A and O-B incompatibilities. The mean age at admission (2.3 $\pm$ 1.2 vs 2.4 $\pm$ 1.7 days; p = 0.25), mean initial TSB (16.0 $\pm$ 7.1 vs 15.0 $\pm$ 7.1 mg/dl; p = 0.59), mean peak TSB (18.8 $\pm$ 6.9 vs 17.9 $\pm$ 7.5 mg/dl; p = 0.61), mean age at peak TSB (3.7 $\pm$ 1.2 vs 4.0 $\pm$ 1.4 days; p = 0.28), duration of phototherapy (6.9 $\pm$ 1.8 vs 7.5 $\pm$ 1.7 days; p = 0.17), and number of patients required for exchange transfusion (6 vs 7; p = 0.57) were similar in both O-A and O-B incompatibility groups. No differences were found in the baseline parameters among patients of both the groups.

None of the patients in either group having hyperbilirubinemia expired. However, four of the six patients having ABE in Rh D group and two of the four in Rh C group had persistent neurological abnormalities at discharge.

4. Discussion

In our study, the commonest etiology of hyperbilirubinemia requiring treatment was blood group incompatibility, followed by sepsis, which was in contrary to the report by Narang et al.⁸ from India who reported G-6PD deficiency in 17.7%, ABO

incompatibility in 6.1%, Rh isoimmunization in 2.9%, and idiopathic disease in 57.7% among 242 cases of neonatal hyperbilirubinemia (TSB >15 mg/dl). The higher incidence of G-6PD deficiency in their study relates to the higher prevalence of G-6PD deficiency in the population being catered. A Canadian study by Sgro et al.¹⁰ reported ABO incompatibility (51.6%) as commonest cause of hyperbilirubinemia, similar to us, followed by G-6PD deficiency (20%), hereditary spherocytosis (7%), and sepsis/urinary tract infection (3%). Immune-mediated hemolysis and hyperbilirubinemia are usually related to Rh D and ABO incompatibilities, and rarely due to other minor blood group incompatibilities, such as anti-C, anti-E, anti-c, anti-e, and anti-Kell.¹⁴ ABO hemolytic disease generally occurs in infants of blood group A or B born to group O mothers. Although ABO incompatibility situation exists in about 15% of pregnancies, only a fraction of infants born in this context develop significant hyperbilirubinemia and often it is difficult to predict, even not strongly predicted by the Coomb's test.²² The diagnosis of ABO hemolytic disease as opposed to ABO incompatibility is generally reserved for those who have a positive Coomb's test and clinical jaundice within first 12–24 h of life. Neonates having O-B incompatibility have been reported to develop hyperbilirubinemia within 24 h of life, more frequently than O-A incompatibility²³; however, the present study did not find such difference.

In the Rh system, there are five major antigens of clinical importance: D, C, E, c, and e. In a study from central India,²⁴ the distribution of five major Rh antigens found was as follows: D-91.6%, C-84%, E-25.3%, c-58.3%, and e-78.5%. There are 18 possible phenotypic combinations; nine belong to Rh D positive, commonest being DCCee (41%), while the other nine belong to Rh D negative, commonest being dceee (5.6%). The most instances of Rh isoimmunization are due to the Rh D antigen. Since the introduction of anti-D immunoglobulin, the cases of Rh-D hemolytic disease and hyperbilirubinemia have

markedly declined; however, it was the case in 14% of patients in our study, reflecting inadequate obstetric care. Sporadic cases of hemolysis and hyperbilirubinemia in neonates have been reported due to minor blood group incompatibility.^{15,25,26} In the present study, Rh C and Rh E incompatibilities were found in 14% and 3% of cases, respectively. In a study of 82 cases of neonatal jaundice requiring exchange transfusion, subgroup incompatibility was the cause in 14 cases (anti-C: 4 cases, anti-E: 3 cases, anti-c: 2 cases, and anti-Kell: 5 cases).¹⁴ Isoimmunization due to non-Rh D antigens is similar to Rh D antigen, but the disease is usually milder, especially with Rh C, Rh E, and Rh e; however, combination of the antigens can cause severe fetal and neonatal hemolytic disease.²⁷ The present study found Rh D incompatibility to be more severe than Rh C and ABO incompatibilities; however, there was no significant difference in severity between Rh C and Rh E.

Sepsis is known to cause hemolysis and hyperbilirubinemia, probably by increasing oxidative stress damaging red blood cells that are susceptible to cell injury.²⁸ Incipient sepsis/bacteremia has been reported as a rare cause of hyperbilirubinemia in the developed world²⁹; however, it accounted for 21% of our cases, reflecting poor perinatal care. Most of our sepsis cases were culture negative, probably because most of them were referred cases and had received prior antibiotics. The etiology of hyperbilirubinemia could not be ascertained in 31 (15.5%) of our neonates. Many authors have also been unable to establish the etiology of hyperbilirubinemia in more than half of the cases in their series.^{8,10} It emphasizes the need for more thorough investigations to find out the cause and further studies to determine the role of environmental factors and genetic interactions, which may exaggerate hyperbilirubinemia when associated with other high-risk conditions.³⁰

Despite recommendation to perform predischarge bilirubin in infants and follow-up,^{7,11} effective anti-D prophylaxis for Rh D disease, and wide availability of effective treatment for hyperbilirubinemia, 10 of our patients had ABE of moderate to severe grade. This may be partly because of early discharge after delivery, lack of screening for hyperbilirubinemia and their follow-up leading to delayed detection of hyperbilirubinemia and referral, and lack of concern about high bilirubin level among parents and healthcare providers.

Although the evidence of hemolysis and positive direct Coomb's test indicates significant immune-mediated hemolytic disease, they may not be helpful because of their poor sensitivity and specificity.^{31–33} Thus, the diagnosis of symptomatic immune-mediated hemolytic disease leading to hyperbilirubinemia should be considered in the context of blood group incompatibility that may or may not be accompanied by a positive, direct Coomb's test and/or evidence of hemolysis. The major limitations of the study were that we have not tested for Rh c and Rh e incompatibilities, including other minor blood group incompatibilities. Also, we could not perform direct Coomb's test in many of the patients, because of logistic reason. The tests for Rh C, Rh E incompatibility, G-6PD deficiency, and hereditary spherocytosis were performed only among those cases that did not demonstrate ABO and/or Rh D incompatibility, which limit to understand its interaction with major blood group incompatibility. Also, because of logistic reason, we could not perform

antibody titer against various blood group antigens and indirect Coomb's test in maternal blood, which would have strengthened the diagnosis of blood group incompatibilities.

In conclusion, the present study found blood group incompatibility and sepsis as common causes of neonatal unconjugated hyperbilirubinemia requiring treatment. Rh incompatibility other than D can lead to severe hyperbilirubinemia, and needs to be investigated further, especially in infants not demonstrating ABO and/or Rh D incompatibility.

Author's contribution

SNS conceived and supervised the study and prepared the manuscript. He will act as guarantor of the study. SKS, AB, and ST collected data, analyzed it, and helped in manuscript writing. MK supervised the study. TC and AK analyzed the samples and critically reviewed the manuscript. The final manuscript was approved by all authors.

Conflicts of interest

The authors have none to declare.

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