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► To cite this version:

Issam Tout, Pietro Lampertico, Thomas Berg, Tarik Asselah. Perspectives on stopping nucleos(t)ide analogues therapy in patients with chronic hepatitis B. *Antiviral Research*, 2021, 185, pp.104992 -. 10.1016/j.antiviral.2020.104992 . hal-03493469

HAL Id: hal-03493469

<https://hal.science/hal-03493469>

Submitted on 15 Dec 2022

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1 **Views on stopping nucleos(t)ide analogue therapy**
2 **in patients with chronic hepatitis B**

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15 **Abstract**

16 Long-term treatment with nucleos(t)ide analogs (NAs) is the current first line therapy
17 for patients with chronic hepatitis B (CHB), recommended by most of the current
18 guidelines. NAs prevent disease progression, liver failure, decrease the risk of
19 hepatocellular carcinoma (HCC), and have favorable safety profiles. However, low
20 rates of on-therapy functional cure (hepatitis B surface antigen [HBsAg] loss), which
21 is regarded as the optimal end point, prevent many patients from stopping NA
22 therapy with the need for a lifelong treatment. The higher likelihood of HBsAg loss
23 associated with stopping as compared to continuing NAs has got a lot of attention
24 lately. Recommendations regarding endpoints allowing for safely stopping NA
25 therapy differ between the different international guidelines. Whereas in HBeAg-
26 positive patients, HBeAg seroconversion with at least one year of consolidation
27 therapy is an accepted endpoint of treatment, the recommendations for HBeAg-
28 negative ones differ. Some guidelines propose ≥ 3 years of HBV DNA undetectability
29 to stop NA while others regard HBsAg loss as the only acceptable endpoint to
30 therapy. Stopping NA can lead to substantial rates of virologic relapses and
31 consequent ALT flares in some of them. Moreover, no reliable predictor(s) of post-NA
32 relapses have been identified so far. Quantitative HBsAg is becoming an increasingly
33 promising marker to predict safe NA cessation. On the other hand, investigating the
34 role of the immune system in mediating sustained virologic responses after NA
35 withdrawal is needed to suggest immunological biomarkers to safely stop NA. In this
36 article, we will review relevant literature regarding NA stopping rules and discuss
37 promising viral and immunological biomarkers to predict antiviral responses and thus
38 to help identify patients who are more likely to achieve HBsAg seroclearance.

39

40 **Highlights**

- 41 • NA therapy can effectively reduce HBV load but cannot prevent the risk of
42 rebound after treatment interruption due to the presence of cccDNA and
43 integrated HBV sequences in infected hepatocytes.
- 44 • NA cessation in HBeAg-positive non-cirrhotic patients is considered after
45 HBeAg seroconversion and consolidation treatment.
- 46 • NA cessation in HBeAg-negative patients has become an interesting approach
47 to reach functional cure.
- 48 • Better definition of patients with a high chance to achieve HBsAg loss after
49 stopping NAs is needed.
- 50 • Retreatment criteria and monitoring frequencies should be standardized.
- 51 • End of treatment HBsAg level is a promising predictor of outcome for safe NA
52 discontinuation.
- 53 • There is an urgent need to investigate immunological predictors of safe NA
54 cessation, such as HBV-specific CD8⁺ T cells.

55 **Keywords**

56 Quantitative HBsAg, relapse, immune control, NA discontinuation, HBV infection.

Abbreviations

AASLD, American Association for the Study of Liver Diseases; ADV, adefovir dipivoxil; AHB, Acute hepatitis B; ALT, alanine aminotransferase; anti-HBc, Hepatitis B core antibody; anti-HBe, Hepatitis B e antibody; anti-HBs, hepatitis B surface antigen antibody; APASL, Asian Pacific Association for the Study of the Liver; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CTL, Cytotoxic T cell; CXCL-10, C-X-C motif

chemokine 10; DC, Dendritic cells; DNA, Desoxyribonucleic acid; EASL, European Association for the Study of the Liver; ETV, entecavir; FASLG, Fas Ligand Gene; HBcAg, Hepatitis B core antigen; HBcrAg, Hepatitis B core-related antigen; HBeAg, Hepatitis B e antigen; HBsAg, Hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFN γ , interferon gamma; IL, Interleukin; LAM, Lamivudine; NA, nucleos(t)ide analogue; NK cells, Natural Killer cells; PD-1, Programmed cell death protein 1; PEG-IFN, pegylated interferon; snp, single nucleotide polymorphism; TAF, tenofovir alafenamide; T-bet; TBV, Telbivudine; TDF, tenofovir disoproxil fumarate; TFV, tenofovir; TGF- β , Tumor growth factor beta; Tim-3, T-cell immunoglobulin and mucin-domain containing-3; TLR, toll like receptor; TNF α , Tumor necrosis factor alpha; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; WHO, World Health Organization.

Electronic word count: 7897 words.

Number of figures and tables: 5 (3 tables and 2 figures).

Declaration of interests

Tarik Asselah has acted as a speaker and investigator for Janssen, Gilead, Roche, and Merck, MYR Pharmaceuticals.

Pietro Lampertico : speaker bureau and/or advisory board: AbbVie, Alnylam, Arrowhead, Bristol-Myers Squibb, Eiger BioPharmaceuticals, Gilead Sciences, GSK, Janssen, MSD, MYR Pharmaceuticals, Roche, and Spring Bank.

57 Thomas Berg has received research support from AbbVie, Bristol-Myers Squibb, Gilead,
58 Intercept, Janssen, Merck/MSD, Novartis, Pfizer, Roche and Sequana Medical; and provided
59 consultancy, and participated in speakers bureaux and advisory boards for AbbVie, Alexion,
60 Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Gilead, GlaxoSmithKline, Intercept,
61 Janssen, Merck/MSD, Merz, Novartis, Sequana Medical and Roche.

Issam Tout declares no competing interests.

Financial support statement: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

64 **1. Introduction**

65 Hepatitis B virus (HBV) infection remains a major global health concern as over 257
66 million individuals are estimated to be carriers of hepatitis B 'surface' antigen
67 (HBsAg), the hallmark of chronic HBV infection (CHB)(European Association for the
68 Study of the Liver, 2017; World Health Organization et al., 2017). CHB has potential
69 adverse outcomes, including cirrhosis, hepatic decompensation, and hepatocellular
70 carcinoma (HCC)(European Association for the Study of the Liver. 2017; Sarin et al.,
71 2016; Terrault et al., 2018). In 2015, hepatitis B resulted in an estimated 887 000
72 deaths from these liver diseases according to the World Health organization
73 (WHO)(World Health Organization et al., 2017). HBsAg correlates with the presence
74 of covalently closed circular DNA (cccDNA), in Hepatitis B 'e' antigen (HBeAg)-
75 positive CHB patients, and is a key marker of infection(Tout et al., 2020). Its
76 clearance is a reasonable therapeutic end point and is also referred to as a functional
77 cure of CHB(Martinot-Peignoux et al., 2014). Although nucleos(t)ide analogues (NA)
78 and immunomodulators (pegylated interferon (PEG-IFN)) are recommended
79 therapies, most patients take NA due to the easy administration, safety, and
80 efficacy(Cornberg et al., 2020). These therapies reduce viral replication and their
81 aims include improving quality of life and decreasing the risk of liver disease-related
82 complications(Marcellin et al., 2019; Papatheodoridis et al., 2018c) but however they
83 rarely lead to HBsAg seroclearance. Long term NA therapy raises some concerns
84 such as adherence, side effects, and, particularly, costs (Lampertico and Berg, 2018;
85 Papatheodoridis et al., 2016). Additionally, a significant proportion of patients may
86 want to discontinue therapy for various reasons. Thus, defining rules to stop NA
87 therapy is of paramount importance and safety without loss of efficacy must be

88 considered(Lampertico and Berg, 2018). The concept of NA discontinuation was first
89 mentioned in 2000 APASL guidelines and the first stopping rule for HBeAg-negative
90 CHB was included in 2008 APASL guidelines, driven by cost and drug-resistance
91 issues (Liaw et al., 2008). These recommendations were based on several earlier
92 studies in Asian patients treated with lamivudine (LAM) showing that the sustained
93 response rate 6-12 months after cessation of LAM therapy was around 50% if
94 patients had achieved a maintained virological response before stopping LAM
95 therapy (Chan et al., 2007; Chien and Liaw, 2006; Fung et al., 2004). This NA
96 cessation concept was later initiated in Western countries with the study by
97 Hadziyannis et al. showing a remarkably high HBsAg loss rate when stopping
98 NAs(Hadziyannis et al., 2012).

99 In this review, we discuss current international guidelines for stopping NA therapy
100 and we evaluate data of HBsAg loss in different categories of CHB patients.
101 Moreover, potential virologic and immunologic predictors of NA cessation may also
102 be considered to precisely determine the outcome of HBV infection, and identify
103 patients who are more likely to achieve HBsAg seroclearance or a sustained
104 response.

105 **2. Goals and outcomes of NA therapy**

106 The goal of therapy for CHB is to improve survival by preventing disease progression
107 and improving quality of life. This is best achieved by long-term NA therapy leading to
108 HBV suppression, associated with a reduction in histological activity, a lower risk of
109 cirrhosis, and reversal of fibrosis(European Association for the Study of the Liver.
110 2017; Marcellin et al., 2019; Marcellin and Asselah, 2013; Sarin et al., 2016; Terrault
111 et al., 2018). NA mechanism of action consists of viral polymerase inhibition, which

112 leads to decreased virion assembly, and thus a reduction of the viral replication and
113 load (Asselah et al., 2019). According to the European Association for the Study of
114 the Liver (EASL)(European Association for the Study of the Liver. 2017) and the
115 American Association for the Study of Liver Diseases (AASLD)(Terrault et al., 2018),
116 the currently approved and first-line recommended NA drugs are tenofovir disoproxil
117 fumarate (TDF), tenofovir alafenamide (TAF), and entecavir (ETV) due to their high
118 barrier to HBV resistance, their high effectiveness, and their well tolerance. Other
119 NAs such as Lamivudine (LAM), Telbivudine (TBV) and adefovir dipivoxil (ADV)
120 should not be used as first-line treatment options due to their low barrier to HBV
121 resistance causing resistance strains and insufficient virologic response. The main
122 advantage of NA treatment is its predictable high long-term antiviral efficacy leading
123 to undetectable HBV DNA levels in the vast majority of compliant patients as well as
124 its favorable safety profile(European Association for the Study of the Liver. 2017; Lok
125 et al., 2016; Terrault et al., 2018). Over 8 years (Papatheodoridis et al., 2018c) and
126 10 years(Marcellin et al., 2019), NAs have a favorable safety profile, are well
127 tolerated, and resulted in continued maintenance of virologic suppression.

128 The ideal endpoint of therapy in HBeAg-positive and HBeAg-negative patients is
129 sustained HBsAg loss with or without seroconversion to anti-HBs, which is usually
130 associated with complete and definitive remission of the activity of CHB (Kim et al.,
131 2014). However, these endpoints are rarely achieved because of the presence of the
132 covalently closed circular DNA (cccDNA) minichromosome, which remains
133 untouched in the nuclei of infected hepatocytes as well as the presence of integrated
134 HBV sequences in hepatocytes genome which can cause reactivation of the disease
135 and relapse of viraemia. Since these goals are rarely reached, surrogate endpoints
136 have been identified to assess efficacy, including improved liver inflammation

137 (biochemical remission), viral suppression (virologic remission) and HBeAg
138 seroconversion (European Association for the Study of the Liver. 2017). Achievement
139 of these objectives has been shown to stop the inflammation cascade and fibrosis
140 progression (Marcellin et al., 2013). NA treatment rarely achieve a functional cure
141 and necessitate lifelong therapy in addition to other concerns such as adherence
142 (Ford et al., 2018), side effects (Fontana, 2009; Marcellin et al., 2019), and,
143 particularly, costs (Aiello et al., 2016). Therefore, the concept of NA treatment
144 cessation is an interesting strategy to consider and further exploration of the
145 recommended rules of stopping NAs before a functional cure and the benefits gained
146 is needed. It is rational to discontinue any therapy if it is of no further benefit to the
147 patient (Hadziyannis and Hadziyannis, 2020).

148 **3. Aim of NA therapy cessation strategy**

149 Over the last years, there has been a growing body of evidence that assessed the
150 potential benefits, the safety, and the efficacy of stopping long-term NA therapy.
151 One of the first pivotal studies regarding the NA cessation approach was a small,
152 prospective cohort study from Greece that assessed the biochemical and virologic
153 effects of stopping long-term NA therapy in HBeAg-negative CHB patients
154 (Hadziyannis et al., 2012). After being treated for 4–5 years, all 33 patients
155 developed virologic relapse once ADV was stopped, 76% had biochemical relapse,
156 and 45% were retreated. Interestingly, among those with sustained biochemical and
157 virologic remission, 39% reached HBsAg loss during 5.5 years of follow-up
158 (Hadziyannis et al., 2012). This is a remarkable finding compared to the very low
159 HBsAg clearance rate during NA therapy. Quantitative HBsAg (qHBsAg) at the time
160 of stopping therapy was < 1000 IU/mL which suggested that monitoring of HBsAg
161 level can guide the timing of stopping NAs. Since then, a series of studies attempted

162 to determine the occurrence of HBsAg loss after stopping NA therapy. Chan et al.
163 reported that 20% of patients achieved HBsAg seroclearance after stopping LAM
164 therapy (Chan et al., 2011). The main predictor of HBsAg seroclearance was end of
165 therapy qHBsAg levels $< 2 \log \text{ IU/mL}$ and $> 1 \log$ reduction from baseline. A
166 randomized study (the FINITE study)(Berg et al., 2017) compared standard TDF
167 therapy continuation (n=21) against its interruption (n=21) in HBeAg-negative
168 patients that had been under NAs for at least 3,5 years. Thirteen out of 21 patients in
169 the NA stopping group remained off-therapy and 4 of them even achieved HBsAg
170 seroclearance after 3 years of follow-up. Those who stopped therapy had greater
171 reduction in qHBsAg compared to those who continued therapy (0.283 vs 0.088 log
172 reduction respectively)(Berg et al., 2017).
173 To evaluate stopping NAs, we must consider what are the benefits of their
174 discontinuation. Since long-term NA therapy can already achieve viral suppression
175 and limit the risk of liver-related diseases, the withdrawal of the treatment should aim
176 to have similar or even better outcomes than treatment continuation. HBsAg loss,
177 indicating immune control with sustained virologic remission (Tout et al., 2020),
178 remains the best indicator of long-term favorable outcomes and therefore of NA
179 cessation. Relapse must also be considered, which occurs frequently off-treatment
180 and may lead to clinically significant flares. Determining precise predictors of relapse
181 or sustained remission is crucial to identify which patients should not stop therapy.

182

183 **3.1 HBV guidelines and recommendations on stopping NA therapy**

184 Three major international scientific guidelines, AASLD, EASL and the Asian Pacific
185 Association for the Study of the Liver (APASL) have proposed stopping rules for

186 different categories of CHB patients under NA(European Association for the Study of
187 the Liver. 2017; Sarin et al., 2016; Terrault et al., 2018) (Table 1). However, there is
188 no clear consensus regarding when and how to stop the administration of NAs
189 without compromising optimal management in the majority of CHB patients. The ideal
190 endpoint for all subgroups of patients is considered to be the sustained loss of
191 HBsAg, with or without subsequent seroconversion to anti-HBs (European
192 Association for the Study of the Liver. 2017; Sarin et al., 2016; Terrault et al., 2018).
193 Therefore, major guidelines recommend that all CHB patients, including those with
194 compensated cirrhosis, can stop NA therapy if they achieve HBsAg loss that persists
195 for at least 1 year, even if this goal is rarely achieved. Alternatively, surrogate
196 endpoints have been determined to safely discontinue the treatment. In non-cirrhotic
197 HBeAg-positive CHB patients, HBeAg seroconversion is a reliable indication of
198 sustained off-NA response, when it is accompanied by inhibition of HBV replication
199 and persistently normal ALT levels. Thus, all three guidelines recommend that these
200 patients can discontinue therapy if they achieve HBeAg seroconversion with HBV
201 DNA undetectability and normal ALT levels, and after they complete at least 12
202 months (up to 3 years according to the APASL) of consolidation therapy.

203 For non-cirrhotic HBeAg-negative CHB patients, APASL guidelines have suggested
204 that NAs can be discontinued after at least 2 years of therapy if serum HBV DNA
205 remains undetectable on 3 separate occasions 6 months apart, which could be
206 mainly driven by cost constraints. Additionally, they also suggest NA withdrawal in
207 the case of HBsAg loss with at least 12 months of a post-HBsAg loss consolidation
208 period even if there was no HBsAg seroconversion (Sarin et al., 2016). Interestingly,
209 the EASL recommendations also suggested that NA discontinuation may be
210 considered in selected non-cirrhotic patients with long-term (≥ 3 years) on-therapy

211 virologic suppression if close post-treatment monitoring is guaranteed for at least 1
212 year (European Association for the Study of the Liver. 2017). AASLD guidelines
213 generally do not recommend NA discontinuation for these patients except in the case
214 of HBsAg seroclearance, but however included a recommendation of NA
215 discontinuation as an option if there is a compelling rationale and under the condition
216 of careful monitoring every 3 months for at least 1 year (Terrault et al., 2016). APASL
217 are the only guideline who consider possible stopping of NA therapy, before HBsAg
218 seroclearance, in patients with compensated cirrhosis, in condition of a careful off-
219 therapy monitoring plan, while both EASL and AASLD recommend indefinite NA
220 therapy in cirrhotic CHB patients who remain positive for HBsAg.

221

222 **3.2 NA cessation in HBeAg-positive CHB patients before HBsAg** 223 **loss**

224 The main guidelines suggest that pretreatment HBeAg-positive patients, without
225 cirrhosis, can stop NA therapy after reaching HBeAg seroconversion with
226 undetectable HBV DNA and completing 1 to 3 years of consolidation therapy. Several
227 reports have evaluated the durability of HBeAg seroconversion with LAM therapy,
228 resulting in a wide range of durable HBeAg seroconversion rates. Song et al.
229 evaluated patients who underwent HBeAg seroconversion with short duration LAM
230 consolidation therapy (2 to 4 months) and observed a relapse with elevated
231 transaminases and reappearance of HBeAg in 49% of patients at 2 years post-
232 treatment (Song et al., 2000). Another study evaluated the durability of serological
233 responses in 40 HBeAg-positive patients who were treated by LAM (Dienstag et al.,
234 2003). Most HBeAg responses achieved during LAM therapy were durable, and most
235 responders experienced prolonged clinical benefit after HBeAg seroconversion and

236 subsequent discontinuation of LAM. The relapse rate after HBeAg seroconversion
237 was only 27% with a median period of 3-year follow up (Dienstag et al., 2003).
238 Similarly, a study from Taiwan showed that a duration of LAM consolidation therapy >
239 48 weeks may be favorable for maintaining durable HBeAg seroconversion (Kuo et
240 al., 2010). Then later, Lee et al. showed that consolidation therapy for more than 1
241 year after HBeAg seroconversion helped maintain the serological response (Lee et
242 al., 2010). A prospective study of 82 patients demonstrated a cumulating relapse rate
243 of 23.4% at 6 months and 29.4% at 4 years after discontinuation of LAM (Wang et al.,
244 2010), and a second study observed a 3-year cumulative relapse rate of 34.4% in 42
245 patients (Jin et al., 2012). Recently, a study from Belgium aimed to determine relapse
246 rates and clinical outcomes in a predominantly Caucasian cohort of 98 CHB patients
247 with NA-induced HBeAg seroconversion (Van Hees et al., 2018). Viral relapses were
248 observed in more than half of patients who discontinued treatment with potential fatal
249 outcomes while none of the patients who continued therapy after HBeAg
250 seroconversion relapsed or developed severe hepatic outcomes (Van Hees et al.,
251 2018). Kang et al. assessed the antiviral response in 157 patients who had
252 discontinued LAM for a mean 72 months of follow-up. The cumulative probabilities of
253 virologic relapse at 1, 24, 48, 96, and 120 months were 10.3%, 55.6%, 62.8%,
254 67.0%, and 67.0%, respectively (Kang et al., 2017). However, most of these studies
255 were long-term retrospective studies that did not correct for selection or
256 measurement bias (Table 2). Therefore, the evidence on NA withdrawal in HBeAg-
257 positive CHB patients is lacking and needs to be based on better designed studies.

258 Among the prospective studies, Jung et al. evaluated virologic relapse in 45 HBeAg-
259 positive CHB patients who were treated with LAM or ETV. Within 1 year or NA
260 treatment, viral relapse occurred in 57,8 % of the patients (Jung et al., 2016). In

261 several studies (Chaung et al., 2012; Pan et al., 2013; Reijnders et al., 2010; Sohn et
262 al., 2014) with various NAs, the off-treatment response was not durable despite
263 consolidation therapy after the HBeAg seroconversion. In the largest prospective
264 study with 10-year follow up, 31% of 138 HBeAg-positive CHB patients experienced
265 virologic relapse, almost all of whom (76%) developed HBeAg seroreversion,
266 attesting that this was not durable at the time of stopping therapy (Liu et al., 2018). In
267 a randomized controlled trial from Toronto, 61% of pretreatment HBeAg-positive
268 patients required retreatment compared with 22% of HBeAg-negative patients (Liem
269 et al., 2019).

270 A systematic review by Papatheodoridis et al., including 25 studies with 1716 patients
271 who discontinued NA and were followed up for > 12 months shows that 51% of
272 HBeAg-positive CHB patients had durable virologic remission at 3 years post-
273 treatment and 76% of them had durable biochemical remission (Papatheodoridis et
274 al., 2016). These rates were higher than in HBeAg-negative CHB patients. Sustained
275 HBeAg seroconversion was found in 95%, 92% and 88% of patients at 6, 12 and 24
276 months, respectively, following discontinuation of NA therapy (Papatheodoridis et al.,
277 2016). Collectively, these data suggest that the risk of relapse requiring retreatment
278 is relatively high in pretreatment HBeAg-positive patients, even with long
279 consolidation therapy after HBeAg loss. Due to the heterogeneity of the studies and
280 their design, it is, therefore, difficult to confirm the advantage of NA discontinuation in
281 pre-treatment HBeAg-positive CHB patients who undergo HBeAg seroconversion.
282 Another factor leading to clear discrepancies between studies is the variable relapse
283 definition (Papatheodoridis et al., 2018a). Although prolongation of NA therapy in
284 HBeAg-positive patients is aiming at functional cure under treatment, this is still a
285 rare event, and HBeAg seroconversion is the currently accepted endpoint despite

286 reported data of high relapse rates. Determining the appropriate duration for
287 consolidation therapy is important to achieve sustained remission in those patients.
288 Thus, there is an urgent need to specifically examine the effect of the duration of
289 consolidation therapy on sustained HBeAg seroconversion in future well designed
290 studies.

291

292 **3.3 NA cessation in HBeAg-negative CHB patients before HBsAg** 293 **loss**

294 Current interest and debates in stopping NA are largely related to finite NA therapy in
295 HBeAg-negative CHB patients. The guidelines recommend that NA therapy in
296 HBeAg-negative CHB patients, without cirrhosis, can be stopped after ≥ 3 years of
297 virologic suppression (EASL 2017)(European Association for the Study of the Liver.
298 2017), after treatment for at least 2 years with undetectable HBV DNA documented
299 on three separate occasions, 6 months apart (APASL 2015)(Sarin et al., 2016), or
300 only after achieving HBsAg loss, except if there is a compelling rationale and under
301 the condition of careful monitoring every 3 months for at least 1 year (AASLD
302 2018)(Terrault et al., 2018). The previously mentioned study by Hadziyannis et al.
303 was one of the first papers that presented data favoring NA withdrawal in HBeAg-
304 negative CHB patients (Hadziyannis et al., 2012). It showed that stopping adefovir
305 therapy in HBeAg negative patients was associated with a 39% HBsAg
306 seroclearance rate. This high HBsAg seroclearance rate was not observed in
307 subsequent studies probably due to shorter follow-up duration or even the ethnicity of
308 the studied cohort. Additionally, whether the initial increase in HBV DNA, after NA
309 withdrawal, settles down to quiescent disease or severe flares varies widely between
310 different studies and patients and is hard to predict. Some studies defining viral

311 relapse after treatment cessation as HBV DNA >60 IU/mL (Shouval et al., 2009; Sohn
312 et al., 2014), HBV DNA >70 copies/mL (Jung et al., 2011), or HBV DNA >200 IU/mL
313 (Chan et al., 2011), found a very high percentage of HBeAg-negative CHB patients
314 going through viral relapse after NA cessation. Other studies have used levels of
315 HBV DNA >2000 IU/mL to define relapse. A prospective study of 184 Asian patients
316 taking ETV for at least 24 months and using the APASL criteria to guide treatment
317 cessation (treatment for at least 2 years with undetectable HBV DNA on 3 separate
318 occasions, 6 months apart) had a cumulative rate of virologic relapse 74.2% and
319 91.4% at 6 and 12 months post-treatment respectively (Seto et al., 2015). In two
320 studies by Jeng W-J et al. on Asian patients treated with ETV (Jeng et al., 2013) or
321 TDF (Jeng et al., 2016), 46% and 52% relapsed respectively. In another study of 45
322 patients of >3years follow-up, LAM, ETV or ADV therapy, the relapse rate was 49%
323 and 73% at 6 and 12 months (Kim et al., 2013). A recent study on 234 HBeAg-
324 negative CHB patients who received ETV and 119 receiving TDF showed that HBV
325 relapse occurs sooner and is more severe after cessation of TDF than after cessation
326 of ETV (Kuo et al., 2019). These results confirmed earlier reports that showed that
327 discontinuation of TDF rather than ETV was associated with earlier relapse (Höner
328 Zu Siederdisen et al., 2018; Jeng et al., 2016; Su et al., 2018). Off-ETV mostly
329 occurred after 6 months vs within 6 month off-TDF (Jeng et al., 2016). These studies
330 clearly show the difference in relapse timing between ETV and TDF. The reason
331 behind this obvious difference in timing needs to be investigated in future studies,
332 perhaps having to do with pharmacokinetics of different NAs or different interactions
333 with immune responses.

334 In HBeAg-negative CHB patients most of the virologic relapse occurs within the first
335 3-9 months after treatment discontinuation (Fung et al., 2004; He et al., 2013).

336 Studies with longer follow-up confirm possible sustained off-treatment response and
337 HBsAg loss in HBeAg-negative CHB patients (Table 3). The daring-B study followed
338 57 patients who discontinued therapy after median virologic remission of 5,3 years
339 and remained under a median of 18 months of follow-up(Papatheodoridis et al.,
340 2018b). For a cutoff of HBV DNA > 2,000 IU/ml, the cumulative virologic relapse
341 rates were 56%, 70% and 72% at 3, 12 and 18 months after therapy. The probability
342 of relapse decreased after month 6.

343 In a systematic review on NA discontinuation, 2% HBsAg loss was observed
344 (Papatheodoridis et al., 2016). The pooled rates of sustained virologic remission at
345 12, 24, 36 months in HBeAg-negative patients were 43.7%, 31.3% and 30.1%
346 respectively (Papatheodoridis et al., 2016). In another systematic review of 22
347 studies including 1732 HBeAg-negative patients, the 1 year off-therapy virologic
348 relapse was < 70%, and clinical relapse (HBV DNA >2000 IU/mL and ALT elevation)
349 occurred in < 50% of patients and <40% required retreatment (M.-L. Chang et al.,
350 2015). Thus, these two recent systematic reviews show that stopping NA is relatively
351 safe with a careful off-therapy monitoring plan. Concerning the safety of NA
352 discontinuation, they suggest that significant complications such as severe flares and
353 decompensation were relatively infrequent and occurred in 0,8% of patients with
354 cirrhosis, while jaundice developed in 2,5%, with 1 death from liver failure (M.-L.
355 Chang et al., 2015; Papatheodoridis et al., 2016). Conversely, a study from Toronto,
356 with majorly Asian patients, found that only 33% of pretreatment HBeAg-negative
357 patients had a sustained off-treatment response, 72 weeks after NA withdrawal (Liem
358 et al., 2019). Although the retreatment criteria in this study was not stringent which
359 allowed to better evaluate the off-NA viral and biochemical kinetics during close-visit
360 monitoring, there were only 27 HBeAg-negative CHB patients which limits the

361 significance of such results. In another recent study of 106 HBeAg-negative patients
362 who stopped TDF therapy (Buti et al., 2019), almost a third of patients experienced a
363 grade 3 or higher laboratory abnormality (ALT elevations) and serious adverse
364 events occurred in 4% of patients. 35% of patients had both HBV DNA
365 concentrations of less than 2000 IU/mL and ALT concentrations less than the ULN at
366 TFFU week 24(Buti et al., 2019). These notable differences between studies may
367 originate from following different guidelines thereby limiting their scope by design,
368 different definitions of virologic relapse, differences in outcome definitions, or
369 heterogeneity of cohorts. Most of the studies, on which the guidelines are based on,
370 consist of Asian cohorts with genotype B and C, leading to a heterogeneity compared
371 to Caucasian patients with a prominent genotype D. Finally, a recent multicenter
372 study including 276 patients from 16 hospitals in Korea, aimed to determine whether
373 NA discontinuation was as safe as NA continuation after NA-induced HBsAg
374 seroclearance. The authors found that discontinuation of NA was not associated with
375 a higher risk of either HBsAg reversion, serum HBV DNA redetection or HCC
376 development compared with NA continuation (Kim et al., 2020).

377

378 **3.4 Stopping NA in patients with cirrhosis before HBsAg loss**

379 Patients with cirrhosis are at risk of developing liver decompensation and stopping
380 therapy in this subgroup is controversial due to the risk of liver failure and ascites
381 (Wen-Juei Jeng et al., 2018). While the EASL and the AASLD guidelines(European
382 Association for the Study of the Liver, 2017; Terrault et al., 2018) do not recommend
383 stopping NA therapy, before HBsAg loss, in patients with cirrhosis, under any
384 circumstance, the APASL guidelines(Sarin et al., 2016) may consider it in

385 compensated cirrhosis with a careful monitoring plan. Therefore, data evaluating NA
386 discontinuation, before HBsAg loss, in patients with pretreatment cirrhosis, come
387 mainly from Asian studies (Jeng et al., 2013; Kuo et al., 2010). In a large study from
388 Taiwan, the cumulative probability of liver decompensation was 3% over a > 5 years
389 follow-up and only 1% of patients died (Wen-Juei Jeng et al., 2018). Chen et al.
390 analyzed 586 HBeAg-negative CHB patients with compensated cirrhosis and 205
391 patients, among them, who stopped ETV based on APASL 2008 guidelines (Chen et
392 al., 2015). These patients were matched by propensity scoring to patients who
393 continued treatment and no significant difference was found in liver related
394 complications/deaths between the two groups after a median follow-up of 59 months
395 (Chen et al., 2015). Another study found, similarly, no statistical significance between
396 patients with and without cirrhosis who stopped LAM therapy (Ming-Ling Chang et al.,
397 2015). Jeng et al. assessed 39 cirrhotic patients receiving TDF and found that
398 relapse occurred in 39.5%. Two patients developed hepatic decompensation(Jeng et
399 al., 2016). In the study by Kuo et al., jaundice occurred in 8,5% of patients all of
400 whom had cirrhosis. Of these 7 patients, 6 improved after LAM retreatment and 1
401 patient with decompensated liver disease died 1 week later(Kuo et al., 2010).
402 Furthermore, the HCC risk decreases beyond year 5 of ETV/TDF therapy in
403 Caucasian CHB patients, particularly in those with compensated cirrhosis
404 (Papatheodoridis et al., 2017). The studies discussing the safety of NA
405 discontinuation in cirrhotic CHB patients are not frequent and are usually
406 retrospective, therefore less rigorous than well-executed randomized control trials
407 showing the benefit of continuing therapy in these patients (Liaw et al., 2004).
408 Therefore, there is a need to perform future studies on NA cessation in cirrhotic
409 patients to determine if there is a benefit of stopping the treatment especially since

410 this is a controversial issue between the different guidelines. Lifelong NA treatment
411 may be an unaffordable burden for healthcare systems in certain countries and thus,
412 the only benefit of stopping therapy in cirrhotic patients could be reducing the costs of
413 treatment.

414 **3.5 Rescue therapy in case of relapse**

415 Cessation of antiviral treatment with NA, before HBsAg loss, may lead to clinical
416 reactivation/relapse, flares, decompensation, overt liver failure and death. Therefore,
417 specific stringent criteria for retreatment are critical for the optimal management of
418 CHB patients who discontinue NA and help preventing significant clinical relapses
419 (Berg et al., 2017; Papatheodoridis et al., 2018a, 2018b). Close post-treatment
420 follow-up with, at least, ALT and HBV DNA determinations is mandatory, and strict
421 and predefined criteria for retreatment are necessary to avoid potential life-long
422 retreatment (Papatheodoridis et al., 2018a). However, the criteria for retreatment
423 have not been standardized resulting in great heterogeneity among different studies
424 (Papatheodoridis et al., 2016, 2018a). For patients with flares, delaying retreatment
425 can be detrimental, while starting retreatment too soon might counteract the potential
426 relapse-associated induction of a functional cure (W.-J. Jeng et al., 2018). Severe
427 hepatic flares require immediate retreatment and it is quite challenging to determine
428 the exact “kind” of flares occurring and base on that observation the decision to
429 retreat patients. Some patients can still be in an intermediate state, neither fulfilling
430 criteria for CHB nor for an inactive carrier state, and therefore require further follow-
431 up for a precise disease activity characterization and to decide whether retreatment
432 should be commenced (Wen-Juei Jeng et al., 2018; Lampertico and Berg, 2018).
433 Therefore, the retreatment decision is based on the severity of HBV flares and the
434 persistence (≥ 3 months) of liver disease activity, taking into consideration,

435 primordially, the patient's safety (Lampertico and Berg, 2018). Interestingly, HBsAg
436 kinetics during clinical relapse have been proposed to guide retreatment decision;
437 retreatment may be required in those with increasing HBsAg levels, whereas
438 retreatment might be unnecessary in those with decreasing HBsAg levels (Chien and
439 Liaw, 2020; Liaw, 2019; Liaw et al., 2018). This rescue therapy induces virologic and
440 biochemical remission in all non-cirrhotic CHB patients who developed HBV related
441 flares (Papatheodoridis et al., 2016). Concerning patients with persistently mild to
442 moderate liver disease activity, the decision of retreatment relies on persistent
443 elevation of HBV DNA and ALT levels for at least 6 months after stopping
444 therapy (European Association for the Study of the Liver, 2017; Terrault et al., 2018).
445 The DARING-B study shows that retreatment is also required in cases of modest ALT
446 elevations ($>3\times$ ULN) and high HBV DNA levels ($>100\ 000$ IU/mL) at the same visit
447 (Papatheodoridis et al., 2018b).

448 Interestingly, the study by Kuo et al., conducted in 342 CHB patients who stopped
449 ETV vs. patients who stopped TDF (Kuo et al., 2019) showed that the rate of off-
450 therapy HBsAg loss was comparable between the two groups after 2 to 3 years of
451 follow-up (Kuo et al., 2019). A large, retrospective cohort study from Taiwan (691
452 HBeAg-negative CHB patients who stopped treatment according to the APASL
453 stopping rule) revealed a 13% HBsAg loss at 6 years off therapy, much higher than
454 that during therapy (Wen-Juei Jeng et al., 2018). The DARING-B study followed 57
455 patients who discontinued therapy after median virologic remission of 5,3 years and
456 remained under a median of 18 months of follow-up (Papatheodoridis et al., 2018b).
457 Cumulative rates of HBsAg loss were 5%, 16% and 25% at 6, 12 and 18 months
458 being higher in patients with lower HBsAg levels at treatment discontinuation.
459 Another recent study found a 5% HBsAg loss among 106 HBeAg-negative patients

460 who stopped TDF therapy (Buti et al., 2019). However, the follow-up duration was 24
461 weeks and therefore is too short to reflect a sustainable HBsAg loss rate especially
462 among a subgroup of patients achieving a low-replicative state and therefore are
463 potential HBsAg loss candidates if they are followed up for a longer duration after NA
464 cessation(Buti et al., 2019).

465 As mentioned before in this section, the “retreatment” or “no retreatment” decision in
466 CHB patients with hepatic flares is governed by the outcomes of these, being
467 detrimental or beneficial to the patients. Nevertheless, it should be noted that the “no
468 retreatment” decision is considered to be a strong predictor of off-NA HBsAg
469 loss(Berg et al., 2017; Chen et al., 2019; Liaw, 2020; Liem et al., 2019). Therefore, a
470 proper assessment is crucial to better determine the type of HBV flares, which will
471 help with the retreatment/no retreatment decision.

472 Figure 1 presents a proposed algorithm for the follow-up of non-cirrhotic CHB
473 patients who stop NA therapy and indications for retreatment.

474 **4. Biomarkers to consider to safely stop NA therapy before HBsAg** 475 **loss**

476 The evaluation of the probability of post-NA remission has been quite challenging
477 due to the great heterogeneity among the studies which have variations in patient
478 populations, designs, definitions of relapse, post-NA management and duration of
479 follow-up (Papatheodoridis et al., 2016). Thus, the identification of reliable serologic,
480 virologic, or immunologic predictors of post-NA remission or relapse is quite
481 interesting and helps refining current rules of NA cessation. CHB outcomes rely on
482 equilibrium between the virus and the host, therefore different virologic and
483 immunological parameters could be considered as predictors of safe NA

484 discontinuation. In recent years, numerous studies have investigated several host
485 and viral factors such as patient characteristics including age (Bonino et al., 2007; Ha
486 et al., 2012; Marcellin et al., 2009) and gender (Bonino et al., 2007; Chen et al.,
487 2014), baseline clinical characteristics including HBV DNA (Huang et al., 2003; Jeng
488 et al., 2013), ALT levels (Bonino et al., 2007; Chien and Liaw, 2006; Marcellin et al.,
489 2009), HBsAg (Chen et al., 2018, 2014, 2014; Liang et al., 2011), treatment-related
490 factors including duration of consolidation therapy (Jeng et al., 2013; Jiang et al.,
491 2015; Kuo et al., 2010; Sohn et al., 2014), and virus-related factors, such as
492 genotype (Chien and Liaw, 2006; He et al., 2013) and viral mutations (Liang et al.,
493 2011; Ryu et al., 2003) have also been considered. However, these factors vary
494 between studies because of the various designs, populations, and patient pre-
495 treatment HBeAg status.

496 In HBeAg-positive CHB patients, the duration of consolidation therapy is the most
497 analyzed predictive factor. Most guidelines consider that prolonged additional
498 administration of NA after HBeAg seroconversion may enhance the durability of
499 sustained response after treatment cessation and most of them consider that a 12-
500 month period offers a more durable sustained response (European Association for
501 the Study of the Liver. 2017; Pan et al., 2013; Ryu et al., 2003; Sarin et al., 2016;
502 Terrault et al., 2018; Wang et al., 2010). A study on LAM cessation showed that
503 consolidation therapy for more than 1 year after HBeAg seroconversion helped
504 maintain the response (Lee et al., 2010). Song et al. showed that high pre-treatment
505 HBV DNA levels were predictive for relapse and that pre-treatment HBV DNA and the
506 duration of consolidation treatment were independent important risk factors for
507 relapse (Song et al., 2000). There have been no meta-analyses examining the effect
508 of the duration of consolidation therapy on sustained HBeAg seroconversion and

509 there is a need to further explore this aspect. On the other hand, studies showed
510 that the combination of young age and prolonged consolidation treatment gives lower
511 relapse rates (Byun et al., 2005; Chen et al., 2014; Lee et al., 2010; Wang et al.,
512 2010). Chien et al. noticed that patients with genotype C had higher relapse rates
513 than those with genotype B (Chien and Liaw, 2006). Cao et al. showed that the level
514 and persistence of off-treatment elevated HBV DNA levels were useful in the
515 prediction of a subsequent biochemical relapse and may guide off-treatment
516 management (Cao et al., 2017). A study reported a negative correlation between
517 baseline ALT levels and relapse rate (Pan et al., 2013) while another one showed
518 that lower ALT baseline levels correlated with higher rates of HBsAg loss (Chen et
519 al., 2014).

520 In HBeAg-negative CHB patients, several predictive factors have been associated
521 with a better chance of sustained off-NA remission such as younger age, higher
522 levels of ALT at baseline and at the end of the treatment, lower serum HBV DNA at
523 baseline, low serum HBsAg levels at the end of treatment as well as decreasing
524 HBsAg levels during therapy (Papatheodoridis et al., 2016). Two studies have
525 demonstrated that patients with sustained response are younger than patients with
526 relapse (Ha et al., 2012; Liu et al., 2011). Higher levels of ALT at baseline and at the
527 end of treatment (Hadziyannis et al., 2012) and lower levels of HBV DNA
528 pretreatment (Hadziyannis et al., 2012; Jeng et al., 2013) predicted a sustained
529 response.

530 In addition to predicting HBsAg loss by its decrease (Martinot-Peignoux et al., 2013),
531 qHBsAg has also been used to predict off-therapy sustained response with NAs, and
532 off-therapy flares (Höner Zu Siederdisen et al., 2016; Martinot-Peignoux et al., 2015,
533 2014; Qiu et al., 2016). Low levels of serum HBsAg at the end of treatment, or

534 decreasing levels during therapy or after its cessation were shown to be positive
535 predictors for sustained response (Chan et al., 2011; Hadziyannis et al., 2012). Chen
536 et al. showed that HBsAg cut-off values of 120 and 200 IU/mL predicted a 79%
537 HBsAg loss and 93% post-treatment sustained response respectively (Chen et al.,
538 2014). Conversely, two studies showed no influence of HBsAg kinetics and relapse
539 rates (Jeng et al., 2016; Seto et al., 2015). Several studies identified serum HBsAg
540 levels of $<2 \log_{10}$ IU/mL to be a reliable predictor of a sustained off-treatment
541 response (Chen et al., 2018; Chi et al., 2019; Wen-Juei Jeng et al., 2018;
542 Papatheodoridi and Papatheodoridis, 2020; Su et al., 2018). In a study of 204
543 HBeAg-negative patients, qHBsAg level ≥ 1500 IU/mL at discontinuation was the only
544 predictive factor of relapse (Ge et al., 2015). qHBsAg $>3 \log$ IU/mL was predictive of a
545 virologic relapse at 1 year in another study (Liang et al., 2011). In the FINITE study,
546 patients who achieved a decrease in HBsAg levels after treatment withdrawal had
547 baseline HBsAg levels $<25\ 000$ IU/mL when NA treatment was discontinued (Berg et
548 al., 2017). A recent meta-analysis found that older age, shorter duration of
549 consolidation therapy, lower levels of baseline and EOT HBsAg levels ≥ 1000 IU/ml
550 were predictive factors of relapse in HBeAg-negative CHB patients who discontinued
551 NA treatment (Y. Liu et al., 2019). Liu et al. showed in a systematic review that an
552 HBsAg <100 IU/mL at EOT is a significant marker for safe NA discontinuation (J. Liu et
553 al., 2019). Two recent studies found that an HBsAg <50 IU/mL (Gao et al., 2019) or
554 HBsAg <40 IU/mL (Tseng et al., 2020) have higher predictive values of better
555 sustained responses in HBeAg-negative CHB patients. More studies outside Asia
556 and therapies using different NAs are needed to define the optimal cut-off levels.
557 Nevertheless, from a practical viewpoint, EOT HBsAg <100 IU/mL, in most studies,
558 seems to be the most important predictor for subsequent functional cure (J. Liu et al.,

559 2019). Additionally, serial HBsAg measurements after EOT are important since
560 decreasing levels are associated with beneficial outcomes and increasing levels are
561 predictors of relapse(Liaw, 2019).

562 Several viral markers have been proposed as potential biomarkers for a safe NA
563 discontinuation. Serum HBV pregenomic RNA (henceforth referred to as HBV RNA)
564 reflects the transcriptional activity of liver cccDNA even during suppression of HBV
565 DNA during NA treatment or very low HBV replication activity (Giersch et al., 2017)
566 and its decline seems to be a good predictor of HBeAg seroconversion (van Bömmel
567 et al., 2015) . Unlike HBsAg, HBV RNA is derived only from cccDNA, and its
568 quantification is not affected by viral antigens or antibody complexes and therefore it
569 may reflect more accurately the transcriptional activity of cccDNA. Levels of serum
570 and intrahepatic HBV RNA have been suggested as a novel marker during NA
571 treatment (Wang et al., 2016; Yu et al., 2018). However, it is commonly undetectable
572 in HBeAg-negative patients (Wang et al., 2016), which makes it interesting only in
573 HBeAg-positive CHB patients. Nevertheless, a recent study suggested that HBV
574 RNA along with another marker can predict outcomes in HBeAg-negative CHB
575 patients suppressed on NA therapy (Carey et al., 2019). In a recent study (Fan et al.,
576 2020) including 127 HBeAg-positive patients who stopped TBV-based therapy after
577 achieving HBeAg seroconversion, HBV RNA was deemed satisfactorily in predicting
578 clinical relapse and HBsAg loss after treatment cessation. Similarly, another study by
579 Seto et al. found serum HBV RNA measurement to be essential for deciding on ETV
580 cessation in CHB patients with low HBsAg levels (Seto et al., 2020).

581 HBV core-related antigen (HBcrAg) includes HBcAg, HBeAg and a pre-core protein
582 (p22cr), and its quantification closely correlates with intrahepatic cccDNA level (Lin
583 and Kao, 2016). It has been also proposed as a novel marker for safe NA

584 discontinuation (Matsumoto et al., 2015, 2007). Low HBcrAg levels were associated
585 with a lower risk of a biochemical relapse following LAM discontinuation (Matsumoto
586 et al., 2015). Furthermore, two studies, including a recent one, suggested that the
587 combination of HBsAg together with HBcrAg can predict safe NA discontinuation
588 (Hsu et al., 2019; Tanaka and Matsumoto, 2014). A retrospective cohort study
589 consisting of 560 patients enrolled in a phase IV, multicenter, randomized, controlled
590 trial treated with NA-based therapy for up to 2 years, reported that baseline anti-
591 HBcAg levels is a strong predictor for HBeAg seroconversion during NA therapy (Fan
592 et al., 2016). Another recent prospective study noticed a trend for an inverse
593 association between anti-HBc levels and clinical relapse in CHB patients off-ETV
594 (Tseng et al., 2018). In summary, some promising virologic markers, such as HBV
595 RNA and HBcrAg, could be useful predictors of response but further cohort
596 validation is still needed.

597

598 **5. Immunological response following NA discontinuation**

599 The humoral and cellular immune responses play a crucial role in clearing the
600 infection during acute hepatitis B (AHB) (Rehermann and Nascimbeni, 2005).
601 However, this adaptive response is severely impaired in CHB patients due to a high
602 and persistent viral antigens exposure. This immune exhaustion is characterized by
603 an upregulation of regulatory cells, increase of IL-10 and TGF- β levels, high viral
604 antigen levels especially HBsAg, and mitochondrial dysfunction (Mansouri et al.,
605 2018; Ye et al., 2015). This leads to a dysfunction of CD4⁺ and CD8⁺ T cells
606 (Fisicaro et al., 2017), decreased numbers and dysfunction of DCs and NKs/NKTs
607 (Fisicaro et al., 2019; Maini and Peppia, 2013; Martinet et al., n.d.; Ouaguia et al.,

608 2019) up-regulated/enhanced expression of regulatory factors, including the immune
609 checkpoint proteins PD-1, CTLA-4, and Tim-3 (Hoogeveen and Boonstra, 2020); and
610 impaired innate immune response (Faure-Dupuy et al., 2017), especially TLR
611 downregulation and dysfunction (Ma et al., 2018; Tout et al., 2018; Zhang and Lu,
612 2015).

613 Multiple studies have shown an increased frequency and improved function of HBV-
614 specific CD4+ and CD8+ T cells in CHB patients after NA therapy and viral load
615 reduction (Boni et al., 2012, 2003). Most outcomes after NA cessation in CHB
616 patients, ranging from functional cure to severe liver inflammation, links intimately to
617 the immune system.

618 Hence, immunologic biomarkers that discriminate between antiviral responses and
619 pathologic inflammation after stopping therapy could facilitate clinical decisions, limit
620 adverse outcomes, and identify mechanisms leading to functional cure. In this
621 context, we will mainly discuss the immunological activity of T cells and NK cells as
622 well as the cytokinic profile after NA discontinuation.

623 **5.1 T cells**

624 Several studies have shown that, after long-term NA treatment discontinuation, most
625 patients remain with a viral response after a long follow-up (Berg et al., 2017;
626 Hadziyannis et al., 2012) and thus we could speculate that the host's immune system
627 is controlling HBV replication. The reduction in viral load induced by the antiviral
628 therapy would restore the function of HBV-specific CD8+ T cells allowing them to
629 control the virus. Patients with viral control are more likely to have a good immune
630 response against the virus (Maini et al., 2000), in comparison to cases with virologic
631 suppression, that have a dysfunctional response. Therefore, changes in the

632 phenotype and functionality of HBV-specific CD8⁺ T cells may account as a valuable
633 predictor of antiviral control and may point us to the patients that can safely stop NA
634 therapy without viral relapse.

635 Previous studies of NA-treated HBeAg-positive patients show that control of virus
636 replication induced by therapy can lead to a transient restoration of T cell function
637 (Boni et al., 2003; Stoop et al., 2007). To investigate the effect of long-term treatment
638 on T cell responses, Boni et al. compared HBV-specific T cell responses of patients
639 treated with NA with those detected in other conditions of HBV control (Boni et al.,
640 2012). They demonstrated that HBV-specific CD8⁺ T cell effector abilities were
641 similar between patients after several years of antiviral treatment and acute resolving
642 cases featured by a PD-1⁺ phenotype. More recently, Rivino et al. studied two
643 cohorts of 29 and 27 HBeAg-negative CHB patients who received NA monotherapy
644 or NA combination. Patients were, then, separated into 2 groups based on the
645 presence or absence of hepatic flares during the 6-month period after the termination
646 of the therapy (Rivino et al., 2018). They demonstrated that the patients who did not
647 relapse after NA cessation featured, during treatment, an increased frequency of
648 functional PD-1⁺ HBV-specific CD8⁺ T cells directed against HBcAg and HBV
649 polymerase. However, peripheral T cell expansion or PD-1 expression did not
650 consistently correlate with HBsAg reduction or biochemical reactivation. Rather,
651 CD38 and Ki-67 expression during ALT elevation correlated with HBsAg loss in the 3
652 patients who achieved functional cure (Rinker et al., 2018). Interestingly, a higher
653 frequency of polymerase-specific CD8⁺ T cells expressed markers indicating more
654 severe T cell exhaustion and exhibited a reduced expansion capacity than HBcAg-
655 specific CD8⁺ T cells (Schuch et al., 2019). This indicates that the molecular
656 mechanisms underlying impaired T cell responses differ with respect to the targeted

657 HBV antigens. Overall, PD-1+ HBV-specific T cells were identified as a potential
658 biomarker for the safe discontinuation of NA therapy for CHB patients (Rivino et al.,
659 2018). In line with these results, it was reported that a high level of anti-HBc at the
660 end of NA treatment was associated with a reduced risk of clinical relapse (Chi et al.,
661 2019) and it would be interesting to determine whether this higher level of anti-HBc is
662 related to the higher HBV-specific T cell activity. As the analysis of PD-1+ HBV-
663 specific T cells is somehow complicated, the development of a practical assay to
664 directly quantify these cells would be of great interest to permit this biomarker to
665 become clinically useful. It would be interesting to know what causes the hepatic
666 flares after the cessation of NA therapy since the magnitude of the HBV-specific T
667 cell responses was low in those patients. Additionally, PD-1 upregulation on HBV-
668 specific CD8+ T cells could either mean an activation before HBV clearance in long-
669 term NA treated CHB patients without HBV flares or exhaustion after persistent and
670 high HBV antigens exposition in untreated CHB patients. Therefore, it is unclear
671 whether anti-PD-1 treatments (Fisicaro et al., 2017, p. 8; Salimzadeh et al., 2018) will
672 enhance HBV clearance in patients with PD-1+ HBV-specific T cells. Further
673 longitudinal investigation of blood and liver samples are needed to determine precise
674 immunological events occurring in patients stopping NA therapy.

675 **5.2 NK cells**

676 The role of NK cells, characterized by the presence of activating and inhibiting
677 receptors, in CHB has thoroughly been debated (Maini and Peppia, 2013). Their
678 presence in the liver several folds higher than the periphery, their contribution to liver
679 damage by killing hepatocytes (Dunn et al., 2007; Maini et al., 2000) as well as their
680 correlation with HBV control makes them interesting cells to study the antiviral
681 response against HBV. On the other hand, multiple studies have shown that NK cells,

682 in CHB, are defective in the production of cytokines like IFN- γ , impairing their
683 noncytolytic antiviral capacity (Oliviero et al., 2009; Peppia et al., 2010). NK cells can
684 also impair antiviral immunity by deleting HBV-specific T cells (Lang et al., 2012;
685 Peppia et al., 2013).

686 A partial restoration of antiviral cytokine production of NK cells was shown, following
687 long-term ETV, compared to naïve CHB patients (Tjwa et al., 2011). To further
688 understand to what extent NA treatment can affect NK cells, Boni et al. studied NK
689 cell phenotype and function in HBeAg-negative patients either untreated or treated
690 with NA. No significant improvement of NK cell function was observed, despite a
691 reduced expression of some activation/proliferation markers, including the death
692 ligand TRAIL indicating a low NK pro-inflammatory phenotype (Boni et al., 2015).
693 This lower inflammatory status of NK cells correlated with a better HBV-specific T cell
694 response (Zimmer et al., 2018). An increase in both degranulation and cytokine
695 production, was found, along with a CD38 upregulation, in correspondence of the
696 ALT flares following NA discontinuation, which was particularly enhanced in the
697 fraction of patients experiencing HBsAg loss (Zimmer et al., 2018). This strongly
698 suggested a role for NK cells in HBsAg seroclearance and in biochemical reactivation
699 following NA discontinuation.
700 These studies not only help to clarify mechanisms of liver inflammation but suggest
701 that NK cells may aid in predicting safe NA discontinuation. Studies in bigger cohorts
702 are needed to confirm this lower inflammatory profile tendency in NK cells in
703 successful NA withdrawal as it can be easily evaluated in laboratory and may reflect
704 an HBV-specific T cell restoration.

705

706 **5.3 Cytokines**

707 An adequate antiviral immune response or, in contrast, liver inflammation, associated
708 with hepatic flares, are strictly affected by a balanced cytokine profile (Rehermann,
709 2013; Schurich et al., 2013; Xia and Protzer, 2017) which could be useful to
710 determine the fate of HBV infection upon NA discontinuation. Successful viral
711 repression induced by NA therapy leads to antiviral response by promoting
712 proinflammatory cytokines such as IFN γ (Chen et al., 2011) and IL-2(Jiang et al.,
713 2010), and by decreasing regulatory signals such as IL-10 (van der Molen et al.,
714 2006) and TGF- β (Zheng et al., 2012). Serum cytokine studies, in small cohorts,
715 could not predict off-therapy relapse (Höner Zu Siederdisen et al., 2016; Tan et al.,
716 2010), but we could theoretically speculate that the measurement of these cytokines
717 together with HBV-specific T cells or NK cells could help to establish a good
718 cessation point for therapy. Among the cytokines that increased after NA withdrawal
719 included IL-12 (Höner Zu Siederdisen et al., 2016), which is important for the
720 induction and recovery of exhausted adaptive T-cell responses (Schurich et al.,
721 2013). In addition, an increase in sST2 in clinical relapse patients might be
722 associated with an inflammation-related immune response after discontinuation of NA
723 treatment (Xie et al., 2019).
724 In a recent study aiming to identify an immune signature associated with off-
725 treatment remission to NA therapy, Kranidioti et al. performed microarray analysis of
726 PBMCs from 6 CHB patients who stopped NA therapy (3 with off-treatment
727 remission, 3 with relapse) compared to 5 CHB patients (inactive carriers) as controls
728 (Kranidioti et al., 2019). They found that IFN γ , IL-8, FASLG and CCL4 levels were
729 significantly lower in the patients with off-treatment remission compared to the

730 patients with relapse. Thus, they identified these 4 genes as potential biomarkers for
731 stopping NA therapy (Kranidioti et al., 2019).

732 Larger studies are required to determine more adequate cytokine candidates.
733 Additionally, several studies have shown that genetic polymorphisms might influence
734 the immune response in CHB (Galmozzi et al., 2014; Ju et al., 2017). Yu et al.
735 showed an effect of the IL-28B (IFN λ 3/4) genotype on the response to LAM at one
736 year (Yu et al., 2013). Among 33 single-nucleotide polymorphisms (SNPs) from 16
737 immune-related genes, a recent study found that genetic variations in the CXCR5
738 gene decrease the risk of clinical relapse after NA discontinuation in CHB patients
739 and thus these genotypes could facilitate optimization of NA discontinuation (Wu et
740 al., 2020, p. 5).

741 An additional important factor is the migration of HBV-specific T cells to the infected
742 liver which is controlled by chemokines and their receptors (Brass and Brenndörfer,
743 2014; Larrubia et al., 2008). Chemokines drive inflammatory infiltrates, possibly
744 induced by HBV replication (CXCL-8) or IFN γ (CXCL-9/10) (Tan et al., 2010). Studies
745 confirmed the associations between liver disease and chemokine production after
746 stopping NA therapy (Höner Zu Siederdisen et al., 2016; Tan et al., 2010). Previous
747 work reported higher levels of CXCL-10 in patients with HBsAg loss during NA
748 therapy (Jaroszewicz et al., 2011). Another study found that CXCL-10 levels started
749 to significantly increase after the 3rd year of treatment with ETV presenting a
750 promising predictor of HBsAg decline (Papatheodoridis et al., 2014).
751 An efficient viral suppression under NA therapy would restore the migration of
752 functional HBV-specific T cells, helped by a lower inflammatory status of NK cells, to
753 clear infected hepatocytes and thus leading to HBsAg loss (Figure 2).
754 More robust immune biomarkers with simple practical applications, remain important

755 to optimize NA cessation strategy. Furthermore, future clinical studies on larger
756 scales, should implement the monitoring of such promising immune biomarkers
757 especially HBsAg-specific CD8+ T cells before and during the study to assess the
758 immune environment in CHB patients who have undergone NA discontinuation which
759 will allow a better understanding of the mechanism of off-NA HBsAg loss. Other
760 immune cells, such as B cells, would be interesting to study during NA cessation in
761 order to understand their role in HBV clearance. HBsAg-specific B cells have gained
762 over the last few years a massive interest and constitute a novel attractive target for
763 antiviral strategies toward functional cure of chronic HBV infection (Neumann-
764 Haefelin and Thimme, 2018; Salimzadeh et al., 2018).

765

766 **6. Future directions and conclusions**

767 Although stopping NA therapy following HBeAg seroconversion is recommended in
768 major guidelines, this approach is associated with relapse in a substantial proportion
769 of patients(Seto et al., 2015). With the relapse rates following HBeAg seroconversion
770 reaching almost 50% of CHB patients, the value of HBeAg seroconversion as a
771 clinical endpoint is a subject of debate and continuing NA therapy in these patients
772 could be more beneficial. Nevertheless, 1 to 3 years of consolidation therapy with
773 normal levels of HBV DNA and ALT are determined to be sufficient to decrease
774 relapse rates.

775 In HBeAg-negative patients, recent reports confirmed the possibility of sustained off-
776 treatment response and higher HBsAg loss rates compared with the current long-
777 term NA strategy. Thus, this Finite strategy can be considered a rational strategy to

778 induce a functional cure in carefully selected non-cirrhotic HBeAg-negative patients
779 and with close follow-up monitoring.

780 It is worthy of mentioning that most of the relevant data so far come from Asia, where
781 the guidelines recommending that NA can be discontinued rather early, are based
782 primarily on reducing costs and not on efficacy. In addition, the systemic evaluation of
783 the results has been quite challenging due to the great heterogeneity of studies.
784 Therefore, prospective well-designed studies with a large number of patients and
785 longer follow-up are needed to confirm the findings and to further refine the criteria
786 for NA withdrawal.

787 Careful selection of candidates to NA withdrawal requires validated serologic,
788 virologic, or immunologic markers and predictors of relapse or on-treatment
789 responses. HBsAg levels and other promising predictors such as anti-HBc, HBV
790 RNA, and HBcrAg should be prospectively validated in larger cohort studies.
791 NA therapy in patients with liver cirrhosis should be lifelong and should be excluded
792 from this Finite therapy strategy due to the risk of subsequent decompensation or
793 even death.

794 Moreover, the definition of relapse strongly affects the off-NA rates of relapse and
795 therefore can have a great impact on the probability of retreatment. Retreatment
796 criteria should be standardized. Based on current knowledge, patients with persistent
797 HBV activity and severe flares should be retreated. EASL guidelines specify that the
798 indication for treatment in patients who have discontinued therapy should be in line
799 with the indications for treatment-naïve patients, specifically those with HBV DNA
800 >2,000 IU/ml and ALT >ULN (European Association for the Study of the Liver. 2017).
801 HBsAg kinetics measurements, after EOT, are a very interesting strategy to
802 distinguish beneficial from detrimental flares as early as possible, which may be

803 extremely challenging to identify(Jeng et al., 2019; Liaw et al., 2018). More frequent
804 HBsAg measurements are required for HBsAg kinetics to be effectively used in
805 retreatment decisions. On the other hand, monitoring frequencies should also be
806 standardized. Monitoring should ideally include measuring ALT and HBV DNA levels
807 monthly for the first 6 months, and thereafter, every 3 months for the first 2 years.
808 Finally, the identification of immunological biomarkers such as HBV-specific T cells,
809 for safe therapy discontinuation, is urgently needed to move forward in the clinical
810 management of CHB patients. A necessary step to translate these findings into the
811 clinic is to develop a reliable, easy to perform, and rapid assay to identify these
812 immune markers in candidates for NA discontinuation. However, the exact
813 mechanism leading to immunological control in these patients is not well understood.
814 The sudden increase in HBV DNA and HBV proteins following NA withdrawal may be
815 an important trigger to re-induce T cell responses against HBV-infected hepatocytes
816 and further studies are needed to clarify these interactions between the virus and the
817 host's immunity.

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823 **Figure legends**

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825 **Fig. 1. Algorithm for the follow-up of non-cirrhotic CHB patients who**
826 **discontinue NA therapy.**

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828 **Fig. 2. Immunological changes upon NA-induced viral suppression**

829 High viral DNA and HBV proteins during chronic HBV infection lead to exhausted
830 HBV-specific CD8+ T cells characterized by a dysfunctional phenotype, high and
831 sustained PD-1 expression, and abrogated antiviral responses. NK cells are defective
832 and are characterized by a high inflammatory phenotype that may reflect NK cell-
833 mediated T cell deletion through death receptors like TRAIL. After long-term NA
834 therapy-induced HBV suppression, HBV-specific PD-1+ CD8+ T cells restore their
835 antiviral function through a direct cytolytic activity on HBV-infected hepatocytes and
836 effective antiviral cytokine secretion (IFN γ and IL-2). NK cells have a low
837 inflammatory profile and can restore their antiviral functions. This immune control
838 phase is also characterized by high levels of CXCL-10 which could point to anti-viral
839 control. HBV-specific PD-1+ CD8+ T cells, low inflammatory NK cells and levels of IL-
840 2, IFN γ and CXCL-10 are promising predictors of safe NA therapy discontinuation.
841 HBV: Hepatitis B virus; CXCL10: C-X-C motif chemokine 10 ; IFN γ : interferon
842 gamma; IL-2: interleukin 2; NA: Nucleos(t)ide analogue; NK cell: Natural killer cell;
843 PD-1: Programmed cell death protein 1.

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Table 1. Recommendations on stopping NAs by the European, American and Asian Pacific Guidelines for Chronic Hepatitis B, stratified by HBeAg status and cirrhosis

Guideline	EASL (2017)	AASLD (2018)	APASL (2015)
HBeAg positive	-HBsAg seroclearance -HBeAg seroconversion and HBV DNA undetectable with at least 12 months of consolidation therapy	-HBsAg seroclearance -HBeAg seroconversion with at least 12 months of persistently normal ALT levels and undetectable HBV DNA levels (close monitoring every 3 months for at least 1 year)	HBeAg seroconversion with undetectable HBV DNA and persistently normal ALT levels with 1-3 years of consolidation therapy
HBeAg negative	-HBsAg seroclearance -May be considered before HBsAg loss in selected patients with undetectable HBV DNA for at least 3 years if guaranteed close post-treatment monitoring for at least 1 year	HBsAg seroclearance (close monitoring every 3 months for at least 1 year for recurrent viremia, ALT flares, and clinical decompensation). - May be considered if there is a compelling rationale and under careful monitoring every 3 months for at least 1 year.	-HBsAg seroclearance with anti-HBs seroconversion -HBsAg loss with at least 12 months of a post-HBsAg loss consolidation period -After treatment for at least 2 years with undetectable HBV DNA on three separate occasions, 6 months apart
Cirrhosis	Not recommended before HBsAg loss	Not recommended before HBsAg loss	May be considered before HBsAg loss in compensated cirrhosis with careful monitoring plan

AASLD, American Association for the Study of Liver Diseases; ALT, alanine aminotransferase;
APASL, Asian Pacific Association for the Study of the Liver; EASL, European Association for the Study of the Liver; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen

866 Table 2. Outcomes in HBeAg-positive CHB patients after NA cessation

Reference	Study design	HBeAg (+) patients (n)	Follow-up (years)	NA (mean duration of treatment)	Consolidation treatment (months)	Sero-reversion (%)	Clinical relapse (%)
(Song et al., 2000)	Retro.	34	2	LAM (9 mo)	2,5	38	49,2
(Kuo et al., 2010)	Retro.	124	2	LAM (6 mo)	9	44,3	68,4
(Lee et al., 2010)	Retro.	178	5	LAM (26 mo)	>12	18	30,2
(Wang et al., 2010)	Prosp.	82	4	LAM (24 mo)	16	42,9	29,4
(Van Hees et al., 2018)	Retro.	62	3,8	LAM, TDF, ADV, ETV, LAM+ADV (5,8 years)	8	9	48
(Jung et al., 2016)	Prosp.	45	1	ETV, LAM (8,4 years)	6	n.a.	18
(Reijnders et al., 2010)	Retro.	42	5	LAM, ADV, ETV (26 mo)	>6	44	69
(Liu et al., 2018)	Prosp.	138	10	LAM, ADV, TBV, ETV (27,5 mo)	21	n.a.	31
(Liem et al., 2019)	RCT	18	1,5	ADV, LAM, TBV, TDF(7,4 years)	40	5	6
(Fan et al., 2020)	Prosp.	127	4	TBV(35 mo)	18	17,2	31,5

867 HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; mo, months; n.a., non-applicable;
 868 NA, nucleos(t)ide analogue; Prosp., prospective; RCT, randomized controlled trial; Retro,
 869 retrospective

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879 Table 3. Outcomes in HBeAg-negative CHB patients after NA cessation

Reference	Study design	HBeAg (-) patients (n)	Follow-up (years)	NA (mean duration of treatment)	HBsAg loss (%)	Clinical relapse (%)	Retreatment (%)
(Seto et al., 2015)	Prosp.	184	1	ETV (36,7 mo)	0	22,8	n.a.
(Kim et al., 2013)	Retro.	45	1	ETV, LAM, ADV (38,4 mo)	n.a.	53,3	n.a.
(Fung et al., 2004)	Retro.	27	1,5	LAM (2 years)	0	30	n.a.
(Wen-Juei Jeng et al., 2018)	Retro.	691	3	ETV, TDF, ADV, LAM, TBV(3 years)	13	61	41
(Papatheodoridis et al., 2018b)	Prosp.	57	1,5	ETV/TDF (≥ 4 years)	25	33	28
(Liem et al., 2019)	RCT	27	1,5	ADV, LAM, TBV, TDF (7,4 years)	4	19	22
(Berg et al., 2017)	RCT	21	3	TDF(>4 years)	19	19 (Vir*)	38
(Gao et al., 2019)	Retro.	10	1	LAM, ADV, TBV, ETV (42 mo)	20	30	30
(Papatheodoridis et al., 2018a)	Prosp.	130	1,3	ETV, LAM, ADV(5 years)	n.a.	40	33
(Cao et al., 2017)	Prosp.	22	2	ETV, TDF(4 years)	6,1	53	53

880 HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; mo, months; n.a., non-applicable;

881 NA, nucleos(t)ide analogue; Prosp., prospective; RCT, randomized controlled trial; Retro,

882 retrospective; Vir*, virologic relapse.

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1504

NA discontinuation before HBsAg loss
(according to major guidelines)

Pretreatment HBeAg-positive CHB patients

Pretreatment HBeAg-negative CHB patients

Follow-up:
ALT and HBV DNA monthly
for 3 months, then every 3
months
HBeAg/anti-HBe every 6
months for up to 2 years

Follow-up:
ALT and HBV DNA monthly
for 3 months, then every 3-4
months up to 1 year

Severe flares +
persistence (≥ 3 months)
of liver disease activity

Mild to moderate liver
disease activity

Detrimental
flares (severe
liver disease)

Beneficial
flares (Immune
reactivation)

Persistent elevated HBV
DNA and ALT levels for
at least 6 months after
stopping NA

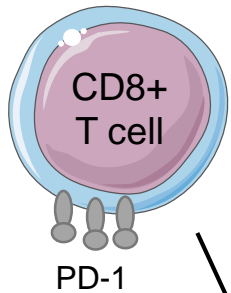
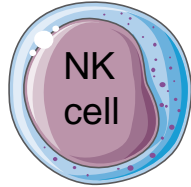
Low HBV DNA levels
(< 2000 IU/mL) and
ALT levels

Retreatment

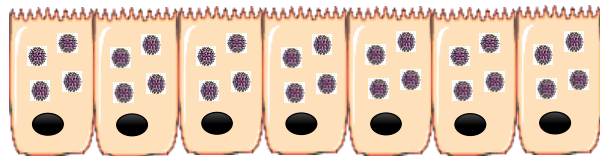
Close monitoring: ALT and HBV DNA levels every 6 months
Liver biopsy every 12 months

Immune Exhaustion

High NK pro-inflammatory phenotype can lead to T cell deletion via death receptors



Exhausted PD-1(hi) HBV-specific CD8+ T cells are dysfunctional and are unable to mount an effective antiviral response



HBV-infected Hepatocytes

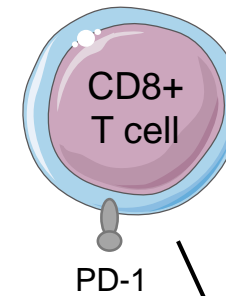
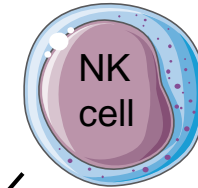
NA therapy



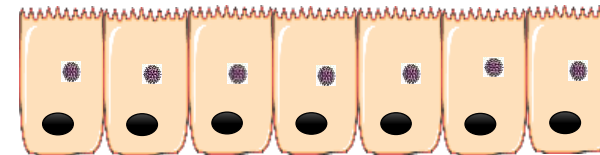
HBV viral suppression

Immune Control

Low NK pro-inflammatory phenotype and restoration of antiviral cytokine production (IFN γ)



Effective PD-1+ HBV-specific CD8+ T cells mediate a robust antiviral response (direct cytolytic activity on HBV-infected hepatocytes and a non-cytolytic activity via IFN γ and IL-2 secretion)



HBV-infected Hepatocytes