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Research Article

High Performance Liquid Chromatography as A Gold Standard Versus Mentzer Index for Screening Beta-Thalassemia Trait: Diagnostic Correlation and Efficacy

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ABSTRACT

INTRODUCTION: Beta-thalassaemia is a hereditary blood disorder characterized by a partial (β^+) or complete (β^0) reduction in the synthesis of beta-globin chains of hemoglobin. The heterozygous state of either mutation results in the beta-thalassaemia trait, in which the hallmark finding is an elevated level of Hemoglobin A_2 (HbA_2), composed of $\alpha_2\delta_2$ chains. Accurate detection of this condition is vital for recessive gene testing and prevention programs.
However, high-performance liquid chromatography (HPLC) has emerged as the recommended method due to its accuracy, reproducibility, and ease of use, though it is currently limited to few laboratories in the country.
MATERIALS AND METHODOLOGY: This cross-sectional study evaluated individuals with microcytic, hypochromic anemia, in whom beta-thalassaemia trait was suspected based on red blood cell indices. A complete blood count (CBC) was performed using an automated hematology analyzer, and the Mentzer Index (MCV/RBC) was calculated for each subject. Hemoglobin analysis was performed using the VARIANT™ system (Bio-Rad Laboratories), a fully automated HPLC platform utilizing the Beta-Thal Short Program.
RESULTS: HPLC proved to be a rapid, technically straightforward, and highly reproducible method for the quantification of HbA_2. Among individuals confirmed as carriers of classical beta-thalassaemia, the HbA_2 level was consistently elevated above 4.0%, with values ranging from 4.5% to 8.1%, and a mean value of 5.9%. These individuals also demonstrated microcytosis and hypochromia, with mean corpuscular volume (MCV) < 75 fL and mean corpuscular hemoglobin (MCH) < 27 pg. MI values < 13 suggested β-thalassaemia trait, while values > 13 were more consistent with iron deficiency anemia.
CONCLUSION: High-performance liquid chromatography (HPLC) is a reliable and efficient confirmatory method for the identification of beta-thalassaemia trait, with an HbA_2 cut-off level > 4.0% being predictive of carrier status. When combined with red cell indices—particularly MCV and MCH—and screening tools such as the Mentzer Index, HPLC offers a robust strategy for the preliminary identification of beta-thalassaemia carriers. This approach facilitates early genetic counseling and guides the need for molecular studies where necessary. Integration of these parameters provides a cost-effective and efficient diagnostic pathway, particularly valuable in regions where molecular testing is limited.

Keywords*: Beta-thalassaemia trait, High-performance liquid chromatography, HbA_2, Mentzer Index, Microcytosis, Carrier screening.*

INTRODUCTION

Thalassemia is a common genetic hemoglobin disorder caused by the reduced or absent synthesis of one or more globin chains. It is recognized as the most prevalent monogenic disorder globally, with the World Health Organization (WHO) estimating that approximately 5% of the world's population are carriers of a thalassemia gene mutation [1]. Globally, around 1.5% of individuals (approximately 80–90 million) carry β-thalassemia traits, and each year, about 60,000 infants are born with β-thalassemia major [2]. India alone contributes nearly 25% of the global burden, with an estimated 100,000 affected individuals and over 40 million carriers. Carrier frequency ranges from 3–4% nationally, but can vary regionally (e.g., 1–6% in Maharashtra and Gujarat, and up to 17% in Sindhi and tribal subgroups) [3,4].

Pathophysiology, Genetics & Clinical Spectrum

Beta-thalassemia is caused by mutations in the HBB gene on chromosome 11, resulting in reduced (β^+) or absent (β^0) synthesis of β-globin chains. This imbalance between α- and β-globin chains leads to ineffective erythropoiesis and chronic hemolytic anemia [5]. Over 350 different β-thalassemia mutations have been reported worldwide. In India, approximately 80 mutations have been documented, with five common variants (e.g., IVS-I-5 G→C, codon 41/42 deletion, 619 bp deletion) accounting for 80–90% of cases [3,6].

The β-thalassemia trait (minor) is typically asymptomatic and presents with microcytic hypochromic anemia, reduced MCV and MCH, and elevated HbA_2 levels (>3.5%) on HPLC [7]. In contrast, β-thalassemia major presents early in life with severe anemia, hepatosplenomegaly, skeletal deformities, growth retardation, and transfusion dependence [5].

Elevated HbA_2 (>3.5%) is a hallmark of the β-thalassemia trait, while HbF is often mildly raised. Low MCV and MCH levels further support the suspicion of thalassemia [7]. Traditional diagnostic methods like cellulose acetate electrophoresis and microcolumn chromatography are labor-intensive and require technical expertise [8]. High-Performance Liquid Chromatography (HPLC) has emerged as the gold standard due to its automation, reproducibility, and precision.

AIMS OF STUDY

The objective of this study is to determine the diagnostic cut-off level of HbA_2 measured via HPLC for the identification of classical beta-thalassemia carriers. Detecting these carriers is critical for preventing homozygous states that result in beta-thalassemia major and lifelong transfusion dependency.

MATERIALS AND METHODOLOGY

This retrospective study included 100 patients with microcytic hypochromic anemia and elevated RBC counts, who were admitted to the inpatient wards of P.D.U. Medical College and Hospital, Rajkot. Laboratory evaluations, including hematological and HPLC testing, were performed in the Department of Pathology between August 1, 2024, and July 31, 2025.

A total of 2 mL venous blood was collected in EDTA-anticoagulated tubes and processed within 6–8 hours. Samples for HPLC were stored at 2–8°C and analyzed within 5 days. Patients who had received transfusions in the prior 3 months were excluded to avoid interference in hemoglobin quantification [9].

Parameters Assessed

- ***Mentzer Index (MI) Calculation***

The Mentzer Index was calculated for each patient using values obtained from a complete blood count (CBC). For this calculation, 2 mL of venous blood was collected in EDTA-anticoagulated tubes and processed within 6–8 hours of collection.

The formula applied was: Mentzer Index (MI) = MCV(fL)/RBC count (millions/μL)
An MI value of less than 13 was considered suggestive of β-thalassemia trait, whereas a value greater than 13 indicated iron deficiency anemia (IDA).

- **High-Performance Liquid Chromatography (HPLC) Analysis**

HPLC was performed as a confirmatory test by using the Bio-Rad VARIANT® β-Thal Short Program to quantify different hemoglobin fractions, including HbA, HbA_2, and HbF. Whole blood samples were lysed to release hemoglobin and injected into the HPLC system equipped with a cation exchange column. Hemoglobin variants were separated based on retention time and visualized as a chromatogram. Interpretation was based on standard cut-offs: HbA_2 >3.5% was diagnostic of β-thalassemia trait, while HbF >10% suggested β-thalassemia major or intermedia, delta β-thalassemia. Normal HbA_2 levels (2.2–3.2%) were considered non-thalassemic and typically indicated iron deficiency. For HPLC, 2 mL of EDTA-anticoagulated whole blood was used, with samples remaining stable for up to 5 days when stored at 2–

8°C. Patients who had received blood transfusions within the previous three months were excluded from HPLC testing to avoid interference in hemoglobin fraction analysis.

Confirmatory Testing

For definitive diagnosis, DNA-based methods such as PCR and sequencing are used to detect β-globin gene mutations. These tests help establish genotype–phenotype correlations and confirm complex or compound heterozygote cases [6,10]. Family studies and genetic counseling are recommended to support informed reproductive choices and disease prevention [4].

INCLUSION CRITERIA:

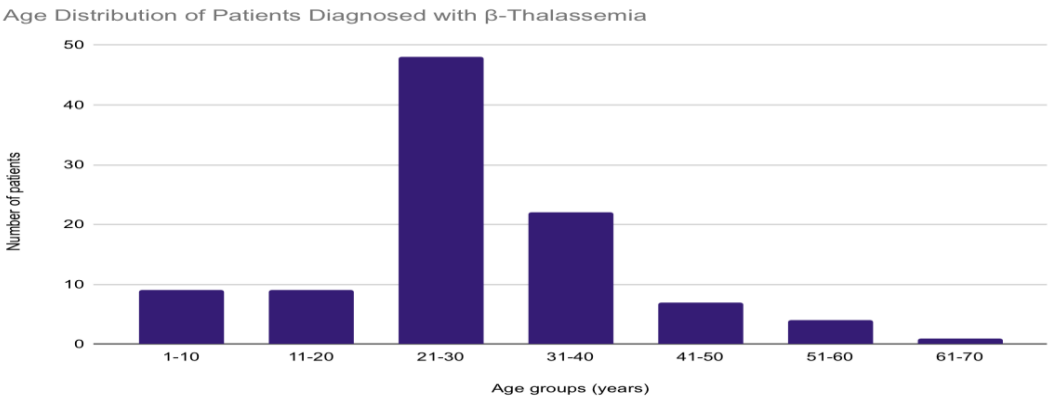
- Age ≥ 1 year
- Individuals presenting with microcytic hypochromic anemia (MCV < 80 fL, MCH < 27 pg)
- Suspected β-thalassaemia trait based on red cell indices or family history
- No prior blood transfusion in the past 3 months

EXCLUSION CRITERIA:

- Iron deficiency anemia (confirmed by serum ferritin or iron studies) not yet corrected
- Presence of other hemoglobinopathies (e.g., HbE, HbS) on HPLC
- Severe systemic illness affecting hematological parameters
- Age < 1 year (HbA_2 levels are not reliable in infants)
- Recent blood transfusion (< 3 months)

RESULTS:

1. **Age-Wise Distribution of β-Thalassemia Cases**

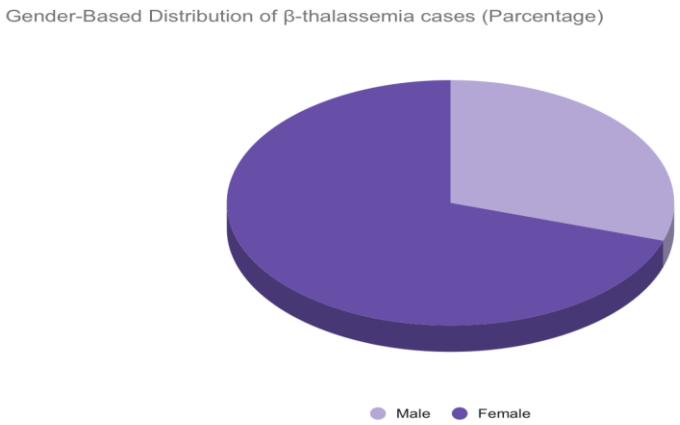


[Figure: 1] Distribution of β-Thalassemia Diagnosed Cases According to Age

This vertical bar chart [Figure 1] displays the number of β-thalassemia cases across different age groups. The majority of cases are concentrated in the 21–30 years age group, accounting for nearly 50 cases, followed by a notable number in the 31–40 years group (~22 cases). Fewer cases are observed in both younger (1–20 years) and older (>40 years) age groups. This distribution suggests that β-thalassemia is most frequently diagnosed or presented clinically in early adulthood, particularly during the third decade of life. The pattern may reflect increased medical evaluation during reproductive or working years, or delayed diagnosis due to lack of early childhood screening. The low number of cases in older age groups could be attributed to earlier mortality in severe forms of the disease or underdiagnosis in older individuals.

2. **Gender Gender-Based Distribution of β-Thalassemia Cases**

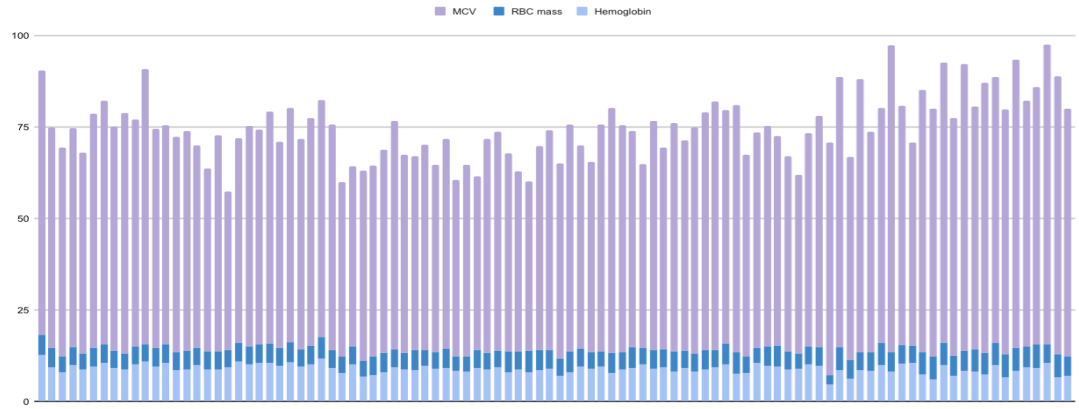
This pie chart illustrates the gender distribution of β-thalassemia cases, expressed as a percentage. It shows a higher proportion of female cases compared to male cases, with females comprising approximately two-thirds of the total and males about one-third. This gender disparity may be attributed to social factors such as more frequent screening of women during antenatal checkups or premarital testing, rather than a true biological difference — since β-thalassemia is an autosomal recessive disorder with no inherent sex-linked transmission. [Figure: 2]



[Figure: 2] Proportion of Male and Female β-Thalassemia cases

3. Comparison of MCV, RBC Mass, and Hemoglobin Levels in β-Thalassemia Cases

This [Figure: 3] shows individual patient data for Mean Corpuscular Volume (MCV), RBC mass, and hemoglobin concentration across a cohort of β-thalassemia cases. MCV (purple bars) varies widely between patients, with most values clustered below the normal reference range (80–100 fL), consistent with microcytosis seen in β-thalassemia. RBC mass (dark blue bars) remains relatively elevated or stable across many cases, which is characteristic of the β-thalassemia trait or intermedia, where the body compensates for anemia by increasing red cell production. Hemoglobin levels (light blue bars) are generally low to normal, reflecting varying severity of anemia among patients. This pattern - low MCV with relatively maintained or elevated RBC mass and reduced hemoglobin — supports the diagnosis of β-thalassemia, highlighting the importance of red cell indices and counts in its screening and differentiation from other microcytic anemias like iron deficiency.



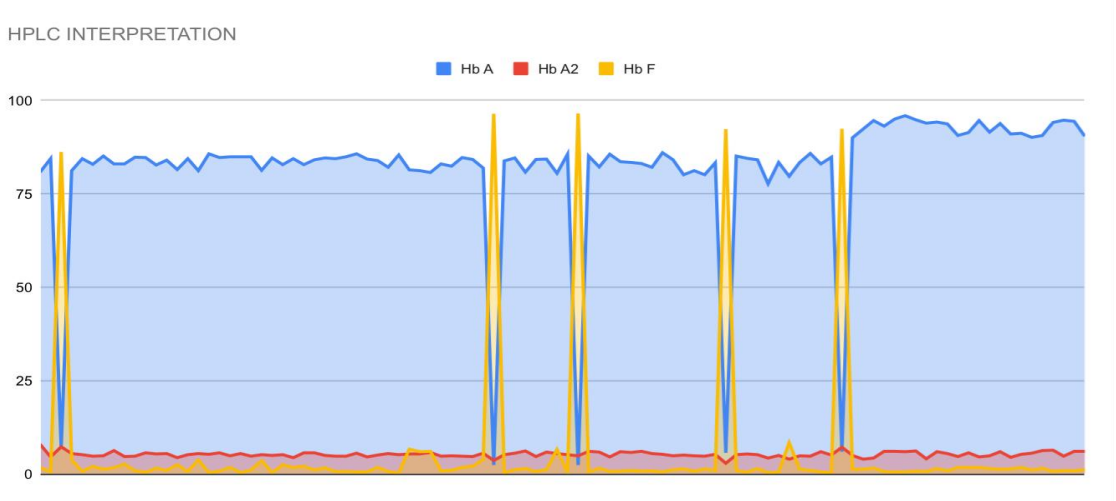
[Figure: 3] Comparative Analysis of MCV, RBC Count, and Hemoglobin Concentration Among β-Thalassemia Cases

4. HPLC Interpretation of Hemoglobin Fractions in β-Thalassemia Cases

This line chart [Figure: 4] illustrates the relative percentages of Hb A (blue), Hb A_2 (red), and Hb F (yellow) measured by HPLC across a series of patients evaluated for β-thalassemia.

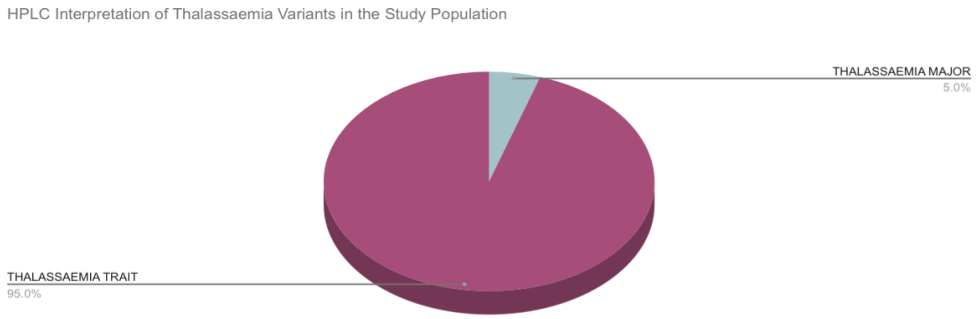
- Hb A (blue area) is the predominant hemoglobin fraction in most cases but shows fluctuations — with occasional sharp drops coinciding with spikes in Hb F.
- Hb A_2 (red line) remains consistently elevated above the normal upper limit (typically >3.5%), which is a hallmark of β-thalassemia trait.
- Hb F (yellow spikes) shows sharp increases in a few cases, suggesting β-thalassemia major or intermedia, where fetal hemoglobin production persists at high levels due to ineffective adult hemoglobin synthesis.

The combination of elevated Hb A_2 with or without raised Hb F on HPLC confirms the diagnosis of β-thalassemia and helps distinguish between carrier states and more severe disease phenotypes.



[Figure: 4] HPLC Interpretation of Hemoglobin Fractions in β-Thalassemia Cases

5. Proportion of Thalassemia Trait and Thalassemia Major Detected by HPLC in the Study Population

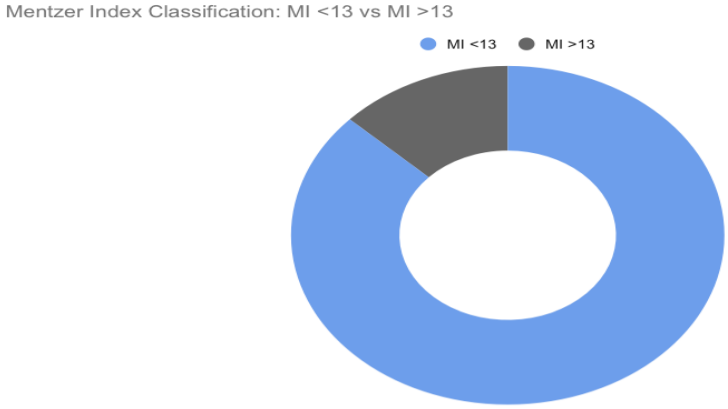


[Figure: 5] Proportion of Thalassemia Trait and Major Cases Identified by HPLC

This pie chart [Figure: 5] shows the distribution of thalassemia variants detected via HPLC. The majority (95%) are thalassemia trait cases, identified by elevated Hb A_2 levels and typically asymptomatic. Thalassemia major accounts for 5%, characterized by high Hb F and reduced Hb A, indicating more severe disease requiring medical intervention. This finding highlights the predominance of thalassemia trait, underscoring the need for community-level screening to identify carriers and prevent severe thalassemia in future generations.

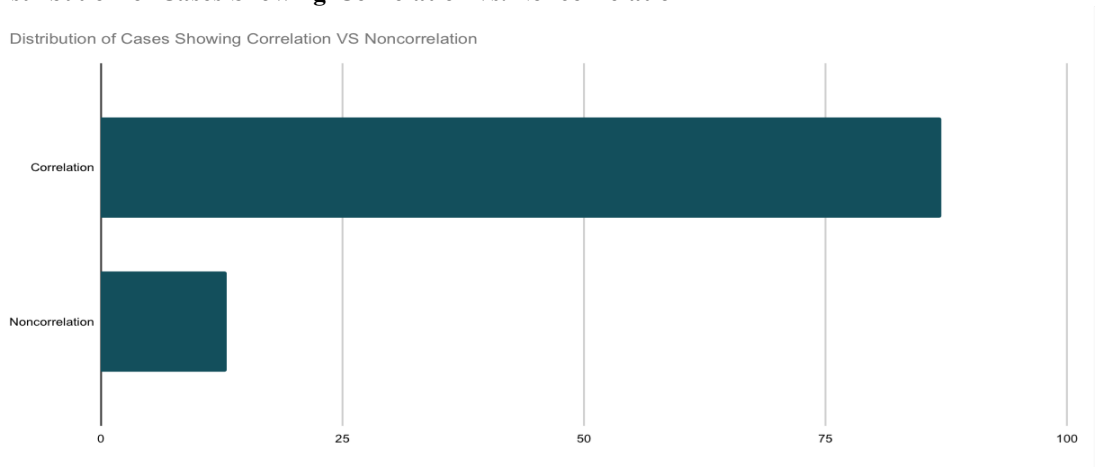
6. Mentzer Index Classification in the Study Population: MI <13 vs MI >13

This Figure: 6 shows most patients with MI < 13 (blue), indicating β-thalassemia trait. Fewer with MI > 13 (gray), suggesting iron deficiency anemia. Highlights Mentzer Index as a simple screening tool for microcytic anemia.



[Figure: 6] Distribution of Patients by Mentzer Index

7. Distribution of Cases Showing Correlation vs. Noncorrelation



[Figure: 7] Correlation Between Diagnostic Parameters

Figure: 7 shows ~85–90% of cases with strong correlation between red cell indices and HPLC findings, supporting β-thalassemia diagnosis. A smaller portion (10–15%) shows noncorrelation, highlighting the need for careful evaluation in such cases.

DISCUSSION

Parameter	**Mentzer Index (MI)**	**High-Performance Liquid Chromatography (HPLC)**
Principle	MCV ÷ RBC count	Quantification of HbA_2, HbF, and Hb variants
Screening/Confirmatory	Screening	Confirmatory
Typical Cut-off	< 13 suggests β-thal trait	HbA_2 > 4.0% confirms β-thal trait
Sensitivity	~85–90% (Meshram et al., 2017)[12]	~95–98% (Amid et al., 2013)[11]
Specificity	~80–85%	~98–100%
PPV (Positive Predictive Value)	~80%	~99%
NPV (Negative Predictive Value)	~85–90%	~98%
Advantages	-Inexpensive - Easily calculated from CBC -Good for population-level screening	- Accurate and reproducible -Detects co-existing variants (e.g., HbE, HbS)
Limitations	- Affected by iron deficiency anemia - Overlaps with other microcytic anemias	- Requires specialized equipment - Costlier and less accessible in rural settings
Turnaround Time	Immediate (part of CBC)	< 10–15 minutes per sample
Used In	Initial screening	Diagnostic confirmation and pre-DNA workup

[Table: 1] Comparative Study of Hematological Indices and HPLC Findings in β-Thalassemia Trait

In our study, HPLC demonstrated excellent correlation (r = 0.97–0.99) and precision (CV 0.5–4.4%) in estimating HbA_2. A cut-off > 4.0% effectively identified β-thalassemia trait, with the Bio-Rad Premier system achieving 100% sensitivity and 99.6% specificity. The Mentzer Index (MI < 13) showed strong agreement with elevated HbA_2 levels, maintaining its reliability even in the presence of iron deficiency.

HPLC is superior to traditional methods for HbA_2 estimation due to its automation, speed, and reproducibility. Characteristic red cell indices (low MCV/MCH) combined with raised HbA_2 offer a practical and cost-effective screening strategy. Together, MI, microcytosis, and elevated HbA_2 support early identification and triaging of carriers for confirmatory molecular testing or genetic counseling, especially in low-resource settings.

CONCLUSION

The combined analysis highlights a high prevalence of β-thalassemia trait, with strong correlation between red cell indices (low MCV, high RBC), Mentzer Index <13, and elevated HbA_2 on HPLC. Most patients were young adults, with more female carriers, likely due to reproductive health screening. HPLC profiles confirmed elevated HbA_2 in trait and increased HbF in thalassemia major cases.

Mentzer Index and HPLC showed strong diagnostic agreement, with few discordant cases possibly due to iron deficiency or transfusions. Using MI as a screening tool, followed by HPLC, offers a reliable, cost-effective approach. HPLC remains the preferred method for its precision, reproducibility, and automation.

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