Stopping nucleot(s)ide analogues in non-cirrhotic HBeAg-negative chronic hepatitis B patients: HBsAg loss at 96 weeks is associated with low baseline HBsAg levels

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Authors' contributions: The study was designed and conducted according to the protocol in collaboration with the principal investigators. SALH, GSB and DA collected the data and monitored the study conduct. SV, SALH and AJT performed the statistical analyses. All authors were involved in

data acquisition and analysis, interpretation of the data, and contributed to drafting the manuscript and critical revision of the manuscript with regards to important intellectual content. All authors approved the final version of the manuscript prior to submission.

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Abstract:

Background and Aims: Current guidelines recommend long-term nucleot(s)ide analogue (NA) therapy for patients with HBeAg-negative chronic hepatitis B (CHB). However, disease remission has been described after stopping NA therapy, as well as HBsAg loss.

Methods: We performed a prospective multi-centre cohort study of stopping NA therapy. Inclusion criteria were HBeAg-negative CHB, the absence of cirrhosis and HBVDNA<lower limit of quantification for ≥18 months. We assessed virological and biochemical outcomes including HBsAg loss, as well as NA restart rates, over 96 weeks.

Results: 110 patients (62% entecavir(ETV); 28% tenofovir(TDF), 10 % other) were enrolled. Median age was 56 years, 57% were male, 85% were Asian, median baseline HBsAg level was 705(214-2325)IU/mL. Virological reactivation occurred in 109/110 patients, median time to detection 8(4-12) weeks, and occurred earlier after stopping TDF vs ETV (median 4 vs 12 weeks P<0.001). At week 96, 77(70%) remained off-treatment, 65(59%) had ALT<2xULN, 31(28%) patients were in disease remission with HBVDNA <2,000IU/mL plus ALT<2xULN and 7(6%) patients had lost HBsAg. Baseline HBsAg≤10IU/mL was associated with HBsAg loss (6/9 vs 1/101 P<0.001). ALT>5xULN occurred in 35(32%); ALT flares were not associated with HBsAg loss. There were no unexpected safety issues.

Conclusion: Virological reactivation was very common after stopping NA therapy and occurred earlier after stopping TDF vs ETV. The majority of patients had ALT<2xULN at week 96, but only one third achieved disease remission and HBsAg loss was rare. Very low HBsAg levels at baseline were uncommon but predicted for HBsAg loss and disease remission.

Introduction:

Nucleot(s)ide analogues (NA) are standard treatment for HBeAg-negative chronic hepatitis B (CHB). The two first-line NA, entecavir (ETV) and tenofovir disoproxil (TDF), are both potent antiviral agents which effect durable viral suppression, reduce hepatic necro-inflammation, fibrosis progression and reduce risk of cirrhosis, liver failure and hepatocellular carcinoma (HCC) [1, 2]. However, HBsAg clearance, or functional cure, is rare, [3, 4] and long-term treatment is recommended [1, 2, 5, 6, 7, 8, 9]. Finite therapy for individuals with HBeAg-negative CHB has been identified as an area of unmet need by expert organisations [2] because of concerns regarding cost, long-term side effects and the risk of the development of drug resistance with indefinite treatment. In the past decade, there has been increasing interest in whether NA therapy can be safely stopped in a subset of patients.

In 2012, Hadziyannis and colleagues published a landmark study evaluating clinical outcomes after stopping NA treatment in a small cohort of non-cirrhotic patients with HBeAg-negative CHB who were long-term responders to adefovir monotherapy [10]. All patients experienced early virological relapse. Virological relapse was associated with biochemical relapse in 76% of patients, with antiviral therapy resumed in 15/33 patients. Eighteen patients remained off-treatment through five years of follow-up and all achieved a sustained response, defined by serum HBV DNA level < 2,000 IU/mL and normal serum ALT level. HBsAg loss was observed in 72% (13/18) of patients who maintained a sustained response off treatment by the end of 5 years of follow-up. HBsAg loss was associated with lower HBsAg level at the time adefovir was stopped, and was more common among participants who did not restart treatment. Among those patients who did not restart NA therapy, baseline ALT was higher in those who achieved HBsAg loss, suggesting a role for immune-mediated cytolysis of HBV-infected hepatocytes. Stopping treatment was safe, with no episodes of liver decompensation reported. Since this initial study, there have been a number of reports of clinical outcomes after stopping long-term NA therapy in patients with HBeAg-negative CHB [4, 11, 12, 13, 14]. The studies have been heterogeneous, often retrospective in design, and with considerable variation between protocols for ethnicity of cohort, inclusion / exclusion of people with cirrhosis, duration of follow-up, as well as criteria for re-starting NA therapy.

There remain important questions about the rate and outcome of virological and biochemical relapse after stopping NA treatment, the safety of this approach, and the rates of HBsAg loss in prospective follow-up. The answers will inform decisions about patient selection for this strategy in clinical practice. We therefore performed a prospective multi-centre study to evaluate clinical outcomes after stopping NA therapy in non-cirrhotic individuals with HBeAg-negative CHB. Here we present the final clinical outcomes after 96 weeks of follow-up.

Methods:

Study Design. We performed an investigator-initiated, prospective, multi-centre, observational cohort study of the clinical outcomes after stopping NA therapy among non-cirrhotic patients with HBeAg-negative CHB and complete virological suppression on-treatment. We present the results after 96 weeks of follow-up. Patients had study visits at weeks 4, 8, 12, 18, 24, 36, 48, 60, 72, 84 and 96. Patients had more frequent study visits if they experienced virological or biochemical flare according to the following criteria: ALT 1.2 – 5 x upper limit of normal (ULN) or HBV DNA > 10^5 IU/mL – study visit at week 4 post-flare, and every 4 weeks until ALT < 1.2 x ULN or HBV DNA < 10^5 IU/mL; ALT > 5 x ULN – visit at week 2 post-flare, and every 2 weeks until ALT < 5 x ULN, then every 4 weeks until < 1.2 x ULN. The ULN for ALT was defined by the local laboratory. The protocol criteria for re-starting NA treatment included: i) Serum HBV DNA > 2,000 IU/mL and serum ALT > 5 x ULN for ≥ 16 weeks or ALT > 10 x ULN for ≥ 8 weeks; ii) Clinical evidence of hepatic decompensation defined by INR ≥ 1.5 or serum bilirubin level > 2 x ULN or ascites or hepatic encephalopathy; or iii) investigator discretion. Each study visit included a physical assessment, standard laboratory testing, and quantification of serum HBV DNA and HBsAg level. Liver stiffness measurement and liver ultrasound was undertaken every 6 months, as was liver ultrasound for HCC screening. All adverse events were recorded at each visit. Serum HBV DNA was measured using the COBAS Ampliprep/COBAS TaqMan platform (Roche Diagnostics lower limit of detection 20 IU/mL) at all sites. Serum HBsAg level was measured using the Elecsys® HBsAg II quant II (Roche Diagnostics) with a lower limit of reporting of 0.05 IU/mL.

Patient selection. Patients, aged over 18 years, with non-cirrhotic e-antigen negative CHB infection were enrolled from 9 centres in Australia from July 2014-May 2019. Key inclusion criteria included: i) continuous NA therapy for at least 2 years with HBV DNA levels < 20 IU/mL on 3 consecutive occasions at least 6 months apart over 18 months (NA therapy could have included monotherapy or combination therapy); ii) HBeAg-negative CHB at the time NA was started; iii) HBsAg-positive, HBeAg-negative and anti-HBe-positive at the time of screening; and iv) the absence of cirrhosis (liver stiffness ≤ 9.5kPa at screening, and if available, liver histology showing METAVIR stage ≤ F3 prior to starting NA). Exclusion criteria included: i) HBeAg-positivity at the time NA were started; ii) diagnosis of cirrhosis at any time (histology showing METAVIR F4 prior to starting NA therapy, previous clinical diagnosis of cirrhosis or liver decompensation events); iii) median liver stiffness > 9.5 kPa at screening; iv) documented or suspected hepatocellular carcinoma (HCC); v) HBV associated extra-hepatic manifestations; vi) co-infection with HIV, hepatitis C virus or hepatitis D virus; vii) concomitant immunosuppression; and (viii) significant alcohol consumption (> 30 g/day for women and > 50 g/day for men).

Outcomes: The pre-specified primary outcome of this trial was the rate of HBsAg loss at week 96. Secondary outcomes included the rate of biochemical remission at week 96 (serum ALT < 2 x ULN); virological remission at week 96 (HBV DNA < 2,000 IU/mL), as well as other biochemical outcomes (rates of ALT > ULN, ALT 2-5 x ULN, ALT 5-10 x ULN, and ALT > 10 x ULN) and virological outcomes (time to HBV DNA detectability, HBV DNA > 2,000 IU/mL, HBsAg decline). Key safety endpoints included the occurrence of ALT flares > 10 x ULN, the occurrence of liver decompensation events, the diagnosis of HCC during the study, as well as the number of participants who restarted NA treatment.

Statistical analysis: Statistical analysis was performed with the statistical package *Stata* (version 16.1; StataCorp, College Station, TX). In all cases, tests of significance were 2-tailed with a p level at <.05. Descriptive data are presented as median (interquartile range) and frequency (percentage). Time to flare (virological and biochemical) and time to HBsAg loss are presented using Kaplan-Meier analysis. Association of baseline characteristics and time to flare was evaluated using Cox proportional hazards regression. Results are expressed as hazard ratio with 95% confidence interval. Logistic regression was used to examine associations with HBsAg loss. Two-tailed Fisher exact test and Mann-Whitney test were used for comparison of categorical and continuous variables between groups. To determine the optimal cutoff of DNA level for predicting ALT flare (> 2x ULN, > 5 x ULN and > 10x ULN), peak DNA level prior to the peak ALT flare (or anytime during 96 weeks for those without a flare) was used. Optimal cutoff was determined using ROC analysis as a point that is closest to perfect sensitivity and specificity (minimum distance from the left-upper corner of the unit square). Sensitivity, specificity, positive and negative predictive value and area under the receiver operating curve (AUC ROC) with 95% confidence interval were calculated for optimal cutoff and for commonly used clinically relevant HBV DNA cutpoints (2,000, 20,000 and 200,000 IU/mL). The same approach was taken to determine the optimal cutoff of baseline HBsAg for predicting HBsAg loss.

Ethics Statement: The study was approved by the St Vincent's Hospital Ethics Committee as the lead site (ID Number 032/14) and at each participating site and was conducted in compliance with the principles of the 1975 Declaration of Helsinki and local regulatory requirements. All patients provided informed consent prior to screening.

Results:

Patients characteristics

One hundred and ten patients were prospectively recruited. The baseline patient characteristics are shown in Table 1. The cohort was predominantly Asian (85%), male (58%) with a median age of 56 years. Sixty-eight patients (62%) were on ETV therapy and 31 patients (28%) were on TDF therapy at baseline. The median HBsAg level was 623 (204 – 1981) IU/mL. Twenty-two patients (20%) had HBsAg levels < 100 IU/mL at baseline. Two patients were lost to follow up at 4 and 12 weeks but were included in our intention-to-treat analysis as having restarted NA from when they left the study.

Virological and biochemical profiles over time

Serum HBV DNA levels over time

Serum HBV DNA was detectable in 109/110 participants (Figure 1a) at a median time of 8 weeks (IQR 4-12 weeks). This occurred earlier in patients who stopped TDF compared to ETV (median 4 vs 12 weeks, p <0.001, Figure 2A), and virological relapse, defined by HBV DNA level > 2,000 IU/mL, occurred earlier in 98 (89%) patients, earlier in patients who stopped TDF than ETV (HR 2.8, 95% CI 1.8, 4.4; p<0.001) with median time to relapse of 7 weeks (95% CI 4-9 weeks) compared to 19 weeks (95% CI 18-26 weeks). On multi-variable analysis, baseline NA (TDF vs ETV) was independently associated with shorter time to virological relapse; the only other baseline predictors of time to virological relapse were baseline HBsAg level and age (Figure 4 and Supplementary Table 2). 12/110 (11%) patients maintained HBV DNA levels < 2,000 IU/mL throughout 96 weeks of follow-up. 46/110 (42%) patients had a peak HBV DNA > 200,000 IU/mL (Supplementary Table 1). Median time to peak HBV DNA was 38 weeks (95% CI 29-55 weeks) overall.

Serum ALT levels over time

A biochemical relapse (ALT > 2 x ULN) was seen in 50% of patients up to week 96. In those who relapsed, median time to biochemical relapse was 20 weeks (95% CI 12-26 weeks). The time to biochemical relapse was shorter among patients who stopped TDF compared to ETV (HR 2.4, 95% CI 1.4, 4.1; p = 0.002, Figure 2B). On multi-variable analysis, baseline NA (TDF vs ETV) was the only predictor of time to biochemical relapse. Peak serum ALT level > 5x ULN was observed in 35 (32%) patients and peak ALT > 10 x ULN was observed in 24 patients (22%) (Supplementary Table 1). The hazard of a severe hepatitis flare with peak ALT > 5 x ULN did not differ after stopping TDF vs ETV (HR 1.20, 95% CI 0.58, 2.48; p=0.632). An association was observed between rising HBV DNA level and subsequent risk of ALT flare (individual patient plots, Supplementary Figure 1B); the optimal HBV DNA

threshold for predicting ALT rise > 5 x ULN at the subsequent time-point in this cohort was 96,730.5 IU/mL (AUROC = 0.68 (0.69, 0.78), Supplementary Table 3). Patients who experienced an ALT flare > 5 x ULN were more likely to start NA therapy (OR 11.15 [4.33 – 28.74], P < 0.001).

HBsAg decline and HBsAg Loss

HBsAg decline > 0.5 log IU/mL was observed in 31 (28%) patients and HBsAg decline > 1.0 log IU/mL was observed in 16 (15%) patients at 96 weeks. Overall there was a small but statistically significant decline in HBsAg levels from baseline to week 96 across the entire cohort (median decline = -0.24 log (-0.59, -0.08) log IU/mL p <0.001, Figure 1C). Sustained HBsAg loss was observed in 7 patients (6%). The rate of HBsAg loss increased over the 96 weeks after stopping NA (Supplementary Figure 3*)*. HBsAg levels at baseline were significantly lower in patients who lost HBsAg compared to patients who remained HBsAg positive at week 96 (3.1 [1.6-10] vs 685 [241-1835], p < 0.001). The optimal cutoff value for HBsAg levels that predicted HBsAg loss at week 96 was 10 IU/mL (sensitivity 86%, specificity 97%) Baseline HBsAg levels ≤ 10 IU/mL were associated with HBsAg loss (6/9 (67%) vs 1/101 (1%), OR 0.005 (0.0005, 0.06) p < 0.001, Figure 4). No other variable was associated with HBsAg loss. (Supplementary Table 5). HBsAg level ≤ 10 IU/mL was also associated with lower risk of virological and biochemical relapse (Figure 4).

Clinical outcomes at week 96:

We considered clinical outcomes at the week 96 timepoint according to the following categories: HBsAg loss, disease remission (HBV DNA < 2,000 and ALT < 2 x ULN), clinical relapse (HBV DNA > 2,000 and ALT > 2 x ULN), indeterminate phenotype (HBV DNA > 2,000 and ALT < 2 x ULN OR HBV DNA < 2,000 and ALT > 2 x ULN) or NA re-treatment (Figure 3). As noted, HBsAg loss was observed in 7 (6%) patients. 31 (28%) patients were in disease remission with both serum HBV DNA level < 2,000 IU/mL and ALT < 2 x ULN at week 96. A total of 65 (59%) patients had a serum ALT level < 2 x ULN at week 96. Twelve (11%) patients maintained HBV DNA levels < 2000 IU/mL throughout the entire 96 weeks of follow-up. A total of 45 (41%) had a persistently low ALT level (< 2 x ULN) throughout 96 weeks of follow-up. Thirty three (30%) patients were restarted on NA therapy by 96 weeks either for a persistent large flare (ALT > 10x ULN) or recurrent flare. Two patients withdrew from the study at time points 4 & 12 weeks; both restarted NA at the time of study withdrawal.

ALT flares after stopping NA treatment may reflect increased immune pressure on HBV, and we were interested in whether ALT flares might be associated with HBsAg loss or the achievement of disease remission at end of study. However, none of the patients who achieved HBsAg loss had an ALT rise >

2 x ULN over 96 weeks, and none of the patients who experienced an ALT > 5 x ULN achieved HBsAg loss (0/35). In fact, the peak ALT was significantly lower among patients who achieved HBsAg loss (0.76 x ULN [0.56-1.71 x ULN] vs 2.21 x ULN [1.12-9.45 x ULN], P = 0.003). No other variable was associated with HBsAg loss. (Supplementary Table 5). There was no association between ALT flare (ALT > 5 x ULN) and the achievement of spontaneous clinical remission – in fact, patients experiencing an ALT flare > 5 x ULN were less likely to achieve clinical remission off-treatment at week 96 OR 0.15 [0.05, 0.46], p = 0.001). (Supplementary Table 4).

Safety

There were no unexpected safety issues through 96 weeks of follow-up. In five patients, bilirubin rose > 2x ULN in the context of an ALT flare, but this settled rapidly as the ALT dropped. Three of these patients were started on NA therapy; in two patients the ALT flare settled spontaneously under observation. There were no liver decompensation events (INR > 1.5, hepatic encephalopathy, ascites) in these non-cirrhotic patients, and no cases of HCC nor development of cirrhosis during the follow up period.

Discussion

We report the results of a large prospective study of clinical outcomes after stopping NA therapy in HBeAg-negative CHB patients. All patients were non-cirrhotic, and 85% of patients were of Asian ethnicity. At 96 weeks after treatment withdrawal, 28% remained off drug therapy and were in disease remission with both serum HBV DNA level < 2,000 IU/mL and ALT < 2 x ULN and 59% had serum ALT < 2 x ULN. Twelve (11%) patients maintained HBV DNA levels < 2000 IU/mL through follow-up. Thirty-three (30%) patients were restarted on NA therapy. Stopping NA was safe in this population and no liver decompensation events were observed.

A key finding in this study was that the rate of HBsAg loss was low (6%) in the first 96 weeks after stopping NA treatment. The rate of HBsAg loss reported in previous studies has varied up to 55% in one study with 6 years of follow-up. There are a number of potential explanations for the variation in HBsAg loss rates observed in the literature. Many prior studies had a retrospective cohort design with risk of ascertainment bias [15]. Emerging data suggest that HBsAg loss rates are higher in Caucasian cohorts compared to Asian cohorts; this may relate to HBV genotype, duration of infection or host genetics. Patients in our cohort were predominantly Asian and assumed to have been infected at birth.

HBsAg loss rates also increase with longer duration of follow-up. In our recent meta-analysis, we reported higher rates of HBsAg loss in studies with ≥ 4 years of follow-up [15] Although the rates of HBsAg loss in this study were low, we believe they were triggered by stopping NA therapy given the extensive literature reporting very low rates of HBsAg loss over time among patients with HBeAg-negative CHB continuing on long-term NA therapy [10, 12, 16].

Very low baseline HBsAg levels were associated with HBsAg loss, with 6/9 patients with baseline HBsAg ≤ 10 IU/mL losing HBsAg at week 96. Low HBsAg levels also predicted lower risk of HBV DNA > 2,000 IU/mL as well as biochemical relapse up to week 96. Low HBsAg levels might therefore be a suitable biomarker to identify patients most suitable for considering stopping of NA therapy, identifying patients with higher likelihood of HBsAg seroclearance, and who are also suitable for a less intensive monitoring schedule after treatment cessation. It was notable that only a small minority of participants in this study had HBsAg levels ≤ 10 IU/mL. The clinical utility of this very low HBsAg threshold for predicting HBsAg loss after stopping NA therapy should be independently validated; if confirmed it would pave the way for a personalised approach to stopping NA therapy

109/110 patients experienced virological reactivation after stopping NA. Virological and biochemical reactivation occurred earlier in patients who stopped TDF than ETV. This observation confirms previous findings from retrospective studies [17, 18]. The difference in time to relapse has a number of potential explanations. It has previously been suggested that nucleotide analogues (TDF) but not nucleoside analogues (ETV), are associated with higher circulating levels of IFN-λ3 in patients, and can induce IFN-λ3 *in vitro* [19]. Early relapse might therefore occur following the withdrawal of nucleotide analogues as both direct antiviral and immune controls are removed. There are also a number of mechanistic differences between the two drugs that may be relevant. Firstly, in hepatocytes, the adenine nucleotide pool in hepatocytes that competes with TDF is much larger than the guanine nucleotide pool competing with ETV, meaning that low residual concentrations of ETV-triphosphate are more effective competitive antagonists of the HBV reverse transcriptase. Secondly, the rate-limiting enzyme for ETV activation is the constitutively active deoxyguanosine kinase whereas for TDF activation is an adenylate kinase, which is inducible but not constitutively active. Finally, ETV-triphosphate can be internally incorporated into host DNA, potentially creating a reservoir of ETV-monophosphate that may be recognised by DNA repair systems, whereas TDF is an obligatory chain terminator with no potential for sequestration in DNA. It is important to note that we were not able to collect detailed past historical details of NA therapy for our cohort, beyond confirming that participants met the inclusion criteria, because often participants had been started on NA therapy by

another doctor many years before. We acknowledge that ETV has been available for longer in Australia than TDF and it possible that median duration of ETV therapy was longer than TDF in our cohort. However, we do not think this is the most likely explanation for Virological and biochemical reactivation occurring earlier in patients who stopped TDF than ETV. If the explanation was simply duration, this would imply that ETV-treated patients had achieved lower levels of intrahepatic cccDNA, and that relapse was purely a function of cccDNA load. However, there was no difference in baseline HBsAg levels between the ETV and TDF cohorts, still the best peripheral surrogate we have for the hepatitis B cccDNA reservoir, nor age, which has been suggested to inversely correlate with cccDNA in the liver. Furthermore, the viral kinetics of relapse after stopping TDF are so different to ETV, and so rapid, that we believe implicate alternative explanations, as proposed above. There are also clinical implications for the recommended monitoring schedule of patients stopping NA. Patients stopping TDF should be monitored closely for virological and biochemical flare from week 4 after stopping, whereas ETV are not likely to experience a flare until week 12 or later.

Biochemical flares were observed after stopping therapy. The timing of HBV DNA reactivation (early vs late) was not associated with the risk of severe ALT flare, but the peak HBV DNA level predicted subsequent ALT > 5x ULN. In this cohort, the optimal HBV DNA cut-off for predicting subsequent ALT > 5 x ULN was HBV DNA > 5 $\log_{10}$ IU/mL. This cut-off value will need validation, but the clinical implication is that patients with HBV DNA level exceeding 5 $\log_{10}$ IU/mL after stopping NA should be monitored more frequently for ALT flare. We were interested in the possibility of a "therapeutic" hepatitis flare after stopping NA therapy. There were a small minority of flare patients in whom there was a reduction in HBsAg level > 1 $\log_{10}$ IU/mL within 3 months of the peak flare (Supplementary Table 6), and this was associated with a higher peak ALT level, but none of these patients had achieved HBsAg seroclearance by week 96. Longer-term follow-up may identify an association with HBsAg loss. However, the majority of patients who experienced ALT flares > 5 x ULN did not achieve HBsAg decline or loss, nor were they more likely to be in clinical remission off-treatment at week 96. These patients should therefore be closely monitored and we recommend a low threshold for restarting NA therapy.

We did not identify any unexpected safety issues. Hepatitis flares were observed, but most were asymptomatic and no liver decompensation events were observed. Patients with cirrhosis were excluded from this study, which we believe was appropriate. Liver-related morbidity and mortality reported in the literature after stopping NA therapy almost always is reported in patients with underlying cirrhosis, and we recommend careful exclusion of cirrhosis before considering this strategy.

Patients were closely monitored in the context of severe ALT flares, another important practice point, and NA should be restarted if ALT flares are prolonged or there is any concern about decompensation.

A question for future studies is whether stopping NA therapy has a role to play in treatment strategies involving combinations of novel antiviral or immunomodulatory therapies aiming for HBV cure. RNA interference can effectively knock down serum HBsAg levels, and multiple candidates have entered phase two development [20, 21]. An important question is whether HBsAg loss rates can be increased by stopping NA therapy after an siRNA candidate has been used to reduce HBsAg levels to low levels. Emerging data has suggested that the addition of an immunomodulator to combination regimens of novel antivirals can be associated with HBsAg loss – future studies should also evaluate whether there is an immunomodulatory benefit for the "stop-flare" in the context of novel antiviral treatment strategies.

In conclusion, virological relapse was universal after stopping NA therapy and occurred earlier in patients who stopped TDF than ETV. Patients require monitoring for hepatitis flares, but stopping NA in non-cirrhotic patients was safe. After 96 weeks, most patients had ALT < 2 x ULN and only a minority had restarted NA. Six percent of patients lost HBsAg after 96 weeks of follow-up of our predominantly Asian patients. HBsAg loss was predominantly seen in patients with very low levels of HBsAg at baseline, which also predicted for the lower likelihood of virological and biochemical relapse throughout 96 weeks. Measuring HBsAg levels may therefore identify patients most suitable for a trial of stopping NA therapy. The timeline for our study was limited to 96 weeks, however, rates of HBsAg loss may increase with longer duration of follow-up.

References:

1 Terrault NA, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM*, et al.* Update on Prevention, Diagnosis, and Treatment of Chronic Hepatitis B: AASLD 2018 Hepatitis B Guidance. Clinical liver disease 2018;**12**:33-4.

2 European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017;**67**:370-98.

3 Van Boemmel FS, K. Heyne, R. Moller, H. Peterson, J. Buggisch, P et al. Response to discontinuation of long-term nucleos(t)ide analogue treatment in HBeAg negative patients: Results of the Stop-NUC trial. J Hepatol 2020;**73**:S118-S9.

4 Berg T, Simon KG, Mauss S, Schott E, Heyne R, Klass DM*, et al.* Long-term response after stopping tenofovir disoproxil fumarate in non-cirrhotic HBeAg-negative patients - FINITE study. J Hepatol 2017;**67**:918-24.

5 Sarin SK, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ*, et al.* Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol Int 2016;**10**:1-98.

6 Chang TT, Liaw YF, Wu SS, Schiff E, Han KH, Lai CL*, et al.* Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B. Hepatology 2010;**52**:886-93.

7 Lai CL, Shouval D, Lok AS, Chang TT, Cheinquer H, Goodman Z*, et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. The New England journal of medicine 2006;**354**:1011-20.

8 Marcellin P, Heathcote EJ, Buti M, Gane E, de Man RA, Krastev Z*, et al.* Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. The New England journal of medicine 2008;**359**:2442-55.

9 Snow-Lampart A, Chappell B, Curtis M, Zhu Y, Myrick F, Schawalder J*, et al.* No resistance to tenofovir disoproxil fumarate detected after up to 144 weeks of therapy in patients monoinfected with chronic hepatitis B virus. Hepatology 2011;**53**:763-73.

10 Hadziyannis SJ, Sevastianos V, Rapti I, Vassilopoulos D, Hadziyannis E. Sustained responses and loss of HBsAg in HBeAg-negative patients with chronic hepatitis B who stop long-term treatment with adefovir. Gastroenterology 2012;**143**:629-36.e1.

11 Jeng WJ, Chen YC, Chien RN, Sheen IS, Liaw YF. Incidence and predictors of hepatitis B surface antigen seroclearance after cessation of nucleos(t)ide analogue therapy in hepatitis B e antigen-negative chronic hepatitis B. Hepatology 2018;**68**:425-34.

12 Yao CC, Hung CH, Hu TH, Lu SN, Wang JH, Lee CM*, et al.* Incidence and predictors of HBV relapse after cessation of nucleoside analogues in HBeAg-negative patients with HBsAg<=200IU/mL. Sci Rep 2017;**7**:1839.

13 Liem KS, Fung S, Wong DK, Yim C, Noureldin S, Chen J*, et al.* Limited sustained response after stopping nucleos(t)ide analogues in patients with chronic hepatitis B: results from a randomised controlled trial (Toronto STOP study). Gut 2019;**68**:2206-13.

14 Papatheodoridis GV, Manolakopoulos S, Su TH, Siakavellas S, Liu CJ, Kourikou A*, et al.* Significance of definitions of relapse after discontinuation of oral antivirals in HBeAg-negative chronic hepatitis B. Hepatology 2018;**68**:415-24.

15 Hall SAL, Vogrin S, Wawryk O, Burns GS, Visvanathan K, Sundararajan V*, et al.* Discontinuation of nucleot(s)ide analogue therapy in HBeAg-negative chronic hepatitis B: a meta-analysis. Gut Published Online First: 07 September 2021. doi: 10.1136/gutjnl-2020-323979

16 Chen CH, Lu SN, Hung CH, Wang JH, Hu TH, Changchien CS*, et al.* The role of hepatitis B surface antigen quantification in predicting HBsAg loss and HBV relapse after discontinuation of lamivudine treatment. J Hepatol 2014;**61**:515-22.

17 Su TH, Yang HC, Tseng TC, Liou JM, Liu CH, Chen CL*, et al.* Distinct Relapse Rates and Risk Predictors After Discontinuing Tenofovir and Entecavir Therapy. J Infect Dis 2018;**217**:1193-201.

18 Höner Zu Siederdissen C, Hui AJ, Sukeepaisarnjaroen W, Tangkijvanich P, Su WW, Nieto GEG*, et al.* Contrasting Timing of Virological Relapse After Discontinuation of Tenofovir or Entecavir in Hepatitis B e Antigen-Negative Patients. J Infect Dis 2018;**218**:1480-4.

19 Murata K, Asano M, Matsumoto A, Sugiyama M, Nishida N, Tanaka E*, et al.* Induction of IFN-λ3 as an additional effect of nucleotide, not nucleoside, analogues: a new potential target for HBV infection. Gut 2018;**67**:362-71.

20 Kadelka S, Dahari H, Ciupe SM. Understanding the antiviral effects of RNAi-based therapy in HBeAg-positive chronic hepatitis B infection. Sci Rep 2021;**11**:200.

21 Yuen MF, Wong DK, Schluep T, Lai CL, Ferrari C, Locarnini S*, et al.* Long-term serological, virological and histological responses to RNA inhibition by ARC-520 in Chinese chronic hepatitis B patients on entecavir treatment. Gut 2021.

Table 1. Patient characteristics at baseline

	NA-Stop n=110
Age (years), Median (IQR	**56 (50-63)**
Male, n (%)	**63 (57%)**
Ethnicity, n (%) **Asian** **Caucasian** **Other**	 **93 (85%)** **10 (9%)** **7 (6%)**
NA Stopped at Baseline, n (%): **ETV** **TDF** **Other**	 **68 (62%)** **31 (28%)** **11 (10%)**
ALT (IU/mL), Median (IQR)	**24 (17-31)**
HBsAg level (IU/mL), Median (IQR) **HBsAg level** **≤ 10 IU/mL** **>10 – 100** **>100 – 500** **>500 – 1000** **> 1000**	**623 (204 – 1981)** **9 (8%)** **12 (11%)** **29 (26%)** **16 (15%)** **44 (40%)**
Cirrhosis, n (%)*	**0 (0%)**
*LSM < 9.5kPa, non-cirrhotic prior to starting NA therapy	

Figure 1. Patient HBV DNA, ALT and HBsAg profiles at each study visit.

Individual patient profiles are plotted for HBV DNA (A), ALT (B) and HBsAg (C). In Figure (C) the dark black lines represent patients who lost HBsAg and the grey lines represent patients who remained HBsAg-positive to the end of 96 weeks of follow-up. In Figure (A), two patients had detectable HBV DNA at baseline, but did have undetectable HBV DNA at screening.

(A) HBV DNA

(B) ALT

(C) HBsAg

Figure 2. Virological reactivation and biochemical relapse occurred earlier after stopping TDF than ETV.

(A) The figure presents time to detection of serum HBV DNA (virological reactivation, HBV DNA level ≥ 10 IU/mL), comparing patients who stopped TDF to patients who stopped ETV. Stopping TDF was associated with shorter time to HBV DNA detectability (median 4 vs 12 weeks, $p < 0.001$). No other clinical variable was associated with time to detection of serum HBV DNA after stopping NA therapy (including age, gender, ethnicity, baseline ALT or baseline HBsAg, data not shown). Note that there

was also no difference in the total number of patients experiencing HBV DNA levels > 2,000 IU/mL up to week 96 (TDF, n = 30 vs ETV, n = 62, P = 0.43). **(B)** The figure presents time to ALT rise > 2 x ULN. Stopping TDF was associated with shorter time to ALT rise > 2 x ULN (HR 2.4, 95% CI 1.4, 4.1; p = 0.002). No other clinical variable was associated with time to ALT (including age, gender, ethnicity, baseline ALT or baseline HBsAg, data not shown).

Figure 3. Clinical outcomes over time

Intention to treat analysis of clinical outcomes in 110 patients who stopped NA therapy. Patients were classified into the following groups: HBsAg loss, Biochemical Remission (ALT < 2 x ULN), Virological Remission (HBV DNA < 2,000), or NA treatment restarted.

Figure 4. Low baseline HBsAg levels were associated with higher likelihood of HBsAg loss and lower rates of Virological and Biochemical Relapse

(A) Baseline HBsAg level ≤ 10 IU/mL was associated with HBsAg loss – 6 of 7 patients who lost HBsAg had a baseline HBsAg level ≤ 10 IU/mL (HBsAg ≤ 10 IU/mL, HBsAg loss = 6/9 (67%) vs HBsAg > 10 IU/mL, HBsAg loss = 1/101(1%), p< 0.001, Figure 5). Low baseline HBsAg levels were also associated with a lower rate of (B) virological relapse (HBsAg level ≤ 10 IU/mL, 3/9 vs HBsAg level > 10, 95/101, HR 0.15 (0.05, 0.47) p-value =0.001) and (C) biochemical relapse (HBsAg level ≤ 10 IU/mL, 1/9 vs HBsAg level > 10, 54/101, HR 0.16 (0.02, 1.13) p=0.066 through 96 weeks of follow-up (Figure 5).

(A)

(B) (C)

Statement of Interests:

Authors' Declaration of Personal Interests: AJT advisory board member – Abbvie, Gilead Sciences, Roche Diagnostics, BMS, Merck, Immunocore, Janssen, Assembly Biosciences, Arbutus, Vir Biotechnology, Eisai, Ipsen, Bayer; speaker – Abbvie, Gilead Sciences, Roche, BMS; research / grant support – Gilead Sciences, Merck, BMS, Abbvie, Roche Diagnostics. KV received research funding from Gilead Sciences and Abbvie, as well as speaker fees from Gilead. AJN received speaker fees/advisory board from Bayer, Eisai and Ipsen; GM received research grants from Gilead and Abbvie; SAL received

funding from Aligos Therapeutics, Assembly Biosciences & ClearB Therapeutics; MTL received Gilead research funding, Dr Falk Speaker fee, Abbvie research support, Bayer speaker fee, MSD ad board, Roche speakers fee; PAR has received research funding from Gilead Sciences and is on the Scientific Advisory Board of Enochian Biosciences; The remaining authors had no conflicts of interest to declare.

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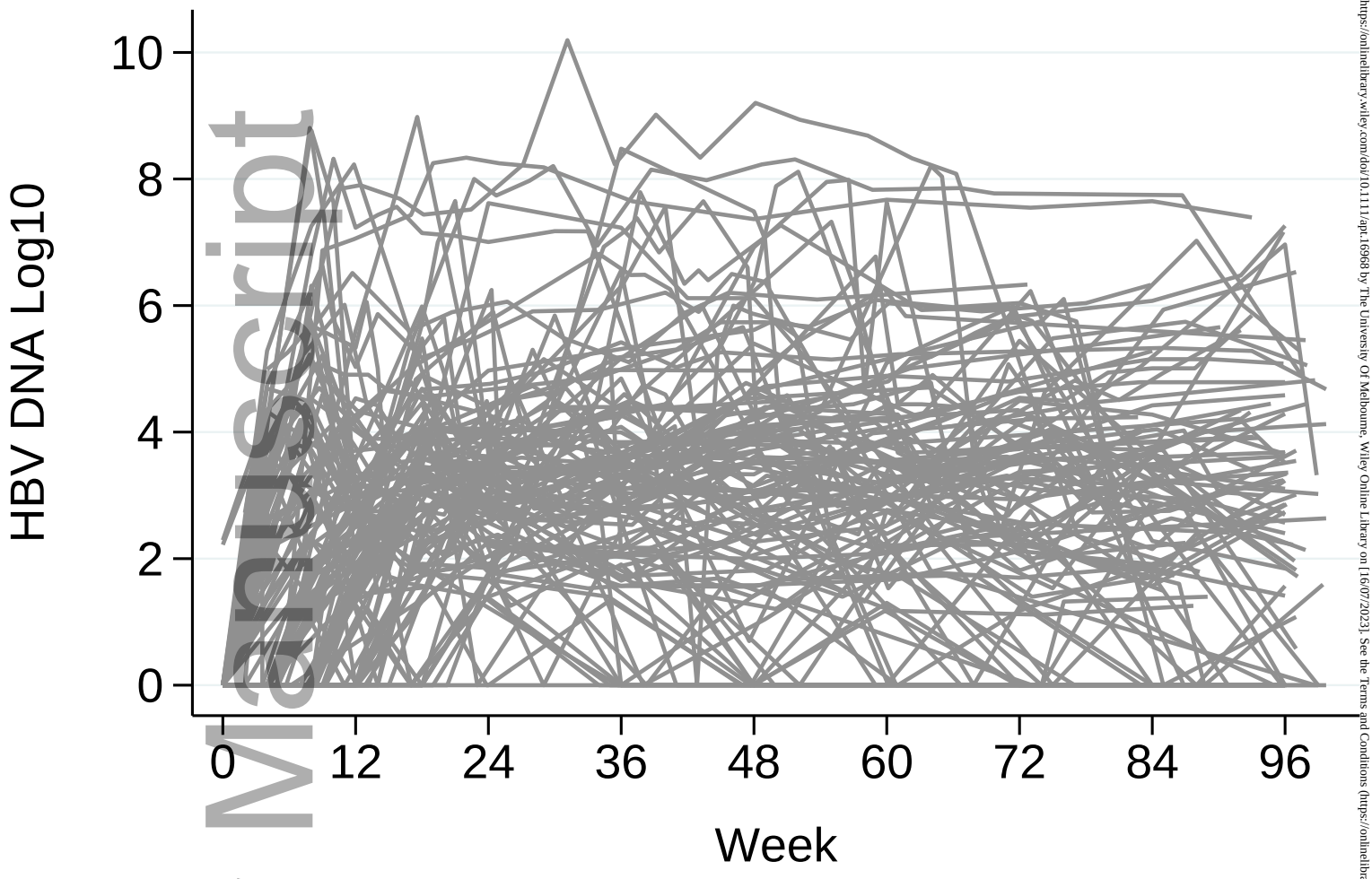

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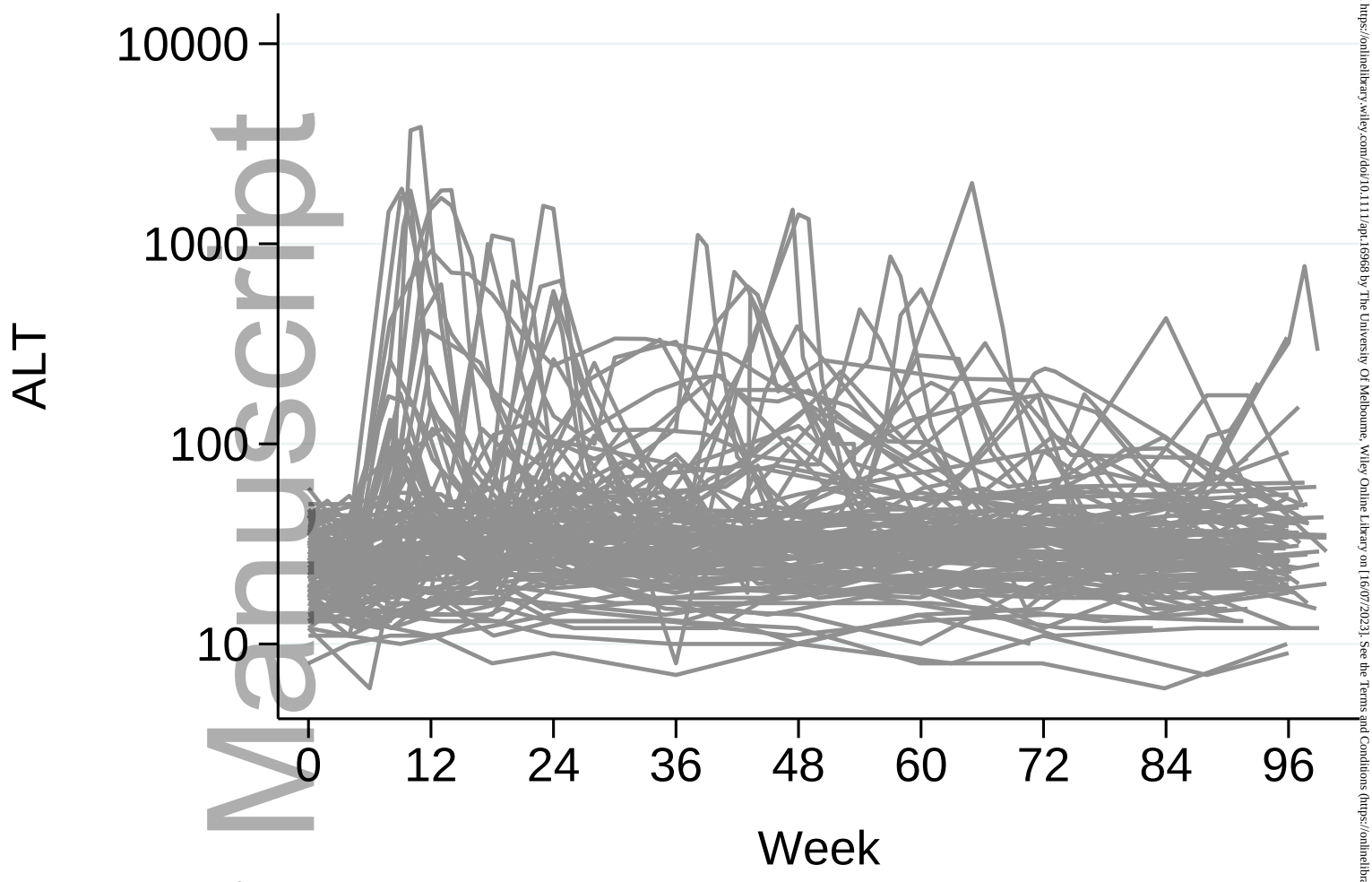

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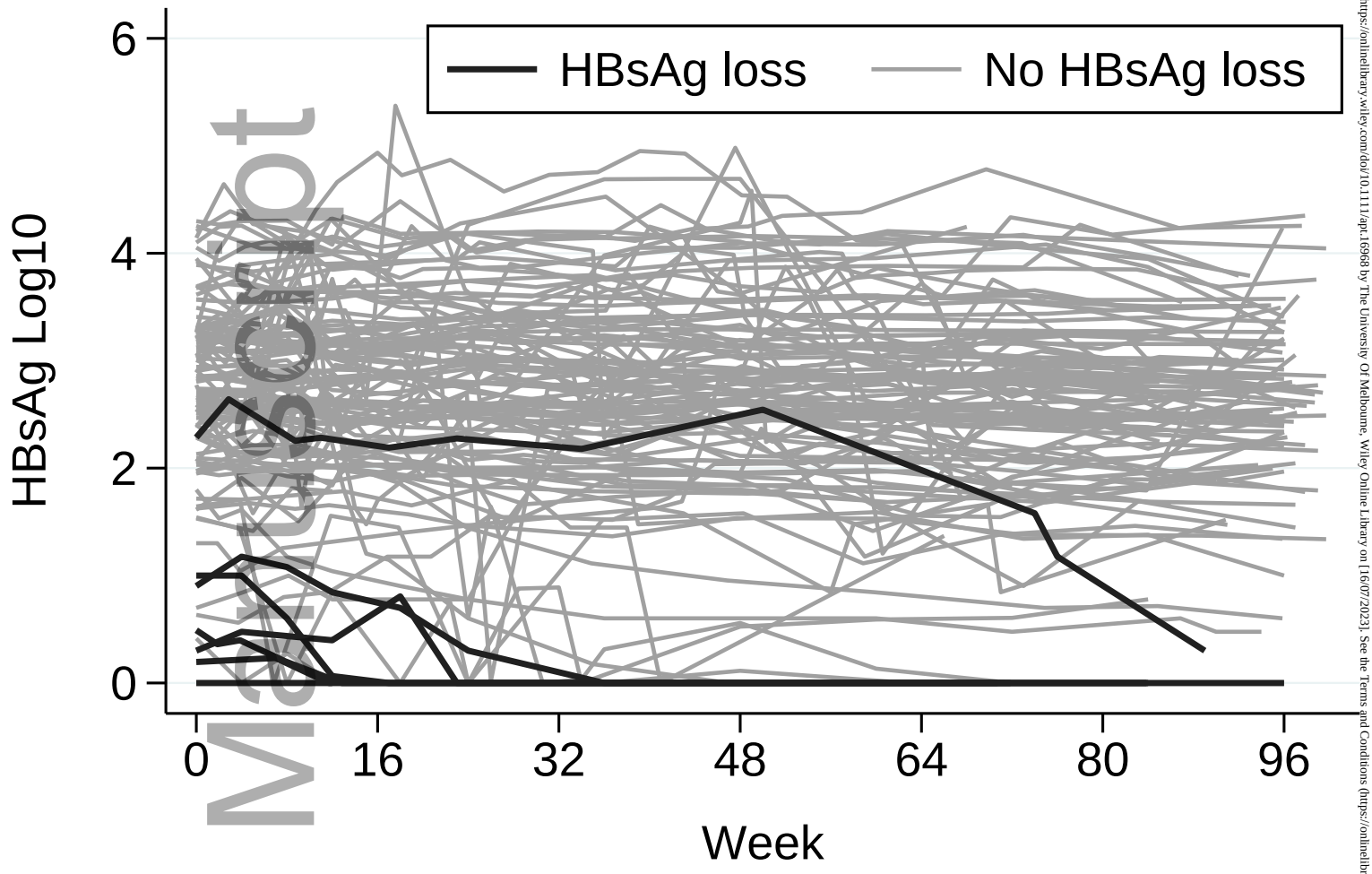

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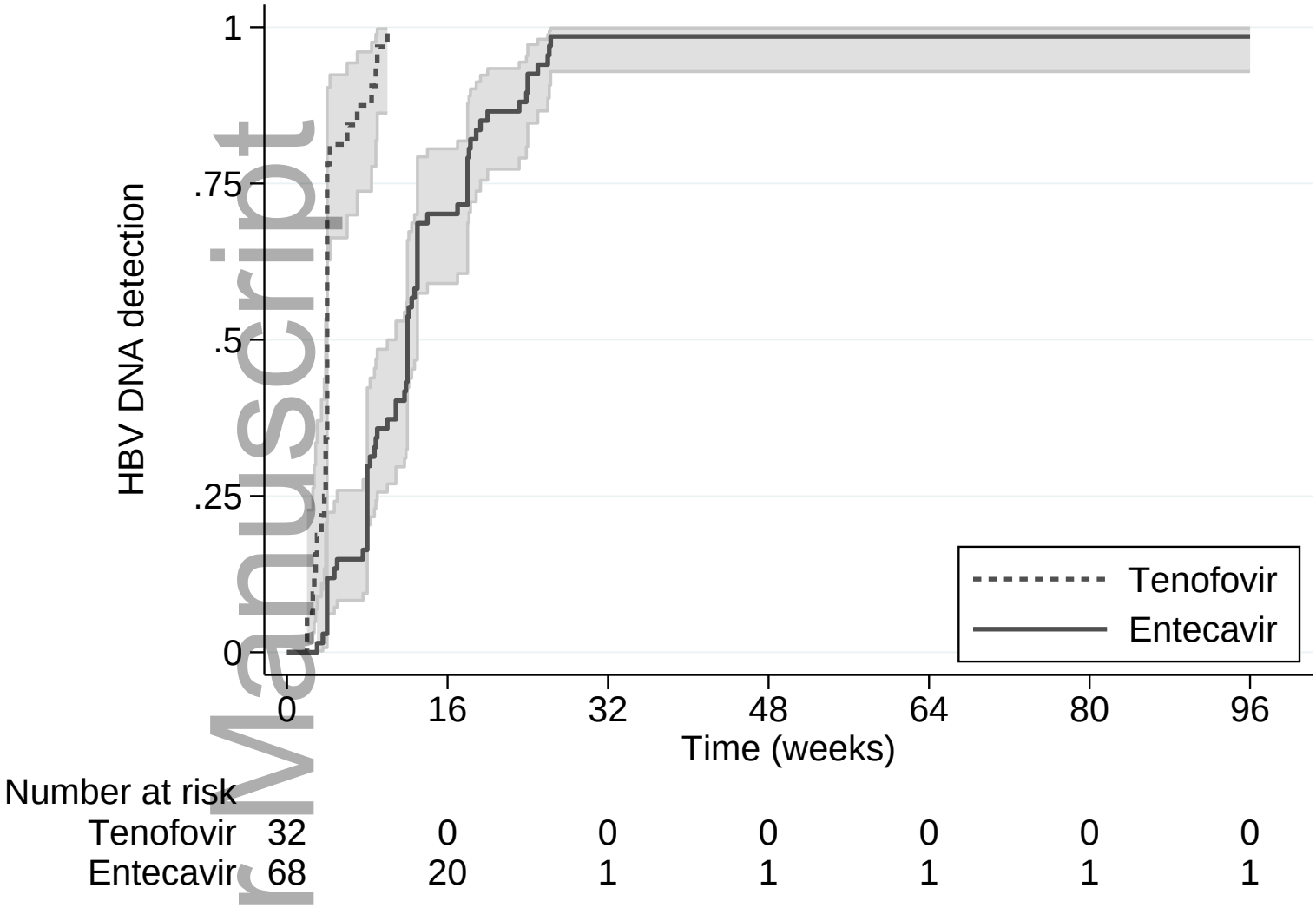

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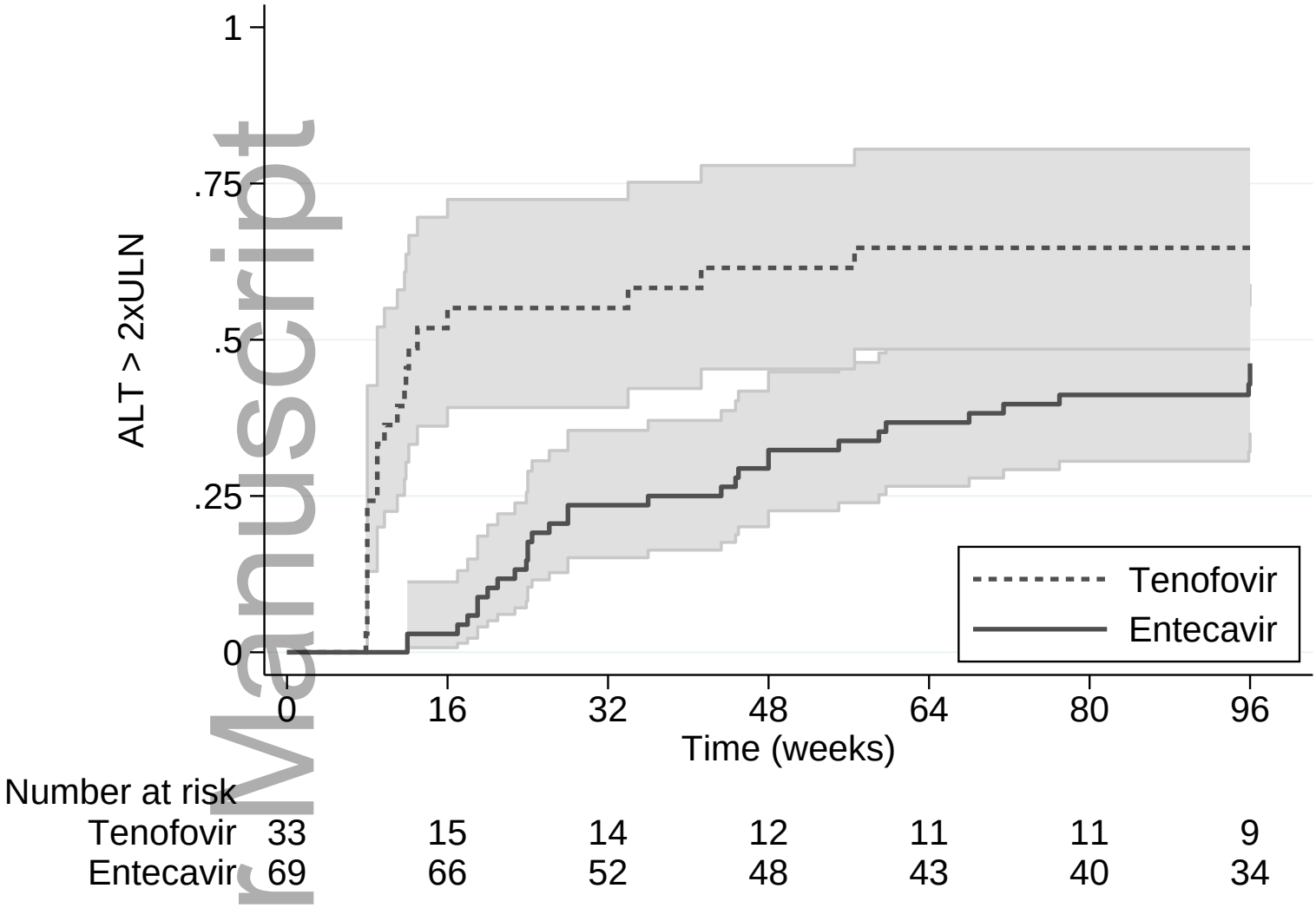

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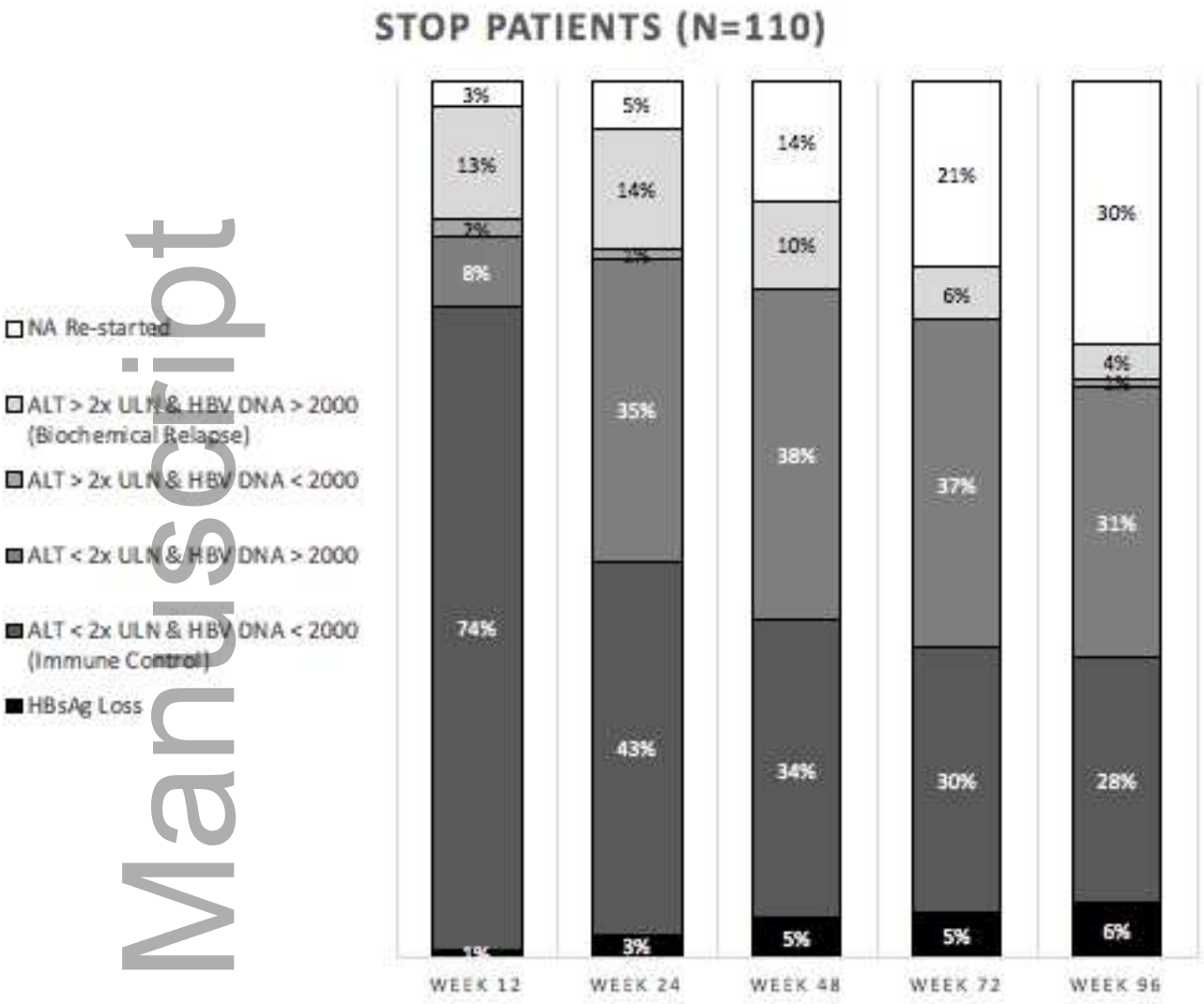

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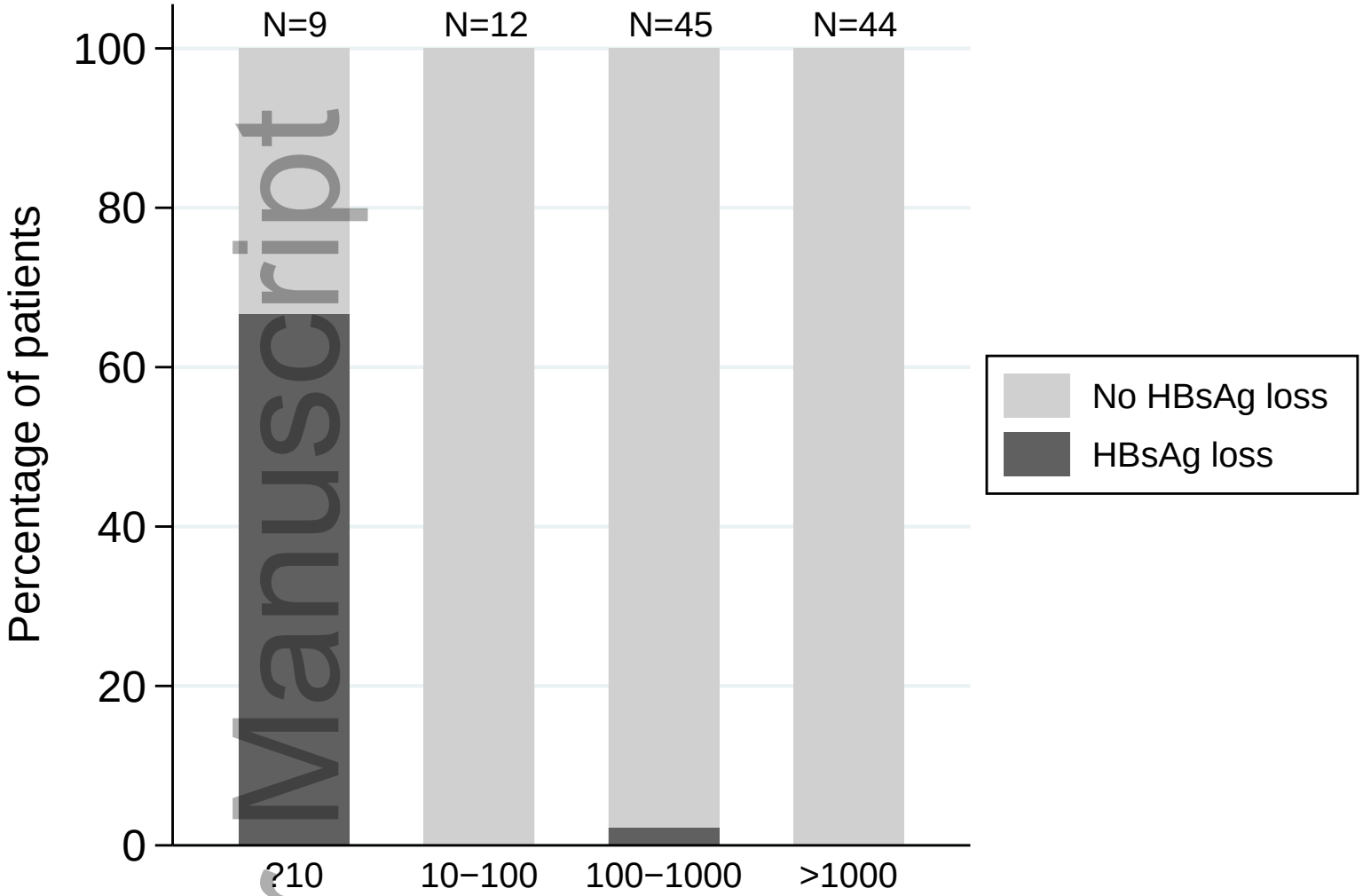

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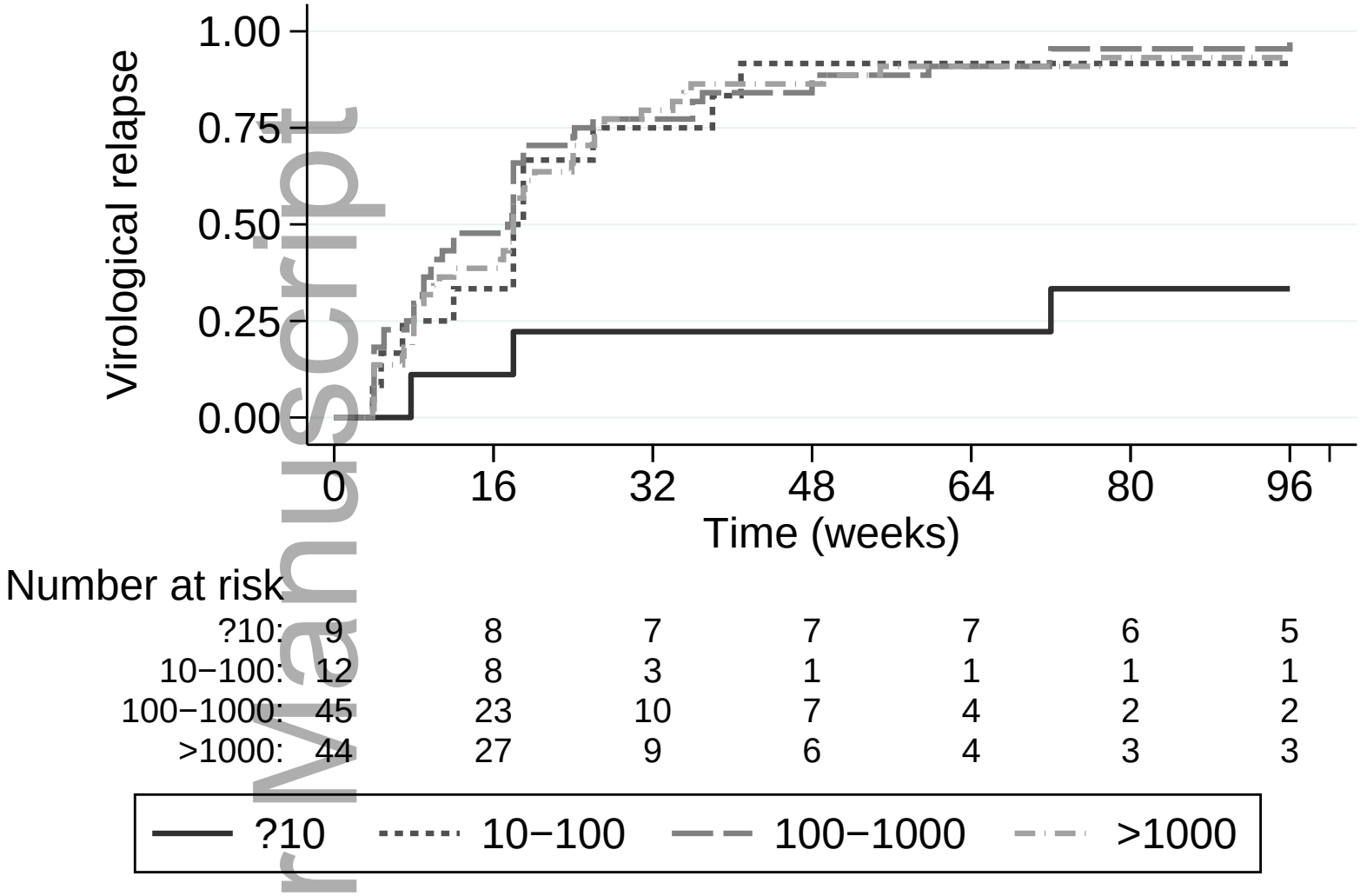

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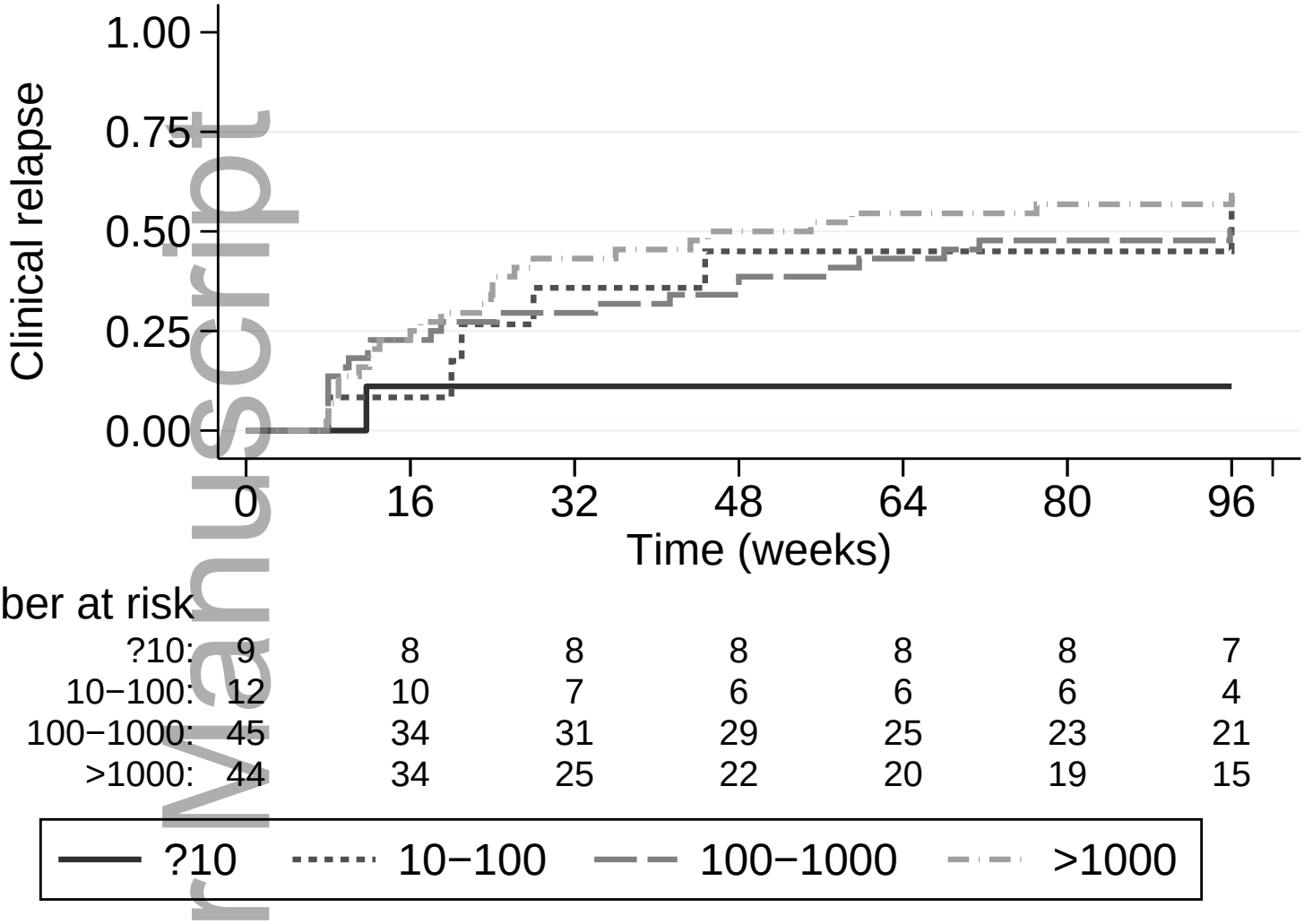

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