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# An Academic Referral Hospital's Evaluation of Three Staining Methods for Liver Fibrosis in Biopsy Samples

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**Abstract:**

Context: The accumulation of extracellular matrix components, especially elastic fibers and collagen, causes liver fibrosis to worsen over time, causing the organ to stop working as it should. In order to diagnose, predict, and treat fibrosis, accurate histological staging is required. Common fibrosis evaluation stains include hematoxylin and eosin (H and E), Masson's trichrome (MT), and orcein (OR), although there is a lack of clarity about the relative efficacy and connection of these stains in everyday practice. Approach: From 2021 to 2023, 76 liver biopsy samples were reviewed in this retrospective analysis. The patients were sent to Taleghani Hospital in Tehran, Iran. We stained all of the samples with H&E, MT, and OR. Skilled pathologists staged the fibrosis using the Ishak grading method. The agreement and correlation between staining procedures were evaluated using statistical analysis, which included Fisher's exact test. Significant results were defined as P values less than 0.05. The results show that with 76 patients (34.2% male, mean age 41.2 years), the finding of fibrosis was most common at Stage 2 for MT (35.5%) and Stage 3 for OR (23.7%). H and E and MT showed significant differences in fibrosis staging ($P < 0.001$), as with MT and OR ($P = 0.006$). At Stage 2, there was a significant difference between MT and OR ($P = 0.003$). Importantly, in 36.8% of instances, OR staining revealed a lower level than MT, but in 23.7% of cases, OR staining revealed a higher stage. When looking at individuals between the ages of 0 and 32 ($P = 0.032$) and males ($P = 0.036$), there were statistically significant differences between MT and OR. In summary: In order to evaluate liver fibrosis, our results show that MT and OR staining techniques provide complementing information. The noticeable differences, particularly in the intermediate phases, indicate that OR staining, when used in conjunction with MT, improves diagnostic precision. For a thorough assessment of fibrosis in liver biopsies, it is advised to routinely utilize both stains.

Liver fibrosis, Masson's trichrome, orcein, hematoxylin and eosin, histopathology

## INTRODUCTION

Chronic liver damage causes an overabundance of extracellular matrix components, especially collagen fibers, which leads to the pathological state known as liver fibrosis. [1] Untreated fibrosis of the liver may alter its normal structure and function, increasing the risk of cirrhosis and liver failure. Therefore, diagnosis, prognosis, and treatment decision-making all depend on an accurate evaluation of liver fibrosis.

among which are the stains that mainly bring attention to collagen fibers: Picrosirius red (SR), hematoxylin and eosin (H and E), and Masson's trichrome (MT). [2] in The structural remodeling of is facilitated by elastic fibers as well as collagen.

Hepatocellular analysis of liver biopsy samples is yet the

gold standard for fibrosis staging. In order to identify and measure fibrotic tissue, many staining techniques have been used,

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hepatic tissue that has become fibrotic. The particular purpose of orcein (OR) staining is to identify elastic fibers; this allows for the separation of new and old fibrosis and provides additional information that collagen-based stains do not. [3] The distribution and deposition of collagen, which is associated with clinical outcomes in chronic liver illnesses, may be precisely measured thanks to advancements in digital image processing, which has further improved the quantification of liver fibrosis. four to six] In addition to histology procedures, noninvasive techniques like transient elastography (FibroScan) have become more popular for evaluating liver stiffness. [7] Research on individuals with chronic hepatitis C has shown that elastography measures correlate with fibrosis stages determined by MT, SR, and OR staining. on lines 8, 9 Combining OR staining with MT has been shown to be useful in improving the identification and characterisation of advanced fibrosis and cirrhosis, according to recent studies. One example is the work of Nguyen et al., who demonstrated that cirrhotic liver tissues may be better assessed for fibrosis changes when stained with OR. [10] The therapeutic importance of elastic fiber quantification was further shown by Leite et al., who discovered a substantial correlation between hepatitis C patients' fibrosis severity determined by elastography and the elastic fiber content revealed by OR staining. the eleventh Regardless of these advancements, there is still a lack of research comparing the efficacy and precision of various histochemical staining techniques, especially when it comes to a wide range of liver illnesses other than hepatitis C. More research is required since there are discrepancies in the results. In order to help refine histopathological examination processes, this research will gather biopsy samples from a referral university hospital and examine the efficacy of H and E, MT, and OR staining methods in identifying liver fibrosis.

## METHODS

### Ethical considerations

This study was conducted in full accordance with the ethical principles outlined in the Declaration of Helsinki. The research protocol was reviewed and approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences (approval code: IR. SBMU. MSP. REC.1401.171). Given the retrospective nature of the study, only archived and anonymized liver biopsy samples were utilized. No direct patient contact or intervention was involved. The requirement for individual informed consent was waived by the ethics committee, as the use of deidentified data posed minimal risk to participants and ensured the protection of patient confidentiality. All procedures involving human tissue were performed in compliance with institutional guidelines and ethical standards. Strict measures were taken to safeguard patient privacy, and all data were handled confidentially. Access to patient records and samples was limited to authorized personnel involved in the study. The study's findings are reported in aggregate form, ensuring that no individual patient can be identified. Data supporting the results of this study are available from the corresponding author upon reasonable request and with appropriate institutional approval.

### Type of sampling and reason of selection

A nonselective, convenience sampling method was used. All available liver biopsy samples archived in the pathology department of Taleghani Hospital from 2021 to 2023 (SBMU, Tehran, Iran) that met the inclusion criteria, specifically, samples suspected of having liver fibrosis or with a confirmed diagnosis of liver fibrosis according to pathology reports, were included in the study. After excluding three samples due to insufficient tissue, a total of 76 cases were analyzed. The reason for selecting this sampling method was the retrospective nature of the study and the practical need to utilize existing, archived biopsy samples. This approach enabled the researchers to access a sufficient and representative number of cases across all stages of liver fibrosis, ensured the feasibility of the study by relying on already available paraffin-embedded tissue blocks, and reflected the real-world spectrum of liver fibrosis encountered in a major referral hospital. Such a method is commonly used in retrospective histopathological studies where the aim is to analyze all eligible cases within a defined period to maximize sample size and enhance the generalizability of the findings to routine clinical practice

### Patient consent statement

This study was conducted using archived, anonymized liver biopsy samples and retrospective review of medical records. No direct patient contact or intervention was involved. All procedures were performed in accordance with institutional guidelines and the ethical standards of Shahid Beheshti University of Medical Sciences. The use of archived tissue samples and retrospective data was approved by the institutional ethics committee, which waived the requirement for individual patient consent due to the deidentified nature of the data and minimal risk to participants. Patient confidentiality was strictly maintained throughout the study.

### Inclusion criteria

The inclusion criteria were liver biopsy samples archived in the pathology department of Taleghani Hospital from 2021 to 2023, with a suspected or confirmed diagnosis of liver fibrosis based on pathology reports, and sufficient tissue available for additional staining and analysis.

### Exclusion criteria

The exclusion criteria were liver biopsy samples with insufficient tissue for additional staining and analysis, as well as any samples with poor staining quality or inadequate specimen integrity. In addition, cases lacking complete pathology reports or relevant clinical data.

### Staining methods

The extracted slides from the archive, all of which were stained by the H and E staining method and some, by the MT staining method as well, were examined to determine if the staining quality and the adequacy of the specimen for OR and MT staining are proper, and the inappropriate samples were excluded. Then, the paraffin-embedded blocks of the tissue samples were selected to undergo microtomy (tissue sectioning) for MT and OR staining.

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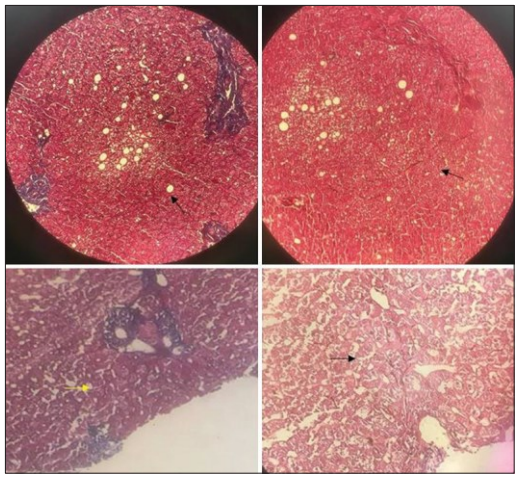
Our MT staining technique was as follows: First, the tissue sections with a thickness of five µm were provided using a microtome and placed on standard microscopic slides. The slides were hydrated using decreasing grades of alcohol and then placed in Bouin solution at 56°C in the laboratory oven for 60 min. Then we washed the slides with running water, and then with distilled water, and we poured Weigert's Hematoxylin solution on the tissue for 10–15 min. After that, the slides were washed with running water for 10 min and then with distilled water, and placed again for 10–15 min in the slide staining jar containing Biebrich scarlet-acid fuchsin solution. After washing them again with distilled water, we poured Phosphotungstic-phosphomolybdic acid on the slide for about 10–15 min and placed them in aniline blue solution for 20–25 min. In the next step, we washed the samples with distilled water and soaked them in 1% acetic acid solution for 2–5 min. In the end, the slides were washed with distilled water and dehydrated with alcohol, and after passing through xylene and placing a coverslip, the slides were finally fixed and ready to be evaluated microscopically.

Our OR staining technique was as follows: first, we hydrated the samples using distilled water, then we placed them in a potassium permanganate solution for 10 min. Then, they were washed in water and immersed in 5% oxalic acid until they became colorless. We washed them again with tap water. Then, 0.5% periodic acid was added. Then, we washed them with tap water and then with distilled water. After that, the samples were immersed into OR solution between 4 and 16 h at room temperature and washed in ethanol. Then, they were dehydrated with ethanol and clarified with xylene. Then, we covered them using glue and coverslips [Figure 1].

**Figure 1:** The top left panel shows marked fibrosis in various regions of the



hepatic lobule, stained with Masson's trichrome (MT). The top right panel, stained with hematoxylin and eosin, demonstrates significantly less visible fibrosis, but highlights fatty changes in the liver tissue (dark arrow). The lower left panel, stained with orcein (OR), reveals a distinct pattern of fibrosis compared to MT. The lower right panel, stained with hematoxylin and MT, also shows less apparent fibrosis (dark arrow)

### Assessment

The samples were evaluated by the MT staining method as the basic staining and OR and H and E staining methods to be compared with MT. After the slides were prepared, our pathologists interpreted them based on the Ishak fibrosis scoring system.

### Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Due to institutional and ethical regulations, the raw data derived from patient records and archived biopsy samples cannot be made publicly available to protect patient confidentiality. Deidentified data sets and additional information relevant to the analyses conducted in this research may be provided by the corresponding author (Dr. Zhaleh Mohsenifar, mohsenifar@ sbmu.ac.ir) upon justified request and with appropriate institutional approval

### Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics software, version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to summarize demographic variables, including age and gender distribution, as well as the frequency of each fibrosis stage as determined by the three staining methods (H and E, MT, and OR). To assess the correlation and agreement between the different staining methods in staging liver fibrosis, Fisher's exact test was employed due to the categorical nature of the data and the relatively small sample size. The concordance between staining methods at each fibrosis stage was evaluated, with particular attention to stages where discrepancies were most pronounced. Pairwise comparisons were conducted between MT and OR, MT and H and E, and OR and H and E staining results. In addition, the directionality of discrepancies was analyzed to determine the proportion of cases in which one staining method indicated a higher or lower fibrosis stage compared to another. Subgroup analyses were performed based on patient gender and age groups to explore potential demographic influences on staining concordance.

All statistical tests were two-tailed, and $P < 0.05$ was considered indicative of statistical significance. Results are presented as frequencies, percentages, and $P$ values. Where relevant, tables and figures were used to illustrate the degree of accordance and discordance between staining methods.

## RESULTS

Of the 76 individuals whose liver samples were examined in our research, 64.21 percent were male and 65.78 percent were female. The average age of the patients was 41.19 years, with men's average age being 42.26 and females' average age being 40.64. H and E staining revealed the following frequencies of fibrosis stages: The percentages are as follows: 7.89% in Stage 0 (absence of fibrosis), 28.94% in Stage 1, 31.57% in Stage 2, 9.21% in Stage 3, 5.26% in Stage 5, and 7.89% in Stage 6. P

occurring more often throughout the first two phases. Fibrosis identification by MT staining occurred most often in Stage 2, with a frequency of 33.52%, in Stage 2, 9.21%, 23.68%, 3.94%, 13.15%, and 10.52% in the following stages. In terms of OR staining, the frequencies were as follows: 5.26% in Stage 0, 21.05% in Stage 1, 19.73% in Stage 2,

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23.68% in Stage 3, 13.15% in Stage 4, 3.94% in Stage 5, and 13.15% in Stage 6, with Stage 3 holding the greatest frequency. The staining techniques were compared statistically based on their ability to identify liver fibrosis. Both the OR and MT staining differences and the MT staining differences between H and E were statistically significant (P = 0.006, P = 0.001, and P = 0.001, respectively). Table 1 displays the degree of agreement between the outcomes of the MT and OR tests. Table 1 shows that between stages 2 and 5, the two staining methods differed the most. To be more precise, about 77.8% of instances in which MT staining revealed Stage 2 fibrosis also exhibited a different stage when OR staining was applied, and 70% of cases in which MT staining revealed Stage 5 fibrosis also showed a different stage when OR staining was applied. When Fisher's exact test was used to assess this difference, it was found to be statistically significant. The difference was significant in Stage 2 (P = 0.028) when studied independently and then put to Fisher's exact test with the other stages. In Stage 5, however, it was not significant (P = 0.731). In Stage 6, there was a perfect agreement between the two approaches, with a p-value of less than 0.001. Additional statistical analysis was carried out to determine whether OR staining indicates a greater stage compared to MT staining, or if the reverse is true. If you look at Table 2, you'll see that while MT staining revealed Stage 2 fibrosis, OR staining indicated a lower stage in 44.4% of instances and a higher stage in 33.3% of cases also. On average, MT staining revealed a higher stage than OR staining in 23.7% of instances, whereas OR staining revealed a lower stage in 36.8% of cases. Statistical analysis using Fisher's exact test also confirmed the significance of this difference (P = 0.012). Statistical examination of the concordance between MT and H&E findings revealed that in about 63% of instances, H&E staining revealed a stage other than 2 when MT staining indicated Stage 2. Furthermore, when H and E staining revealed a stage other than 1, 71.4% of the time MT staining had already recognized Stage 1. Using Fisher's exact test, we found no statistical significance (P = 0.503) for this difference. In 59.2% of instances, H and E staining indicated a lower stage than MT staining, while in 1.3% of cases, H and E staining showed a higher stage than MT staining. This was determined by further investigation to see whether H and E staining reveals a higher or lower level. Similarly, Fisher's exact test analysis of this difference did not reveal a statistically significant difference (P = 0.572). Table 3 summarizes these results. Statistical investigation comparing the findings of H and E staining with those of OR staining revealed that when OR staining identified Stage 2, H and E staining indicated the same stage in 53.9% of instances and a lower stage than OR in 44.4% of Stage 2 cases. H and E staining revealed a lower stage in 44.7% of instances, but in 1.3% of cases it revealed a greater stage than OR staining. Moreover, this distinction was were tested using Fisher's exact test; the results were inconclusive. In Table 4 you can see the outcomes. Stages 0 and 6 showed no difference in MT and OR staining techniques in our male patients' specimens (Table 3), however stage 1 showed a difference of 100%, stage 2 a difference of 71.4%, and stage 3 a difference of 66.4%.

**Table 1: Accordance between Masson's trichrome and orcein results**

| MT grade | Different OR | Same OR | *P* |
|---|---|---|---|
| 0.00 | 1 (33.3) | 2 (66.7) | 0.558 |
| 1.00 | 4 (57.1) | 3 (42.9) | >0.99 |
| 2.00 | 21 (77.8) | 6 (22.2) | 0.028 |
| 3.00 | 11 (61.1) | 7 (38.9) | >0.99 |
| 4.00 | 2 (66.7) | 1 (33.3) | >0.99 |
| 5.00 | 7 (70) | 3 (30) | 0.731 |
| 6.00 | 0 | 8 (100) | <0.001 |
| Total | 46 (60.5) | 30 (39.4) | |

Details agreement between MT and OR staging. Discordance is highest at Stage 2. Statistical analysis reveals significance at Stage 2 (*P*=0.028). Concordance at Stage 6 is perfect. These differences suggest variability in intermediate fibrosis stages and the need for multiple staining methods. MT: Masson's trichrome, OR: Orcein

**Table 2: Comparison between Masson's trichrome and orcein results**

| MT grade | MT > OR | MT < OR | MT=OR | *P* |
|---|---|---|---|---|
| 0.00 | 0 | 1 (33.3) | 2 (66.7) | 0.45 |
| 1.00 | 2 (28.6) | 2 (28.6) | 3 (42.9) | 0.89 |
| 2.00 | 12 (44.4) | 9 (33.3) | 6 (22.2) | 0.063 |
| 3.00 | 7 (38.9) | 4 (22.2) | 7 (38.9) | 0.975 |
| 4.00 | 2 (66.7) | 0 | 1 (33.3) | 0.612 |
| 5.00 | 5 (50) | 2 (20) | 3 (30) | 0.687 |
| 6.00 | 0 | 0 | 8 (100) | <0.001 |
| Total | 28 (36.8) | 18 (23.7) | 30 (39.4) | |

The direction of staging differences between MT and OR. OR showed a lower stage in 36.8% and a higher stage in 23.7% of cases. Stage 2 shows significant variability (*P*=0.012). The choice of stain influences perceived fibrosis severity. MT: Masson's trichrome, OR: Orcein

**Table 3: Number of the cases with differentresults between Masson's trichrome and orcein in male and female samples**

| MT grade | Male | Female |
|---|---|---|
| 0.00 | 0 | 1 (33.3) |
| 1.00 | 2 (100) | 2 (40) |
| 2.00 | 5 (71.4) | 16 (80) |
| 3.00 | 4 (66.7) | 7 (58.3) |
| 4.00 | - | 2 (66.7) |
| 5.00 | 3 (60) | 4 (80) |
| 6.00 | 0 | 0 |
| Total | 14 (53.8) | 34 (68) |
| *P* | 0.036 | 0.149 |

Compares MT and H and E staging. When MT detected Stage 2, H and E differed in 63% of cases. H and E often underestimated fibrosis. The differences were not statistically significant (*P*=0.503). H and E alone is insufficient for fibrosis assessment. MT: Masson's trichrome, OR: Orcein

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**Table 4: Number of the cases with different results by Masson's trichrome and orcein in different age groups**

| 0-32 | 32-56 | >56 |
|---|---|---|
| 0 | 1 (50) | - |
| 1 (33.3) | 2 (100) | 1 (50) |
| 12 (85.7) | 6 (60) | 3 (100) |
| 3 (100) | 5 (50) | 3 (60) |
| 0 | 2 (100) | - |
| 2 (66.7) | 3 (60) | 2 (100) |
| 0 | 0 | 0 |
| 18 (69.2) | 19 (52.8) | 9 (64.3) |
| 0.032 | 0.121 | 0.229 |

Evaluates OR and H and E agreement. H and E often underestimated fibrosis. Higher concordance in advanced stages suggests H and E is more reliable then. These results support routine use of special stains, including OR, for liver fibrosis evaluation. OR: Orcein

66.7% in Stage 3. This difference between the two methods in different stages is statistically significant ($P = 0.036$) in male cases. In female samples, only in Stage 6 did the two diagnostic methods not differ, and in the rest of the stages, the MT and OR methods differed between 33.3% and 80%; however, these differences were not significant. Statistical analysis of the MT and H and E method results showed that the difference between the results of the two methods in different stages of fibrosis in male cases ($P = 0.584$) and female cases ($P = 0.373$) was not statistically significant. According to Table 4, comparing the results of MT and OR staining in different age groups showed that in the age group of 0–32 years, there was no difference between MT and OR methods in stages 0, 4, and 6. In Stage 3, 100% and in Stage 2, 85.7% of the cases showed a stage difference between MT and OR results. This difference was measured using Fisher's exact test and was statistically significant ($P = 0.032$).

Comparing the difference between the stages assigned by the two staining methods of MT and H and E showed no significant difference in the different age groups of our study sample. A representative microscopic image illustrating the differences between MT (left) and OR staining (right) in the assessment of liver fibrosis is provided in Figure 1.

## DISCUSSION

In this research, 76 liver biopsy samples were examined using MT, OR, and H and E staining procedures, spanning all phases of fibrosis. Since various staining methods disclose diverse collagenous components of liver fibrosis, our approach was nonselective and included a wide range of fibrosis severity. We wanted to examine their diagnostic performance and concordance. There was a statistically significant difference between H&E and MT staining when it came to identifying fibrosis, according to our data. Based on these findings, it seems that MT staining in addition to H&E staining is essential for a thorough evaluation of liver biopsy samples, since using only one approach might result in an inaccurate assessment of the existence and degree of fibrosis. Akin to that, the

There was a notable disparity between OR and MT staining in 60.5% of instances, indicating a considerable divergence between the two approaches. Stage 2 had the most noticeable disparity, with a statistically significant difference [Table 1]. When comparing MT and OR staining, the largest disparities were seen in males and patients aged 0–32. Table 2 further shows that there was a significant directionality of differences in the stages indicated by OR staining compared to MT in 36.8% of cases and 23.7% of cases, respectively, with a p-value of 0.012. In particular for intermediate stages and certain demographic groupings, our results highlight the significance of using OR staining in conjunction with H and E and MT. We found that H and E often under-estimated fibrosis compared to MT, particularly at Stage 2 (63% of cases varied from MT) [Table 3]. The tendency implies that H and E may not be enough for reliable fibrosis evaluation, even if these changes were not statistically significant. H and E continued to understate fibrosis in early stages, however their agreement with OR was greater in later stages [Table 4]. Both our findings and those of other researchers in the area build upon earlier research. The therapeutic relevance of noninvasive fibrosis testing, especially transient elastography, in chronic liver disease was emphasized by both Bojanic et al. and Lee et al. Researchers have shown that elastography results correspond well with those from MT, Sirius Red (SR), and OR staining procedures, which is important. on lines 8, 9 Discrepancies can arise, but when they do, the findings of OR staining are more in line with elastography than with MT or SR. This indicates that OR may be a better indicator of the elastic fiber content and actual severity of fibrosis, especially in late stages of the illness. [9] The use of OR as a supplemental stain in advanced fibrosis was the subject of the 2023 research by Nguyen et al. Their research showed that whereas MT used collagen deposition to classify patients as either advancing or regressing, OR added further information by drawing attention to elastic fiber content, which could differentiate between active and regressing fibrosis. [10] Among their cohort, MT verified 18 out of 22 cases that progressed, whereas OR confirmed regressing cases as having thin elastin septa devoid of collagen fibers. We agree with their findings that OR staining is useful for complex assessment of fibrosis dynamics, even though their research only included patients with severe fibrosis and a history of hepatitis therapy. [10] Similarly, Leite et al. compared SR, H and E, and OR stains with elastography as a reference technique to assess liver samples from hepatitis C patients. There was a strong correlation between elastography results and OR staining, and they discovered that higher levels of fibrosis were linked to a larger elastic fiber content. Our findings that OR is most instructive in later stages of fibrosis are in agreement with theirs that it is useful in severe cases and may have prognostic implications. the eleventh Our research stands out because we included a wider range of patients, not only those with hepatitis C or severe fibrosis, and we showed that there are substantial disparities in staining.

approaches for all demographic groups and stages of fibrosis. We conclude that OR staining, together with H&E and MT, should be regularly included in liver biopsy evaluations. It is possible that this multimodal technique is especially useful for identifying the development or improvement of liver disease, since it offers a more precise and thorough evaluation of fibrosis by catching components of both elastic fibers and collagen. Since OR staining reveals the type of collagenous components of fibrosis and its grade, it appears necessary to use it in conjunction with routine staining methods to improve the accuracy of diagnosing liver fibrosis in patients with various

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underlying causes. Our results add to the growing body of evidence supporting this claim.

## CONCLUSIONS

According to the results of our study, in order to evaluate liver fibrosis in different liver samples, the use of OR staining along with routine staining methods such as MT and H and E, seems necessary, since it provides us with useful information about the presence of fibrosis and its grade, even in different sex and age groups. It is suggested that more extensive studies be conducted considering more variables, including the type of underlying diseases that cause fibrosis and the type and duration of the previous treatments, as well as the follow-up of the patients. Furthermore, studies on the comparison between the staining results and imaging results are suggested to further investigate the application and effectiveness of these diagnostic methods in clinical decision-making.

### Outcome of the study

This study found significant differences among H and E, MT, and OR staining methods in staging liver fibrosis. MT and OR showed the greatest discrepancy at Stage 2. Overall, results indicate that using both MT and OR staining improves diagnostic accuracy compared to relying on a single method.

### Rationale of the study

Liver fibrosis is a major cause of chronic liver disease, and accurate staging is essential for patient management. Although various histological staining methods such as H and E, MT, and OR are routinely used, their relative efficiency and agreement in diagnosing and staging liver fibrosis remain unclear. This study aims to compare these methods to optimize fibrosis assessment in clinical practice.

### Limitation of the study

The insufficient amount of tissue in some samples led to their exclusion due to the impossibility of staining, and as a result, reduced our sample size.

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