A Study on Association between Neutrophil to Lymphocyte Ratio and Steatohepatitis and Fibrosis in Patients with Non-Alcoholic Fatty Liver Disease

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ABSTRACT

BACKGROUND
Non-alcoholic fatty liver disease (NAFLD) is seen worldwide and is the most common liver disorder in industrialized countries. Non-alcoholic steatohepatitis (NASH) is the severest form of NAFLD and is associated with inflammation and oxidative stress. Human neutrophil peptides have the ability to enhance hepatic fibrosis. We wanted to study the association between neutrophil to lymphocyte ratio and steatohepatitis and fibrosis in patients with non-alcoholic fatty liver disease.

METHODS
This is an analytical cross-sectional study conducted among 50 cases and 50 controls attending the hospital attached to BMCRI. The neutrophil lymphocyte ratio was calculated and compared between cases and controls.

RESULTS
Our study results showed that 7 patients had grade 1, 19 had grade 2, 24 had grade 3 hepatic steatosis and 13 patients had fibrosis. The mean neutrophil lymphocyte ratio (NLR) was significantly higher in cases than controls (3.6 +/- 1.83 and 1.72 +/- 0.57, P - 0.00). However, there was no statistical significance (F: 2.06, P value: 0.14) in the utility of NLR in detecting higher grades of steatosis (grade 1:2.41 +/- 0.76, grade 2:4.01 +/- 2.46, grade 3:3.62 +/- 1.3). The mean NLR was significantly higher (P - 0.001) in patients with fibrosis (3.72 +/- 2.1) compared with controls (1.46 +/- 0.51).

CONCLUSIONS
The neutrophil lymphocyte ratio can be used to detect the presence of steatohepatitis and fibrosis in patients with NAFLD but cannot be used to predict the presence of higher grades of hepatic steatosis.

KEY WORDS
Non-Alcoholic Fatty Liver Disease, Non-Alcoholic Steatohepatitis, Fibrosis, Neutrophil-Lymphocyte Ratio
Non-alcoholic fatty liver disease is one of the most common causes of chronic liver disease.\textsuperscript{1} Non-alcoholic fatty liver disease encompasses a spectrum of pathologic conditions, ranging from simple fatty infiltration termed as steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis.\textsuperscript{2} This entity has now reached epidemic proportions and is now one of the leading indication for liver transplantation in Western countries.\textsuperscript{3} Though the pathological picture resembles that of alcohol induced liver injury, it occurs in patients who do not consume alcohol or consume alcohol in quantities not considered to be harmful to the liver (less than 20 g/day or <140g/week).\textsuperscript{4}

NASH is characterized by deposition of triglyceride in the liver greater than 5 % of the total liver weight without a history of excessive alcohol intake or known aetiologies of liver disease.\textsuperscript{5,6} Approximately 20 to 30 % of adults in the general population in Western countries have NASH, and its prevalence increases to 70 to 90 % among persons who are obese or have diabetes.\textsuperscript{7} The overall prevalence of NASH is 15 to 40 % in western countries while 9 - 40 % in Asian countries.\textsuperscript{7}

Diabetes mellitus (DM), obesity and hyperinsulinemia are associated with NASH.\textsuperscript{8} There has been an exponential increase in the incidence of DM, obesity, and insulin resistance in India in the last two decades.\textsuperscript{9,10} Prevalence of NASH based on the ultrasound is 18.9 % in adults according to a population-based study in western India.\textsuperscript{7}

Non-alcoholic steatohepatitis is the most severe form of non-alcoholic fatty liver disease and is associated with inflammation and oxidative stress. Although, dysregulated lipid accumulation occurs across the non-alcoholic fatty liver disease spectrum, the features of liver cell injury, such as hepatocyte ballooning, cytoskeletal changes and hepatocyte apoptosis occur predominantly in NASH and distinguish NASH from simple steatosis.\textsuperscript{11}

Simple steatosis without fibrosis or inflammation has a benign clinical course. NASH, however, has a more progressive course and can lead to cirrhosis in 10 - 15 % of the patients. A liver biopsy remains the gold standard to establish extent of liver damage and fibrosis and is an invasive and expensive procedure. Imaging studies have been used to diagnose NASH with less sensitivity and specificity. Hence there is a great need for identifying non-invasive markers for predicting NASH and significant fibrosis. Blood neutrophil-lymphocyte ratio is a simple marker of subclinical inflammation and can be easily calculated from the differential WBC counts. The N/L ratio has been used to predict outcomes in patients with coronary artery disease and cancer.

Neutrophil-lymphocyte ratio integrates information on the inflammatory milieu and physiological stress. A prominent feature of inflammation is neutrophil accumulation. Neutrophil derived myeloperoxidase can increase macrophage cytotoxicity and induce neutrophil activation in a NASH mouse model.\textsuperscript{12}

In the foz/foz NASH metabolic syndrome model, a reduction in the hepatic cholesterol stores was associated in ameliorated liver injury, apoptosis, macrophage, and neutrophil accumulation. Neutrophil dysfunction was also associated with liver fibrosis and cirrhosis in NASH.\textsuperscript{13} Human neutrophil peptides have the ability to enhance liver fibrosis in fatty liver disease by inducing hepatic stellate cell proliferation.\textsuperscript{14}

This ratio integrates information on two different pathways - the neutrophil that is responsible for ongoing inflammation and the lymphocytes that represent the regulatory pathway. The N/L ratio is an indicator of overall inflammatory status of the body and higher ratios may be found in patients with NAFLD patients with advanced disease.\textsuperscript{14} The neutrophil-lymphocyte ratio can hence be used as a marker for NASH and fibrosis in patients with non-alcoholic fatty liver disease and can be a novel, non-invasive marker to predict advanced disease.

We wanted to study the association between neutrophil to lymphocyte ratio and steatohepatitis and fibrosis in patients with non-alcoholic fatty liver disease.

\section*{METHODS}

This is an analytical cross-sectional study conducted from November 2016 to October 2018. Data was collected from the patients visiting the outpatient department and in-patients at hospitals attached to Bangalore Medical College and Research Institute.

\section*{Sample Size}

Based on a previous study by Naim Alkhouri et al. neutrophil value was $4.1 \pm 1.4$ for cases and assumed equal standard deviation for controls and minimum expected difference is 0.8.\textsuperscript{15}

\begin{equation}
\begin{align*}
n &= \frac{2(Z_\alpha + Z_1-p)^2\sigma^2}{d^2} \\
&= \frac{2(1.96 + 0.84)^2(1.4)^2}{(0.8)^2} \\
&= 48 \approx 50
\end{align*}
\end{equation}

50 cases and 50 age and sex matched healthy controls were considered for the study.

\section*{Inclusion Criteria}

1. Patients willing for study
2. Age $\geq 18$ years,
3. Non-alcoholics
4. Raised liver enzymes – AST, ALT at least 1.5 times above upper limit of normal
5. USG examination showing - Diffuse fatty infiltration

\section*{Diffuse Fatty Infiltration will Be Graded as Follows -}

1. Mild/grade 1 (mild steatosis) - slightly increased liver echogenicity with normal vessels and absent posterior attenuation
2. Moderate/grade 2 (moderate steatosis) - moderately increased liver echogenicity with partial dimming of vessels and early posterior attenuation
3. Severe /grade 3 (severe steatosis) - diffusely increased liver echogenicity with absence of visible vessels and heavy posterior attenuation.

**Exclusion Criteria**

1. Positive test for HBsAg and anti HCV antibodies
2. Alcohol intake > 20gm/day
3. Past history of chronic liver disease of proven aetiology.
4. Patients on medications that can induce fatty liver like methotrexate, oestrogen, amiodarone, tamoxifen, corticosteroids and tetracyclines
5. Hepatocellular carcinoma
6. History of gastrointestinal bypass surgery
7. Patients with active infection.
8. Patients not consenting for the study.

Written informed consent was taken from the patients. After taking informed consent from patients, a detailed history and clinical examination was performed to identify all the necessary symptoms and signs.

All the routine investigations such as
- Complete blood counts
- Liver function tests
- Renal function tests
- Blood sugars
- Viral markers (HBsAg, anti - HCV antibodies)
- Ultrasound abdomen
- Other relevant investigations

Non-alcoholic fatty liver disease was graded as per ultrasonographic criteria.

USG examination showing - diffuse fatty infiltration. Diffuse fatty infiltration will be graded as follows: 16
- Mild/grade 1 (mild steatosis) - slightly increased liver echogenicity with normal vessels and absent posterior attenuation
- Moderate/grade 2 (moderate steatosis) - moderately increased liver echogenicity with partial dimming of vessels and early posterior attenuation
- Severe/grade 3 (severe steatosis) - diffusely increased liver echogenicity with absence of visible vessels and heavy posterior attenuation
- Fibrosis was evidenced as increase in the echotexture of the liver with surface nodularity.

Neutrophil-lymphocyte ratio is calculated from the differential count as reported in CBC and was compared with that of controls.

**Statistical Analysis**

The data was entered in Microsoft excel sheet and was analysed using statistical package for social sciences (SPSS) version 22 software. The categorical data was represented in the form of frequency and percentage. Chi-square/Fisher's exact test was used to test the significance for categorical data.

Continuous data was represented as mean and standard deviation. Student - t test and analysis of variance (ANOVA) was used for comparison between groups. P value < 0.05 was considered statistically significant.

**RESULTS**

Table 1 shows distribution of the subjects based on age. Equal distribution was considered in both cases and controls with higher distribution of subjects in 41 to 60 years age group - (20 out of 100). Equal distribution was considered in both cases and controls with respect to gender with maximum subjects being females - 68 out of 100.

| Age | Case | Control | Total |
|-----|------|---------|-------|
| ≤ 20 | 2 | 2 | 4 |
| 21 - 40 | 17 | 50.0 % | 50.0 % | 100.0 % |
| 41 - 60 | 10 | 50.0 % | 50.0 % | 100.0 % |
| > 60 | 1 | 50.0 % | 50.0 % | 100.0 % |

**Table 1. Distribution of the Subjects Based on Age and Gender**

| Symptoms | Frequency | Cases |
|----------|-----------|-------|
| Malaise | 41 | 82.0 |
| Abdominal discomfort | 27 | 54.0 |
| Jaundice | 7 | 14.0 |
| Abdominal distension | 3 | 6.0 |
| Fever | 2 | 4.0 |

**Table 2A. Distribution of the Subjects Based on Symptoms**

Out of 50 cases, 23 (46 %) patients had diabetes and 8 (16 %) cases had hypertension. Out of 50 (100 %), 43 (86 %) had BMI ≥ 25 followed by 5 (10 %) had BMI in the range of 18.5 - 22.9 and the least number of subjects – 2 (4 %) were in the BMI range of 23 - 24.9. Cross-tabulation of gender and waist circumference showed that out of 34 (68 %) females, 23 (95.8 %) were obese and 11 (42.3 %) were non-obese whereas - as out of 16 (32 %) males, 15 (57.7 %) were non-obese and 1 (4.2 %) was obese.

| BMI (Asian Classification) | Frequency | Cases |
|---------------------------|-----------|-------|
| 18.5 - 22.9 | 5 | 10.0 |
| 23 - 24.9 | 2 | 4.0 |
| > 25 | 43 | 86.0 |
| Total | 50 | 100.0 |

**Table 2B. Distribution of the Subjects Based on BMI**

Other signs observed among cases showed that hepatomegaly was high among cases (54 %), followed by pedal oedema (42 %). The mean haemoglobin of cases and controls was 12.02 ± 1.94 and 12.9 ± 1.35 respectively.

The difference between mean haemoglobin and the groups was significant (P < 0.000). The mean total count of cases and controls was 9.636.02 ± 2,990.82 and 7,920.80 ± 2,276.91.
The mean neutrophils of cases and controls was 71.15 ± 8.35 and 57.58 ± 7.38 respectively. The difference between neutrophils and the groups was significant (P = 0.000). The mean lymphocytes of cases and controls was 22.33 ± 6.13 and 35.27 ± 6.48 respectively. The difference between lymphocytes and the groups was significant (P = 0.000). The mean platelet of cases and controls was 231 ± 85 and 293 ± 99 respectively. The difference between mean platelet count and the groups was significant (P = 0.000).

The mean MCV of cases and controls was 84.85 ± 8.41 and 84.23 ± 11.93 respectively. The difference between mean MCV and the groups was not significant (P = 0.77). The mean RBS of cases and controls was 181.36 ± 108.82 and 111.66 ± 112.97 respectively. The difference between mean RBS and the groups was significant (P = 0.000). The mean urea of cases and controls was 7.38 ± 10.21 and 23.24 ± 6.89 respectively. The difference between mean urea and the groups was significant (P = 0.01). The mean creatinine of cases and controls was 0.78 ± 0.34 and 0.69 ± 0.20 respectively. The difference between mean creatinine and the groups was not significant (P = 0.11).

The mean total bilirubin of cases and controls was 1.55 ± 0.85 and 0.59 ± 0.26 respectively. The difference between mean total bilirubin and the groups was significant (P = 0.000). The mean direct bilirubin of cases and controls was 0.65 ± 0.46 and 0.20 ± 0.15 respectively. The difference between mean direct bilirubin and the groups was significant (P = 0.00). The mean total protein of cases and controls was 6.22 ± 0.8 and 7.12 ± 0.6 respectively. The difference between mean total protein and the groups was significant (P = 0.00).

The mean albumin of cases and controls was 2.98 ± 0.77 and 3.81 ± 0.47 respectively. The difference between mean albumin and the groups was significant (P = 0.00). The mean globulin of cases and controls was 3.23 ± 0.82 and 3.27 ± 0.54 respectively. The difference between mean globulin and the groups was not significant (P = 0.77).

The mean AST of cases and controls was 86.66 ± 39.7 and 22.84 ± 9.23 respectively. The difference between mean AST and the groups was significant (P = 0.000). The mean ALT of cases and controls was 91.50 ± 25.34 and 25.14 ± 25.20 respectively. The difference between mean ALT and the groups was significant (P = 0.000). The mean ALP of cases and controls was 157.46 ± 45.61 and 84.24 ± 27.16 respectively. The difference between mean ALP and the groups was significant (P = 0.000). The mean NLR of cases and controls was 3.6 ± 1.83 and 1.72 ± 0.57 respectively. The difference between mean NLR and the groups was significant (P = 0.000).

Mean distribution of grading of HS and NLR showed higher mean with grade 2 (4.01 ± 2.46) followed by grade 3 (3.62 ± 1.3) and grade 1 (2.41 ± 0.76). ANOVA was applied to find the statistical difference among the grading of HS. ANOVA showed no statistically significant difference among the groups for mean NLR (P = 2.06; P = 0.14).

| Grade of HS | N | Mean | Std. Deviation | Minimum | Maximum | F Value* | P Value |
|------------|---|------|----------------|---------|---------|---------|---------|
| 1          | 7.00 | 2.41 | 0.76 | 1.73 | 4.00 | 2             | 0.14 |
| 2          | 19.00 | 4.01 | 2.46 | 1.61 | 12.10 | 2.06 | 0.14 |
| 3          | 24.00 | 3.62 | 1.30 | 2.01 | 8.00 | 2.06 | 0.14 |
| Total      | 50.00 | 3.60 | 1.83 | 1.61 | 12.10 | 2.06 | 0.14 |

*ANOVA

Mean distribution of fibrosis and NLR showed higher mean with fibrosis (3.72 ± 2.1) as compared to mean NLR without fibrosis (1.46 ± 0.51). Independent sample t-test was applied to find the statistical difference between the fibrosis. There was statistically significant difference between the groups for mean NLR (T = 9.065; P = 0.001).

| Fibrosis | N | Mean | Std. Dev. | Minimum | Maximum | t Test | P Value |
|----------|---|------|-----------|---------|---------|--------|---------|
| Yes      | 13 | 3.27 | 0.50 | 2.1 | 4.50 | 5 | 0.001 |
| No       | 13 | 1.46 | 0.51 | 0.97 | 2 | 21 | 0.001 |

**DISCUSSION**

Chronic liver disease is one of the leading causes of significant morbidity and mortality accounting to 800,000 deaths per year. Non-alcoholic fatty liver disease is one of the most common causes of liver disease in modern industrialized economies. NAFLD is highly prevalent in modern societies and 10 - 25 % cases develop hepatic fibrosis leading to cirrhosis, end stage liver disease and hepatocellular carcinoma. Liver biopsy is the "gold standard test" for diagnosis and staging of the disease. It is invasive and cumbersome. Studies on non-invasive methods in identifying severe disease is an area of active research and the search for a novel non-invasive marker continues.

Neutrophil lymphocyte ratio could be an important marker of systemic inflammation. The increase in NLR could be explained by the "Two hit hypothesis." The first hit is excessive triglyceride accumulation, and the second hit is activated pro-inflammatory pathways. NLR has been related to various inflammatory conditions and cancers and has been associated with poorer outcomes. In our study conducted at Bangalore medical college and research institute and attached hospitals, 50 cases of NAFLD and 50 age and sex matched controls were compared. It was found that 37 (74 %) patients belonged to the age group between 20 - 60 years of age proving that the disease prevailed in the young and middle aged with maximum patients in the age group between 40 - 60years. 32 % cases were males and 68 % patients were females. In a population-based study by Deepak Amarapurkar et al. it was found that the mean age of the patients with NAFLD was 39.08 ± 12.3 years and the disease was prevalent amongst the middle aged. However, the...
prevalence of NAFLD was higher in males compared to females. 41 (82 %) patients complained of “malaise” which was the predominant symptom followed by abdominal discomfort in 27 (54 %) patients and loss of appetite in 9 (18 %) patients, proving that patients presented more commonly with nonspecific symptoms. Hepatomegaly was observed in 27 (54 %) patients followed by pedal oedema in 21 (42 %) patients. 11 (22 %) patients had jaundice. According to a review article by Basaranoglu and Neuschwander –Tetri,25 the most frequent symptoms are fatigue and right upper quadrant pain or dullness, although most patients may be asymptomatic, which is similar to the observations in our study. Mild to moderate hepatomegaly is one of the most common physical examination findings.

Non-alcoholic fatty liver disease has been increasing in prevalence in the last decade and has been found to have a strong association with metabolic risk factors of diabetes, dyslipidaemia and obesity. NAFLD is seen in more than 70 % of patients with diabetes and coexistent obesity.

In a study by Deepak Amarapurkar et al.7 a population-based study, diabetes was evident in 22 % cases of NAFLD, 52 % cases had obesity and 38 % patients had abdominal obesity.7 In a study by Singh SP et al.26 464 consecutive patients with NAFLD were studied for associated risk factors, diabetes was seen in 15.2 % of cases, hypertension in 17.4 % of the cases. The mean BMI observed in cases was 26.25 +/−3.80 which is in accordance with our study.

In our study, 2.3 (46 %) patients had diabetes and 8 (16 %) cases had hypertension as existing comorbidities. 86 % patients had body mass index (BMI) greater than 25 kg/m² and 4 patients were overweight and mean BMI was 27.652 kg/m², indicating that obesity is a major risk factor for the development of NAFLD. 24 (48 %) patients had higher waist circumference with majority of patients being females with abdominal obesity. A study by Ramesh Kumar et al.27 205 cases of NAFLD were studied, it was observed that 68.8 % patients were obese and 18 % were overweight.

White blood cell (WBC) count is a simple, global and inexpensive marker of inflammation and is a useful marker for infection and inflammation. Higher levels of WBC count have been associated with diabetes and metabolic syndrome linked to chronic low-grade inflammation and insulin resistance. Insulin resistance is a key feature in the pathogenesis of NAFLD.

In our study, significant statistical difference (P < 0.05) was noted between cases and controls with respect to total counts, neutrophil counts, lymphocyte counts and platelet count. Mean total count was 9,636 ± 2,990.82 in cases and 7,920.80 ± 2,276.91 respectively. The total counts were significantly higher in cases than controls. In a cross-sectional study by Yong-jae Lee et al.28 a positive association was found between WBC counts and NAFLD prevalence in a dose response manner. In a study by Kuppan et al.29 it was found that the leucocyte count was significantly higher in patients with NAFLD compared with those without (7.8 ± 1.4 × 10^9/l vs. 6.9 ± 0.9 × 10^9/l; P value < 0.001).

The mean neutrophil percentage was 71.15 ± 8.35 % and 57.58 ± 7.38 % in cases and controls respectively and was found to be higher in cases compared to controls and was statistically significant. The mean lymphocyte percentage in cases and controls were 22.33 ± 6.13 % and 35.27 ± 6.48 % respectively and it was statistically significant. Lower lymphocyte counts were found in cases compared to controls. The mean platelet count in cases and controls were 2.31 ± 0.85 lakh/mm^3 and 2.93 ± 0.99 lakh/mm^3 respectively and was found to be statistically significant in our study.

The cases had a lower mean platelet count compared to the controls. The peripheral platelet production is regulated by thrombopoietin which is synthesized in the liver. In a prospective cohort study by Liu et al.30 at five years of follow-up, there was significant reduction in the platelet count from 220 ± 40.70 (10^9/L) to 208.41 ± 43.26 (10^9/L) in patients with NAFLD even after adjustment for confounding variables.

The mean total bilirubin was 1.55 ± 0.85 mg/dl and 0.59 ± 0.26 mg/dl in cases and controls respectively. The mean direct bilirubin of cases and controls was 0.65 ± 0.46 mg/dl and 0.20 ± 0.15mg/dl respectively. The mean total bilirubin and direct bilirubin was significantly higher in cases and controls.

In a study by Chang Y et al.31 it was found that higher direct bilirubin levels were significantly associated with a lower risk of developing NAFLD. On the contrary our study shows significantly higher levels of total and direct bilirubin levels in cases compared to controls.

Mean albumin level was 2.98 ± 0.77 g/dl in cases and 3.81 ± 0.47 g/dl in controls and albumin levels were significantly lower in cases. However, there was no significant statistical difference for globulin levels. Younossi et al.32 proved that hypoalbuminemia, high total bilirubin, and prolonged prothrombin time in patients with diabetes mellitus and NAFLD was associated with poorer outcome.

Cases had significantly higher mean AST, ALT and ALP levels compared to controls and ALT levels were higher than AST levels. In a study by Chang et al.33 it was observed that in apparently health non-diabetic Korean men, increasing ALT concentration, even within its reference interval, was an independent predictor of incident NAFLD.

In our study, it was observed that 7 patients had grade 1 hepatic steatosis, 19 patients had grade 2 hepatic steatosis and 24 patients had grade 3 hepatic steatosis with 13 patients having evidence of fibrosis on ultrasonography. The mean neutrophil - lymphocyte ratio of cases and controls was 3.6 ± 1.83 and 1.72 ± 0.57 respectively. The difference between mean NLR and the groups was significant (P < 0.001). Mean distribution of grading of HS and NLR showed higher mean with grade 2 (4.01 ± 2.46) followed by grade 3 (3.62 ± 1.3) and grade 1 (2.41 ± 0.76).

ANOVA showed no statistically significant difference among the groups for mean NLR (F = 2.06; P = 0.14). Mean distribution of fibrosis and NLR showed higher mean with fibrosis (3.72 ± 2.1) as compared to mean NLR without fibrosis (1.46 ± 0.51). There was statistically significant difference between the groups for mean NLR (T = 9.065; P = 0.001).

Observations in our study shows that the mean NLR was higher in cases compared to controls and it was statistically significant. Hence, NLR can be used to detect the presence of steatohepatitis in patients with NAFLD.

However, there was no statistical significance in the utility of NLR in detecting higher grades of hepatic steatosis. The mean NLR was significantly higher in patients with fibrosis compared with controls and hence can be used as a marker to identify fibrosis, which is advanced disease in patients with NAFLD.
In a study conducted by Naim Alkhouri et al., it was found that the patients with NASH had higher NLR compared to the non-NASH group [2.5 (1.9 - 3.3) and 1.6 (1.2 - 2.0)] with P value < 0.001 and patients with advanced fibrosis had elevated NLR [2.9 (2.0 - 3.9)] which was statistically significant which is similar to the observation in our study.

In a study by N.K. Kadaraman et al., where the NLR was used to assess the severity of NASH in diabetics, it was found that neutrophil-lymphocyte ratio increases with increasing grade of steatosis in NALFD patients with diabetes and can be used as a convenient marker to follow progression of non-alcoholic fatty liver disease. Our study failed to correlate with the increasing grade of steatosis but showed higher mean NLR in cases compared to controls and can be used as a non-invasive marker to detect the presence of NAFLD and to detect advanced disease.

CONCLUSIONS

Neutrophil lymphocyte ratio can be used to detect the presence of steatohepatitis in patients with NAFLD. The neutrophil lymphocyte ratio cannot be used to predict the presence of higher grades of hepatic steatosis.

Data sharing statement provided by the authors is available with the full text of this article at jemds.com.

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