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**microRNA-200a: A Stage-Dependent Biomarker and Predictor of Steatosis and Liver Cell Injury in Human Immunodeficiency Virus Patients**

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Nonalcoholic fatty liver disease contributes to liver-related mortality and has a high prevalence among patients with human immunodeficiency virus (HIV). The early detection of steatosis could prevent disease progression through lifestyle changes. However, as the common serum markers are nonspecific and the gold standard for the detection of nonalcoholic fatty liver disease remains the invasive liver biopsy, its verification is limited. Therefore, the search for novel biomarkers is essential. Several studies have emphasized the role of microRNAs (miRNAs) as biomarkers for certain liver diseases. With our study, we aimed to investigate the potential of miR-200a as a biomarker for liver injury, fibrosis, and steatosis in HIV patients. The study cohort consisted of 89 HIV patients. Clinical and laboratory parameters were assessed twice, within a median follow-up period of 12 months. miR-200a serum levels were determined by real-time polymerase chain reaction and normalized to spiked-in RNA (SV40). miR-200a serum levels showed a significant correlation with the patients’ controlled attenuation parameter scores and their body weight at baseline and with alanine aminotransferase serum levels at follow-up. At baseline, we observed a stage-dependent increase in miR-200a serum levels according to the degree of steatosis. More importantly, patients with higher baseline levels of miR-200a recorded a progression of steatosis at follow-up. Remarkably, miR-200a not only reveals a prognostic value for steatosis but possibly also for liver damage and metabolic adaptations as patients with an increase in alanine aminotransferase/aspartate aminotransferase serum levels over time also recorded higher baseline miR-200a levels.

**Conclusion:** Our study reveals miR-200a not only to be a stage-dependent biomarker of steatosis but also to be a predictor of steatosis progression and probably liver cell injury in HIV patients. (Hepatology Communications 2017;1:36-45)

**Introduction**

The implementation of combined antiretroviral therapy (cART) caused a drastic decline in acquired immunodeficiency syndrome-related mortality.1 However, the increase in life expectancy among human immunodeficiency virus (HIV) patients has placed an emphasis on the adverse effects of medication, comorbidities, and the patients’ lifestyle.2 Among other diseases, liver disease has emerged as a severe threat and contributes to 7%-14% of nonacquired immune deficiency syndrome (AIDS)-related deaths in HIV patients.3 Remarkably, nonalcoholic fatty liver disease (NAFLD), emerging in the Western world,4,5 shows a high prevalence of up to 65% among HIV patients.6 Especially older antiretroviral drugs have been linked to the development of steatosis.7,8 They can affect glucose metabolism and disturb fatty acid oxidation in the liver, resulting in decreased insulin sensitivity, increased triglyceride and

**Abbreviations:** AIDS, acquired immunodeficiency syndrome; ALT, alanine-aminotransferase; APRI, AST/thrombocyte-ratio-index; AST, aspartate-aminotransferase; CAP, controlled attenuation parameter; cART, combined antiretroviral therapy; FIB4, fibrosis-4; HCC, hepatocellular carcinoma; HIV, human immunodeficiency syndrome; miRNA, microRNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RT-PCR, real-time PCR; ULN, upper limit of normal.

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cholesterol levels, and a change in lipoprotein composition.\(^{(9-11)}\) NAFLD patients show a 6-fold increased liver-related mortality rate compared to healthy individuals.\(^{(12)}\) This can be explained by the progression of NAFLD to nonalcoholic steatohepatitis (NASH) and complications, such as cirrhosis and hepatocellular carcinoma (HCC).\(^{(13-15)}\)

Screening for NAFLD and NASH is challenging, and the commonly used serum markers, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are unspecific.\(^{(14)}\) The gold standard for the diagnosis of NAFLD remains the histologic analysis of liver biopsies. Nevertheless, liver biopsies have drawbacks: they are inaccurate through sampling variability,\(^{(16)}\) and small sample size and they foster complications.\(^{(17)}\)

The research for reliable biomarkers has revealed microRNAs (miRNAs) as specific indicators for liver injury. miRNAs are short, noncoding nucleotide fragments with gene-regulatory properties. They have gained importance in different aspects of liver disease, such as fibrosis/cirrhosis, HCC, and NAFLD/NASH.\(^{(18-21)}\)

In the present study, we focused on the role of miRNAs in hepatic injury, fibrosis, and steatosis. miR-200a has been demonstrated to be up-regulated in NAFLD and is associated with fibrosis within NAFLD, mainly in experimental models.\(^{(22-24)}\)

Therefore, we analyzed the serum levels of miR-200a in an HIV patient cohort. We found miR-200a to function as a stage-dependent biomarker of steatosis and as a predictor of steatosis progression; we also found it has an association with increased aminotransferases.

**Materials and Methods**

**STUDY DESIGN**

The serum levels of miR-200a were determined in samples of 89 HIV-positive patients at baseline, of which 7 had a co-infection with hepatitis B virus (Fig. 1). The miR-200a levels were correlated to the patients’ clinical parameters and laboratory values, which were obtained at baseline and after a median follow-up of 12 months (8-30 months).

**HUMAN SAMPLE COLLECTION AND ETHICAL APPROVAL**

The patient cohort consisted of 89 HIV individuals who made use of medical attendance in the outpatient clinic at the Bonn University Hospital. The patients' blood samples were collected from September 2013 until March 2016. The samples for the assessment of miR-200a levels were collected within a timeframe of...
3 months. Serum samples from 42 healthy donors served as controls. Informed consent was provided in written form by each patient. The local ethics committee permitted the study in agreement with the Declaration of Helsinki (No. 069/10).

CLASSIFICATION OF STEATOSIS AND DEFINITION OF LIVER FIBROSIS

We determined the controlled attenuation parameter (CAP) by means of transient elastography measurement (FibroScan, Echosens, France) and classified the degree of steatosis according to the work of Sasso et al.\(^{25}\) CAP values were divided into four groups according to the degree of steatosis: S0, < 11% (<238 dB/m); S1, 11%-33% (238-259 dB/m); S2, 34%-66% (260-291 dB/m); S3, ≥67% (≥292 dB/m).

Liver fibrosis was determined by FibroScan measurement and calculation of the fibrosis-4 (FIB4) index and aspartate aminotransferase-to-platelet ratio index (APRI) score as described\(^{26,27}\):

\[
\text{FIB4 index} = \frac{[\text{age} \times \text{AST}]}{[\text{platelet count} \times \sqrt{\text{ALT}}]}, \quad \text{where age is years, AST and ALT are IU/L, and platelet count is } 10^9/L.\]

\[
\text{APRI score} = \frac{[\text{AST/upper limit of normal [ULN]}]}{[\text{platelet count}]} \times 100, \quad \text{where AST is IU/L and platelet count is } 10^9/L.\]

Liver fibrosis was regarded as relevant with either a FibroScan value of ≥7.1 kPa, an FIB4 index of ≥ 1.45, or an APRI score of ≥1.1.

miRNA ISOLATION AND QUANTIFICATION BY REAL-TIME POLYMERASE CHAIN REACTION

RNA isolation and quantification was performed as described\(^{18,28}\). Briefly, RNA was isolated with QIAzol Reagent (Qiagen, Hilden, Germany) and spiked-in RNA (SV40) was used for later normalization. SV40 was used in favor of other control miRNAs, which can possibly be altered following liver damage.\(^{29}\) The quantity of total RNA was assessed by measurement of the spectrophotometric absorbance at 260 nm with the ND1000 Nanodrop (NanoDrop, Wilmington, DE) according to the manufacturer’s instructions. The quality of RNA was determined by microcapillary electrophoresis (2100 BioAnalyser; Agilent Technologies, Waldbronn, Germany). Reverse transcription of RNA was conducted with the miScript Reverse Transcription Kit (Qiagen). Real-time polymerase chain reaction (PCR) was carried out on a CFX thermocycler (Bio-Rad, Berkeley, CA), using the miScript SYBR Green PCR Kit (Qiagen) for quantification. The analyses of miR-200a and SV40 were conducted in duplicate in accordance with the manufacturer’s instructions. Expression levels were calculated by applying the delta delta Ct method. The miR-200a/SV40 spiked-in RNA ratio was multiplied by a factor of 10,000 to compensate for higher SV40 concentrations. Data are shown as relative units.

STATISTICS AND GRAPHICS

Nonparametric tests were used for all analyses. The correlation analysis was performed using the Spearman’s correlation test. The Mann-Whitney test was applied for comparison between two groups, and the Kruskal-
Wallis test, as a one-way analysis of variance, was used for the comparison of more than two groups. The standard deviation is shown as the mean ± SEM. Analysis of the area under the receiver operating characteristic curve (AUROC) was used to determine potential cut-off values for predicting steatosis grade S1 or higher, S2 or higher, and S3 or severity of steatosis. In all tests, a P value of < 0.05 was regarded as statistically significant. Data analysis was carried out with SPSS Statistics v.23 for MAC (SPSS Inc., Chicago, IL) and GraphPad Prism (GraphPad Software, La Jolla, CA), which was also used for graphical visualization.

Results

PATIENTS’ CHARACTERISTICS

Table 1 portrays the baseline characteristics of our patient cohort recorded at the initial date of study participation. The study cohort consisted of 89 HIV patients with an average age of 52 years, of which 74 were male patients and 15 were female patients. Most of the patients had undergone cART treatment, and the majority of these patients responded to the treatment, indicated by a viral load of below 40 copies/mL.

CLINICAL PARAMETERS

The clinical examinations and blood collections were performed at two different points in time within a median follow-up period of 12 months. miR-200a serum levels were determined at baseline (Table 2). HIV patients had significantly higher serum levels of miR-200a compared to HIV-negative controls (Supporting Fig. S1).

The mean body mass index (BMI) of 25.8 kg/m² (19-37 kg/m²) within the cohort indicated that, on average, participants were low-grade overweight at baseline measurement. The CAP values showed an average of 245.2 dB/m (100-375 dB/m), reflecting a degree of steatosis of 11%-33% with a minor increase over time. Remarkably, the highest CAP values indicated steatosis levels of more than 67% (≥292 dB/m). Most patients showed an increased CAP value at follow-up, and one patient showed a higher steatosis grade at follow-up (Supporting Fig. S2).

Aminotransferases are often used to investigate the occurrence of liver damage but they may also represent metabolic adaptions. The group’s average AST and ALT values were comparable to those of healthy individuals (AST, 27.3 U/L; ALT, 38 U/L), although the highest liver aminotransferase levels showed values of triple the ULN (norm: 35 U/L women, 50 U/L men). Furthermore, the analysis of serum lipid parameters revealed increased triglyceride serum levels of above 200 mg/dL on average. However, the low-density lipoprotein–cholesterol levels resided within a normal range of below 150 mg/dL. To investigate the occurrence of liver fibrosis, we measured liver stiffness by means of a FibroScan. We observed a significant increase in stiffness over time. Finally, we determined additional predictors of fibrosis. In contrast to the FibroScan, neither FIB4 nor APRI scores were elevated significantly at follow-up.

CORRELATION ANALYSIS

In a first approach, we correlated the miR-200a serum levels with the patients’ clinical parameters measured at baseline and at follow-up. Table 3 shows the results of the Spearman’s correlation analysis. At baseline, a significant positive association was obtained between the patients’ serum levels of miR-200a with their CAP scores and their body weight (Supporting Fig. S3A,B). The analysis of parameters at follow-up revealed an even stronger association with the patients’ body weight, and we observed a significant positive association with the patients’ ALT serum levels (Supporting Fig. S3C,D). No significant associations were observed between fibrosis and miR-200a. To strengthen the validity of the CAP score as a marker of steatosis, we investigated a correlation of CAP itself with...
other clinical parameters related to the body’s fat composition and liver function. The analysis revealed a significant positive correlation between the CAP scores and parameters reflecting the patients’ body fat composition, such as body weight, BMI, serum triglyceride, and cholesterol levels. The accumulation of fat in the liver tissue is not a threat to a patient’s health by itself. However, as steatosis can progress to fibrosis, more severe liver injury can result. In our study, we observed a significant positive correlation between the patients’ CAP scores and the FIB4 values.

TABLE 2. Clinical Parameters at Baseline and Follow-Up

| Clinical Parameters at Baseline | Measurement at Baseline | n | Measurement at Follow-up | n | P |
|-------------------------------|-------------------------|---|--------------------------|---|---|
| BMI (kg/m²)                  | 25.8 (19-37)            | 86 | 25.7 (17-34)             | 59 | 0.97 |
| CAP (dB/m)                   | 245.2 (100-375)         | 65 | 254.2 (131-389)          | 71 | 0.357 |
| FibroScan (kPa)              | 5.5 (1.7-23.4)          | 82 | 6.4 (1.9-29.1)           | 79 | 0.0453 |
| Laboratory values            |                         |   |                          |   |    |
| miR-200a (ratio to SV40*10^5)| 9.9 (0.1-122.9)         | 89 |                         | - |    |
| AST (U/L)                    | 27.3 (11-147)           | 82 | 28.4 (13-145)            | 86 | 0.1896 |
| ALT (U/L)                    | 38.0 (17-149)           | 88 | 40.5 (17-122)            | 87 | 0.2488 |
| GammaGT (U/L)                | 66.1 (18-273)           | 88 | 69.7 (21-290)            | 87 | 0.9863 |
| ChE (U/L)                    | 14,197 (2,877-25,760)   | 77 | 14,705 (6,411-23,960)    | 79 | 0.5023 |
| Albumin (g/L)                | 44.0 (32-54)            | 78 | 44.9 (31-54)             | 78 | 0.0826 |
| Triglyceride (mg/dL)         | 242.5 (34-1199)         | 85 | 211.5 (53-909)           | 82 | 0.5225 |
| Cholesterol (mg/dL)          | 204.1 (104-317)         | 85 | 198.0 (87-315)           | 82 | 0.4112 |
| LDL (mg/dL)                  | 124.6 (34-203)          | 79 | 124.0 (35-204)           | 80 | 0.826 |
| HDL (mg/dL)                  | 46.8 (18-123)           | 79 | 48.2 (25-101)            | 80 | 0.3517 |
| FIB4 score                   | 1.0 (0.4-4.1)           | 82 | 1.1 (0.4-4.5)            | 85 | 0.6508 |
| APRI score                   | 0.3 (0.1-1.1)           | 82 | 0.3 (0.1-0.9)            | 85 | 0.7416 |
| Platelet count (g/L)         | 218.6 (61-332)          | 88 | 221.4 (89-331)           | 86 | 0.7991 |
| CD4 cell count (cells/mm³)   | 591 (171-1478)          | 87 | 607 (153-1475)           | 82 | 0.8219 |
| Leukocytes (G/L)             | 5.8 (3.0-10.0)          | 88 | 6.1 (3.0-14.0)           | 86 | 0.5729 |

Data shown as Spearman correlation coefficient ($R$). *$P<0.05$, **$P<0.01$.

TABLE 3. Spearman Correlation of Patients’ miR-200a Serum Levels and CAP Scores With Clinical Parameters

| miR-200a | Clinical Parameters at Baseline | R | P | n |
|----------|---------------------------------|---|---|---|
| CAP (dB/m) | 0.326** | 0.008 | 65 | 0.177 | 0.14 | 71 |
| Body weight (kg) | 0.216* | 0.046 | 86 | 0.305* | 0.017 | 61 |
| ALT (U/L) | 0.177 | 0.099 | 88 | 0.214* | 0.046 | 87 |

Data shown as Spearman correlation coefficient ($R$). *$P<0.05$, **$P<0.01$.
miR-200a CORRELATES TO THE DEGREE OF STEATOSIS AND PREDICTS ITS PROGRESSION

The determination of CAP scores allows the classification of steatosis into different stages. Accordingly, we compared the patients’ serum levels of miR-200a between distinct groups of stage-dependent steatosis (Fig. 2A). The results verify the outcome of our correlation analysis. We found a linear cohesion between the serum levels of miR-200a and the group-specific CAP values. With an increase in the stage of steatosis, we observed increased miR-200a levels. A significant difference ($P = 0.0157$) was obtained between the patient group with the lowest degree ($<11\%$, $<238$ dB/m) of steatosis and the group with the highest degree of steatosis ($\geq67\%$, $\geq292$ dB/m). Similarly, levels of miR-200a were significantly higher in patients with severe steatosis (S2-S3) compared to patients without steatosis or with mild steatosis (S0-S1) (Fig. 2B). In contrast to miR-200a levels, no other clinical parameter showed a stage-dependent increase that discriminates the different steatosis stages (Supporting Fig. S4). Although the AUROC curves were
significant for the prediction of the steatosis grades, the results of the areas under the curve were only moderate (Supporting Fig. S5).

Next, we analyzed the serum levels of miR-200a in relation to the change of the CAP score over time (Fig. 2C,D). The distribution of the miR-200a levels over the steatosis grades at follow-up was comparable to those at baseline, with even the statistical value (Fig. 2C). Furthermore, we observed significantly higher miR-200a levels in patients stagnating or increasing toward higher levels of steatosis (≥34%, ≥260 dB/m) compared to patients remaining at or decreasing toward lower stages of steatosis (<34%, <260 dB/m) (Fig. 2D). Other clinical parameters reflected the progression of steatosis grade only partially (Supporting Fig. S6, Supporting Tables S2, S3).

**miR-200a SERUM LEVELS PREDICT LIVER DAMAGE AND RELATE TO THE BODY’S FAT COMPOSITION**

Subsequently, we analyzed miR-200a for further predictive properties. We compared the serum levels of miR-200a between patients with a relevant decrease or increase in aminotransferase levels over time. The ULN was used as a fixed point. Patients with an increase in serum ALT (Fig. 3A) and AST (Fig. 3B) levels over time showed elevated miR-200a serum levels. Accordingly, we examined lower miR-200a serum levels in patients with a decrease in aminotransferase levels during the follow-up period. In addition, we compared the miR-200a expression pattern in patients with aminotransferase levels below and above the
ULN (Fig. 3C). At follow-up, we found significantly higher miR-200a levels in patients with AST levels above the ULN compared to patients with AST levels below the ULN ($P = 0.0204$). To investigate the validity of miR-200a as a marker for the body's fat composition, we analyzed its association with different serum lipid markers. The analysis revealed a relevant trend ($P = 0.0576$) for higher miR-200a levels in patients with triglyceride levels above the group's mean value (242 mg/dL) compared to individuals with values below the mean (Fig. 3D).

Discussion

With the present study, we are the first to show the relevance of miR-200a as a stage-dependent biomarker of steatosis and its predictive value for the progression of steatosis and liver cell injury in HIV patients.

In former research approaches, miR-200a was indicated to play a role in steatosis.$^{(22-24)}$ A recent study by Leti et al.$^{(31)}$ found miR-200a to be present among the 10 most up-regulated miRNAs in NAFLD patients as analyzed in liver tissue samples by means of high-throughput screening. Likewise, miR-200a showed elevated expression levels in patients with NASH,$^{(21)}$ a condition in which proinflammatory cytokines, such as interleukin (IL)-6, are up-regulated.$^{(32)}$ In accordance with their finding, a previous study of our collaborators demonstrated a positive correlation of miR-200a with IL-6, of which increased levels were linked to higher morbidity and mortality rates in HIV patients receiving cART.$^{(33)}$ While the role of miR-200a in liver fibrosis is controversial in recently published studies,$^{(34,35)}$ we could not find an association of miR-200a with liver fibrosis.

The present study is distinguished from the aforementioned studies by investigating the association of miR-200a in detail within a cohort of 89 HIV patients and by describing its predictive values, especially with regard to steatosis.

NAFLD often develops as a consequence of metabolic syndrome, consistent with adiposity and type 2 diabetes.$^{(36)}$ We demonstrated this and could support our hypothesis by showing an association of miR-200a serum levels with hepatic steatosis, measured by CAP scores and the patients’ body weight. Our findings are supported by two independent experimental approaches using rats. Rats fed a fatty rich diet developed steatosis, demonstrated by means of histologic analysis.$^{(24,37)}$ In these studies, miR-200a was found to be gradually up-regulated as the liver fat content increased. Therefore, our human data confirm that miR-200a is involved in the pathogenesis of NAFLD.

The interaction of miRNAs with signaling cascades of fatty acid metabolism was shown for certain miRNAs.$^{(38)}$ In the case of miR-200a, it has been described to interact with different gene targets of cholesterol and fatty acid metabolism, such as oxysterol binding protein and adenosine monophosphate-kinase.$^{(21)}$ These pathways are relevant to our present study as we found a link between elevated triglyceride and miR-200a levels. Furthermore, miR-200a expression was increased in hepatocytes in vitro with steatosis induction.$^{(24)}$ This suggests that damaged hepatocytes might release miR-200a on induction of steatosis to affect lipid metabolism. However, further studies are needed to describe the exact underlying mechanism.

Based on the CAP-determined steatosis classification,$^{(25)}$ our work revealed miR-200a to be a stage-dependent biomarker of liver steatosis in humans. With this finding we confirmed the results of the above-mentioned experimental settings in which miR-200a correlated to the degree of steatosis in rats,$^{(24,37)}$ and were able to transfer it for the first time to a human context.

More importantly, we are the first to show the ability of miR-200a to predict progression and severity of steatosis for up to 30 months of follow-up in HIV patients. Furthermore, miR-200a was, in contrast to the observed clinical parameters, the only marker to reflect the stage of hepatic steatosis accurately. Other tested markers that are associated with NAFLD or liver injury either did not have a linear correlation with stages of steatosis (e.g., gamma-glutamyltransferase) or alterations were too small (e.g., BMI). This finding might be of extraordinary importance for several clinical settings. Metabolic alterations are increasingly seen in the management of HIV patients, influenced by comorbidities, life style, and adverse effects of medication.$^{(2)}$ As a biomarker, miR-200a might stratify the risk of aggravation of steatosis in HIV patients in the area of modern cART.

When NAFLD progresses to NASH, irreversible liver cell injury occurs and the severity of liver disease increases. At this point, the development of cirrhosis or HCC becomes likely.$^{(15)}$ Our study reveals a prognostic value of miR-200a for the occurrence of liver damage. We found elevated baseline miR-200a levels to predict increased aminotransferase levels at follow-up.

A biomarker with these abilities might facilitate the management of patients at risk of developing NASH.
Circulating miR-200a levels could allow surveillance and a risk assessment for the development and progression of steatosis and liver injury in a noninvasive but accurate way. Certainly, an early intervention could prevent disease progression and improve the outcome for HIV and NAFLD patients generally. The interventions might not only involve medication but also nutritional counseling and physical activity advice for the patients at risk.

miR-200a represents an accurate and noninvasive biomarker determined in the patient’s blood. This possibly makes miR-200a technically easier to apply than the common gold standard, which involves an invasive liver biopsy and requires medical indication not generally given in the early stages of steatosis. However, when miR-200a predicts a high-grade steatosis, liver biopsy might still be useful to differentiate between different etiologies of steatosis or to deliver more details regarding histology.

The present study has some limitations. In our cohort, we observed steatosis in HIV patients who did not suffer from severe liver disease. As biopsies require medical indication, we could not relate miR-200a levels to histologic results. Further studies of patients with severe NAFLD could prove the association of miR-200a and steatosis by the additional collection of biopsies. In addition, insulin levels and homeostasis assessment of insulin resistance could not be assessed for this study. However, with the CAP parameter we used a relatively reliable parameter as it is not biased by fibrosis and cirrhosis and represents a nonoperator and machine-dependent method. (25)

The use of a spike-in for normalization of miR-200a could be regarded as a limitation; however, there is no consensus about the right normalization strategy. Another restriction is that the average serum aminotransferase levels (ALT/AST) of our study cohort were equal to those of healthy individuals and we merely found an increase in overall aminotransferase levels over time. In our setting, this was inevitable as we selected our patients based on HIV infection rather than liver cell injury.

An interesting approach for future research would also be the inclusion of cART-naive patients as a control group. This could help to quantify the direct effect of antiretroviral treatment on the development of steatosis. However, as most HIV patients receive therapy, the power of a control group and the usefulness of biomarkers in this context will always be limited. Furthermore, there are some hints for a mechanistic explanation behind the increased miR-200a levels and possible targets of miR-200a, which need to be confirmed in additional studies.

In summary, steatosis is often seen in HIV patients. Early diagnosis is an important step in the prevention of its progression. We consider miR-200a as a potential stage-dependent marker of steatosis and show its applicability to predict progression of NAFLD and liver cell injury in the setting of HIV patients.

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