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RESEARCH ARTICLE

Tumour necrosis factor-α and soluble Fas ligand as biomarkers in non-acetaminophen-induced acute liver failure

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Abstract

Objectives: Cytokines as prognostic markers in acute liver failure (ALF) have not been evaluated in the Indian subcontinent with hepatitis E as the commonest aetiological agent. We investigated the clinical significance of proinflammatory/apoptotic cytokines soluble Fas ligand (sFasL) and tumour necrosis factor (TNF)-α in ALF of specific aetiology.

Methods: A total of 82 cases, 37 ALF and 45 acute hepatitis (AH), and 60 healthy controls were recruited. Serum levels of sFasL and TNF-α were determined at admission and death/recovery.

Results: Mean sFasL and TNF-α serum levels at admission were significantly higher (p <0.001) in patients with ALF than AH, but no marked difference was observed between ALF-E (expired, n = 23) and ALF-S (survivors, n = 14), although the former had comparatively higher levels. ALF-E had higher than baseline TNF-α and sFasL concentrations at death, while in the ALF-S group the samples obtained from the patients as soon as they came out of encephalopathy, showed either lower or similar TNF-α and sFasL levels as found at admission.

Conclusion: The high levels of sFasL and TNF-α are associated with ALF. Following the trend of these cytokines can be useful in predicting death and timely referral to a transplant centre.

Keywords: *Acute liver failure; TNF; soluble Fas ligand; non-acetaminophen; hepatitis E*

Introduction

Despite aggressive medical management acute liver failure (ALF) is a substantial cause of morbidity and mortality all over the world. In western countries acetaminophen (paracetamol) is the commonest cause of ALF followed by other drugs and hepatitis B (Vickers et al. 1988, Lidofsky et al. 1992, Sallie et al. 1994, Schiodt et al. 1999, Ostapowicz et al. 2002) while in India, viruses, the commonest being hepatitis E, are the primary cause of ALF (Acharya et al. 1996, 2000, Kar et al. 1997, Dhiman et al. 1998).

Liver tissue repair, inflammation, regeneration and fibrosis may all be triggered by apoptosis (Rust & Gores 2000, Jaeschke et al. 2002) The Fas ligand (FasL)/Fas receptor and tumour necrosis (TNF)-α/TNF type I receptor (TNFRI) apoptotic pathways are the two major mechanisms regulating this phenomenon. Many studies (Ogasawara et al. 1993, Galle et al. 1995, Ryo et al. 1996, 2000, Rivero et al. 2002, Mita et al. 2005, Rutherford et al. 2007) barring one (Kasahara et al. 2000) revealed that the FasL/Fas system is involved in massive hepatocyte destruction in patients with ALF. A biologically active soluble form of FasL (sFasL) might also interact with Fas-bearing cells, thus contributing to systemic tissue injury during inflammation.

The role of apoptotic factor TNF-α in ALF has been investigated with variable results (Muto et al. 1988, De la

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Mata et al. 1990, Sekiyama et al. 1994, Streetz et al. 2000, Masahito et al. 2000, Rutherford et al. 2007). Most of the studies involved ALF caused by either acetaminophen or hepatitis B infection. But so far, there is no report from India on the role of Fas and TNF as prognostic markers in ALF, which is induced mainly by the hepatitis E virus that is highly prevalent in this part of the world. Therefore, the present study has been designed to evaluate the significance of TNF-α and sFasL in ALF in a specific patient population and aetiology.

Materials and methods

Study subjects

The present study included a total of 82 cases comprising 37 patients with ALF and 45 patients with acute hepatitis (AH). The patients were recruited from the emergency and outpatient department during the period from February 2005 to April 2006. The ALF disease group was further divided on the basis of outcome of the disease into expired (ALF-E, $n=23$) and survived (ALF-S, $n=14$). Sixty volunteer (replacement and altruistic) blood donors served as healthy controls. ALF/fulminant hepatic failure was diagnosed as defined by Trey and Davidson (1970) and Tandon et al. (1999), while acute viral hepatitis was defined as those patients, who had an acute self-limiting disease and a serum AST elevation at least fivefold that of the upper limit of normal, or exhibited jaundice, or both (Smedile et al. 1982).

Informed consent was obtained from the patient or the nearest relative in all the cases and controls. The study was carried out in accordance with principles laid down by the Helsinki agreement.

Sample collection and analysis of biochemical and viral markers

After a complete physical examination, biochemical profile and serological studies were carried out for all patients and controls. Sera were separated and stored at -80°C for serological analysis and quantification of serum TNF-α and sFasL levels. Biochemical parameters including detailed liver function tests were carried out at days 0, 1, 2, 5, 10 and 15 and repeated whenever clinically indicated. All the cases were screened for hepatitis virus markers: IgM anti-HAV, HBsAg and IgM HBcAb, anti-HCV and IgM anti-HEV. Acute HBV and HCV infection was confirmed with HBV-DNA polymerase chain reaction (PCR) and HCV-RNA PCR, respectively. All patients were followed up and monitored for the development of any complications and the final outcome. A second blood sample was taken from 20 ALF-E patients at death and 12 ALF-S patients when they recovered from encephalopathy. The patients with any evidence of chronic liver disease were excluded from the study. Serum ceruloplasmin, antinuclear antibody and serum ferritin levels were checked to screen for Wilson's disease, autoimmune hepatitis and haemochromatosis, respectively.

In vitro quantification of sFasL (BMS260/2; Bender Med Systems, Austria) and TNF-α (Diaclone Research, Besancon, France) was done using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) for Windows (version 12.0) was used. The χ^2 test/Fisher's exact test (for smaller numbers on subgroup analysis) was also used to compare the different parameters between cases and controls. The non-parametric Spearman rank correlation coefficient was applied to determine the correlation between quantitative parameters. Sample size was calculated for 80% power and a 5% type I error rate.

Results

Demographics, clinical features, haematological and biochemical parameters and liver function tests of ALF (ALF-E vs ALF-S) and AH cases are presented in Table 1. Of the pregnant women who had hepatitis ($n=14$), there was a higher percentage in the ALF group 9/14 (64.3%) compared with the AH group 5/14 (35.7%) and similarly in the ALF-E group 6/9 (66.7%) compared with the ALF-S group 3/9 (33.3%), but the differences did not attain statistical significance.

Distribution of viral and non-viral aetiologies

The percentage distribution of various hepatotropic viruses in the patients is depicted in Table 2. HEV was found to be the commonest aetiological agent among all the groups. The overall mortality observed in ALF cases was 23/37 (62.2%), whereas the mortality in HEV-related ALF cases was 7/15 (46.7%), which is lower than that presented by all other groups combined, i.e. 16/22 (72.7%).

Serum TNF-α and sFasL: comparisons among groups

The serum levels of TNF-α and sFasL in both the cases and controls are depicted in Table 3. In the ALF group the mean sFasL levels (1.05216 ng ml^{-1}) were significantly higher than in the AH group (0.33533 ng ml^{-1}) ($p<0.001$). This significant difference could not be found between mean sFasL concentrations in the ALF-E group (1.10609 ng ml^{-1}) and the ALF-S group (0.96357 ng ml^{-1}),

Table 1. Demographics and clinical profile of study subjects.

	ALF (n = 37)	Acutehepatitis (AH) (n = 45)	ALF vs AH**	ALF-E(n = 23)	ALF-S(n = 14)	ALF-E vs ALF-S***
Age (years), n (%)						
12–20	7 (18.9)	6 (13.3)	0.781	3 (13)	4 (28.6)	0.402
20–40	25 (67.6)	32 (71.1)		16 (69.6)	9 (64.3)	
>40	5 (13.5)	7 (15.6)		4 (17.4)	1 (7.1)	
Sex distribution, n (%)						
Male	17 (45.9)	24 (57.8)	0.286	9 (39.1)	8 (57.1)	0.286
Female	20 (54.1)	21 (42.2)		14 (60.9)	6 (42.9)	
Clinical features, n (%)						
Pedal oedema	5 (13.5)	3 (6.7)	0.458	5 (21.7)	0	0.135
Splenomegaly	0	2 (4.4)	0.499	0	0	
Ascites	4 (10.8)	5 (11.1)	0.99	4 (17.4)	0	0.276
Liver span ≤ 4 cm	25 (67.6)	0	<0.001	19 (82.6)	6 (42.9)	0.012
Haematological and biochemical parameters[a]						
Haemoglobin(g dl^{-1})	10.10 (4.5–16.2)	11.50 (7.6–15.7)	0.114	10.0 (4.5–16.2)	11.50 (8–16)	0.328
WBC (cells mm^{-3})	13727 (1900–39800)	8324 (4200–18000)	0.001	13813 (1900–39800)	13585 (4800–25400)	0.699
ESR(mm 1st h)	19.54 (4–112)	8.73 (4–25)	<0.001	23.83 (4–112)	12.50 (4–28)	0.122
BUN (mg dl^{-1})	50.11 (20–112)	33.22 (12–87)	0.005	59.7 (22–112)	34.36 (20–56)	0.014
Creatinine (mg dl^{-1})	1.13 (0.4–6.8)	0.7 (0.4–1.8)	0.007	1.4 (0.5–6.8)	0.67 (0.4–0.9)	0.003
Glucose (mg dl^{-1})	108 (50–279)	127 (62–243)	0.009	111 (50–183)	103 (60–279)	0.360
Liver function tests[a]						
Bilirubin (T) (mg dl^{-1})	14.98 (6.4–38)	8.9 (2.3–25)	<0.001	15.0 (6.4–38)	14.8 (8.2–29)	0.632
Bilirubin (D) (mg dl^{-1})	10.28 (3.2–24.7)	6.23 (1–19)	<0.001	10.09 (3.2–24.7)	10.6 (5–24)	0.817
T/D	1.49 (1.1–2.5)	1.49 (1.1–2.6)	0.859	1.51 (1.2–2.5)	1.46 (1.1–2.2)	0.588
AST (U l^{-1})	1844.81 (51–7480)	1217.22 (80–7500)	0.034	2004.35 (51–7480)	1582.71 (190–4697)	0.817
ALT (U l^{-1})	2010.46 (59–7110)	1453.18 (86–8300)	0.063	2017.22 (59–7110)	1999.36 (198–4950)	0.793
ALP (kU l^{-1})	23.27 (5–56)	18.2 (5–44)	.031	22.74 (5–56)	24.14 (9–44)	.567
PT prolongation (s)	50.27 (1–286)	7.61 (0–34)	<0.001	72.00 (10–286)	14.57 (1–38)	<0.001

[a]Median (min–max.); ALF, acute liver failure; WBC, white blood cells; ESR, erythrocyte sedimentation rate; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; PT, prothrombin time.
**p-Value calculated from Wilcoxon Mann–Whitney test clinical parameters between ALF and AH.
***p-Value calculated from Wilcoxon Mann–Whitney test for the comparison of various clinical parameters between ALF-E and ALF-S.

although the level was higher in the former group. Similarly in the ALF group the mean TNF-α concentration (170.22 pg ml^{-1}) was significantly higher than in the AH group (83.82 pg ml^{-1}) (p <0.05), but a significant difference could not be found between the mean TNF-α concentration in the ALF-E group (181.74 pg ml^{-1}) and the ALF-S group (151.29 pg ml^{-1}), although the level was higher in the former. Also, mean sFasL and TNF-α concentrations were found to significantly (p<0.01) higher in all the hepatitis groups than in the control group (0.12331 ng ml^{-1} and 15.31 pg ml^{-1}, respectively). A highly significant (p<0.001) positive correlation (correlation coefficient (CC) 0.398) was established between TNF-α and sFasL serum concentrations in all the case groups.

Follow up of TNF-α and sFasL in ALF

A follow-up study of patients in the ALF-E and ALF-S groups revealed that in the ALF-E group, sFasL (1.40 ± 0.201 ng ml^{-1}) and TNF-α (196.44 ± 23.34 pg ml^{-1})

levels were higher at death when compared with admission mean sFasL (1.12 ± 0.173 ng ml^{-1}) and TNF-α (181.74 ± 37.03 pg ml^{-1}) concentrations. In the ALF-S, sFasL (0.84 ± 0.301 ng ml^{-1}) and TNF-α (142.44 ± 33.14 pg ml^{-1}) levels at recovery were lower than the admission mean sFasL (0.96 ± 0.162 ng ml^{-1}) and TNF-α (151.29 ± 41.61 pg ml^{-1}) concentrations. The results were not statistically significant.

Table 2. Percentage distribution of aetiologies among all study groups.

	ALF (n = 37)	ALF-E (n = 23)	ALF-S (n = 14)	Acute hepatitis (n = 45)
Hepatitis A	1 (2.7 %)	0	1 (7.1 %)	3 (6.7 %)
Hepatitis B	4 (10.8 %)	4 (17.4 %)	0	5 (11.1 %)
Hepatitis C	1 (2.7 %)	1 (4.3 %)	0	0
Hepatitis E	15 (40.5 %)	7 (30.4 %)	8 (57.1 %)	23 (51.1 %)
Co-infection[a]	3 (8.1%)	1 (4.3%)	2 (14.3%)	2 (4.4%)
EBV[b]	3 (8.1%)	2 (8.6%)	1 (7.1%)	4 (8.8%)
Drugs[c]	0	0	0	3 (6.7%)
No aetiology[b]	10 (27.0 %)	8 (34.7 %)	2 (14.3 %)	5 (11.1 %)

ALF, acute liver failure; EBV, Epstein–Barr virus. [a]HAV and HBV co-infection: one each in ALF-E, ALF-S and acute hepatitis; HEV and HBV co-infection, one each in ALF-S and acute hepatitis. [b]IgM serology for EBV was positive. [c]Isoniazid and rifampicin.

Correlation of TNF-α and sFasL with other parameters

TNF-α at admission had a correlation with total bilirubin (CC = 0.307, p = 0.005), direct bilirubin (CC = 0.269, p = 0.014), SGOT/ALT (CC = 0.391, p <0.001) and prothrombin time difference (CC = 0.057, p = 0.005). A similar correlation was also obtained for total bilirubin (CC = 0.381, p = <0.001), direct bilirubin (CC = 0.358, p = 0.001), SGOT/ALT (CC = 0.301, p <0.006) and prothrombin time difference (CC = 0.582, p = <0.001) with serum levels of sFasL. In addition, ALP was also found to be positively correlated (CC = 0.235, p = 0.033) with sFasL levels in serum.

Serum levels of TNF-α and sFasL varies in relation to aetiologic agent

The ALF and AH groups were combined to evaluate the effect of aetiology on cytokine levels (Figures 1 and 2).

Table 3. Serum levels of tumour necrosis factor (TNF)-α (pg ml^{-1}) and soluble Fas ligand (sFasL) (ng ml^{-1}) in the study groups.

	ALF (n = 37)	AH (n = 45)	p-Value	ALF-E (n = 23)	ALF-S (n = 14)	p-Value	Controls
TNF-α (pg ml^{-1}), mean ± SE	170.22± 27.632	83.82± 11.845	0.033	181.74± 37.029	151.29± 41.611	0.526	10.22 ± 2.13
sFasL (ng ml^{-1}),mean ± SE	1.052± 0.123	0.335± 0.030	<0.001	1.106± 0.173	0.964± 0.162	0.817	0.085 ± 0.012

ALF, acute liver failure; AH, acute hepatitis.
p-Value calculated from Wilcoxon Mann–Whitney test for the comparison of TNF-α and sFas ligand serum levels.

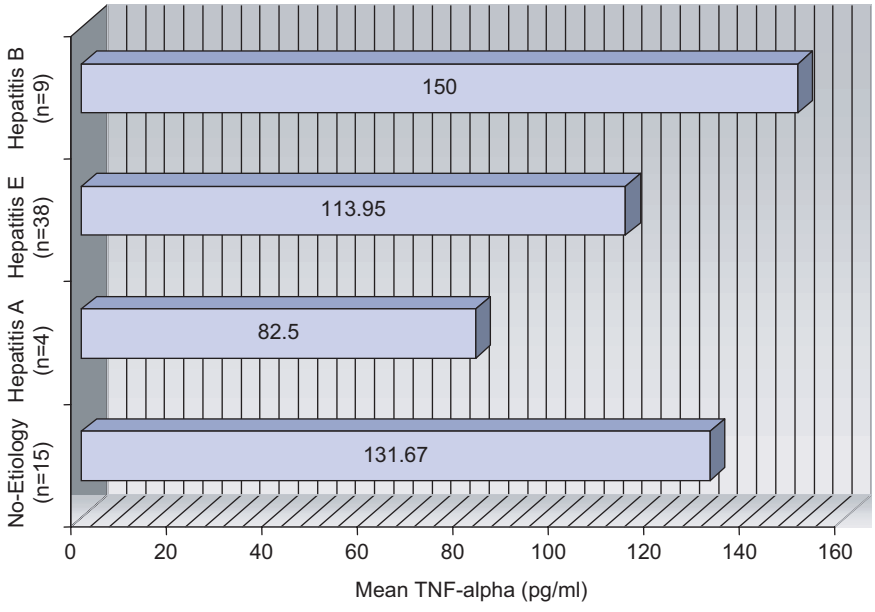

Figure 1. Mean tumour necrosis factor (TNF)-α levels in relation to aetiological agent.

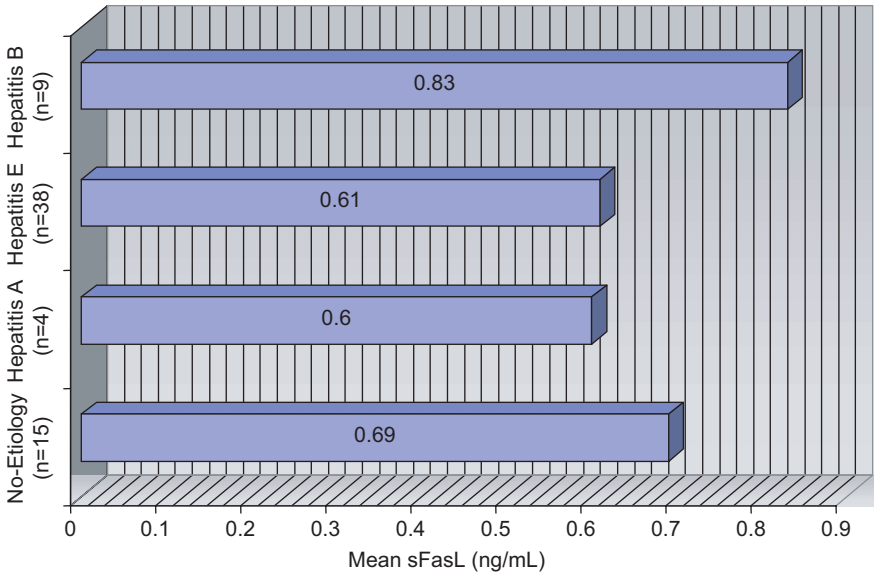

Figure 2. Mean sFas ligand (FasL) levels in relation to aetiological agent.

HBV-related cases had the highest serum levels for both TNF-α and sFasL followed by HEV-infected cases and then HAV-infected cases. While for the 'no aetiology' group, intermediate serum levels for both TNF-α and sFasL were observed between the HBV- and HAV/HEV-infected groups. However, the differences were statistically not significant.

Serum TNF-α and sFasL in HEV-related groups

In HEV-related patients, in the ALF group ($n=15$), mean sFasL (1.03 ± 0.101 ng ml^{-1}) and TNF-α (166.53 ± 13.24 pg ml^{-1}) were significantly higher than in the AH group ($n=23$) (sFasL 0.25 ± 0.091 ng ml^{-1}; TNF-α 73.23 ± 16.12 pg ml^{-1}). Again the levels in the ALF-E group were higher (sFasL 1.21 ± 0.112 ng ml^{-1}; TNF-α 178.22 ± 22.23 pg ml^{-1}) than in the ALF-S group (sFasL 0.89 ± 0.201 ng ml^{-1}; TNF-α 154.53 ± 16.34 pg ml^{-1}) but the differences were not significant.

Discussion

Our findings revealed that the clinical presentation and aetiology of ALF in India markedly differs from those of Western population. Also, no significant association could be established between age, bilirubin levels in serum and mortality in ALF cases which does not conform with the previous reports (Acharya et al. 1996). Prothrombin time difference (PT-D), which is an established biochemical prognostic marker for ALF (Bernuau et al. 1986, O'Grady et al. 1989, Acharya et al. 1996, Dhiman et al. 1998) was found to be significantly higher (p <0.001) in the ALF group compared with that of the AH group and also of the ALF-E group vs ALF-S group (p<0.001), thereby predicting mortality. On the other hand the jaundice–encephalopathy interval was found to have no impact on mortality, which is in agreement with other studies (Acharya et al. 1996, Dhiman et al. 1998). Also BUN and creatinine were significantly elevated in ALF-E when compared with ALF-S. We found that a liver span ≤ 4 cm at admission can be a valuable early clinical indicator of mortality but due to possible interobserver variation its usefulness is uncertain. We found mortality rates to be lower in the age group 12–20 years (42%) when compared with the >20 years age group (66.6%). There was a higher percentage of pregnant females in the ALF group suggesting pregnancy is a risk factor for the development of ALF in patients with AH. There was no significant difference in mortality rates of pregnant (66.7%) vs non-pregnant (72.7%) women with ALF which is consistent with the reports from Indian subcontinent (Bhatia et al. 2008).

Serum level of sFasL, an apoptotic marker, was found to be significantly (p<0.001) elevated in the ALF group in comparison to the AH group. This is similar to a study by Ryo et al. (2000) whereas no such marked difference was observed by Tokushige et al. (2000). Serum sFasL levels were higher in the ALF-E group than in the ALF-S group, but the difference was not statistically significant. In addition, sFasL serum levels were found to be positively correlated with other biochemical parameters such as serum bilirubin, AST, ALP and PT-D.

The differential effect of aetiology of ALF (acetaminophen vs non-acetaminophen) on TNF-α has already been described (De la Mata et al. 1990) thus making it important to study the effects of this cytokine in our population where aetiology markedly differs from the

West. Serum TNF-α levels were significantly higher in the ALF vs AH group ($p=0.033$), but this association could not predict mortality. Also, the TNF-α levels in all disease groups were higher than those described earlier in literature (Streetz et al. 2000, Masahito et al. 2000). This discrepancy can be attributed to different aetiology whereby virus-induced ALF causes greater stimulation of TNF-α production in comparison to acetaminophen-induced ALF as evident from higher TNF-α levels in the serum of the former (De la Mata et al. 1990). On the other hand the effects of different viral aetiologies on TNF-α levels reveal that it were found to be highest in HBV-infected hepatitis which is in agreement with an earlier report (Tokushige et al. 2000). This was followed by HEV and HAV infections. Similar to sFasL expression, the TNF-α levels in serum were found to be positively correlated with bilirubin, AST and most importantly with PT-D.

In addition, sFasL level was found to be significantly ($p<0.001$) associated with TNF-α. In a separate study (Singhal et al. 2008) we showed that upregulation of Fas and TNF-I receptors on hepatocytes of liver biopsies from patients with ALF supports the role of these cytokines in liver injury. Clearly liver injury in ALF is an interplay of various cytokines involved in the TNF family of death receptors leading to activation of intrinsic caspase 8 or effect on NF-κB which is not evaluated in this study.

Follow-up data of a few cases of ALF ($n=32$) revealed that the serum levels of sFasL and TNF-α either remained unchanged in cases recovering from encephalopathy or decreased, while an increasing trend was observed in patients who died. Therefore, it can be concluded that the levels are directly associated with the severity of the disease which is supported by other studies (Wang et al. 1999, Streetz et al. 2000). Serial monitoring of these cytokines could be one of the criteria for the prognosis of ALF. As our observation was based on only two time points, further studies are needed to elucidate their role with respect to ALF. As our analysis was based on only two readings of TNF-α levels, it is worthwhile mentioning that there could be multiple factors affecting the production of this cytokine such as demographics, aetiology, time of presentation and association with HLA as well as presence of multiple polymorphisms in TNF-α or nearby genes, all these factors can possibly confound some of the results.

To conclude, decreased liver span, elevated BUN, creatinine and PT difference can be useful in predicting mortality in ALF. Serum levels of TNF-α and sFasL are increased significantly more in ALF compared with AH and can be useful in differentiating ALF from AH, but are unable to predict mortality in non-acetaminophen-induced ALF and also in the HEV-induced ALF subgroup. Following the trends of sFasL and TNF-α serum levels can be of help in guiding timely referral to a transplant centre for patients with ALF in the Indian subcontinent.

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