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Corrected integrated density: a novel method for liver elastic fibers quantification in chronic hepatitis C

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Abstract

Background: Elastic fibers deposition is triggered during liver fibrosis and is related to worse clinical prognosis in chronic hepatitis C patients. This study aimed to verify if a new method for elastic fiber quantification can be used to discriminate between different degrees of fibrosis in liver biopsies of patients with hepatitis C.

Methods: Individuals presenting with different degrees of fibrosis in liver biopsy were included. Slides of liver samples were stained with orcein with and without prior oxidation. Morphometric analysis was proceeded, and quantification accomplished by corrected integrated density.

Results: Twenty-seven patients, mean age 52 years-old, 59% women, were included. Elastic fibers density was higher in advanced fibrosis patients and there was a positive correlation with Metavir score (*Spearman* $r = 0.609$, $p < 0.001$), as well as with the noninvasive scores Fib-4 (*Pearson* $r = 0.46$, $p = 0.029$) and APRI ($r = 0.52$, $p = 0.01$).

Conclusion: Morphometric analysis by corrected integrated density demonstrates that elastic fibers abundance is higher in advanced stage of fibrosis in patients with hepatitis C.

Keywords: Liver fibrosis, Chronic hepatitis C, Elastic fibers

Background

Chronic hepatitis C affects 71 million people worldwide, and is a leading cause of cirrhosis and liver transplantation in most countries (Collaborators 2017). The grade of hepatic fibrosis is an important prognostic factor (Manns et al. 2017). To this purpose noninvasive methods have been developed, such as transient elastography, magnetic resonance elastography, and scores based on laboratory tests (Paranaguá-Vezozzo et al. 2017). Histological analysis of liver biopsy is the gold standard and allows for better staging, through the evaluation of fibrosis by the Metavir score (Bedossa and Poynard 1996).

During the development of liver fibrosis, new elastic fibers are deposited in the extracellular matrix, conferring

tissue resistance. Elastin is an amorphous protein highly resistant to degradation; it is believed to be the most stable component among the extracellular matrix. It is degraded slowly and late in the reversion of fibrosis, and may lead to irreversibility of the process (Chen et al. 2019).

Elastic tissue is physiologically present in several human tissues, especially where greater elasticity is needed. In the healthy liver it is observed in small amounts, mainly in the wall of arterioles and in portal tracts (Kanta 2016). In these locations it exerts a structural function, providing stability to the bile ducts and vessels under conditions of high ductal pressure (Mederacke et al. 2013).

Elastic fibers are composed of two proteins, elastin and fibrillin, which are separated from each other instead of constituting bundles as is the case of collagen (Kielty et al. 2002). Fibrillin forms microfibrils, the mold to elastic fibers; elastin forms the fibers' core (Houghton et al. 2006). Chronic hepatitis C causes a cellular inflammatory response

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as a result of oxidative stress and immune response to hepatocytes expressing viral epitopes (Neumann-Haefelin and Thimme 2013). This response generates release of cytokines and growth factors that activate stellate cells in myofibroblasts, which synthesize extracellular matrix (Geerts 2001, Franceschini et al. 2012). In this matrix small amounts of elastic fibers are found (Burt et al. 1990). Therefore, fibrogenesis results in deposition of elastin (Baiocchini et al. 2016). The immature (or oxytalan) fibers are present only as microfibrils in physiological conditions; however, during late elastogenesis, they generate mature elastic fibers. On histopathology, orcein is one of the selective methods for staining these fibers. When oxidation by peracetic acid or permanganate is accomplished, oxytalan fibers are visible (Nakayama et al. 2008, Kanta 2016).

An association of hepatic elastin with poor prognosis in patients with chronic hepatitis C has been found by some authors (Kendall et al. 2018). It is not well established if quantification of the fibers is directly related to fibrosis stage (Yasui et al. 2019).

We hypothesize that the hepatic inflammatory damage caused by the hepatitis C virus might lead to the deposition of elastin in the extracellular matrix. In this study, we aim at assessing whether a new method for elastic fiber quantification can be used to discriminate between different degrees of fibrosis in liver biopsies of patients with hepatitis C, thus confirming the quantitative relationship between elastin and fibrosis.

Methods

Patients

This was a retrospective study. Inclusion criteria were: chronic hepatitis C confirmed with positive hepatitis C virus PCR and anti-HCV test lasting for more than 6 months; ultrasound-guided liver biopsy performed between January 2010 and December 2012 at the Hospital de Clínicas de Porto Alegre (HCPA). Individuals with another liver disease or current alcohol abuse were excluded, as well as cases with samples measuring less than 0.7 cm in the longer axis (which are considered nondiagnostic). A total of 33 cases were included.

This study was approved by Ethics Committee of the HCPA number 3.227.637. Informed consent was waived due to its retrospective design, but authors signed a data usage responsibility term. The study was performed according to the guidelines of the Declaration of Helsinki, 1975.

Study variables

Demographic data, clinical history and biochemical tests were obtained from electronic records. Non-invasive fibrosis scores were calculated as follows: FIB-4 (Fibrosis-4) = age (years) x AST (IU/L) / platelets (10^9/L) x $\sqrt{}$ ALT (IU/L); APRI (AST to platelets Ratio Index) = AST (UI/L) / ALT upper limit of normal × 100 / platelets (10^9/L) (Trang et al. 2008).

Histopathological procedures

The paraffin-embedded liver samples were sectioned into 4 μm-thick sections, deparaffinized and stained according to the standard procedures of the Department of Surgical Pathology of the HCPA: one slide was stained with hematoxylin-eosin, the other with Sirius Red staining, for Metavir score. They were reviewed by a second expert pathologist. The blocks were reprocessed for staining with orcein to verify mature elastic fibers, and a second slide was submitted to oxidation by potassium permanganate to highlight oxytalan fibers. The elastic fibers distribution (restricted or not to portal tracts) was verified. Those slices were submitted to morphometry.

Morphometric analysis

The slides were analyzed with Olympus BX41 microscope in a magnification of 200X. Following microscope calibration, each fragment was photographed in its entire length with the Olympus DP73 microscope-attached camera and the CellSens® software. Five hundred images were generated and saved in TIFF format to allow for better resolution. The images were converted on ImageJ 1.8.0_112 to grayscale 8-bit images (Schneider et al. 2012) and analyzed morphometrically as follows: to find the optical density of the elastic fibers, the gray value threshold was determined manually for each image to distinguish the particles (that is, the areas with gray values within the threshold range) from the background. The micrometer-to-pixel scale was calculated from the scale generated by CellSens® when each image was obtained.

Area and mean gray value (MGV) of each particle were obtained with ImageJ. The corrected density in absorbance-micrometer^2 per particle was obtained by the formula (255-MGV)*area (Hartig 2013). Artifacts and white areas were manually removed using the "wand" tool with a tolerance of 10. The sum of the corrected densities of all particles (corrected integrated density, CID; also known as corrected absorbance) corresponds to the quantification of elastic fibers in that region of the fragment, and the sum of CIDs of all the photographs corresponds to the sum of all densities in that individual slide. The division of the summed CIDs by the number of photographs informs the mean CID of elastic fibers per photograph, which was used in our analysis. The same procedures were repeated by a second blinded observer.

Statistical analysis

To detect difference in the presence of elastic fibers outside portal tracts, a minimum of six early and advanced

fibrosis patients were needed (Nakayama et al. 2008). Categorical variables were shown as number and proportion in percentage, and continuous variables were presented as mean and standard deviation. To compare between groups, quantitative variables presented symmetric distributions and Student t-test was performed. To assess correlation between two continuous or ordinal variables, Pearson or Spearman's rank-test were performed. A 95% interval confidence calculated by Bland-Altman was used to verify concordance between observers. Statistical significance was defined as $p < 0.05$.

Results

A total of 33 patients were included, 5 were excluded due to insufficient sample (scarce material after reprocessing), and 1 due to the presence of concomitant hepatocellular carcinoma, comprising 27 individuals with ultrasound-guided needle biopsy. The mean age was 52 years, with a predominance of women. Arterial hypertension was the most frequent comorbidity (Table 1).

Elastin was verified in orcein-stained slides as described in methods (example in Fig. 1). Elastic fibers

Table 1 Baseline sample characteristics

	Total 27
Age (years)	52.3 (11.15)
Female (%)	16 (59%)
White (%)	25 (92.5%)
Hypertension (%)	7 (26%)
Former alcohol abuse (%)	8 (29.6%)
Genotype	
1	12
2	2
3	11
4	0
Coinfection 1 + 3	1
FIB-4	2.58 (1.77)
APRI	1.3 (0.9)
Platelets (10^3/mm^3)	191.11 (66.97)
INR*	1 (0.07)
Total bilirrubin (mg/dL)	0.74 (0.22)
Albumin (mg/dL)	4.23 (0.26)
Fibrosis Grade - Metavir	
F0	4
F1	5
F2	6
F3	5
F4	7

* *INR* international normalized ratio, *FIB-4* Fibrosis-4, *APRI* AST to platelets Ratio Index, percentage or standard deviation between parenthesis
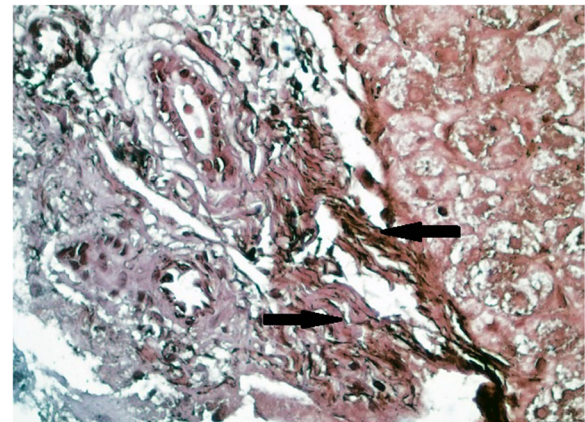
Fig. 1 Representative case of F4 fibrosis, stained with orcein, 400x magnification. Elastic fibers are thin structures (black arrows), surrounded by collagen (thicker and lighter color)

outside portal tracts were detected in only one of 15 patients F0–2 and in 8 of 12 advanced fibrosis ($p = 0.12$).

The mean elastic fibers density was significantly higher in the group with advanced fibrosis (Metavir F3-F4) compared to other individuals (Fig. 2). A higher mean was also observed in this group when analyzing oxytalan fibers, $p = 0.027$ (Fig. 3).

We also graded the quantification according to terciles. None of the F0-F2 patients was in the highest tercile, while 50% of the F3-F4 patients were in this group ($p = 0.003$). Descriptive analysis was undertaken. The group of patients with elastic fibers outside portal tracts had a higher fibers' density than the other individuals ($p = 0.015$).

The mean of difference in quantification between observers was -414.72×10^3 and was not significant (IC 95% -342.8 to 259.86, $p = 0.3$). The Metavir fibrosis stage correlated linearly with the elastic fibers' density (Fig. 4). There was no significant correlation between oxytalan fibers and degree of fibrosis (Spearman correlation coefficient $r = 0.38$ $p = 0.063$). Activity score and genotype did not correlate to elastic fibers density.

We also analyzed noninvasive markers of fibrosis. The Fib-4 score showed a positive significant correlation with the elastic fibers' quantification (Pearson correlation coefficient $r = 0.46$, $p = 0.029$), as well as APRI ($r = 0.52$, $p = 0.01$). There was a correlation in the limit of significance between Fib-4 and oxytalan fibers ($r = 0.453$, $p = 0.052$), but it was not observed for APRI ($r = 0.366$, $p = 0.12$).

All included cirrhotic individuals were classified clinically as Child-Pugh A. Only four among those had cirrhosis with complications (three cases of medium or large esophageal varices and one hepatocellular carcinoma). The mean elastic fibers density of this subgroup compared to others with advanced fibrosis were higher, but
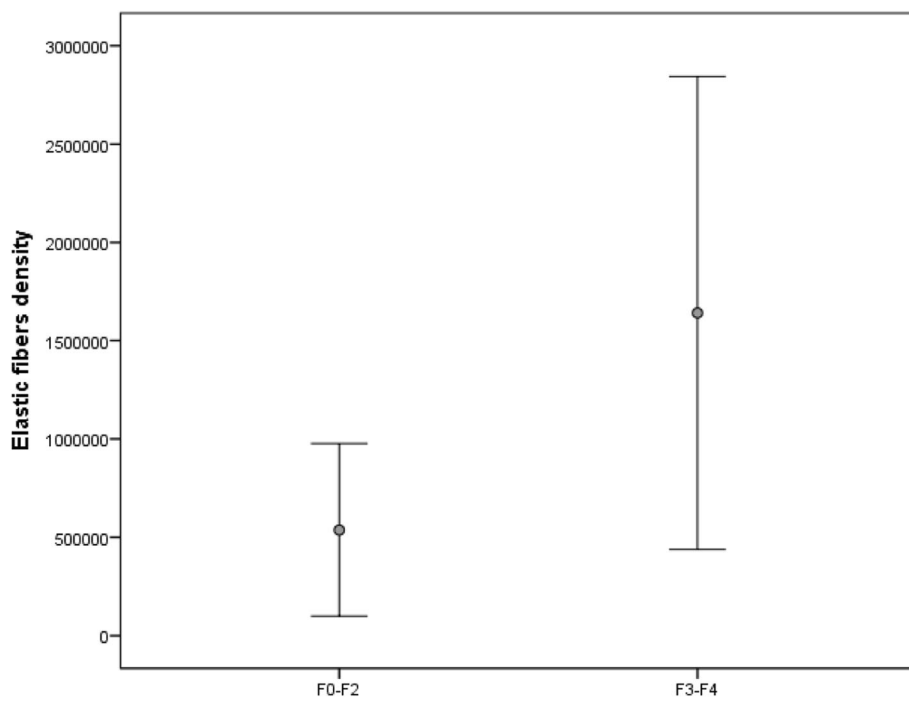
Fig. 2 Elastic fibers density in groups of early and advanced fibrosis. F0-F2: 536.72×10^3 absorbance-micrometer^2 (438.83×10^3) X F3-F4: 1641.04×10^3 absorbance-micrometer^2 (1202.75×10^3), $p = 0.001$
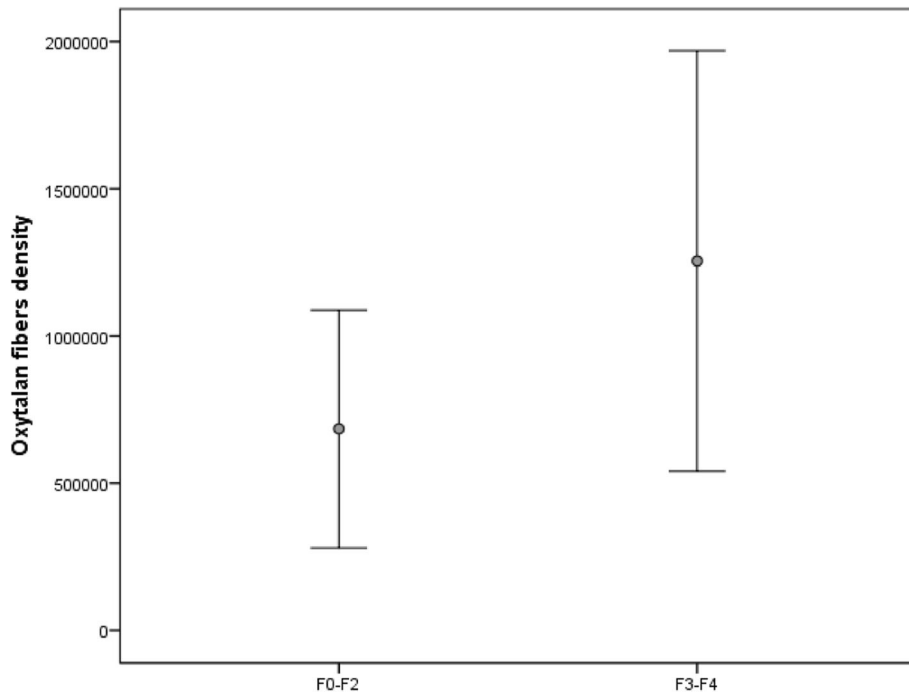
Fig. 3 Oxytalan fibers density in groups of early and advanced fibrosis. F0-F2: 683.87×10^3 absorbance-micrometer^2 (404.35) X F3-F4: 1254.4×10^3 absorbance-micrometer^2 (714.08×10^3), $p = 0.027$

Fig. 4 Correlation of stage fibrosis and elastic fibers density, Spearman test, $r = 0.609$, $p < 0.001$
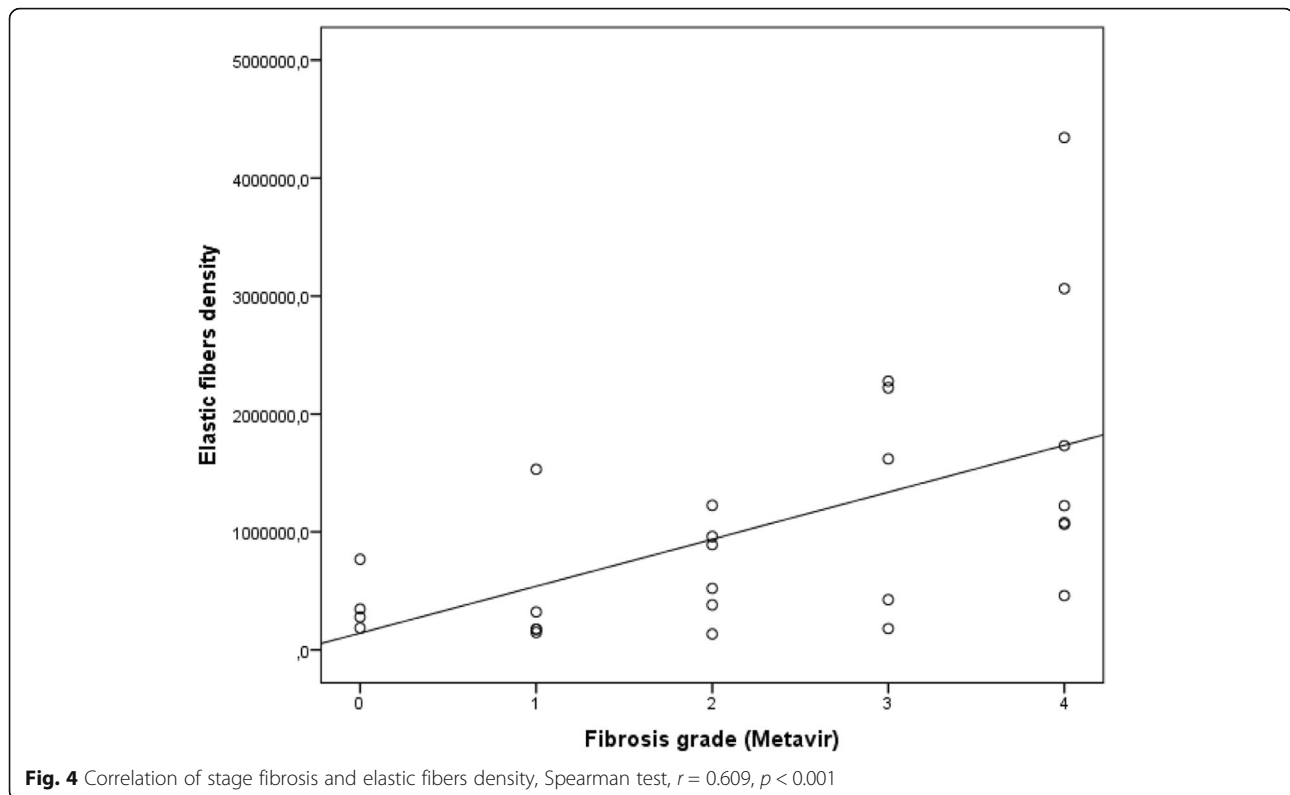
not significant: 2214.96×10^3 absorbance-micrometer^2 (standard deviation = 1845.73) versus 1449.74×10^3 absorbance-micrometer^2 (standard deviation = 986.26), $p = 0,1$.

Discussion

This study revealed a positive correlation between the grade of hepatic fibrosis and density of elastic fibers, especially those with amorphous material (elastin). We found that noninvasive scores may also presume the degree of deposition of hepatic elastin. Fibrosis scores such as Metavir are ordinal or semi-quantitative. Morphometric analysis allows a more reliable evaluation of the deposition of extracellular matrix structures in fibrogenic processes, since it is quantitative (Goodman et al. 2007). Other advantages over conventional visual assessment are the objectivity, reproducibility, and the ability to detect changes not apparent to the naked eye (Hamilton and Allen 1995). In hepatitis C patients who are non-responders to treatment, morphometric analysis demonstrated relevant worsening in fibrosis (80% increase of collagen content) in 4 years (Goodman et al. 2009). Only by direct measurement of fibrosis this change can become apparent, because histological stages are neither a continuous function nor a sensitive measure.

Hepatic elastic fibers have been first evaluated in non-alcoholic fat liver disease, with a higher amount found in the zone three of patients with advanced fibrosis (Nakayama et al. 2008). These fibers have emerged as new focus of research due to its possible relationship with worse prognosis in chronic hepatitis C and advanced fibrosis (Houghton et al. 2006, Kendall et al. 2018). Contrary to what was previously assumed, elastic fibers have been found in greater quantity in liver biopsies with F3 fibrosis in relation to F2, but there was no evidence of a linear relation with a fibrosis score by Metavir (Yasui et al. 2019). In these work, authors have quantified elastic fibers by measuring the percentage in which they occur in the total area of the fragment (Abe et al. 2013). In our work, on the other hand, we measure the absorbance by corrected integrated density. This measure is more reliable and accurate because it verifies not only the affected area, but also the elastic fiber concentration or density, which may explain the difference of our findings. As in previous published literature, we found a positive correlation between non-invasive fibrosis scores and the fibers quantification (Masugi et al. 2018, Yasui et al. 2019).

The reported association of elastin with poor prognosis (Kendall et al. 2018) might be explained by different stages of fibrosis in cirrhotic patients. The quantification of elastin allows more information than just the grade in Metavir, which does not reflect the heterogeneity of patients in the subgroup F4.

Our study has as limitation the non-evaluation by the other quantification method, the percentage of affected area, to compare the findings. This technique would make this study more expensive, mostly because of the

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need of a scanner. However, we believe that the rigorous methodology makes our findings reliable. Also, the descriptive analysis correlated to our quantification. Another limitation was the small sample, which did not allow us to adequately measure statistical significance to evaluate the prognosis of the individuals. However, this was not a primary goal; we plan to conduct these analyses in another study with an increased sample. In addition, due to the cross-sectional design, this study does not provide information on more specific aspects of fibrosis triggers and elastic fibers synthesis.

Conclusion

The elastic fibers are in higher concentration in advanced stages of fibrogenesis. Our quantification method adds relevant information to liver pathology study in chronic hepatitis C. Although labor-intensive, this method has the advantage of being more precise than simple histopathological evaluation or less complex methods of quantification, mainly because it concatenates two dimensions of elastin deposition – the area occupied by elastic fibers and the density of these fibers in such areas – thus being a good fit for more complex experiments and for precision medicine. This method is also inexpensive and potentially applicable to other substances than elastin.

Due to its relationship with worse prognosis and possibly irreversibility of fibrosis, we believe that elastin may play an important role in understanding the pathogenesis and the predictability of prognosis in viral cirrhosis.

Abbreviations
ALT: Alanine aminotransferase; APRI: AST to platelets ratio index; AST: Aspartate aminotransferase; CID: Corrected integrated density; FIB-4: Fibrosis-4; HCPA: Hospital de Clinicas de Porto Alegre; INR: international normalised ratio; MGV: Mean gray value; PCR: Polymerase chain reaction

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Not applicable.

Informed consent
The authors signed a declaration for Ethics Committee and are committed to keep patients' data confidential. Formal informed consent was waived by the HCPA Ethics Committee.

Authors' contribution
CL: design of study, participated in the acquisition of clinical data, analysis and interpretation of data, participated in writing manuscript. RTS: participated in digital analysis conceptualization and execution, and writing manuscript. ENT, CTSC, MRAS, MRMT: responsible for the conception and design of the study, besides writing manuscript. All authors read and approved the final manuscript.

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
This study was approved by Ethics Committee of the HCPA number 3.227.637.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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