Universitat Autònoma de Barcelona

Cinètica de l'antigen de superfície del virus de l'hepatitis B durant i després del tractament antiviral en pacients amb hepatitis crònica B antigen e negatiu

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TESI DOCTORAL

“Cinètica de l’antigen de superfície del virus de l’hepatitis B durant i després del tractament antiviral en pacients amb hepatitis crònica B antigen e negatiu”

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ABREVIATURES

VHB: virus de l'hepatitis B

ADN: àcid desoxiribonucleic

ADN rc: ADN circular parcialment relaxat

HBeAg: Antigen e

ADNccc: ADN circular tancat covalentment

IFN: interferó

HBsAg: antigen de superfície

OMS: Organització Mundial de la Salut

AIS: àrea integral de salut

CHC: carcinoma hepatocel·lular

UI: unitats internacionals

ml: mil·lilitre

Anti-HBs: anticòs contra l'antigen de superfície

AN: anàlegs de nucleòs(t)ids

IFN-peg: interferó pegilat

LSN: límit superior de la normalitat

LAM: lamivudina

ADV: adefovir

TBV: telbivudina

ETV: entecavir

TDF: tenofovir fumarat disoproxil

TAF: tenofovir alafenamida

VIH: virus de la immunodeficiència humana

HBcrAg: antigen relacionat amb el core

VPP: valor predictiu positiu

VPN: valor predictiu negatiu

S: sensibilitat

Sp: especificitat

AUROC: àrea sota la corba "receiver operating characteristic"

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RESUM

Aquesta tesi es composa de 2 estudis realitzats en pacients amb hepatitis crònica per VHB, HBeAg negatiu que és la forma més freqüent en el nostre medi, per tal d'avaluar la cinètica de l'HBsAg durant i després del tractament antiviral.

El primer estudi és un estudi retrospectiu en que es va analitzar la cinètica de l'HBsAg durant el tractament antiviral amb anàlegs de nucleos(t)ids (AN). Vam demostrar que el descens és molt lent, però que una proporció de pacients (14%) poden presentar una cinètica de descens accelerat i que la reducció de l'HBsAg > 0,3 log UI/ml als 3 anys, identificava els pacients amb probabilitat de perdre l'HBsAg en els següents anys.

El segon estudi va avaluar la cinètica de l'HBsAg abans i després de la retirada de tractament amb AN. La cinètica de l'HBsAg durant el tractament pot predir la pèrdua d'HBsAg després de la retirada de tractament. En el nostre estudi, l'11,5% del pacients van perdre l'HBsAg durant el primer any després de parar el tractament. La meitat dels pacients amb una disminució >1 log UI/ml durant el tractament poden eliminar l'HBsAg durant el primer any després de la retirada de l'AN. D'altra banda, la parada de l'AN en pacients que havien rebut tractament durant més de 6 anys va provocar un descens accelerat de l'HBsAg (3 vegades superior) respecte a l'any previ a la parada.

ABSTRACT

This thesis consists of 2 studies, performed in HBeAg negative chronic hepatitis B patients, which is the most common form in our setting, evaluating the kinetics of HBsAg during and after antiviral treatment.

The first study is a retrospective study evaluating the kinetics of HBsAg before and during antiviral treatment with nucleos(t)ides analogues (NA). We showed that HBsAg decline is very slow during treatment, but a small proportion of patients (14%) may have an accelerated decline. The reduction at 3 years of HBsAg> 0,3 log IU/mL, identified patients with probability of losing HBsAg in the following years.

The second study evaluated the kinetics of HBsAg before and after treatment withdrawal. The kinetics of HBsAg during treatment may predict the loss of HBsAg after treatment withdrawal. In our study, 11,5% of patients lost HBsAg in the first year after stopping treatment. Half of the patients with a decrease> 1 log IU/ml during treatment eliminated HBsAg during the first year after withdrawal of NA. On the other hand, stopping the NA in patients who had been treated for more than 6 years resulted in an accelerated decrease in HBsAg (3 times higher) compared to the year before treatment cessation.

1. INTRODUCCIÓ

1.1. El Virus de l'Hepatitis B

El virus de l'hepatitis B (VHB) és un virus d'àcid desoxiribonucleic (ADN) que pertany a la família dels Hepadnaviridae[1] . En l'hoste, el virus replica exclusivament en els hepatòcits, i els virions s'alliberen a través de les via secretora cel·lular. L'embolcall del virus envolta una nucleocàpsida icosaèdrica que conté el genoma d'ADN bicatenari circular parcialment relaxat (ADNrc) de 3,2K bases. La capacitat de codificació del genoma del VHB ve determinada per quatre marcs de lectura oberts parcialment superposats, denominats P (polimerasa), S (superfície), C (core) i X (proteïna HBx) que codifiquen 7 proteïnes: l'antigen e (HBeAg) que és una proteïna dimèrica, l'antigen del core que és una proteïna de la càpsida viral, la Pol/RT polimerasa, amb activitat transcriptasa inversa, les proteïnes de l'embolcall de mida petita (S), mitjana (M) i gran (L) que són glicoproteïnes, i la HBx que és una proteïna reguladora de la transcripció que és necessària per a l'inici de la infecció[2,3].

Després de l'entrada del virus als hepatòcits, la nucleocàpsida del VHB es transporta al nucli per alliberar el genoma d'ADNrc. En el nucleoplasma, l'ADNrc es converteix en un ADN circular tancat covalentment (ADNccc), que és embolicat per histones i que serveix com a codi per a totes les transcripcions que es tradueixen en les diferents proteïnes virals[4]. A més dels virions infecciosos complets, les cèl·lules infectades produeixen partícules esfèriques o filamentoses subvirals lliures i no infeccioses[2]. La integració del genoma viral en el genoma de l'hoste no és necessària per a la replicació viral, però és un dels mecanismes implicats en la transformació dels hepatòcits i en la carcinogènesi[5]. Les anàlisis filogenètiques de les soques del VHB aïllades han identificat deu genotips principals (A – J) que tenen una distribució diferent a nivell mundial[6,7].

1.2. Infecció pel VHB i resposta immune de l'hoste

L'edat de l'hoste en el moment de la infecció és un factor crucial per al desenvolupament de la infecció crònica. Les vies de transmissió del VHB son principalment la sang i els fluids corporals, incloent la transmissió vertical de mare a fill, la via sexual i la via parenteral. Més del 90% dels nadons amb transmissió perinatal desenvolupen una infecció crònica mentre que menys del 5% de les infeccions en l'edat adulta es fan cròniques.

En les infeccions agudes limitades, la resposta del sistema immunitari innat i adaptatiu al VHB és eficient i oportuna. L'eliminació viral implica la inducció d'una reacció robusta de cèl·lules T adaptatives que indueix tant un efecte antiviral citolític mitjançant l'expressió de citocines antivirals, com la inducció de cèl·lules B que produeixen anticossos neutralitzants que impedeixen la propagació del virus[8,9]. La necrosi hepatocitària de les cèl·lules infectades provoca la dilució de l' ADNccc. Quan la infecció aguda es fa crònica, hi ha un deteriorament progressiu de la funció específica de les cèl·lules T depenent de l'edat de l'hoste[8]. Diversos estudis han demostrat que la infecció per VHB provoca una disfunció de cèl·lules T per múltiples mecanismes reguladors[8,10] . Les cèl·lules T específiques del VHB són més disfuncionals dins del fetge que a la perifèria[11]. En pacients amb infecció crònica per VHB, es detecta una elevada càrrega antigènica i un elevat nombre d'hepatòcits infectats el que provoca una exposició persistent a l'antigen que és una característica clau de l'esgotament de les cèl·lules T[8]. L'estimulació crònica d'antigen s'associa amb l'expressió sostinguda de PD-1 (programmed death 1) i altres molècules co-inhibidores de cèl·lules T específiques del VHB, incloses TIM-3, CTLA-4, 2B4, CD160, LAG-3[8,11]. Tot i que les molècules co-inhibidores es poden expressar de manera feble i transitòria en cèl·lules T funcionals, la seva expressió elevada i sostinguda és el tret distintiu de l'esgotament de les cèl·lules T[12].

Una vegada que les cèl·lules T específiques del VHB arriben al fetge, se sotmeten a l'efecte inhibidor del microambient hepàtic que pot induir l'esgotament de nutrients importants necessaris per a la proliferació i funció de les cèl·lules T, així com una acumulació de metabòlits tòxics que poden perjudicar encara més la resposta de les cèl·lules T[13]. Aquest "milieu" de citocines pot perjudicar no només la funció de les cèl·lules T sinó també de les cèl·lules NK que gairebé no poden produir interferó (IFN)-γ en pacients amb infecció crònica per VHB[13].

1.3. Epidemiologia de la infecció crònica per VHB

La infecció crònica per VHB es defineix com la detecció en sèrum de l'antigen de superfície (HBsAg) 6 mesos després de la infecció. L'Organització Mundial de la Salut (OMS) estima que hi ha 257 milions de persones infectades pel VHB en el món (3,5% de la població) i que l'any 2015 va causar 887.000 morts, principalment per cirrosi i càncer[14].

La prevalença de la hepatitis B varia en diferents zones geogràfiques. És més elevada en la regió del Pacífic occidental i a l'Àfrica, on al voltant del 6% de la població adulta està infectada. En les regions del Mediterrani oriental, Àsia sud-oriental i Europa, s'estima que la prevalença és inferior al 3,3%. Espanya s'havia classificat tradicionalment com un país d'endemicitat intermitja, definida per una prevalença de l'HBsAg en la població general d'entre un 2 i 7%[15]. Tanmateix, des de la introducció de la vacunació a la dècada de 1990, la incidència ha disminuït notablement i en l'actualitat, Espanya se situa entre els països de baixa endemicitat amb una prevalença al voltant del 0,5-0,8% de la població general[16]. En un estudi recent de cribratge poblacional d'hepatitis virals a Catalunya, la prevalença de la infecció crònica per VHB va ser del 0,5%. Cal remarcar, que el 53% dels pacients diagnosticats d'infecció per VHB eren migrants de regions d'alta endemicitat[16].

L'Hospital del Mar, atén la població de l'àrea integral de salut (AIS) Barcelona Litoral Mar. Aquesta AIS te una població de referència d'aproximadament 300.000

persones. Segons dades de la Generalitat de Catalunya de l'any 2019, el 24,1% de la població atesa era població migrant. En l'estudi esmentat anteriorment[16], la prevalença d'infecció crònica per VHB en l'AIS Litoral Mar va ser del 1.5%.

1.4. Història natural de la infecció crònica per VHB

La infecció crònica per VHB és un procés dinàmic que reflexa la interacció entre el VHB i el sistema immunològic. La història natural es pot dividir en diferents fases[1,17–19] que reflecteixen aquest procés dinàmic. (Fig 1). La seva evolució és molt variable des de la infecció crònica, a l'hepatitis crònica, amb diferents graus de fibrosi, la cirrosi i el desenvolupament de carcinoma hepatocel·lular (CHC). En pacients no tractats, s'estima que la incidència de cirrosi varia entre un 2 i un 5,4 per 100 persones i any, amb una incidència acumulada als 5 anys d'entre el 8 i el 20%[17].

La primera fase, que es produeix durant les primeres dècades després de la infecció, s'anomena infecció crònica HBeAg positiu i es caracteritza per una càrrega viral molt elevada (en general amb ADN $>10^7$ unitats internacionals (UI) per mil·lilitre (ml)), positivitat de l'HBeAg en sèrum, nivells normals de transaminases, histologia hepàtica pràcticament normal i una progressió de la fibrosi molt lenta o absent.

La segona fase, s'anomena hepatitis crònica HBeAg positiva. Sol ocórrer en la tercera o quarta dècada de la vida, però apareix de forma més ràpida en pacients infectats en l'edat adulta. Pot durar de setmanes a anys. En aquesta fase, la resposta immune contra els hepatòcits infectats, mediada per limfòcits T, provoca una citòlisi amb elevació de les transaminases i una reducció dels nivells d'ADN. La intensitat de la resposta immunològica varia amb el temps, provocant que els nivells de transaminases siguin fluctuants. Hi ha un augment de la inflamació i un major risc de progressió de la fibrosi. La taxa anual de seroconversió de l'HBeAg sense tractament antiviral és d'aproximadament un 15% anual[1]. Els pacients que es mantenen durant anys en aquesta fase, pels brots repetits de necrosi hepatocel·lular, tenen risc de progressar a

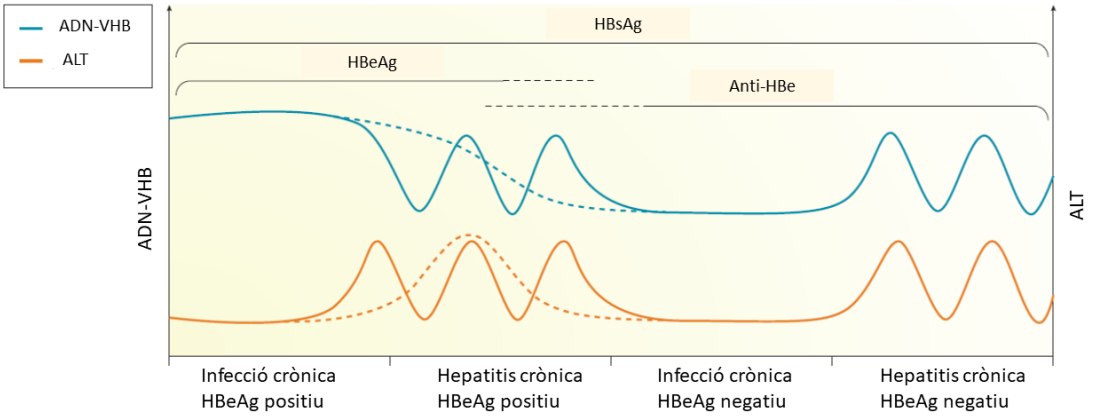
cirrosi i CHC. Aquesta fase, finalitza amb una reducció dels nivells d'ADN i la negativització de l'HBeAg amb seroconversió o aparició d'anticossos contra l'HBeAg (anti-HBe).

Fase d'hepatitis crònica HBeAg negativa. En alguns pacients amb infecció crònica l'activitat inflamatòria persisteix tot i tenir un HBeAg negatiu. Aquest fenomen, sol ocórrer principalment per virus que presenten mutacions precore que no expressen l'HBeAg. Aquestes mutacions són més freqüents a Àsia[20] i en la regió mediterrània[21] i és la forma més comú d'hepatitis crònica en la nostra àrea. En aquesta fase, s'observen períodes d'elevació de transaminases, precedits d'elevacions dels nivells d'ADN i que se segueixen de períodes de remissió de durada variable. Aquests episodis s'associen a progressió de la fibrosi.

Fase d'infecció crònica antigen e negativa. Aquests pacients, típicament mostren nivells normals de transaminases i ADN del VHB baixos (habitualment <2.000 UI/ml), però hi ha pacients que presenten nivells d'ADN entre 2.000 i 20.000 UI/ml. El pronòstic a llarg termini dels pacients en aquesta fase en general és bo[22], tot i que en alguns casos es produeixen reactivacions de la infecció amb elevació dels nivells d'ADN i ALT.

Per últim, s'ha definit la cura funcional, com la pèrdua de l'HBsAg amb o sense seroconversió de l'anticòs contra l'HBsAg (anti-HBs) i que es caracteritza per la presència de valors normals de transaminases i nivells indetectables d'ADN. No obstant, persisteix, a nivell hepàtic l'ADNccc[23]. La pèrdua de l'HBsAg en pacients sense fibrosi avançada s'associa a un risc mínim de cirrosi, descompensació i CHC i a una millora de la supervivència[23]. Actualment, es considera la cura funcional com l'objectiu òptim dels tractaments de l'hepatitis B[18], però aquest objectiu s'aconsegueix poc freqüentment amb els tractament antivirals disponibles.

Figura 1: Fases de la infecció crònica per VHB (adaptat de [1])



1.5. Marcadors serològics en l'hepatitis B

- **ADN del VHB**: la quantificació de l'ADN sèric del VHB és crucial en l'avaluació de pacients amb infecció crònica per VHB i en l'avaluació de l'eficàcia del tractament antiviral. La majoria dels assajos utilitzats en la pràctica clínica utilitzen la tecnologia de reacció en cadena de la polimerasa en temps real amb una sensibilitat de 5-10 UI/ml i un rang dinàmic de fins a 7 log10 UI/ml. Els nivells d'ADN són fluctuants pel que les determinacions seriades són més importants que qualsevol punt de tall arbitrari tant en el pronòstic com en la determinació de la necessitat de tractament.

  En general, els pacients amb infecció crònica HBeAg negatiu tenen nivells d'ADN <2.000 UI/ml i aquells amb hepatitis crònica tenen nivells d'ADN> 20.000 UI/ml. Els pacients HBeAg negatiu tenen nivells més baixos que els HBeAg positius. Cal remarcar que el punt de tall de 20.000 UI/ml és un valor arbitrari que reflecteix la detecció límit dels assajos històrics amb la reacció en cadena no polimerasa[24]. D'altra banda en pacients amb cirrosi i CHC els nivells d'ADN poden ser més baixos, pel que és important interpretar els nivells d'ADN conjuntament amb altres factors com l'edat, la durada de la infecció, els nivells de transaminases, el grau de necroinflamació i l'estadi de fibrosi[25].

- **Antigen de superfície o HBsAg:** és el marcador que confirma la infecció per VHB. Està format per tres proteïnes de diferent mida, que formen l'embolcall viral, la petita (S), la mitjana (M) i la gran (L)[26]. La via secretora de l'HBsAg i la via de replicació de l'ADN viral són dos processos diferents però creuats dins de l'hepatòcit infectat[27]. Les tres proteïnes de l'embolcall tenen una forma glicosilada responsable de la secreció de partícules virals. Les proteïnes superficials també s'acoblen per generar partícules subvirals no infeccioses que es troben en una quantitat molt més elevada, respecte als virions. El paper de les partícules subvirals en la patogènesi de la infecció crònica pel VHB encara no és del tot conegut, però podrien actuar com un mecanisme d'evasió del sistema immunitari bloquejant els anticossos neutralitzants de l'hoste (anti-HBs), promovent així la propagació i la persistència de la infecció[28].

  En general, s'ha descrit que en la història natural de la infecció per VHB, els nivells d'HBsAg varien en les diferents fases, essent més elevats en pacients amb infecció crònica HBeAg positiu, disminuint en la fase d'hepatitis crònica HBeAg positiu, i baixant encara més en pacients amb hepatitis crònica HBeAg negatiu i en pacients amb infecció crònica HBeAg negatiu[29,30]. Per tot això, els nivells d'HBsAg s'han considerat una forma indirecte d'avaluar el control per part del sistema immunològic[30,31]. La primera tècnica de quantificació estandarditzada d'HBsAg, en unitats de pes per volum, està descrita fa més de 40 anys[32]. Actualment es disposa de tècniques per a la quantificació automatitzada, que detecten les tres formes d'HBsAg sintetitzades a partir de l'ADNccc i de l'ADN integrat del VHB. Tanmateix, els anticossos utilitzats en aquests immunoassaigs enzimàtics no permeten distingir entre les diferents proteïnes o d'on prevenen. Hi ha almenys tres tests comercialitzats, l' Architect QT (Abbott Laboratories), l'Elecsys HBsAg II Quant assay (Roche Diagnostic) i DiaSorin Liaison XL. Els tres test estan àmpliament avaluats i es correlacionen estretament entre ells[33–35].

- **Anticòs contra l'Ag de superfice o anti-HBs**: és un anticòs neutralitzant i confereix protecció contra la infecció. Apareix després de l'eliminació d'una infecció aguda i no es

desenvolupa en la majoria de pacients que tenen infecció crònica. També es genera després de la vacunació i la seva presència en sèrum indica immunitat després d'una infecció aguda o vacunació.

- **Antigen e o HBeAg:** és una proteïna accessòria del VHB, no necessària per a la replicació viral però important per a la infecció natural *in vivo* [36]. En les fases inicials de la infecció se sol detectar en sang i això va acompanyat d'una elevada replicació viral. Tanmateix, existeixen formes del VHB anomenades mutants precore, que presenten una mutació G1896A que provoca una parada translacional que evita la formació de l'HBeAg sense afectar la replicació del VHB. Aquesta variant precore és la més freqüent en l'àrea mediterrània. A la zona euromediterrània i a l'Àfrica la gran majoria (>85%) dels pacients que presenten activitat bioquímica i histològica són HBeAg negatius, mentre que a l'Àsia, el nord d'Europa i als Estats Units, predomina l'hepatitis crònica HBeAg positiu[37].
- **Anticòs anti-e o anti-HBe:** és l'anticòs contra l'antigen e. En pacients amb hepatitis crònica HBeAg positiu, la seroconversió a anti-e positiu, s'associa a un control immunològic de la malaltia i a millor pronòstic, i és un dels objectius del tractament antiviral[18].
- **Ag relacionat amb el core o HBcrAg:** està constituït per tres antígens, l'antigen del core (HBcAg), l'antigen e, i el p22cr. Teòricament, tots els components proteics inclosos en l'HBcrAg provenen de la traducció de l'ADNccc, a diferència de l'HBsAg que es tradueix tant de l'ADNccc com de l'ADN integrat, i per tant, l'HBcrAg reflectiria exclusivament l'activitat transcripcional de l'ADNccc[38,39]. Actualment hi ha un sistema comercialitzat (LUMIPULSE®, Fujirebio Inc.) que mesura l'HBcrAg en sèrum utilitzant un immunoassaig enzimàtic de quimioluminescència. El límit inferior de detecció és de 2,0 log U/mL, amb un rang lineal de 3,0 a 7,0 log U/mL (1 kU/mL equival a 1.000 U/mL o a 3 log U/mL).

1.6. Tractament de l'hepatitis crònica per VHB

Amb els tractament actuals, no s'aconsegueix l'erradicació de la infecció pel VHB degut a la persistència d'ADNccc en els hepatòcits, pel que l'objectiu principal del tractament en els pacients amb infecció crònica per VHB és millorar la supervivència i la qualitat de vida, evitant la progressió de la malaltia, la descompensació de la cirrosi, la necessitat de trasplantament hepàtic i el desenvolupament de CHC[18,19,24,40]. Addicionalment, altres objectius del tractament són prevenir la transmissió de mare a fill, evitar la reactivació del VHB en pacients en tractament immunosupressor, reduir el risc de CHC en pacients amb antecedents familiars de tumors relacionats amb el VHB, reduir el risc de recidiva de la infecció en els pacients sotmesos a un trasplantament hepàtic, reduir el risc de recurrència del tumor en pacients amb CHC i la prevenció i tractament de les manifestacions extra-hepàtiques.

La probabilitat d'assolir aquests objectius depèn del moment en que s'inicia el tractament dins la història natural de la malaltia, però també del grau de fibrosi i de l'edat d'inici del tractament. La regressió de la fibrosi i la cirrosi es pot considerar un objectiu addicional del tractament. La inhibició de la replicació del VHB **(resposta virològica)** amb el tractament antiviral ha demostrat disminuir o eliminar l'activitat necroinflamatòria crònica i el procés de fibrosi hepàtica en la gran majoria de pacients, reduint també el risc d'aparició de CHC i de complicacions de la cirrosi[41–43]. Actualment, és l'objectiu principal dels tractament antivirals disponibles. D'altra banda, la pèrdua de l'HBeAg amb el tractament i la seroconversió a anti-HBe es caracteritza per la inducció d'un control immunitari parcial que condueix a una fase de baixa replicació pel que pot representar un bon objectiu en pacients HBeAg positius. La **resposta bioquímica**, definida com la normalització de les transaminases, es podria considerar un objectiu addicional i s'aconsegueix en la majoria de pacients amb una supressió prolongada de la replicació viral. Per últim, la **cura funcional**, s'ha definit com la pèrdua de l'HBsAg (amb o sense seroconversió de l'anti-HBs) i es caracteritza per la presència de valors normals d'ALT i indetectables d'ADN del VHB. La pèrdua d'HBsAg es considera l'objectiu òptim amb els

tractaments disponibles[44]. La pèrdua d'HBsAg en pacients sense fibrosi avançada s'associa amb un risc mínim de cirrosi[45], descompensació o desenvolupament d'HCC, i està relacionada amb una millora de la supervivència[46]. No obstant, amb els tractament disponibles, la probabilitat d'aconseguir la cura funcional és baixa. En una cohort multicèntrica que va rebre ETV o TDF (n=4.769), la taxa de pèrdua de l'HBsAg als 10 anys va ser només del 2,1% i la incidència anual va ser del 0,22%[47].

1.6.1. Indicacions de tractament de l'hepatitis crònica B

La indicació del tractament antiviral en pacients amb infecció crònica per VHB es basa en 3 paràmetres: el valor de transaminases, el nivell d'ADN del VHB i el grau de la lesió hepàtica valorat per biòpsia o per mètodes no invasius. Actualment, hi ha dues opcions de tractament antiviral amb indicacions diferents, els anàlegs de nucleòs(t)ids (AN) o l'interferó pegilat (IFN-peg). Les guies internacionals recomanen iniciar el tractament antiviral en pacients amb infecció crònica per VHB en les següents situacions clíniques[18,24,40].

- Tots els pacients amb hepatitis crònica HBeAg positiu o negatiu que presenten ADN del VHB per sobre de 2.000 UI/ml amb ALT per sobre del límit superior de la normalitat (LSN) i/o almenys necroinflamació hepàtica o fibrosi moderades.
- Pacients amb ADN del VHB > 20.000 UI/ml i ALT >2 vegades LSN, haurien de començar tractament independentment del grau de fibrosi.
- Pacients amb infecció crònica per VHB HBeAg positiu amb nivells d'ADN del VHB elevats i ALT persistentment normal poden ser tractats si tenen més de 30 anys independentment de la gravetat de les lesions histològiques hepàtiques.
- Pacients amb infecció crònica HBeAg positiu o negatiu i antecedents familiars d'HCC o cirrosi o que presentin manifestacions extrahepàtiques es poden tractar encara que no es compleixin les indicacions típiques del tractament.

1.6.2. Anàlegs de nucleòs(t)ids

Els AN aprovats a Europa són la lamivudina (LAM), l'adefovir (ADV), la telbivudina (TBV), l'entecavir (ETV), el tenofovir fumarat disoproxil (TDF) i el tenofovir

alafenamida (TAF). Els AN es poden classificar segons si el seu nivell de barrera a les resistències és baix (LAM, ADV, TBV) o alt (ETV, TDF, TAF). Els AN amb una alta barrera a les resistències són els fàrmacs d'elecció perquè el risc de que apareguin mutacions del VHB durant el tractament que puguin escapar a la seva acció és pràcticament nul. Els AN inhibeixen només la transcripció inversa de l'ARN pregenòmic i per tant són fàrmacs amb una elevada eficàcia en el control de la replicació viral i la resposta bioquímica, però els canvis a nivell transcripcional, especialment en la via secretora de l'HBsAg, no són esperables[27]. La taula 1 resumeix els resultats d'eficàcia del tractament antiviral.

Taula 1: Eficàcia dels tractaments de primera línia en adults amb hepatitis crònica B (no són comparacions directes i el temps de seguiment és diferent)

	ETV[48,49]	**TDF**[50]	**TAF**[51,52]
HBeAg positiu			
Resposta virològica (%)	94	98	73
Resposta bioquímica (%)	80	78	
Pèrdua HBeAg (%)	23	52	22
Pèrdua HBsAg (%)	1.4	4.9	1
HBeAg negatiu			
Resposta virològica (%)	96	100	90
Resposta bioquímica (%)	80	83	81
Pèrdua de l'HBsAg (%)	4.6	3.4	<1

Els resultats d'eficàcia mostren que tot i que el tractament a llarg termini amb ETV o TDF aconsegueix una resposta virològica en gairebé tots els pacients, les taxes de negativització de l'HBsAg són molt baixes, especialment en els pacients HBeAg negatiu. Els pocs estudis que han pogut mesurar l'HBsAg durant el tractament antiviral amb AN mostren que el descens dels nivells d'HBsAg és molt lent i que per tant, aquests pacients requeririen dècades de tractament amb AN per aconseguir la pèrdua de l'HBsAg[53,54].

A nivell de seguretat, en general són tractaments molt ben tolerats i tenen un bon perfil de seguretat. Els principals efectes secundaris a tenir en compte, són a nivell ossi

i renal. Aquests, es van descriure principalment en cohorts de pacients infectats amb el virus de la immunodeficiència humana (VIH)[55]. Tant TDF com ETV es metabolitzen a nivell renal i s'han d'ajustar en pacients amb filtrat glomerular inferiors a 50 ml/min per 1,73 m2. Els mecanismes de toxicitat renal del TDF no es basen en la funció glomerular sinó en el dany a les cèl·lules tubulars causat per altes concentracions de TDF a nivell intracel·lular. La manifestació més greu de la toxicitat tubular per TDF és la síndrome de Fanconi, amb resolució després de la retirada del TDF[56]. També, s'ha descrit un augment dels nivells de creatinina sèrica en un 5% dels pacients tractats amb TDF durant 10 anys i episodis d'hipofosfatèmia en l'1,7% dels casos[50]. En relació als efectes ossis del tractament amb TDF, s'ha descrit una disminució relativa de la densitat mineral òssia en pacients en tractament amb TDF, que s'ha observat que és reversible en els estudis de canvi de tractament a TAF[57].

1.6.3. Interferó pegilat

La formulació d'interferó aprovada a l'hepatitis B crònica és IFN-peg α-2a que presenta una doble activitat, antiviral i immunoestimulant, pel que aconsegueix induir un control immunològic persistent de la infecció amb un tractament de durada limitada. Com a conseqüència, les taxes de negativització de l'HBeAg i de l'HBsAg que s'obtenen amb IFN-peg són superiors a les obtingudes amb els AN. En pacients HBeAg positiu, tractats durant 12 mesos, les taxes de resposta sostinguda (definida com la pèrdua de l'HBeAg i DNA <2.000UI/ml als 6 mesos post-tractament) va ser d'un 20-30%[58] i les taxes de pèrdua d'HBsAg als 12 mesos de tractament van ser d'un 3-7%. En pacients HBeAg negatiu, les taxes de pèrdua de l'HBsAg als 12 mesos de tractament van ser d'un 4%[59]. No obstant això, els seus inconvenients són nombrosos, entre els quals cal destacar que l'administració és subcutània, té una activitat antiviral modesta i sobretot, provoca efectes adversos molt freqüentment i té un nombre elevat de contraindicacions, el que fa que el seu ús sigui restringit i que durant els darrers anys aquesta opció terapèutica hagi perdut gran part del seu paper en el tractament de l'hepatitis B crònica. Tot i això,

la resposta sostinguda després de la interrupció del tractament en pacients HBeAg positiu s'acompanya d'una millora histològica, un descens en el risc de desenvolupar complicacions de la malaltia hepàtica i d'un augment de la supervivència global[19].

Teòricament, la combinació d'un AN i IFN-peg podria tenir efectes sinèrgics però son pocs els estudis que han avaluat aquest efecte. Estudis de prova de concepte amb un número molt reduït de pacients han suggerit que l'addició d'IFN-peg a pacients en tractament amb AN podria augmentar les taxes de pèrdua de l'HBsAg[60,61]. Un estudi recent amb més de 700 pacients inclosos[62] ha demostrat que el tractament combinat pot aconseguir una major taxa de negativització de l'HBsAg (9,1%) que la monoteràpia (0% amb TDF i 2,8% amb IFN-peg). Cal destacar que la majoria de pacients que van perdre l'HBsAg estaven infectats pels genotips A o B.

1.6.4. Durada del tractament amb anàlegs de nucleòs(t)ids

Actualment, les guies clíniques, de les principals societats científiques, sobre el tractament de l'hepatitis crònica B difereixen en les recomanacions sobre la durada del tractament amb AN.

En pacients no cirròtics HBeAg positiu, les guies de les principals societats científiques[18,24,40] suggereixen que el tractament amb AN es pot interrompre quan s'aconsegueix la seroconversió de l'HBeAg i es realitza un tractament de consolidació després de la seroconversió de mínim 12 mesos. A la guia de la "Asia-Pacific Association for the study of the liver" (APASL) del 2015[40] se suggereix que la teràpia de consolidació es podria allargar a tres anys, basant-se en estudis que han demostrat una disminució del risc de recaiguda virològica en comparació amb un any de consolidació[63,64]. En una actualització recent d'aquesta guia[65] se suggereix que en pacients HBeAg positiu, el tractament pot ser retirat si han estat tractats un mínim de 3 anys, i que mantinguin l'HBeAg negatiu durant 1 any. D'altra banda, la guia de la "American Association for the study of liver diseases" (AASLD) del 2018[24] suggereix que

la teràpia amb AN es podria mantenir fins aconseguir la pèrdua de l'HBsAg en aquests pacients.

En el punt en què hi a més divergències entre les guies clíniques és en la retirada del tractament en els pacients HBeAg negatiu que no han negativitzat l'HBsAg. La guia de la APASL va introduir l'any 2008[66] la possibilitat d'aturar la teràpia amb AN en pacients HBeAg negatius després d'almenys 2 anys amb ADN indetectable documentat en tres ocasions separades amb 6 mesos de diferència. Aquest enfocament d'interrupció es va impulsar principalment per polítiques locals de reemborsament i els primers estudis publicats van ser retrospectius. Al 2012, Hadzydiannis et al.[67] van publicar un estudi prospectiu amb 33 pacients als que es retirava el tractament amb ADV i s'observava una taxa elevada de pèrdua de l'HBsAg (39% als 5,5 anys de seguiment). Al 2017, el primer assaig controlat aleatoritzat, l'estudi FINITE[68] va confirmar que l'estratègia d'aturar l'AN augmentava la taxa de pèrdua de l'HBsAg en comparació amb mantenir el tractament (19% vs. 0% als 3 anys de seguiment). A partir d'aquests estudis, la guia de la "European Association for the study of the liver" (EASL) del 2017[18] suggeria que els AN es podien parar en pacients no cirròtics HBeAg negatius després de 3 anys de supressió viral. La guia de la AASLD, no recomana retirar el tractament antiviral en aquests pacients.

Per tant, en pacients sense cirrosi amb hepatitis crònica HBeAg negatiu, no queda clara quina és la durada òptima del tractament amb AN ni quins son els pacients que es podrien beneficiar d'un tractament limitat.

1.6.5. Utilitat de la quantificació de l'HBsAg

La probabilitat de perdre l'HBsAg durant el tractament amb AN és molt baixa, pel que no hi ha factors predictors clarament definits. Hi ha estudis que han avaluat la utilització de l'HBsAg quantificat com a marcador de la resposta al tractament antiviral en pacients HBeAg positiu o negatiu tractats amb IFN-peg[58,69]. A més, s'ha descrit que

el tractament amb IFN-peg produeix un descens més marcat dels nivells d'HBsAg que la LAM[70].

En pacients tractats amb AN s'ha proposat que la variació dels nivells d'HBsAg (cinètica) durant el tractament pot proporcionar informació addicional en la monitorització del tractament antiviral. S'ha descrit que el descens dels nivells d'HBsAg en pacients tractats amb LAM és un factor predictor de l'eliminació de l'HBsAg[54,71]. La disminució de HBsAg va ser similar en pacients HBeAg positius i negatius, al voltant de -0,104 log UI/ml/any. També s'ha descrit que els nivells baixos d'HBsAg (<1000 UI/ml) previs a l'inici del tractament i la seva reducció durant el tractament (>0,166 log UI/ml/any), te un valor predictiu negatiu (VPN) del 98% per predir la pèrdua de l'HBsAg durant el tractament. L'estudi BE-LOW va demostrar, en pacients en tractament amb ETV durant 2 anys, un descens més marcat de l'HBsAg en pacients HBeAg positiu que en HBeAg negatiu[72] . Per això, s'ha postulat que els AN produeixen un descens molt lent de l'HBsAg i que caldrien molts anys per aconseguir la negativització de l'HBsAg durant el tractament[53,54].

2. HIPÒTESI

La pèrdua de l'HBsAg, és difícil d'aconseguir amb els tractaments que disposem actualment però s'han descrit casos després de molts anys de tractament amb AN. D'altra banda els pacients tractats durant anys amb AN solen tenir valors de transaminases normals i nivells d'ADN del VHB indetectables, pel que no disposem de marcadors que ens permetin identificar els pacients amb major probabilitat de perdre l'HBsAg.

La nostra hipòtesi de treball va ser que la determinació quantitativa de l'HBsAg i especialment la seva cinètica durant el tractament podria ser una eina útil per a monitoritzar el tractament antiviral dels pacients amb hepatitis crònica HBeAg negatiu i identificar aquells amb més probabilitat d'assolir la pèrdua de l'HBsAg durant el tractament o després de la seva retirada.

3. OBJECTIUS

3.1. Objectiu principal

- Avaluar els nivells de l'HBsAg i la seva cinètica durant el tractament antiviral i després de la parada del tractament en pacients amb hepatitis crònica HBeAg negatiu

3.2. Objectius secundaris

- Identificar variables basals o durant el tractament amb AN que permetin predir els pacients que presentaran nivells baixos de l'HBsAg (HBsAg<120UI/ml) o que el negativitzaran durant el tractament.
- Avaluar la influència de la cinètica de l'HBsAg durant el tractament amb AN en la resposta després de la parada de tractament i la probabilitat de perdre l'HBsAg.

4. COMPENDI DE PUBLICACIONS

4.1. Article 1

Broquetas T, Garcia-Retortillo M, Hernandez JJ, Puigvehí M, Cañete N, Coll S, et al. Quantification of HBsAg to predict low levels and seroclearance in HBeAg-negative patients receiving nucleos(t)ide analogues. PLoS ONE. 2017;12(11).

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RESEARCH ARTICLE

Quantification of HBsAg to predict low levels and seroclearance in HBeAg-negative patients receiving nucleos(t)ide analogues

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Abstract

Background

HBeAg-negative chronic hepatitis B patients require long-term nucleos(t)ide analogues (NAs) because loss of surface antigen (HBsAg) is unusual. Low quantitative HBsAg (qHBsAg) levels can identify patients with higher probability of seroclearance. The aim of our study was to evaluate qHBsAg in HBeAg-negative patients receiving NAs to predict a reduction of HBsAg levels and seroclearance.

Methods

Retrospective analysis of qHBsAg in HBeAg-negative patients before and at years 1, 3, 5, 8 and over of NAs treatment.

Results

From 1999 to 2015, HBsAg was quantified in 358 serum samples from 95 HBeAg-negative patients. Low qHBsAg (<120 IU/mL) was identified at baseline or during follow-up in 14% of patients and HBsAg loss in 4%. No baseline variables predicted seroclearance and only treatment duration predicted low qHBsAg. The annual decline of qHBsAg was -0.102 log IU/mL and the median time to HBsAg loss was 6.04 years. The decline was greater in patients achieving low HBsAg levels (-0.257) than in those who did not (-0.057)($p<0.001$). The diagnostic accuracy (ROC curve, 95%CI) of qHBsAg delta at year 3 was 0.89 (0.81–0.97), with cut-off >0.3 log IU/mL showing a positive and negative predictive value of 42% and 100% to identify patients achieving low levels of HBsAg.

Conclusions

Reduction of qHBsAg is slow in HBeAg-negative patients receiving NAs, although low levels or faster qHBsAg decline may occur in 14%. A qHBsAg reduction >0.3 log IU/mL at year 3

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can identify patients with a higher probability of achieving low levels and HBsAg seroclearance.

Introduction

The therapeutic endpoint in chronic hepatitis B (CHB) is the functional cure defined as sustained off-drug suppression of serum hepatitis B surface antigen (HBsAg), hepatitis B virus (HBV)-DNA and covalently close circular (ccc)DNA[1]. However, this endpoint is rarely achieved with the available therapies in HBeAg-negative. Serum HBsAg is the glycosylated envelope protein of the mature HBV which is produced by transcription and translation of the surface genes[2]. It has been suggested that serum HBsAg could be considered as a surrogate marker of cccDNA and a marker of host immune control of HBV infection[3]. HBsAg levels significantly vary during HBV infection showing a reduction from HBeAg-positive to HBeAg-negative infection[4].

HBsAg quantification (qHBsAg) has recently been proposed as an important tool in clinical practice to identify HBeAg-negative infected patients (former inactive carriers). Brunetto et al. have shown that the combination of qHBsAg <1000 IU/mL and HBV-DNA ≤2000 IU/mL can recognize most inactive carriers[5]. Another use of qHBsAg may be monitoring antiviral response in HBeAg-positive or -negative patients receiving pegylated interferon (Peg-IFN)[6, 7]. Peg-IFN has immune-mediated antiviral activity which modulates HBsAg production and secretion[8]. Moreover, it has been proposed that qHBsAg during nucleos(t)ide analogues (NAs) therapy can provide additional information to HBV-DNA levels. However, the decline of qHBsAg during NAs therapy is less pronounced than that observed in patients receiving Peg-IFN[8, 9]. A rational explanation is that NAs block viral reverse transcriptase, inhibiting HBV-DNA, but this does not affect either cccDNA or HBsAg production[10].

The qHBsAg has been proposed as a predictor of HBsAg clearance in patients receiving NAs[11–13]. Seto et al. have shown that 10% of patients receiving LAM achieved HBsAg-clearance[12] during the follow-up. Moreover, the decline of qHBsAg was similar in HBeAg-positive and -negative patients, being around -0.104 log IU/mL/year. These authors identified that HBsAg-clearance occurred in patients with a low baseline qHBsAg (<1000 IU/mL) and high on-treatment reduction (>0.166 log IU/mL per year) with a negative predictive value (NPV) of 98%. The BE-LOW study demonstrated similar results using entecavir (ETV)[14]. However, the reduction in qHBsAg was greater in HBeAg-positive than in -negative patients. Recently, a large European cohort receiving tenofovir (TDF) showed that a reduction of qHBsAg levels <1log at weeks 12 and 24 had a NPV to identify HBsAg-loss of 94% and 97%, respectively[15]. Additionally, Chen et al. have demonstrated that qHBsAg could be useful in finite therapies with NAs, to select patients with low risk of relapse after discontinuation. A qHBsAg level <120 IU/mL at the end of treatment (EOT) could predict 79.2% of HBsAg loss in HBeAg-negative patients in whom LAM was discontinued[13].

In contrast, other studies have concluded that NAs produce a very slow decline of qHBsAg and several years are necessary to identify HBsAg-clearance [12, 16]. In a recent Spanish prospective registry, the HBsAg loss rate in HBeAg-negative patients under tenofovir (TDF) or entecavir (ETV) treatment was only 0.3% after five years of follow-up[17]. Therefore, the role of qHBsAg in patients receiving NAs remains unclear with limited data in HBeAg-negative patients.

Thus, the aim of our study was to evaluate qHBsAg in HBeAg-negative CHB patients receiving NAs. Our secondary aim was to identify baseline and on-treatment variables to identify low qHBsAg levels (<120 IU/mL) as a good predictor of HBsAg loss[13].

Materials and methods

Serum samples and study population

This is a retrospective study evaluating cryopreserved serum samples of HBeAg-negative CHB patients, receiving NAs for a minimum of 12 months, from February 1999 to October 2015. All the serum samples had been extracted in fasting conditions and centrifuged at 3000 rpm before preservation at -30°C. The serum samples were part of the private collection (C.0000956) of the IMIM (Hospital del Mar Medical Research Institute) and were identified with a number. All the data were collected and tabulated in a database with an access code to ensure patient confidentiality. Patients enrolled from 1999 to May 2006 gave verbal informed consent for the use of serum samples in biomedical research, and this consent was registered in clinical history and electronic medical records. Those enrolled from May 2006 to October 2015 provided written informed consent. The study protocol was approved by the Ethical Committee of our institution "Comitè Ètic d'Investigació Clínica—Parc de Salut Mar", study reference 2014/5779/I, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

All patients included were adult (>18 years old), had a baseline liver biopsy or had clinical signs of portal hypertension before antiviral treatment. We excluded patients with co-infection (human immunodeficiency virus, hepatitis C or hepatitis D), those with hepatocellular carcinoma, patients who had previously received interferon or Peg-IFN, inactive carriers, patients receiving treatment as a prophylaxis of reactivation, patients who voluntarily stopped NAs during follow-up and those with a baseline liver biopsies of less than 15 mm (length) and/or less than 6 portal triads.

Baseline characteristics and HBsAg quantification

Demographic data of the patients, liver function tests and fibrosis stage, DNA levels, and serological status (HBsAg, HBeAg and antibodies) were retrospectively collected using the clinical history and electronic medical records.

Liver fibrosis stage was evaluated by liver biopsy before antiviral treatment. Liver biopsy was percutaneously performed using a 16-gauge Tru-Cut needle and guided by abdominal ultrasound. Samples were processed at the Pathology Department and stained with hematoxylin-eosin and Masson's trichrome. Fibrosis was staged according to the METAVIR classification[18](F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with few septa; F3 = portal fibrosis with many septa and F4 = cirrhosis). Those patients with clinical signs of portal hypertension were considered as cirrhotic patients.

Antiviral treatment with NAs was based on clinical guidelines and on the physician's decision [19–22]. Patients included in the study had been receiving NAs for more than 1 year, and NAs therapy was stopped in patients with confirmed HBsAg loss.

The HBsAg was quantified in frozen serum samples collected before antiviral treatment (baseline) and at years 1, 3, 5, 8 and over. Samples were tested and quantified for HBsAg by Electro-chemiluminescence immunoassay Elecsys® HBsAgII (Roche Diagnostic, Rotkreuz, Switzerland) according to the manufacturer's instructions. The assay ranged from 0.05 to 52000 IU/mL but in highly concentrated samples above the upper limit a further manual dilution step was necessary to achieve results within the measuring range and multiply by the dilution factor later.

Genotype of HBV was performed by INNO-LIPA® HBV Genotyping/28708v1 (Fujirebio Diagnostics, Goteborg, Sweden) in basal cryopreserved serum samples. The lower limit of detection, according to the manufacturer's instructions was <500 IU/mL. In those samples in which genotype could not be obtained at the first assessment, the procedure was repeated.

Statistical analysis

Quantitative variables were expressed as medians and ranges. Categorical variables were expressed as proportions. Continuous variables were compared by the Mann–Whitney *U* test. Categorical variables were compared by the Pearson chi-square test or the Fisher exact test. Differences between patients who achieved low levels of HBsAg (<120 IU/mL) and those who did not (>120 IU/mL), were analyzed by univariate analysis. Variables showing a P value <0.05 were included in a multivariate forward stepwise logistic regression analysis to determine independent predictors of low levels of qHBsAg and seroclearance. The diagnostic accuracy of qHBsAg (log IU/mL) at each time point to identify patients reaching low levels of HBsAg was assessed using the area under the receiver operator characteristic(AUROC) curve and the 95% confidence interval (95%CI). The optimal cut-off values were selected on the basis of sensitivity (S), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) to identify low levels of qHBsAg (<120 IU/mL) and HBsAg loss. We estimated the linear slope of qHBsAg for each group of patients (HBsAg <120 IU/mL vs. HBsAg >120 IU/mL) using a longitudinal mixed model (LMM) for repeated measurements. Cumulative incidences of low qHBsAg and seroclearance were analysed by the Kaplan-Meier method with a log-rank test. All statistical tests were two-sided and a *P* value<0.05 was considered statistically significant. The statistical analyses were performed with the SPSS® 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics of the patients

The qHBsAg (IU/mL) was evaluated in 485 frozen serum samples of 128 HBeAg-negative CHB patients collected from February 1999 to October 2015. Thirty-three patients did not fulfill the inclusion criteria and were excluded from the study: inactive carriers (n = 5), poor quality liver biopsy or basal serum not available (n = 4), treatment with interferon or pegIFN (n = 17), duration of treatment shorter than 1 year (n = 3) and indication as prophylaxis or acute reactivation (n = 4). Thus, 358 frozen serum samples of 95 HBeAg-negative CHB patients were considered for the analysis. Table 1 shows the baseline characteristics of the patients included; 73% were males, with a median age of 43; 55% were Caucasian (born in Spain or other European countries), 26% from South-East Asia and 9% from sub-Saharan Africa. Differences between European (n = 52) and Non-European (n = 43) patients are shown in Table 1. European patients were older (age: 48 vs. 37, p = 0.008), with a lower proportion of men (58% vs. 91%, p<0.001) and longer treatment duration (years: 7.1 vs. 5.2, p<0.001). However, there were no differences in fibrosis stage, alanine aminotransferase (ALT), HBV-DNA or qHBsAg levels and HBV-genotype.

The first antiviral treatment included TDF in 44% of patients, ETV in 32%, LAM in 9%, adefovir (ADV) in 9% and telbivudine (LdT) in 5%. Among 9 patients receiving LAM, 7 switched to ADV and later to TDF and 1 to ETV during follow-up. Among 9 patients treated with ADV, 8 switched to TDF and 1 to ETV. The 5 patients receiving LdT switched to TDF. Only one patient with TDF switched to ETV due to renal failure.

Predictors of low qHBsAg levels and seroclearance

The aim of our study was to identify baseline and on-treatment predictive variables to achieve low levels of qHBsAg (<120 IU/mL) as a good predictor of HBsAg loss in HBeAg-negative CHB patients receiving NA as reported by Chen et al.[13]. Thirteen patients out of 95 (13.68%) achieved qHBsAg <120 IU/mL during follow-up. The median level of qHBsAg at baseline was 4108 IU/mL (3.6 log IU/mL). On comparing patients with qHBsAg >1000 IU/

Table 1. Baseline characteristics of the patients included in the study.

	Total cohort	Patients from Europe	Non-European	P
	N = 95	n = 52	n = 43	
Baseline				
Age, years	43 (18–77)	47 (19–77)	37 (18–65)	0.008
Males, n (%)	69 (73)	30 (58)	39 (91)	<0.001
Fibrosis				
F0-F1	70 (74)	37 (71)	33 (77)	0.5
F≥2	25 (26)	15 (29)	10 (23)	
ALT, IU/mL	39 (11–252)	40 (11–213)	37 (13–252)	0.9
DNA, IU/mL	23101 (10–1.68·10^8)	19148 (10–1·10^8)	28450 (49–1.68·10^8)	0.2
<20000	45 (47)	28 (54)	17 (40)	0.2
>20000	50 (53)	24 (46)	26 (60)	
qHBsAg, IU/mL	4108 (2.61–187330)	3701 (2.6–187330)	5493 (234–33702)	0.3
<1000	14 (15)	9 (17)	5 (12)	0.4
>1000	81 (85)	43 (83)	38 (88)	
Genotype, n(%)*				
A	14 (19)	8 (21)	6 (17)	0.7
D	39 (54)	22 (58)	17 (49)	
Others (B, C, E, F, G)	20 (27)	8 (21)	12 (34)	
IL28, n(%)**				
CC	35 (44)	16 (36)	19 (54)	0.01
CT	37 (46)	27 (60)	10 (29)	
TT	8 (10)	2 (4)	6 (17)	
Antiviral treatment				
ETV/TDF	72 (76)	33 (64)	39 (91)	0.002
Others (LAM,ADV,LdT)	23 (24)	19 (36)	4 (9)	
Treatment initiation				
<2006	13 (14)	12 (23)	1 (2)	0.003
>2006	82 (86)	40 (77)	42 (98)	
Follow-up				
Time of treatment, years	5.95 (1–15)	7.1 (2–15)	5.2 (1–12.4)	<0.001
< 6 years, n (%)	49 (52)	20 (38)	29 (67)	0.005
> 6 years, n (%)	46 (48)	32 (62)	14 (33)	
HBsAg<120 IU/mL, n (%)	13 (14)	12 (23)	1 (2)	0.003
HBsAg seroclearance, n (%)	4 (4)	4 (7)	0 (0)	0.06

Quantitative variables shown as median and ranges, ALT alanine aminotransferase, LAM lamivudine, ADV adefovir, TDF tenofovir, ETV entecavir, LdT telbivudine

*Data available of 73 patients

**Data available of 80 patients

https://doi.org/10.1371/journal.pone.0188303.t001

mL (n = 81) to those with qHBsAg <1000 IU/mL (n = 14) at baseline, there were no differences regarding age, gender, fibrosis stage, HBV DNA, HBV-genotype, ALT levels or HBsAg seroclearance (p>0.05 in all cases)(data not shown).

Comparison of patients with low qHBsAg or those who achieved a reduction during follow-up (n = 13) with patients not achieving a reduction (n = 82) showed that 92% vs. 49% were Europeans (p = 0.003), had received NAs for a longer period of time (8 vs. 5.9 years; p = 0.001) of more than 6 years (77% vs. 44%; p = 0.003) with LAM (31% vs. 6%, p<0.009) or ADV (23% vs 7%, p = 0.009)(Table 2). The rate of patients with baseline qHBsAg below 1000

IU/mL was higher in those achieving low levels (<120 IU/mL)(39% vs. 11%; p = 0.02). However, among 5 patients with qHBsAg <1000 IU/mL, 3 already showed low qHBsAg (<120 IU/mL) at baseline, limiting its use as a predictive variable. Thus, these 3 patients (two over 62 years of age, with advanced fibrosis and receiving NAs during 5.3 years) were excluded from the kinetics analysis. After excluding these 3 patients, there were no differences between those with qHBsAg lower or higher than 1000 IU/mL to predict low levels during follow-up (20% vs. 11%; p = 0.3). On multivariate analysis, only the median treatment duration was longer in patients achieving low qHBsAg (8 years) compared to those who did not (5.9 years)(OR 1.2; CI 95% 1–1.45; p = 0.04)(Table 2).

During follow-up, 4 patients (4.21%) achieved HBsAg clearance. The median time to HBsAg clearance was 6.04 years (range: 1.7–8.1). All patients with HBsAg clearance were European (p<0.05), 3 were women (p<0.05), 2 had received ADV, and 2 TDF. The NAs therapy was withdrawn in all patients that achieve HBsAg seroclearance and HBsAg did not reappear during the follow-up.

There were no differences in age, fibrosis stage, HBV DNA, ALT and qHBsAg at baseline (data not shown). We could only determine HBV-genotype in 2 of these 4 patients, one was genotype A and the other was a genotype G. The patients presenting HBsAg clearance showed a greater qHBsAg (log IU/mL) reduction (median), compared to those who did not show it at years 1 (0.34 vs. 0.03, p = 0.047) and 3 (1.12 vs. 0.1, p<0.001). In all the patients with HBsAg clearance the decline in qHBsAg at year 3was >0.3 log IU/mL. In contrast, none of the patients with a reduction of HBsAg>0.3 log IU/mL after 5 years of NAs therapy achieved HBsAg seroclearance.

Kinetics of HBsAg levels and regression model during NAs therapy

Considering the lack of baseline characteristics to predict low levels of HBsAg (<120 IU/mL) we evaluated the kinetics of qHBsAg during follow-up. The reduction of qHBsAg (log IU/mL) at years 1 (n = 92), 3 (n = 81), 5 (n = 55), and 8 (n = 23) was 0.04, 0.13, 0.28, and 0.6 (p<0.001), respectively. However, patients showed two different kinetics of qHBsAg (Fig 1A and 1B). The median qHBsAg (log IU/mL) of patients achieving low levels (<120 IU/mL)(n = 10) versus those who did not (n = 82) were different after the first year: baseline (3.42 vs. 3.69; p = ns), year 1 (2.75 vs. 3.59; p<0.001), year 3 (2.39 vs. 3.52; p<0.001), year 5 (1.93 vs. 3.40; p<0.001), and year 8 (1.27 vs. 3.36; p<0.001).

In order to demonstrate the presence of two different kinetics of qHBsAg decline we used the LMM for repeated measurements of HBsAg levels (log IU/mL). The decline of qHBsAg (log IU/mL x year) during therapy was -0.102 and the median time to achieve HBsAg loss was 6.04 years. However, the slope was significantly greater in patients who achieved low HBsAg levels during follow-up (-0.257) compared to those who did not (-0.057) (p<0.001). The median levels of qHBsAg and the complete mathematical mixed model are shown in Fig 1A and 1B for better understanding.

Diagnostic accuracy of qHBsAg decline to predict low HBsAg levels and seroclearance

The speed of qHBsAg decline was evaluated as the differences of qHBsAg values (log IU/mL) from baseline to year 1 (Delta1, Δ1), 3 (Delta3, Δ3) and 5 (Delta5, Δ5). Patients achieving low HBsAg levels (<120 IU/mL) during follow-up showed higher Δ1 (0.12), Δ3 (0.45) and Δ5 (0.82) values compared to those who did not achieve them (Δ1 of 0.03, Δ3 of 0.12 and Δ5 of 0.21) (p<0.05 in all cases). The diagnostic accuracy (AUROC, 95%CI) to identify patients who achieved qHBsAg<120 IU/mL was 0.77 (0.64–0.9) for Δ1 and 0.89 (0.81–0.97) for Δ3 (Fig 2A).

Table 2. Characteristics of the patients included according to qHBsAg during follow-up.

	qHBsAg <120 IU/mL	qHBsAg >120 IU/mL	P
Baseline	**N = 13÷**	**N = 82**	
Age, years	52 (19–77)	41 (18–66)	0.33
Males, n (%)	9 (69)	63 (73)	0.8
Fibrosis (Metavir)			
F0-F1	8 (62)	63 (73)	0.3
F≥2	5 (39)	23 (27)	
ALT, IU/mL	33 (17–177)	40 (11–252)	0.9
DNA, IU/mL	2864 (10–1·10^8)	32998 (49–1.68·10^8)	0.13
<20000	9 (69)	36 (44)	0.09
>20000	4 (31)	46 (56)	
qHBsAg, IU/mL	1822 (137–187330)	4863 (245–54924)	0.14
<1000	5 (39)	9 (11)	
>1000	8 (61)	73 (89)	0.02
Genotype, n(%)**			
A	1 (17)	13 (19)	0.87
D	2 (33)	37 (55)	
Others (B,C,E,F,G)	3 (50)	17 (26)	
IL28, n(%)***			
CC	5 (56)	30 (42)	0.7
CT/TT	4 (34)	41 (58)	
Origin, n(%)			
European	12 (92)	40 (49)	0.003
Non-European	1 (8)	42 (51)	
Treatment initiation			
<2006	5 (38)	8 (10)	0.05
>2006	8 (62)	74 (90)	
Antiviral treatment			
ETV/TDF	5 (38)	67 (82)	0.001
Others (LAM,ADV,LdT)	8(62)	15 (18)	
Follow-up	**N = 13÷**	**N = 82**	
Length of treatment, years	8(2–15)	5.9 (1–14)	0.001
< 6 years, n(%)	3 (23)	46 (56)	0.003
> 6 years, n(%)	10 (77)	36 (44)	
Delta (Δ) of HBsAg (Log IU/mL)	**N = 10***	**N = 82**	
1 year	0.12	0.03	0.008
3 years	0.45	0.12	<0.001
5 years	0.82	0.21	<0.001
8 years	1.2	0.29	0.001
10 years	1.8	0.59	0.009
HBsAg seroclearance, n (%)	4 (40)	0 (0)	<0.001

Quantitative variables shown as median and ranges, ALT alanine aminotransferase, LAM lamivudine, ADV adefovir, TDF tenofovir, ETV entecavir, LdT telbivudine. Thirteen out of 95 patients (13.68%) achieved low levels of qHBsAg (< 120 IU/mL). However, 3 patients had low levels of qHBsAg (<120 IU/mL) at baseline and were excluded from the kinetic analyses of qHBsAg(*).

** Data available of 73 patients

***Data available of 80 patients

https://doi.org/10.1371/journal.pone.0188303.t002

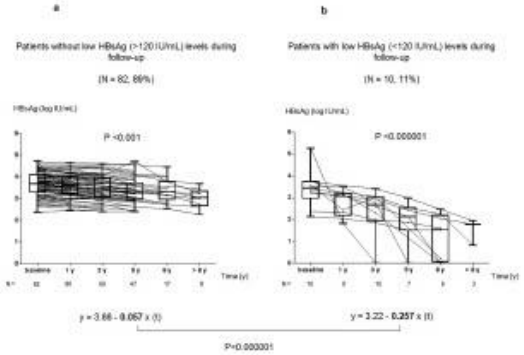

Fig 1. A and B. Different kinetics of qHBsAg in HBeAg-negative CHB patients receiving NAs. Fig 1A shows the kinetics of qHBsAg (log IU/mL) in patients who did not achieve low levels (>120 IU/mL) during follow-up (n = 82). Fig 1B shows the kinetics of qHBsAg in patients who achieved low levels (<120 IU/mL) during follow-up (n = 10). Using a mathematical mixed model for repeated measurements, the slope in patients who achieved low levels of HBsAg (y = 3.22–**0.257**x(t)) was significantly greater compared to those who did not (y = 3.66–**0.057**x(t)) (p <0.001).

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A cut-off of Δ3>0.3 log IU/mL had a S of 100%, Sp of 81%, PPV of 42% and NPV of 100% to identify patients with HBsAg <120 IU/mL during follow-up. We analyzed the baseline variables to predict the faster qHBsAg decline at third year (Δ3>0.3 log IU/mL) and only the median HBV DNA level was higher in those patients who achieved faster reduction (313372 IU/mL) compared to those who did not (16799 IU/mL)(p = 0.03)(data not shown). The diagnostic accuracy (AUROC, 95%CI) of qHBsAg decline from baseline (delta) to predict HBsAg seroclearance was 0.79 (0.6–0.98) at year 1 (Δ1) and 0.96 (0.92–1) at year 3 (Δ3)(Fig 2B). A cut-off of qHBsAg Δ3 >0.3 log IUmL-1 showed a S and NPV of 100% (in both) to predict HBsAg loss, and a Sp of 74% and a PPV of 17%.

Probability of low HBsAg levels and seroclearance according to qHBsAg decline

The cumulative probability (1-cumulative survival) to achieve low qHBsAg (<120 IU/mL) during follow-up in patients with a Δ3 >0.3 log/IUmL (n = 24) after 5, 8 and 10 years of NAs therapy was 22%, 45% and 56%, respectively (log-rank<0.001) (Fig 3). Moreover, the cumulative rate (1-cumulative survival) of HBsAg loss in patients who had achieved low qHBsAg (n = 13) was 17%, 29% and 43% at 5, 8 and 10 years, respectively (log-rank<0.001) (Fig 4A).

The cumulative rate (1-cumulative survival) of HBsAg seroclearance in patients achieving a Δ3>0.3 log IU/mL was 9%, 21% and 32% after 5, 8 and 10 years of antiviral treatment with NAs, respectively, according to the Kaplan-Meier analyses (log-rank = 0.01) (Fig 4B).

Discussion

This study was a long-term retrospective follow-up analysis evaluating the kinetics of HBsAg levels in frozen serum samples of HBeAg-negative CHB patients receiving NAs in clinical practice. The study clearly showed, a very slow decrease of HBsAg levels (-0.1log IU/mL x year) in most patients, similar to previous reports[10, 14, 23].Thus, HBsAg seroclearance occurred in only 4% of our patients after a median time of 6 years of NAs therapy. However, it is important to note that in around 14% of the HBeAg-negative CHB patients receiving NAs, low levels or a faster decline of qHBsAg occurred, showing a greater probability to achieve seroclearance.

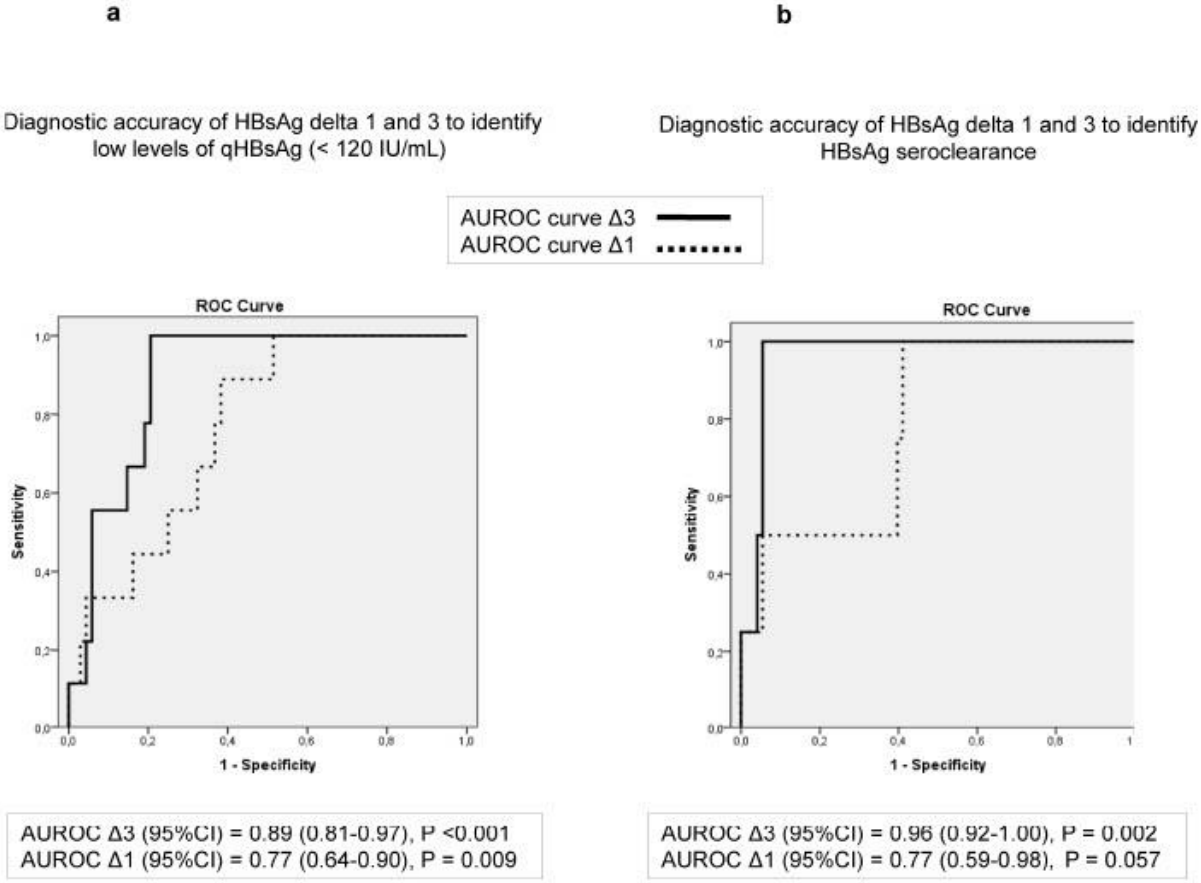

Fig 2. A and B. Diagnostic accuracy of qHBsAg delta at 1 and 3 years to identify low levels of HBsAg (Fig 2A) and seroclearance (Fig 2B) during follow-up. AUROC curve of delta at year 1 (Δ1) is depicted as a dotted line and delta at year 3 (Δ3) as a solid line.

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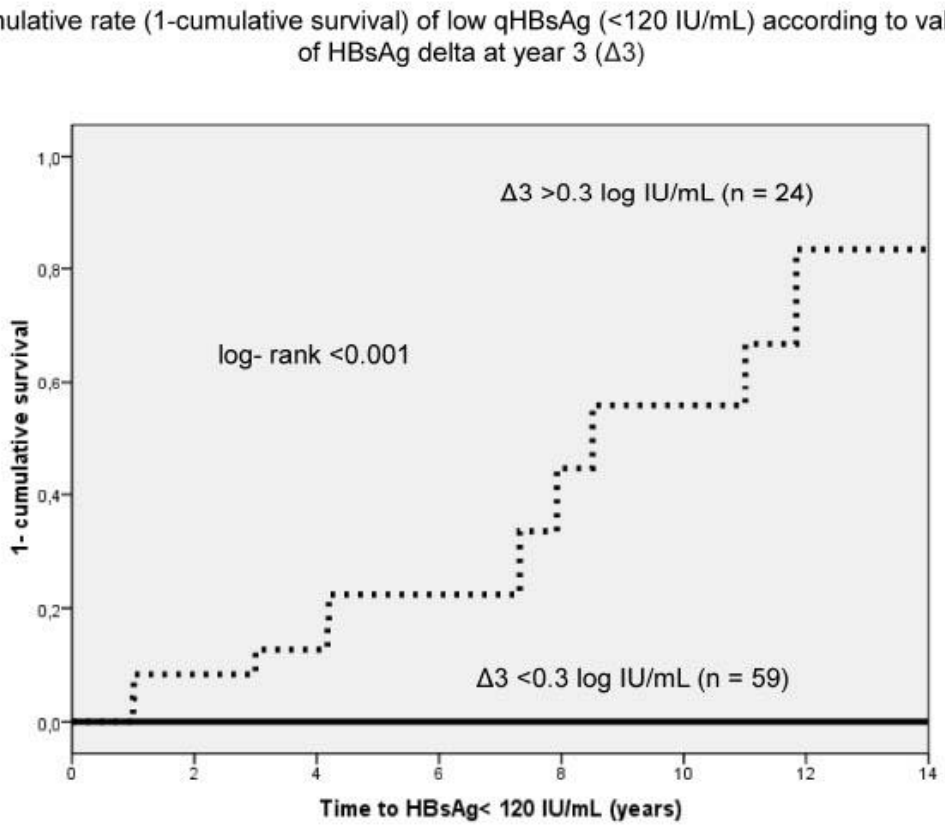
One of the strengths of our study is that it is focused on HBeAg-negative patients who represent the vast majority of cases in many areas, including Europe[22]. Moreover, the study describes the kinetics of HBsAg levels over a long period of treatment with NAs, with a median time of 6 years and a maximum of 15 years. Regarding the origin of our patients, 55% were Europeans. Non-European patients were younger, with a higher proportion of men and received a shorter duration of treatment which is probably explained by recent migratory movements. However, there were no differences in fibrosis stage, baseline ALT, HBV-DNA, HBV-genotype and qHBsAg levels. Genotype D was the most frequent in both groups.

Functional cure of HBV, with HBsAg seroclearance remains the therapeutic endpoint in the treatment of CHB. However, our study has demonstrated a lack of baseline characteristics to predict low levels of HBsAg (<120 IU/mL) or HBsAg loss during follow-up. Previous studies have proposed that the kinetics of HBsAg during peg-IFN therapy is more accurate to predict HBsAg seroclearance than baseline qHBsAg, HBV-DNA or ALT levels in both HBeAg-positive[7] and-negative[6] patients. However, the utility of qHBsAg during NAs therapy is less



	2 y	4 y	6 y	8 y	10 y	12 y	14 y
Δ3 >0.3 log IU/mL (patients at risk, n)	0.08 (22)	0.13 (18)	0.22 (11)	0.45 (5)	0.56 (4)	0.83 (1)	0.83 (1)
Δ3 <0.3 log IU/mL (patients at risk, n)	0.00 (56)	0.00 (47)	0.00 (23)	0.00 (6)	0.00 (4)	0.00 (3)	0.00 (1)

Fig 3. Cumulative probability (1-cumulative survival) of low qHBsAg according to values of HBsAg delta at the third year. The cumulative probability of patients with delta at year 3 (Δ3) >0.3 log IU/mL (n = 24) is depicted as a dotted line and those with Δ3 <0.3 log IU/mL (n = 59) as a solid line.

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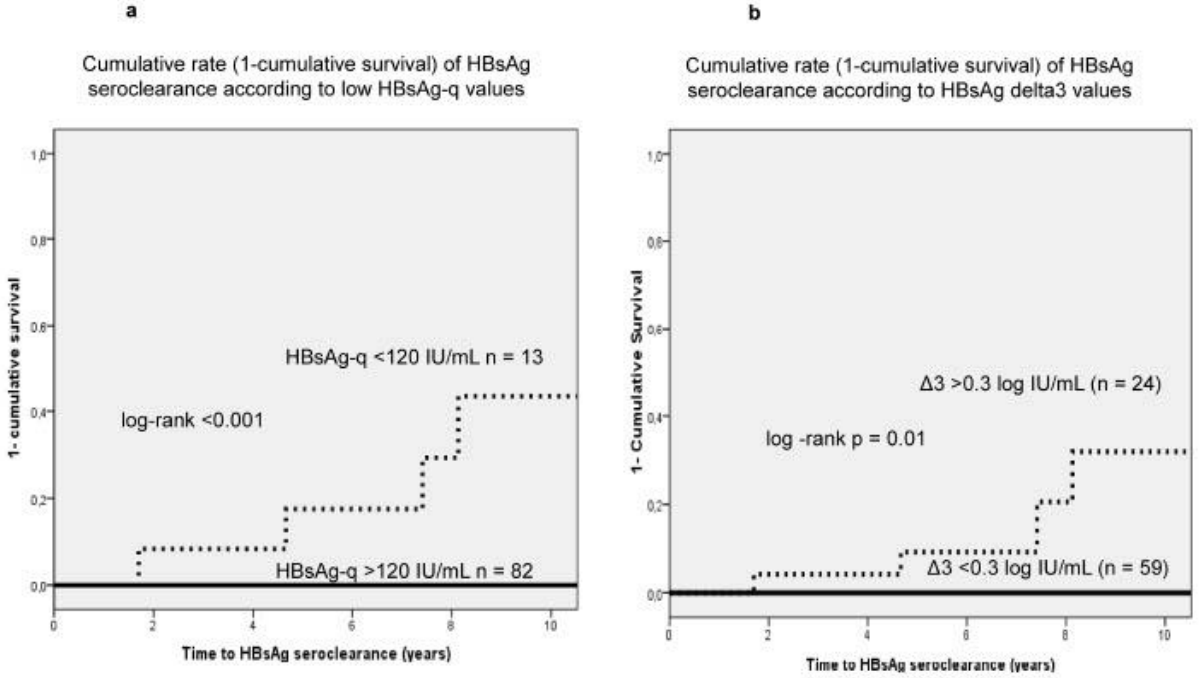
clear. Seto et al.[12] found lower median baseline HBsAg levels in patients achieving HBsAg seroclearance. In contrast, our study found no differences in baseline characteristics to predict HBsAg loss, and only a greater HBsAg reduction during the first years of antiviral treatment identified patients who would achieve lower HBsAg levels and seroclearance during follow-up. It is important to note that the study of Seto et al.[12] included 61% of HBeAg-positive patients.



	2 y	4 y	6 y	8 y	10 y
HBsAg <120IU/mL (patients at risk, n)	0.08 (11)	0.08 (10)	0.17 (9)	0.29 (6)	0.43 (4)
HBsAg >120 IU/mL (patients at risk, n)	0.00 (73)	0.00 (59)	0.00 (30)	0.00 (9)	0.00 (6)

	2 y	4 y	6 y	8 y	10 y
Δ3 >0.3 log IU/mL (patients at risk, n)	0.04 (23)	0.04 (20)	0.09 (14)	0.21 (7)	0.32 (6)
Δ3 <0.3 log IU/mL (patients at risk, n)	0.00 (56)	0.00 (47)	0.00 (23)	0.00 (6)	0.00 (4)

Fig 4. A and B. Cumulative rate (1-cumulative survival) of HBsAg seroclearance according to low levels of qHBsAg or delta at year 3. Fig 4A. The cumulative probability of patients with low qHBsAg (<120 IU/mL) is depicted as a dotted line and those with high levels (>120 IU/mL) as a solid line. Fig 4B. The cumulative probability of patients with delta at year 3 (Δ3) >0.3 (log IU/mL) is depicted as a dotted line and those with Δ3 <0.3 (log IU/mL) as a solid line.

https://doi.org/10.1371/journal.pone.0188303.g004

It has been proposed that the slow decline of HBsAg levels in HBeAg-negative patients receiving NAs might be explained by its association with the cccDNA and the independence of viral replication[14]. A previous study suggested that a qHBsAg decline at 2 years was predictive of HBsAg loss[24]. Similarly, we observed an annual reduction of qHBsAg of -0.102log IU/mL. This reduction was identical to that described by Seto et al.[12], despite the inclusion of only HBeAg-negative patients in our study. Interestingly, we found that 3 patients had low levels of qHBsAg (<120 IU/mL) at baseline. Two of these patients had advanced fibrosis and were 63 and 77 years old, and none of the 3 cleared HBsAg during follow-up. These results are consistent with those of Jang et al. who reported lower qHBsAg in elderly patients and in those with more advanced disease[25]. In our study, we did not find differences regarding levels of qHBsAg according to HBV-genotype. These results are similar to those recently published by Marcellin et al.[26] in which the HBV-genotype did not have impact in qHBsAg levels of patients receiving NAs therapy.

We also investigated the kinetics of qHBsAg during NAs therapy and found low levels or a faster qHBsAg decline during the first 3 years of NAs therapy in 14% of HBeAg-negative

patients. We analyzed the kinetics of qHBsAg to reach low HBsAg levels (<120 IU/mL) during follow-up since this has been proposed as a good predictor of HBsAg loss in HBeAg-negative patients after discontinuation of LAM treatment [13]. In patients who achieved low HBsAg levels (<120 IU/mL) the reduction of qHBsAg (log IU/mL) was accelerated. In order to demonstrate the presence of two different kinetics of qHBsAg decline (negative slope) we used the LMM for repeated measurements to identify the slope of qHBsAg (log IU/mL x year) during therapy. This negative slope was significantly greater in patients who achieved low levels of HBsAg during follow-up (-0.26) compared to those who did not (-0.06). Moreover, a difference of qHBsAg greater than 0.3 log IU/mL from baseline to the third year (Δ3) showed good accuracy (AUROC of 0.89) with a PPV of 42% to identify patients with low HBsAg levels (<120 IU/mL) during follow-up. Thus, patients who presented a faster HBsAg decline, greater than 0.3 log IU/mL during the first 3 years, had a probability of 22% to achieve low values of HBsAg after 5 years of antiviral therapy. It is important to note that the only baseline variable related to the faster decline was the median HBV-DNA levels. However, DNA levels did not predict the HBsAg seroclearance.

Additionally, our study shows that all patients with HBsAg loss during follow-up showed a reduction of qHBsAg>0.3 log IU/mL during the first 3 years of NAs therapy. Similarly, Seto et al. showed that an on-treatment reduction of HBsAg >0.166 log IU/mL/year was the optimal cut-off to predict HBsAg seroclearance[12]. However, our results have shown that the accuracy of Δ3 (AUROC of 0.96) is better than that at year 1 (AUROC of 0.79) to identify patients with HBsAg loss, with a probability of 21% after 8 years of therapy. In contrast, none of the patients with a decline of qHBsAg lower than 0.3log IU/mL or those who reduced this level after 5 years of NAs therapy achieved HBsAg seroclearance.

To identify patients with a rapid drop of HBsAg levels during NAs therapy have clinical implications[3]. 1) The reduction of HBsAg levels during NAs therapy is a good predictor of on-treatment HBsAg seroclearance[12]. 2) Duration of NAs is the best predictive variable to identify patients with sustained virological response 12 months after NAs discontinuation[27] 3) Low levels of HBsAg before NAs discontinuation has been described as good predictive variable to identify patients with higher probability of HBsAg seroclearance[13]. 4) The newest European Clinical Practice Guidelines of Hepatitis B recommend to consider discontinuation of NAs long-term therapy in non-cirrhotic HBeAg-negative patients with virological response [28]. Thus, our study clearly shows that patients with a significant decrease of HBsAg levels during the first 3 years after NAs will achieve low levels of HBsAg during the next years being possible to evaluate the discontinuation of NAs. In contrast, those patients without decrease of HBsAg levels at year 3 after of NAs will not achieve HBsAg clearance during follow-up being necessary to maintenance therapy chronically or to evaluate new antiviral drugs to achieve HBsAg clearance[29].

Our study has some limitations. It is a retrospective study with non-homogeneous treatment duration with NAs. The majority of patients were receiving ETV or TDF (76%), but those with longer follow-up started treatment with LAM or ADV. However, it does reflect the real clinical practice in Europe and the evolution of chronic hepatitis B treatments during the last decades[22]. Another limitation is that we could not determine the HBV-genotype in all of our patients. However, we did not find differences in qHBsAg at baseline or during the follow-up in those patients with genotype determination (77%) as it has been recently reported[26].

Conclusions

Our results confirm that qHBsAg decline is very slow, and the probability of HBsAg seroclearance in HBeAg-negative patients receiving NAs is low. Nevertheless, 14% of these patients

showed low levels or a faster reduction in qHBsAg. These results suggest that monitoring qHBsAg levels at year 3 of therapy with NAs could be more useful to predict treatment response than baseline variables in HBeAg-negative patients. In patients with a qHBsAg decline lower than 0.3 log IU/mL at year 3 the probability of achieving HBsAg seroclearance after a long period of antiviral treatment with NAs is very low.

Supporting information

S1 Table.
(XLSX)

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4.2. Article 2

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On-therapy HBsAg kinetics can predict HBsAg loss after nucleos(t)ide analogues interruption in HBeAg-negative patients. The cup is half full and half empty

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ABSTRACT

Background: Nucleos(t)ide analogues withdrawal may improve HBsAg loss rates. However, conditions to select patients are not well established.
Aims: to evaluate the impact of HBsAg kinetics before treatment interruption on post-treatment response.
Methods: Longitudinal, ambispective study in non-cirrhotic chronic hepatitis B HBeAg-negative patients, analysing on-treatment and post-treatment HBsAg kinetics. On-treatment HBsAg kinetics diagnostic accuracy (AUROC) to identify HBsAg loss was evaluated.
Results: 52 HBeAg-negative patients stopped treatment after 8.2 years, and 6 (11.5%) achieved HBsAg loss one year after withdrawal. Multivariate analysis showed that on-treatment HBsAg kinetics was related to HBsAg loss (OR=0.10; 95%CI=0.016–0.632; $p = 0.014$) with a high diagnostic accuracy (AUROC=0.935). A significant HBsAg decline ≥1 log10 IU/mL showed a positive and negative predictive value of 50% and of 97.6%, respectively. After treatment interruption, HBsAg decline speed (log10 IU/mL/year) accelerated in patients treated >6 years (from -0.06 to -0.20, $p<0.05$) and remained stable in treated <6 years (from -0.12 to -0.12 p=ns).
Conclusions: On-treatment HBsAg kinetics can predict post-treatment HBsAg loss rate. Half of patients with a significant HBsAg decline can eliminate HBsAg the first year after withdrawal. Post-treatment HBsAg decline is faster not only in patients who lost the HBsAg but also in those who remain HBsAg-positive.

1. Introduction

The hepatitis B virus (HBV) infection remains a global public health problem. In chronic hepatitis B (CHB), long-term administration of nucleos(t)ide analogues (NA) with high barrier to resistance, i.e., entecavir (ETV) or tenofovir disoproxil fumarate (TDF), is the treatment of choice [1]. The optimal therapeutic endpoint is the hepatitis B surface antigen (HBsAg) loss, which indicates suppression of HBV replication and viral protein expression. However, in CHB e antigen (HBeAg)-negative patients the decline of HBsAg during NA therapy is very slow (−0.1 log10 IU/mL/year) and HBsAg loss very infrequent (0.6–4.6%) [2–5]. Therefore, European Association for the study of the Liver (EASL) and American Association

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; NA, nucleos(t)ides analogues; HBV, hepatitis B virus; OR, odds ratio; CI, confidence interval; CHB, chronic hepatitis B; ETV, entecavir; TDF, tenofovir disoproxil fumarate; EoT, end of treatment; TE, transient elastography; VR, virological relapse; CR, clinical relapse; ALT, alanine aminotransferase; ULN, upper limit of normality; IL, interleukin; IQR, interquartile range; Peg-IFN, pegylated interferon; AUROC, area under receiver operating characteristic; S, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; +LR, positive likelihood ratio; -LR, negative likelihood ratio.

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for the Study of Liver Diseases (AASLD) guidelines recommended during years to maintain NA until HBsAg loss [6,7] and to monitor on-treatment HBsAg kinetics [2,5,8].

The Asian-Pacific consensus suggested in 2008 to discontinue NA therapy in CHB HBeAg-negative patients with undetectable HBV-DNA in three consecutive determinations separated by 6 months [9]. Important studies, not only in Asian population [10] but also in Caucasian patients [11,12] have shown that NA withdrawal after years of viral suppression, can improve HBsAg loss rates. Thus, EASL guidelines accepted in 2017 that NA could be discontinued in HBeAg-negative patients after 3 years of DNA suppression [1]. Recent studies evaluating NA withdrawal have shown that low HBsAg levels, at baseline and at the end of treatment (EoT), are related to HBsAg loss [10,13,14]. However, the optimal duration of therapy before discontinuation remains unclear and there are not well-established conditions to select these patients [15].

The hypothesis of our study was that HBsAg kinetics during NA therapy could affect the HBsAg kinetics after NA withdrawal. Thus, the primary aim was to evaluate HBsAg decline before and after treatment withdrawal in non-cirrhotic CHB HBeAg-negative patients. Secondary aim was to evaluate the influence of on-treatment HBsAg kinetics on post-treatment responses.

2. Materials and methods

2.1. Patients and study design

This is a single centre, longitudinal, ambispective study analysing HBsAg levels in non-cirrhotic CHB HBeAg-negative patients during NA therapy and after withdrawal. Patients were eligible if they had received a stable NA dose during a minimum of 3 years and achieved virological response (HBV-DNA below the limit of quantification <13 IU/mL).

Recruitment period was from December 2017 to October 2019. Exclusion criteria were: CHB HBeAg-positive patients, human immunodeficiency virus or hepatitis D virus coinfection, immunosuppressive therapy, history of hepatocellular carcinoma, transient elastography (TE) >9.4 kPa [16], absence of HBsAg determination before NA treatment, or inability to perform a close follow-up. HBsAg levels were determined before NA treatment, at year 1 and 3 after initiation and 1 year before withdrawal. Protocol visits were at EoT, and at weeks 4, 12, 24 and 48 after interruption.

The study protocol was approved by the Ethical Committee of our Institution "Comitè Ètic d'Investigació Clínica - Parc de Salut Mar", study reference 2018/7939/I, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. Variables and clinical definitions

The HBV-genotype was collected from electronic data. Demographic data and TE were assessed at EoT. After NA cessation, liver function, HBV-DNA, HBsAg levels, HBeAg, anti-HBe and anti-HBs were assessed at every protocol visit.

HBV-DNA was measured by polymerase chain reaction with a limit of quantification of 13 IU/mL (Versant HBV DNA 1.0®, Siemens Medical Solutions Diagnostics, New York, USA). Serum HBsAg quantification was introduced in our laboratory in July 2014 and was evaluated by Electro-chemiluminescence immunoassay Elecsys® HBsAgII (Roche Diagnostic, Rotkreuz, Switzerland). The assay ranged from 0.05 to 52,000 IU/mL. In highly concentrated samples above the upper limit, the value of manual dilution was multiplied by the dilution factor [17]. In patients who started NA treatment before July 2014, the HBsAg was analysed in cryopreserved serum samples part of the private collection (C.0000956) of the IMIM (Hospital del Mar Medical Research Institute) and were extracted in fasting conditions and centrifuged at 3000 rpm before preservation at −30 °C [5].

The on-treatment HBsAg kinetics was evaluated at different time points, calculated as delta of HBsAg levels from NA initiation to year 1 (Delta_1), year 3 (Delta_3) and to EoT (Delta_EoT) and the off-treatment HBsAg kinetics as delta of HBsAg from EoT to one year after interruption. Virological relapse (VR) was defined as positive HBV-DNA at any time point. Significant virological relapse (SVR) as HBV-DNA above 2000 IU/mL. Clinical relapse (CR) as an elevation of alanine aminotransferase (ALT) above 2 times the upper limit of normality (ULN) and HBV-DNA >2000IU/mL at any time point [18]. Sustained off-treatment response was defined as persistent ALT<2xULN and HBV-DNA<2000IU/mL and patients in "grey-zone" as ALT>2xULN or DNA>2000IU/mL at week 48 after withdrawal. Retreatment with NA was indicated if patient fulfilled any of the following criteria: severe flare (ALT>10xULN in two consecutive blood test for 2 weeks), moderate flare (ALT 5–10xULN in two consecutive blood test for 4 weeks) or mild persistent flare (ALT 2–5xULN and DNA>2.000 IU/ml persisting for more than 6 months).

2.3. Statistical analysis

Quantitative variables were expressed as medians and interquartile ranges (IQR, Q1-Q3). Categorical variables were expressed as proportions. Continuous variables were compared by the Mann–Whitney *U* test, Wilcoxon or Kruskall-Wallis when appropriate and categorical by the Pearson chi-square test or Fisher test. Differences between patients who achieved HBsAg loss and those who did not, were analysed by univariate analysis. Variables showing a *P* value<0.05 were included in a multivariate forward stepwise logistic regression analysis to determine independent predictors of HBsAg loss and expressed as odds ratio (OR) and 95% confidence interval (95%CI). The diagnostic accuracy of HBsAg decline to identify patients at risk of losing HBsAg was assessed using the area under the receiver operator characteristic (AUROC) curve (95%CI). The optimal HBsAg decline cut-off value to identify HBsAg loss was selected on the basis of sensitivity (S), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR) and negative likelihood ratio (-LR). Bootstrapping was used to perform an internal validation of the HBsAg kinetic diagnostic accuracy by generating 1000 resampling sets with random replacement. The results of the internal bootstrap validation gave estimation for the AUROC with the median (Percentile 5-Percentile 95). The correlation between treatment duration (years) and HBsAg decline (log 10 IU/mL) was evaluated by Pearson's coefficient (r). The cumulative HBsAg loss rate was evaluated by the Kaplan-Meier method (Breslow and Log-rank tests).

All statistical tests were two-sided and a *P* value<0.05 was considered significant. Analyses were performed with the SPSS® 25.0 (SPSS Inc., Chicago, IL, USA) and the AUROC of HBsAg decline and Bootstrapping were calculated with MedCalc® v19.1.3 (MedCalc Software).

3. Results

3.1. Study population and baseline characteristics

From January 1999 to December 2017, 148 CHB HBeAg-negative patiens started NA treatment in our hospital. Twenty-seven (18.2%) were lost during follow-up and 9 (6.2%) lost the HBsAg during therapy (6 under NA treatment [5] and 3 under pegylated interferon [peg-IFN] add-on strategy [17]). Therefore, 112 patients were evaluated, and sixty were excluded: 20 (17.8%) with cirrhosis, 6

Table 1
HBsAg kinetics and NA treatment duration.

	N = 52	NA treatment duration 3–6 years (*n* = 11, 21,2%)	NA treatment duration 6–9 years (*n* = 22, 42,3%)	NA treatment duration >9 years (*n* = 19, 36,5%)	P value
Before antiviral treatment					
Males, n (%)	39 (75)	7 (63,6)	17 (77,3)	15 (78,9)	ns
Caucasian, n (%)	30 (57,7)	3 (27,3)	10 (45,5)	17 (89,5)	<0,001
HBV Genotype, n (%)					ns
A	13 (25)	3 (27,3)	6 (27,3)	4 (21,1)	
B	2 (3,8)	0 (0)	1 (4,5)	1 (5,3)	
C	2 (3,8)	0 (0)	1 (4,5)	1 (5,3)	
D	32 (61,5)	8 (72,7)	12 (54,5)	12 (63,2)	
E	3 (5,8)	0 (0)	2 (9,1)	1 (5,3)	
HBsAg (IU/mL)	3821 (1587–7144)	5493 (1698–7005)	3206 (1558–8092)	4084 (1700–7007)	ns
HBsAg (IU/mL), n (%)					ns
<1000	10 (19,2)	2 (18,2)	4 (18,2)	4 (21,1)	
1000–10,000	34 (65)	8 (72,7)	15 (68,2)	11 (57,9)	
>10,000	8 (15,4)	1 (9,1)	3 (13,6)	4 (21,1)	
During NA treatment					
NA treatment, n (%)					ns
Tenofovir	34 (65)	8 (72,7)	15 (68,2)	11 (57,9)	
Entecavir	17 (33)	3 (27,3)	7 (31,8)	7 (36,8)	
Lamivudine	1 (2)	0 (0)	0 (0)	1 (5,3)	
Treatment duration (years)	8,17 (6,5–10,3)	3,98 (3,5–5,1)	7,95 (6,8–8,4)	11,25 (10,2–13,6)	<0,001
Delta_1 HBsAg (log10 IU/mL)	−0,01 (0,03-(−0,09))	−0,03 (−0,19-(−0,02))	0,01 (−0,05–0,07)	−0,02 (−0,17–0,01)	ns
Delta_3 HBsAg (log10 IU/mL)	−0,12 (−0,09(−0,24))	−0,15 (−0,44-(−0,07))	−0,07 (−0,17–0,03)	−0,15 (−0,28-(−0,04))	ns
Delta_1pre-EoT HBsAg (log10/mL)	−0,09 (−0,15-(−0,01))	−0,12 (−0,17-(−0,01))	−0,09 (−0,14-(−0,07))	−0,07 (−0,16-(−0,02))	ns
Delta of HBsAg per year (log10IU/mL/year)	−0,06 (−0,11-(−0,03))	−0,06 (−0,14- (−0,02))	−0,06 (−0,11-(−0,02))	−0,06 (−0,10 -(−0,04))	ns
End of treatment (EoT)					
Age (years)	52 (43–59)	44 (42–53)	53 (43–61)	55 (50–59)	ns
HBsAg (IU/mL)	817 (197–2486)	1350 (332–2639)	817 (221–3721)	558 (57–1628)	ns
HBsAg (IU/mL)					0,053
<100	8 (15,4)	0 (0)	2 (9,1)	6 (31,6)	
100–1000	19 (36,5)	4 (36,4)	10 (45,5)	5 (26,3)	
>1000	25 (48,1)	7 (63,6)	10 (45,5)	8 (42,1)	
Delta_EoT HBsAg (log 10 IU/mL)	−0,51 (−0,93–0,21)	−0,29 (−0,88-(−0,07))	−0,45 (−0,74-(−0,15))	−0,82 (−1,3-(−0,51))	0,017
Delta_EoT HBsAg, n (%)					0,105
<−1 log10 (IU/mL)	42 (80,8)	10 (90,9)	19 (86,4)	13 (68,4)	
≥−1 log10 (IU/mL)	10 (19,2)	1 (9,1)	3 (13,6)	6 (31,6)	
48 Weeks after NA interruption					
HBsAg (IU/mL)	364 (30–1973)	1022 (339–2394)	590 (104–2584)	130 (0–602)	0,097
HBsAg (IU/mL), n (%)					0,017
<100	13 (26,5)	1 (10)	4 (19)	8 (44,4)	
100–1000	18 (36,7)	3 (30)	9 (42,9)	6 (33,3)	
>1000	18 (36,7)	6 (60)	8 (38,1)	4 (22,2)	
Delta_1post-EoT HBsAg (log10 UI/mL)	−0,19 (−0,57-(−0,08))	−0,12 (−0,18-(−0,02))	−0,19 (−0,41-(−0,09))	−0,41 (−1,30-(−0,06))	ns
NA reintroduction, n (%)	3 (5,8)	1 (9,1)	1 (4,5)	1 (5,3)	ns
Sustained off-treatment response, n (%)	21 (40,4)	4 (36,4)	13 (49,1)	4 (21,1)	ns
HBsAg loss, n (%)	6 (11,5)	0 (0)	0 (0)	6 (31,6)	0,003

Quantitative variables are expressed as median (IQR). Qualitative variables are expressed as n (%). HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; NA: nucleos(t)id analogue; EoT: end of treatment; Sustained off-treatment response (ALT<2xULN and HBV-DNA<2000IU/mL).

(4.1%) without baseline serum sample, and 34 (30.4%) declined their participation. Fifty-two patients were included, 75% were males, the median (IQR) age was 52 (43–59), 65% received TDF, and treatment duration was 8.17 (6.51–10.29) years. HBV-genotype was evaluated in all patients and 32 (61.5%) were infected by genotype D. Main characteristics of included patients are depicted in Table 1.

3.2. HBsAg kinetics during therapy and after withdrawal

The HBsAg level (IU/mL) was 3821 (1587–7144) before antiviral treatment, 817 (197–2486) at EoT, and 364 (30–1973) 48 weeks after withdrawal. The HBsAg decline during NA therapy (log10 IU/mL) was −0.01 (0.03-(−0.09)) at year 1, −0.12 (−0.09(−0.24)) at year 3 and −0.51 (−0.93-(−0.21)) at EoT. The speed of HBsAg decline during NA therapy (log10 IU/mL/year) was −0.06 (−0.11-(−0.03)). We observed a correlation between treatment duration (years) and HBsAg decline during treatment (log 10 IU/mL)(r= −0.51; p<0.001). Therefore, the Delta_EoT (log 10 IU/mL) was higher as longer the treatment was: −0.29 in patients treated from 3 to 6 years (n = 11), −0.45 in treated from 6 to 9 (n = 22), and −0.82 in those treated >9 years (n = 19)(p = 0.017) (Table 1 and Fig. 1). However, the speed of HBsAg decline was the same in the three treatment periods −0.06 vs. −0.06 and −0.06 log10 IU/mL/year; p=ns).

The decline of HBsAg one year after NA interruption was −0.19 log10 IU/mL. Therefore, the speed of HBsAg decline after stopping treatment was faster than during therapy (−0.19 vs. −0.06 log10 IU/mL/year; p<0.001). The HBsAg decline after NA withdrawal compared to one year before, was similar in patients treated from 3 to 6 years (−0.12 vs. −0.12; p=ns) but accelerated in those treated from 6 to 9 (−0.19 vs. −0.09; p = 0.015), or >9 years (−0.41 vs. −0.07; p = 0.01)(Table 1 and Fig. 1). After NA interruption, the speed of HBsAg decline was faster not only in patients with HBsAg loss (−1.33 vs. −0.14 log10 IU/mL/year; p = 0.046) but also in those with persistence of HBsAg (−0.18 vs. −0.05 log10 IU/mL/year; p<0.001).

3.3. Predictors of HBsAg loss

Differences between patients who achieved HBsAg loss (n = 6) and those who did not (n = 46) were evaluated by univariate

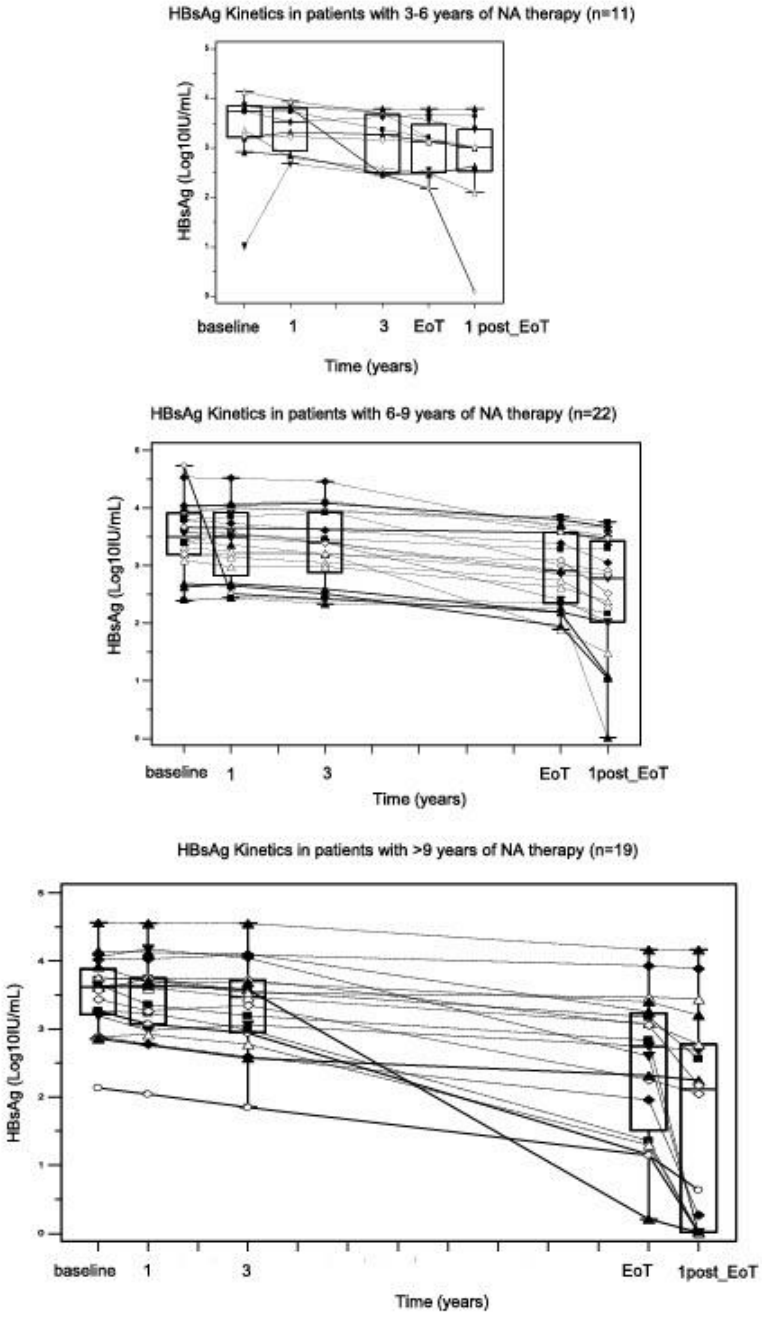


Fig. 1. HBsAg kinetics and treatment duration.
The kinetics of HBsAg (Delta_EoT HBsAg)(log 10 IU/mL) are depicted according to treatment duration in Fig. 1a (from 3 to 6 years; $n = 11$), Fig. 1b (from 6 to 9; $n = 22$) and Fig. 1c (longer than 9 years; $n = 19$). Each line represents one patient, and distribution of HBsAg values in each time point is represented by Box and whisker plots. The decline of HBsAg was higher as longer the treatment was, but the speed of HBsAg decline was the same independently of treatment duration. After stopping treatment, the speed of HBsAg decline was faster than during therapy, especially in those receiving NA longer than 6 years.

analysis (Table 2). HBsAg level before NA therapy was similar between groups (3062 and 3822 IU/mL; p=ns) as the Delta HBsAg at first and third year of treatment (−0.01 vs. −0.01 and −0.12 vs. −0.19 log 10 IU/mL, respectively, p=ns). However, treatment duration was longer in patients with HBsAg loss compared who did not (12.8 vs. 7.9 years; $p = 0.001$). All patients with HBsAg loss (100%) were treated longer than 9 years, compared with 28.3% of those without HBsAg loss ($p = 0.004$). Moreover, the speed of HBsAg decline was faster in patients with HBsAg loss compared with those who did not (0.14 vs. 0.05 log10 IU/mL/year; $p = 0.006$). As

Table 2
HBsAg kinetics and variables associated with HBsAg loss.

	N = 52	HBsAg+ (n = 46, 88,5%)	HBsAg- (n = 6, 11,5%)	P value	OR (95% CI), p value
Before antiviral treatment					
Males, n (%)	39 (75)	34 (73,9)	5 (83,3)	ns	
Caucasian ethnicity, n (%)	30 (57,7)	24 (52,2)	6 (100)	0,026	
HBV Genotype D, n (%)	32 (61,5)	28 (60,9)	4 (66)	ns	
HBsAg (IU/mL)	3821 (1587–7144)	3822 (1578–7159)	3062 (1822–4495)	ns	
HBsAg (IU/mL)				ns	
<1000	10 (19,2)	9 (19,6)	1 (16,7)		
1000–10,000	34 (65)	30 (65,2)	4 (66,7)		
>10,000	8 (15,4)	7 (15,2)	1 (16,7)		
During NA treatment					
Antiviral treatment, n (%)				ns	
Tenofovir	34 (65)	30 (65,2)	4 (66,7)		
Entecavir	17 (33)	15 (32,6)	2 (33,3)		
Lamivudine	1 (2)	1 (2,2)	0 (0)		
Treatment duration (years)	8,17 (6,5–10,3)	7,9 (5,9–10,1)	12,8 (10,3–16,2)	0,001	
Treatment duration, n (%)				0,004	
3–6 years	11 (21,1)	11 (23,9)	0 (0)		
6–9 years	22 (42,3)	22 (47,8)	0 (0)		
>9 years	19 (36,6)	13 (28,3)	6 (100)		
Add-on Peg-IFN, n (%)	19 (36,6)	18 (39)	1 (16,7)	ns	
Delta_1 HBsAg (log 10 IU/mL)	−0,01 (0,03-(−0,09))	−0,01 (−0,08–0,03)	−0,01 (−0,19–0,03)	ns	
Delta_3 HBsAg (log 10 IU/mL)	−0,12 (−0,09(−0,24))	−0,12 (−0,20- (−0,01))	−0,19 (−0,33-(−0,04))	ns	
Delta_1pre-EoT HBsAg (log10 IU/mL)	−0,09 (−0,15-(−0,01))	−0,09 (−0,14-(−0,02))	−0,16 (−0,29-(−0,01))	ns	
Delta_EoT HBsAg/year (log10IU/mL/year)	−0,06 (−0,11-(−0,03))	−0,05 (−0,10-(−0,02))	−0,14 (−0,16-(−0,11))	0,006	
End of treatment (EoT)					
Age (years)	52 (43–59)	51 (43–60)	54 (53–56)	ns	
HBsAg_EoT (IU/mL)	817 (197–2486)	1176 (258–2957)	21,4 (14,3–401)	0,002	
HBsAg (IU/mL)				0,001	
<100	8 (15,4)	4 (8,7)	4 (66,7)		
100–1000	19 (36,5)	17 (37,0)	2 (33,3)		
>1000	25 (48,1)	25 (54,3)	0 (0)		
Delta_EoT HBsAg (log 10 IU/mL)	−0,51 (−0,93–0,21)	−0,47 (−0,87- (−0,15))	−1,75 (−2,11- (−1,45))	<0,001*	**0.10 (0–016–0.632), p = 0.014**
Delta_EoT HBsAg, n (%)				0,001	
<−1 log10 (IU/mL)	42 (80,8)	41 (89,1)	1 (16,7)		
≥−1 log10 (IU/mL)	10 (19,2)	5 (10,9)	5 (83,3)		
48 Weeks after NA interruption					
HBsAg (IU/mL)	364 (30–1973)	590 (135–2489)	<0,05 (BLD)	0,001	
Delta_1post-EoT HBsAg/year (log10IU/mL/year)	−0,19 (−0,57-(−0,08))	−0,18 (−0,40-(−0,07))	−1,33 (−2,60-(−1,16))	0,02	

Quantitative variables are expressed as median (IQR). Qualitative variables are expressed as n (%). HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; NA: nucleos(t)id analogue; Peg-IFN: pegylated interferon; EoT: end of treatment; BLD: below limit of detection.

consequence, patients with HBsAg loss showed greater Delta_EoT HBsAg (−1.75 vs. −0.47 log10 IU/mL; p <0.001) and lower HBsAg levels before interruption (21.4 vs. 1176 IU/mL; $p = 0.002$).

Multivariate analysis including HBsAg_EoT, treatment duration and Delta_EoT, showed that only the Delta_EoT was associated with HBsAg loss after NA interruption (OR=0.10; 95%CI=0.016–0.632; $p = 0.014$).

3.4. Diagnostic accuracy of HBsAg decline to predict HBsAg loss

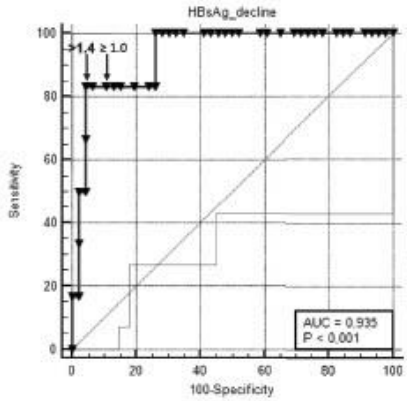
The diagnostic accuracy of HBsAg kinetics during antiviral treatment was excellent to identify patients at risk of losing HBsAg after treatment withdrawal. The AUROC (95% CI) of HBsAg decline was 0.935 (0.83–0.98) (Fig. 2). The bootstrap method showed a median AUROC (Percentile 5-Percentile 95) for the HBsAg kinetics of 0.75 - 0.99 to identify HBsAg loss.

The optimal Delta_EoT cut-off to identify patients at risk of losing HBsAg was > −1.4 log10 IU/mL (S = 83.3%, Sp= 95.7%, PPV=71.4% and NPV=97.8%). Other cut-offs were evaluated for an easier applicability in real clinical practice (Fig. 2). The Delta_EoT cut-off ≥ −1 log10 IU/mL showed good accuracy to identify HBsAg loss (S = 83.3%, Sp = 89.1%, PPV = 50% and NPV = 97.6%). Characteristics of the included patients according to the optimal (−1.4 log10 IU/mL) and useful (−1 log10 IU/mL) cut-offs to identify patients at risk of losing HBsAg are depicted in the Supplementary Tables 1 and 2, respectively.

3.5. Probability of HBsAg loss according to HBsAg kinetics during antiviral treatment

A Delta_EoT ≥ −1 log10 IU/mL was observed in 10 (19.2%) patients (Supplementary Table 2). Patients with a Delta_EoT ≥ −1 log10 IU/mL were usually Caucasian (90% vs. 50%, $p = 0.02$) but HBsAg levels before NA were similar between groups (3330 vs. 3891 IU/mL; p=ns). Patients with a Delta_EoT ≥ −1 log10 IU/mL had a longer treatment duration (10.2 vs. 7.9 years; $p = 0.02$) and faster HBsAg decline (−0.15 vs. −0.05 IU/mL/year; $p = 0.001$). Thus, HBsAg at EoT was lower in patients with a Delta_EoT ≥ −1 log10 IU/mL (114 vs. 1277 IU/mL; $p = 0.01$). Therefore, 5 (50%) out of 10 patients with a Delta_EoT ≥ −1 log10 IU/mL showed HBsAg <100 IU/mL before interruption and 5 achieved HBsAg loss during the first year after withdrawal (p<0.001 in both cases).

The cumulative rate of HBsAg loss at weeks 4, 12, 24, 36 and 48 after withdrawal was 1.9%, 1.9%, 5.8%, 9.6% and 11.5%. The probability of HBsAg loss one year after NA interruption was 50% in patients with a Delta_EoT ≥ −1 log10 IU/mL and 2.4% in those with Delta_EoT < −1log10 IU/mL (log-rank p<0.001; Breslow p<0.001) (Fig. 3). Moreover, patients with a Delta_EoT ≥ −1log10 IU/mL



HBsAg decline during treatment (Log10 IU/mL)	S (95% CI)	Sp (95% CI)	PPV (95% CI)	NPV (95% CI)	+LR	-LR
≥ 1.0	83.3 (36-99)	89.1 (76-96)	50.0 (29-71)	97.6 (87-99)	7.67	0.19
> 1.2	83.3 (36-99)	89.3 (82-95)	62.5 (35-84)	97.7 (88-99)	12.78	0.18
> 1.4	83.3 (36-99)	95.6 (85.2-99)	71.4 (38-91)	97.8 (88-99)	19.17	0.17

Fig. 2. Diagnostic accuracy of HBsAg decline to identify patients at risk of losing HBsAg.
Diagnostic accuracy (AUROC, 95% CI) of HBsAg decline to identify patients at risk of losing HBsAg after treatment interruption, Evaluation of different cut-offs according to sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio, AUROC; area under receiver operating characteristic; CI; confidence interval; S; sensitivity; Sp; specificity; PPV; positive predictive value; NPV; negative predictive value; +LR; positive likelihood ratio; -LR; negative likelihood ratio,

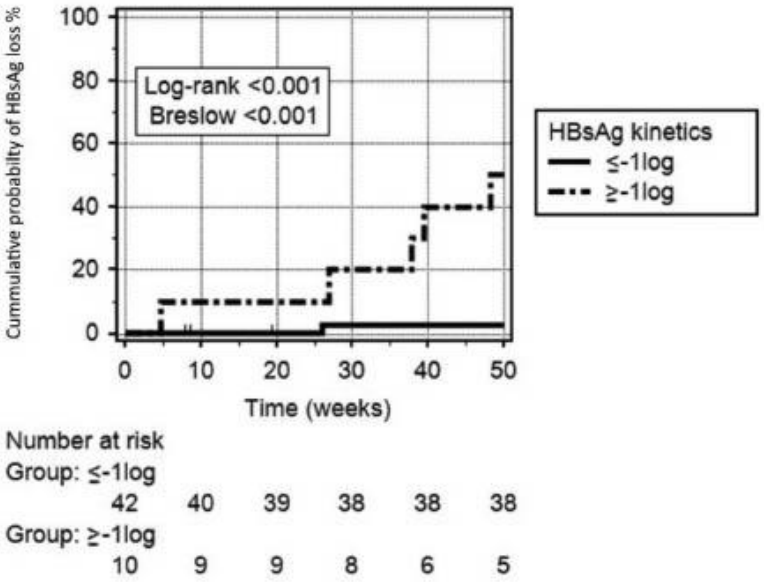


Fig. 3. Cumulative probability of HBsAg loss after NA interruption according to HBsAg kinetics during NA therapy.
Patients with a Delta_EoT HBsAg ≥ −1log10 IU/mL showed an HBsAg loss cumulative probability of 50% one year after NA interruption compared to 2,5% in those with a Delta_EoT HBsAg < −1log10 IU/mL (log-rank= p<0,001; Breslow p=<0,001).

that one year after treatment interruption persisted with HBsAg-positive showed lower HBsAg levels compared to those with a Delta_EoT < −1log10 IU/mL (103 vs. 766 IU/mL; $p = 0.002$).

3.6. Response after NA withdrawal

The HBsAg loss was observed in 6 (11.5%) patients and seroconversion (positive anti-HBs) in 4 (66.7%) of them. There were no differences in baseline characteristics or in on-treatment HBsAg kinetics between patients with and without seroconversion. However, the 4 patients who developed positive anti-HBs had been treated with TDF and the two others with ETV. Virological relapse was identified in 51 (98.1%), SVR in 24 (46.2%) and CR in 5 (9.6%) patients. Severe flare leading to NA reintroduction was observed in 3 (5.8%) but neither acute liver failure nor hepatic decompensation occurred. Patients with NA reintroduction were Caucasian ($n = 1$), Asian ($n = 1$) and Hispanic ($n = 1$), males ($n = 3$), 2 infected by genotype A and 1 genotype D, all of them treated with TDF during 4.7, 6 and 15 years. The three patients showed an HBsAg decline < 1 log10 IU/mL and HBsAg levels before interruption of 1781, 1632 and 807 IU/mL, respectively. Patients were retreated with the same NA and rapidly achieved virological response. Differences on response rates according the HBsAg kinetics during NA therapy are depicted in Supplementary Table 2 and Supplementary

Figure 1. Considering the HBsAg-positive patients without NA retreatment at week 48 ($n = 43$), 21 (48.8%) remained in sustained off-treatment response and 22 (51.2%) in grey zone. Among patients in grey zone, 20 (91%) showed a SVR and only 2 (9%) a CR. Four out of 5 (80%) patients with an HBsAg decline ≥ 1 log10 IU/mL that one year after NA withdrawal persisted with HBsAg-positive remained in sustained off-treatment response (persistent ALT<2xULN and HBV-DNA<2000IU/mL), compared to 48% (18 out of 38) of patients with HBsAg decline < 1 log10 IU/mL ($p = 0.17$).

3.7. HBsAg kinetics according to HBV genotype and NA therapy

The HBsAg kinetics was evaluated before and after treatment withdrawal according to HBV genotype D vs. other genotypes (Supplementary Table 3). Patients infected by genotype D ($n = 32$) showed lower HBsAg levels (IU/mL) before treatment initiation compared to those infected by other genotypes (2497 vs 5708; $p = 0.03$). However, there were no differences in the on-treatment HBsAg kinetics at year 1, at year 3, at EoT or in the speed of HBsAg decline. After treatment interruption, there were no differences between patients according to HBV genotype.

Regarding the type of NA therapy, we did not find differences between patients treated with TDF ($n = 34$) or ETV ($n = 17$) on treatment duration (8.0 vs. 8.5 years; p=ns), levels of HBsAg_EoT (697 vs. 1143 IU/mL; $p = 0.31$), Delta-EoT (−0.62 vs. −0.48 log10 UI/mL; p=ns), Delta_1post-EoT HBsAg (−0.18 vs. −0.19 log10 UI/mL; p=ns), rates of HBsAg loss (11.8 vs. 11.8%; p=ns), VR (97.1 vs. 100%; p=ns), SVR (65 vs. 35.3%; p=ns), CR (11.8 vs. 5.9%; p=ns), or retreatment (8.8 vs. 0%; p=ns). However, patients treated with TDF compared to those treated with ETV showed earlier VR (4 vs. 12 weeks; $p<0.001$), SVR (12 vs. 30 weeks; $p<0.001$) and CR (10 vs. 48 weeks; $p = 0.14$). In patients who had received Peg-IFN ($n = 19$) [17] the add-on therapy was finished a median of 2.3 years before EoT and no differences in HBsAg loss rate were found (5.3 vs. 15.2%; p=ns). However, patients with add-on Peg-IFN showed faster (−0.10 vs. −0.05 log10 IU/mL/year; $p = 0.02$) and greater HBsAg decline (−0.74 vs. −0.46 log10IU/mL; $p = 0.056$).

4. Discussion

Our longitudinal study in non-cirrhotic HBeAg-negative patients with NA treatment withdrawal has shown that on-treatment HBsAg kinetics can predict theHBsAg decline after treatment interruption and the frequency of HBsAg loss .

Our study has shown a high incidence of HBsAg loss during the first year after treatment interruption (11.5%). Studies on Asian population have shown lower HBsAg loss rates (1.8%) [10,19] compared to European cohorts (from 9% to 22%) [11–14]. A recent systematic review of 25 studies showed a 2% HBsAg loss rate. However, only two studies included Caucasian patients, and most were Asian patients infected by HBV genotypes B or C [20]. In contrast, Kuhnhenn et al. have recently described low levels of HBsAg in HBeAg-negative patients infected by genotype B or D [21]. In our cohort, the 61.5% of patients were infected by genotype D. We have confirmed that patients with genotype D had lower levels of HBsAg at the initiation of NA treatment. However, we could not demonstrate differences between patients on treatment HBsAg kinetics or in the rate of HBsAg loss according to HBV genotypes.

Our study has shown a significant correlation between treatment duration and HBsAg decline. Therefore, the HBsAg decline was higher as longer the treatment was. The annual decline of HBsAg during NA treatment was very stable and slow (0.06 log10IU/mL/year) as we have previously reported [5]. The HBsAg decline during first 3 years of treatment was very low and HBsAg kinetics was not associated with HBsAg loss. However, patients with longer treatment duration had more probabilities of clearing the HBsAg during the first year after interruption. A recent meta-analysis has shown that antiviral therapy duration can be crucial to achieve a persistent viral remission after treatment interruption in HBeAg-negative patients [20]. Therefore, we consider that treatment longer than 3 years could be beneficial before interruption [1,9,15].

Patients with HBsAg loss, showed a greater on-treatment HBsAg decline and lower HBsAg values before interruption. The on-treatment HBsAg decline was independently associated with HBsAg loss and was a good predictor of HBsAg loss (AUROC=0.935). The optimal HBsAg decline cut-off was > 1.4 log10 IU/mL. However, to make the use of HBsAg kinetics easier in real clinical practice, we also evaluated the cut-off ≥1 log10 IU/mL that showed good PPV (50%) and excellent NPV (97.6%). Therefore, half of patients with an HBsAg decline ≥1log10 IU/mL achieved the HBsAg loss during the first year after NA withdrawal. Moreover 40% of them remained in sustained off-treatment response with low HBsAg levels (103 IU/mL) and no patients needed to be retreated. On the other hand, despite only 2.4% of patients with an HBsAg decline < 1 log10 IU/mL achieved the HBsAg loss, the 40% of them persisted in sustained off-treatment response.

t is important to note that after NA interruption the speed of HBsAg decline accelerated in patients treated longer than 6 years. It has been recently postulated that long-term HBV-DNA suppression can reinvigorate exhausted CD8+ T cells and restore the immune control against infected hepatocytes after withdrawal [14,22,23]. In consonance, our study has clearly shown that the speed of HBsAg decline after treatment interruption is 3 times faster than during therapy, not only in patients who lost the HBsAg but also in HBsAg-positive patients, being another argument in favour to stop treatment in these patients.

In terms of safety, only three patients developed a severe flare during the first 12 weeks after withdrawal and were retreated with the same NA showing an excellent response. In contrast, more than 90% of HBsAg-positive patients remained without antiviral treatment. Patients receiving ETV showed later virological relapse. Similarly, recent Asian studies [24,25] have reported that patients treated with ETV can develop the clinical or virological relapse later than those treated with TDF [26]. Thus, we consider that NA type should be considered for the monitoring after interruption. Another interesting point of our cohort was that 37% added-on Peg-IFN two years before EoT [17]. Patients with add-on Peg-IFN showed faster HBsAg decline despite no differences in HBsAg loss rate were found. Hence, an add-on strategy with Peg-IFN could be useful to accelerate HBsAg kinetics and to short NA duration before interruption [17,27].

Our study has some limitations. The limited number of included patients compared to Asian cohorts [10,19], a short time of follow-up, and the lack of an external validation. Nevertheless, it is important to note that these limitations have been compensated performing an internal validation of the HBsAg kinetics diagnostic accuracy and evaluating all included patients without any loss during follow-up. Moreover, the number of our included patients are similar to the previous European studies [11–14]. On the other hand, our study has several strengths. All included patients were HBeAg-negative at the NA initiation, who are the patients with less evidence on NA interruption. The long NA therapy has allowed to identify a significant correlation between treatment duration and HBsAg decline and to analyse the HBsAg kinetics before and after NA interruption in three different treatment time periodstime. Moreover, the evaluation of HBsAg kinetics before and after therapy has demonstrated an accelerated effect after withdrawal.

In conclusion, on-treatment HBsAg kinetics can predict the post-treatment HBsAg decline and the frequency of HBsAg with high accuracy. Half of patients with a significant HBsAg decline (≥ 1 log10 IU/mL) can eliminate HBsAg during the first year

after withdrawal compared to only few patient who did not show this kinetics. Importantly, after NA interruption HBsAg decline is faster not only in patients who lost the HBsAg but also in those who remain HBsAg-positive.

Conflict of interest

None declared.

Financial support statement

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Ethics approval statement

The study protocol was approved by the Ethical Committee of our Institution "Comitè Ètic d'Investigació Clínica - Parc de Salut Mar", study reference 2018/7939/I.

Patients consent statement

All patients provided a written informed consent.

Guarantor of the article and final version of the manuscript

Jose A. Carrión Rodríguez certifies that all the authors have approved the final version of the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dld.2021.12.017.

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5. RESUM GLOBAL DELS RESULTATS

En el **primer estudi**[73] vam avaluar els nivells de l'HBsAg en 95 pacients amb hepatitis crònica HBeAg negatiu, abans de l'inici del tractament antiviral amb AN i durant el tractament amb una mediana de temps de gairebé 6 anys (5,95 anys). En aquest estudi vam observar que el descens anual dels nivells de l'HBsAg amb els AN és molt lent (0,1 log10 UI/ml/any). La pèrdua de l'HBsAg es va produir en un 4% dels pacients després de 6 anys de seguiment. No vam identificar variables a nivell basal que fessin predir la pèrdua de l'HBsAg. Malgrat això, un 14% dels pacients va presentar un descens accelerat i/o nivells baixos de l'HBsAg (< 120 UI/mL) durant el tractament. Aquestes variables (descens accelerat i/o nivells baixos d'HBsAg) durant el tractament es van relacionar amb la pèrdua de l'HBsAg. El descens de l'HBsAg, al tercer any de tractament va mostrar una bona precisió diagnòstica (AUROC= 0,89) per identificar els pacients que durant el tractament assolien nivells baixos d'HBsAg. Un descens al tercer any de l'HBsAg > 0,3 log UI/ml va mostrar un valor predictiu positiu (VPP) del 42% i un VPN del 100% per identificar els pacients que arribarien a nivells d'HBsAg < 120 UI/mL. Els pacients que presentaven aquest descens de l'HBsAg >0,3 log UI/mL/3 anys durant el tractament tenien una probabilitat del 22% d'aconseguir nivells baixos d'HBsAg < 120 UI/mL als 5 anys de tractament. En canvi, aquesta probabilitat era del 0% per als pacients que no presentaven aquest descens inicial de l'HBsAg (<0,3 log UI/mL/3 anys).

En el **segon estudi**[74] es va retirar el tractament a 52 pacients, després d'una mediana de 8,2 anys de tractament amb AN. Un any després de la retirada del tractament, l'11,5% dels pacients va perdre l'HBsAg. A la nostra cohort, el 61,5% dels pacients estaven infectats pel genotip D i tenien nivells més baixos d'HBsAg abans del tractament però no es van observar diferències en la cinètica de l'HBsAg durant el tractament ni en la taxa de pèrdua de l'HBsAg després de la parada. Aquest estudi va validar els resultats del primer respecte al descens lent de l'HBsAg durant el tractament amb AN, amb una mediana anual de descens de 0,06 log10 UI/ml/any. Vam identificar una bona correlació (r=-0,51) entre la durada del tractament i el descens de l'HBsAg, pel

que el descens de l'HBsAg va ser més elevat en els pacients tractats durant més temps (-0,29 log10UI/ml en tractats de 3 a 6 anys; -0,45 log10UI/ml en els tractats de 6 a 9 anys i -0,82 log10UI/ml en tractats més de 9 anys), però la velocitat de descens va ser la mateixa en els tres períodes (-0,06 log10UI/ml/any). En canvi, un cop retirat el tractament, la velocitat de descens de l'HBsAg va ser de -0,19 log10UI/ml/any, superior a la observada durant el tractament (p=0,01). Aquesta velocitat va ser més ràpida tant en els pacients que van perdre l'HBsAg (-1,33 vs.-0,14 log10UI/ml/any; p=0,046) com en els que van mantenir l'HBsAg positiu a l'any de parar el tractament (-0,18 vs.-0,05 log 10UI/ml/any; p<0,001). Seguidament, vam analitzar les variables que es relacionaven amb la pèrdua de l'HBsAg. En l'anàlisi univariant, les variables que es van relacionar amb la pèrdua de l'HBsAg van ser el temps de tractament (12,8 vs.7,9 anys; p=0,001), el descens de l'HBsAg al final del tractament (-1,75 vs.-0,47 log10UI/ml; p<0,001) i els nivells de l'HBsAg al moment de la parada (21,4 vs.1.176 UI/ml; p=0,002). El descens de l'HBsAg als 3 anys de tractament no es va relacionar amb la pèrdua de l'HBsAg al parar el tractament però 2 (22%) pacients amb un descens de l'HBsAg > 0,3 log UI/ml al tercer any va aconseguir perdre l'HBsAg al parar l'AN . En l'anàlisi multivariant, només la cinètica de l'HBsAg de l'inici de tractament a la parada es va relacionar de forma independent amb la pèrdua de l'HBsAg (OR=0,10; IC 95%= 0,016-0,632; p=0,014). La corba ROC va demostrar que la cinètica de l'HBsAg durant el tractament mostrava una fiabilitat excel·lent (AUROC 0,935) per a predir la pèrdua de l'HBsAg i la seva validació interna pel mètode de "bootstrap" va confirmar la seva precisió diagnòstica (AUROC percentil 5-95: 0,75-0,99). El punt de tall òptim de descens de l'HBsAg va ser -1,4log10 UI/ml però per tal de fer més fàcil el seu us a la pràctica clínica diària vam escollir el punt de -1log10 UI/ml que mantenia una bona fiabilitat diagnòstica (S=83,3%, Sp= 89,1%, VPP=50% i VPN=97,6%). La probabilitat acumulada de perdre l'HBsAg en parar el tractament en pacients amb una cinètica de l'HBsAg favorable (descens >1log10 UI/ml) va ser del 50%, i només del 2,4% en pacients que no havien assolit aquest descens. A més a més, després de parar el tractament, el 80% dels pacients que persistien amb

l'HBsAg positiu però que havien presentat una cinètica favorable (descens >1log10UI/ml) durant el tractament, es mantenien amb resposta sostinguda (ALT<2 x LSN i ADN-VHB <2.000UI/ml) en comparació al 48% dels pacients que no havien presentat aquest descens ($p<0,05$).

6. RESUM GLOBAL DE LA DISCUSSIÓ

L'hepatitis crònica per VHB és una malaltia d'elevada prevalença a nivell mundial. Els tractaments dels que disposem actualment han demostrat una elevada eficàcia en el control de la replicació viral i en la disminució de la progressió de la malaltia, així com en la reducció de les complicacions i la mortalitat[46]. L'objectiu ideal amb els tractaments dels quals disposem en l'actualitat és la cura funcional, definida com la pèrdua de l'HBsAg. En els pacients amb hepatitis crònica HBeAg negatiu, s'ha descrit que el tractament amb AN requereix d'una durada molt llarga de tractament, inclús indefinida, per aconseguir la pèrdua de l'HBsAg[53], pel que és important aprofundir en el coneixement de la malaltia per a intentar identificar grups de pacients amb major probabilitat d'assolir la cura funcional i avaluar noves estratègies terapèutiques que permetin augmentar les possibilitats d'aconseguir la pèrdua de l'HBsAg. Els nivells de l'HBsAg es correlacionen amb les diferents fases de la infecció crònica pel VHB i nivells baixos d'HBsAg s'han relacionat amb el control per part del sistema immunològic de l'hoste pel que conèixer la seva cinètica pot ser molt important per a la monitorització del tractament antiviral.

En el **primer estudi** hem realitzat una avaluació dels nivells de l'HBsAg en pacients amb hepatitis crònica HBeAg negatiu, abans de l'inici del tractament antiviral i posteriorment durant el tractament amb una mediana de seguiment de 5,95 anys. En aquest estudi hem confirmat que el descens dels nivells de l'HBsAg al llarg dels anys és molt lent (0,1 log UI/ml/any). Aquestes dades són similars a les publicades en altres estudis[54,72,75,76] i confirmen que la probabilitat de perdre l'HBsAg amb el tractament amb AN és baixa. En el nostre estudi, la pèrdua de l'HBsAg es va produir en un 4% dels pacients després d'una mediana de 6 anys de tractament i no vam poder identificar variables basals que fessin predir els pacients que perdrien l'HBsAg durant el seguiment. En canvi, en l'estudi de Seto et al.[54] els autors van identificar que els nivells de l'HBsAg previ al tractament podien predir la pèrdua de l'HBsAg en el seguiment. Una dada

important del nostre estudi és que vam observar que una petita proporció de pacients (14%) presenten una cinètica diferent amb un descens accelerat de l'HBsAg durant el tractament, i són aquests pacients els que tenen més probabilitat de perdre l'HBsAg amb els AN. Per tant, en base als nostres resultats, sembla que més enllà de les variables basals a l'inici del tractament, el comportament o cinètica de l'HBsAg durant els primers 3 anys de tractament pot ser un marcador important a tenir en compte per identificar els pacients amb més probabilitat de perdre l'HBsAg durant el tractament. S'ha descrit que la teràpia amb AN produeix una disminució de l'ADNccc[77]. Tot i això, més important que la disminució de l'ADNccc, és la disminució de la seva activitat transcripcional, però la seva determinació és complexa. S'ha de tenir en compte que per a la determinació de l'ADNccc es requereix una biòpsia hepàtica i la majoria dels assaigs que quantifiquen directament l'ADNccc no poden determinar la seva activitat transcripcional[78]. És per això que s'han investigat les proteïnes virals com l'HBsAg com a marcadors de l'activitat transcripcional de l'ADNccc. En pacients HBeAg positiu sense tractament els nivells de l'HBsAg s'han relacionat amb els nivells d'ADN del VHB[79]. En canvi, en pacients HBeAg negatiu aquesta correlació entre l'HBsAg i l'ADN viral no s'ha demostrat i s'ha suggerit que és degut a una reducció molt marcada de l'activitat transcripcional de l'ADNccc en les fases HBeAg negatiu[80] i que la majoria de l'HBsAg procedeix de l'ADN integrat en l'hepatòcit[81]. Per tant, identificar els pacients que presenten un descens accelerat dels nivells d'HBsAg durant la teràpia amb AN pot ser un bon predictor de la pèrdua de l'HBsAg durant el tractament, però també pot ser útil per definir la durada del tractament, ja que diversos estudis han demostrat que nivells baixos d'HBsAg abans de la parada de l'AN poden ser necessaris per aconseguir la pèrdua de l'HBsAg[82–84]. En el nostre estudi vam observar que un descens del l'HBsAg, al tercer any, superior a 0,3 log UI/ml era una eina fiable (AUROC= 0,89) per identificar pacients que assolirien nivells molt baixos de l'HBsAg (<120 UI/ml) durant el tractament, amb un valor predictiu positiu del 42% i una probabilitat acumulada als 5 anys de tractament del 22%. En canvi, en aquells pacients que no s'assolia el descens de 0,3 log UI/ml, el valor predictiu negatiu era del

100% i cap pacient assolia nivells <120 UI/ml en el seguiment. Aquests resultats, estan en consonància amb l'estudi de Seto et al.[54] realitzat en població asiàtica, en el que els autors van observar que la reducció de 0,166 log UI/ml anual, va ser un bon marcador per a predir la pèrdua de l'HBsAg.

El primer estudi té algunes limitacions. Es tracta d'un estudi retrospectiu, amb una durada no homogènia del tractament antiviral i amb número limitat de pacients. Tanmateix, la recollida de sèrums en els nostres pacients, va ser prospectiva i amb uns intervals molts regulars de seguiment. Respecte al tractament antiviral, tot i que la majoria de pacients havien començat amb ETV o TDF (76%), els pacients amb major seguiment ho havien fet amb LAM o ADV. D'altra banda, tots els pacients inclosos eren HBeAg negatiu a l'inici del tractament i tots van fer un seguiment molt regular sense diferències en l'HBsAg a nivell basal.

Per tant, el primer estudi ha demostrat que la disminució significativa dels nivells de l'HBsAg durant els primers 3 anys de tractament amb AN és un bon marcador per identificar pacients que assoliran nivells baixos d'HBsAg durant el tractament. En canvi, aquells pacients sense disminució de l'HBsAg durant els primers anys de tractament no mostraran un descens significatiu de l'HBsAg amb AN ni aconseguiran l'eliminació de l'HBsAg en els següents anys i per tant, se'ls hauria de plantejar mantenir el tractament de forma crònica o avaluar nous fàrmacs antivirals o noves estratègies terapèutiques per a assolir la cura funcional.

Per això, en **l'estudi inclòs en l'annex 1** de la tesi[85], un cop avaluats els resultats anteriors, ens vam plantejar realitzar una estratègia terapèutica que pogués augmentar les taxes de pèrdua de l'HBsAg. Dels tractament disponibles per a l'hepatitis crònica B, el tractament amb IFN-peg durant 48 setmanes havia demostrat unes taxes de pèrdua de l'HBsAg del 4% que eren superiors a les descrites amb AN[59] i és la primera estratègia que vam avaluar. En aquest nou estudi, vam analitzar la cinètica de l'HBsAg amb l'addició d'IFN-peg durant 48 setmanes comparat amb el tractament amb AN en monoteràpia. Com objectiu secundari vam avaluar la taxa de pèrdua de l'HBsAg en els

dos grups de tractament. En aquest estudi també vam avaluar la cinètica de l'HBcrAg ja que s'ha demostrat que té una bona correlació amb l'activitat transcripcional de l'ADNccc en les diferents fases de la infecció[38,86]. L'estudi va confirmar de manera prospectiva, que els nivells d'HBsAg disminueixen molt lentament en els pacients tractats amb AN en monoteràpia. Durant les 96 setmanes de l'estudi, la mediana de descens de l'HBsAg va ser de -0,12 log10 UI/ml i per tant, la proporció de pacients amb nivells baixos d'HBsAg (< 100 UI/ml) a les setmanes 24, 48 i 96 no va canviar respecte al basal, i cap pacient va aconseguir la pèrdua de l'HBsAg. En canvi, l'addició d'IFN-peg va augmentar el descens de l'HBsAg a la setmana 96 fins a -0,44 log10 UI/ml, la taxa de pacients amb nivells baixos d'HBsAg (< 100UI/ml) va augmentar a les setmanes 24 (16,7%) i 48 (29,6%) respecte al basal, i tres pacients (12,5%) van perdre l'HBsAg durant el tractament. Els resultats d'aquest estudi, descrit l'annex 1 de la tesi, estan en línia amb els resultats dels estudis de "prova de concepte" que s'havien publicat fa uns anys amb un número molt limitat de pacients[60,61] en que els nivells de l'HBsAg van disminuir de forma més marcada en els pacients als que es va afegir l'IFN-peg. Durant la realització d'aquest estudi, s'han publicat els resultats de l'estudi francès PEGAN[87] en el que s'han comparat 90 pacients amb l'addició d'IFN-peg i 93 que mantenien l'AN en monoteràpia. Aquest estudi ha demostrat una taxa major de pèrdua de l'HBsAg a la setmana 48 en pacients amb tractament combinat en comparació a la monoteràpia (7,8% vs. 0%) i a setmana 96 quan es van analitzar els pacients que havien rebut la dosi completa d'IFN-peg (n=65) (11% vs. 3,2%; p=0,04). L'estudi va demostrar un descens dels nivells d'HBsAg més marcat en els pacients amb tractament combinat, Posteriorment, l'estudi HERMES[88] en pacients infectats pel genotip D, ha mostrat resultats similars, amb un descens de l'HBsAg més pronunciat en pacients als que s'afegia IFN-peg. Recentment, s'ha publicat l'estudi SWAP[89]en població asiàtica, en el que es compara la monoteràpia amb AN amb l'estratègia d'afegir IFN-peg (add-on) o canviar l'AN per IFN-peg (switch). Aquest estudi ha demostrat en pacients asiàtics HBeAg negatiu que l'addició o el canvi de IFN-peg augmenta les probabilitats de perdre l'HBsAg en comparació a la

monoteràpia amb AN (10,1% o 7,8% vs. 0%). El descens més marcat dels nivells de l'HBsAg en afegir l'IFN-peg als AN es podria explicar pel seu efecte immunomodulador activant les cèl·lules immunitàries de l'hoste i induint la producció de gens que codifiquen proteïnes amb acció antiviral directa[90]. L'IFN-peg pot inhibir la transcripció del VHB i reduir la producció d'antígens virals mitjançant la degradació de l'ARN pregenòmic i de les partícules del core o modificant la regulació epigenètica de l' ADNccc[91,92]. A nivell immunològic, l'IFN-peg té un efecte divergent. D'una banda, promou la resposta immune innata, especialment la capacitat antiviral de les cèl·lules NK CD56 *bright,* però condueix a un esgotament sostingut de cèl·lules T CD8+ i no millora la reactivació precoç de cèl·lules T específiques del VHB[93,94]. En canvi, els AN no afecten directament a la funció de les cèl·lules NK però un tractament de llarga durada aconsegueix la supressió viral, afavorint la recuperació parcial de les cèl·lules T específiques del VHB[95]. Per tot això, sembla que la supressió viral que s'aconsegueix amb els AN podria millorar la resposta immunològica aconseguida posteriorment amb l'IFN-Peg i això podria explicar l'efecte sinèrgic dels dos tractaments[96].

Cal destacar també de **l'estudi inclòs en l'annex 1** de la tesi, que l'acceptació a rebre tractament amb IFN-peg, en els nostres pacients crònicament tractats amb AN, va ser baixa i només un 40% dels pacients elegibles va acceptar el tractament combinat. A més, l'aparició d'efectes adversos associats a l'INF-peg va ser freqüent i el 22% dels pacients no va completar el tractament. Aquesta taxa d'efectes adversos és similar als estudis prèviament descrits en que s'ha utilitzat l'estratègia d'addició d'IFN-peg[87–89]. Per tot això, si bé el tractament amb IFN-peg ha demostrat disminuir els nivells d'HBsAg i augmentar les taxes de pèrdua de l'HBsAg en pacients HBeAg negatiu, és difícil de poder-lo introduir en la pràctica clínica habitual, encara que podria ser una estratègia pont per reduir els nivells d'HBsAg i aconseguir nivells baixos abans de plantejar altres alternatives terapèutiques o la parada del tractament. A l'estudi, també es van analitzar els nivells de l'HBcrAg. Aquest marcador es va mantenir estable durant les 96 setmanes

sense diferències entre els dos grups de tractament i sense correlació amb els nivells d'HBsAg o la pèrdua de l'HBsAg. Aquests resultats negatius es podrien explicar per la baixa sensibilitat actual de la tècnica (2 log U/mL) i perquè els nivells d'HBcrAg a l'inici del nostre estudi van ser molts baixos, provocant que una proporció elevada dels pacients (31%), tinguessin valors indetectables basalment probablement degut a que eren pacients HBeAg negatius tractats durant un llarg període. Resultat d'això, no hem pogut demostrar una utilitat de l'HBcrAg per a la monitorització d'aquests pacients. En consonància, un estudi recent de pacients amb IFN-peg i AN[97] que ha comparat l'HBcrAg, l'HBsAg, i l'ARN del VHB per a predir la pèrdua de HBsAg ha demostrat que el marcador serològic amb millor capacitat predictiva és l'HBsAg i la seva cinètica.

En el **segon estudi** de la tesi, en base a les dades anteriors, als estudis publicats en l'última dècada[67,68,71], i a les recomanacions de les guies internacionals[18,40], vam plantejar la possibilitat d'analitzar la cinètica de l'HBsAg en pacients HBeAg negatiu després de parar el tractament amb AN per saber si la cinètica de l'HBsAg durant el tractament podia predir la resposta després de la parada del tractament. És per això, que vam avaluar la cinètica abans i després de la retirada del tractament en 52 pacients HBeAg negatiu sense cirrosi que portaven una mediana de tractament amb AN de 8 anys. Un any després de la retirada del tractament amb AN, l'11,5% dels pacients va perdre l'HBsAg. Aquesta prevalença, és superior a la que s'ha publicat en estudis de població asiàtica[98,99], i més similar a la reportada en estudis europeus (del 9% al 22%) [67,68,84,100]. Recentment, l'estudi RETRACT-B, un estudi multicèntric i multiètnic, que inclou el major número de pacients HBeAg negatius amb parada de l'AN (n= 1.552)[82] ha demostrat una pèrdua de l'HBsAg en el 13% del pacients als 4 anys de seguiment. Una revisió sistemàtica[101] de 25 estudis havia mostrat una taxa de pèrdua de l'HBsAg del 2%, però només en dos estudis es van incloure pacients caucàsics, i la majoria de pacients eren asiàtics infectats pels genotips B o C del VHB. A la nostra cohort el 58% eren pacients caucàsics i el 61,5% estaven infectats pel genotip D. Els nostres pacients

amb genotip D van mostrar nivells més baixos d'HBsAg a l'inici del tractament però no vam observar diferències en la cinètica de l'HBsAg durant el tractament o en la taxa de pèrdua de l'HBsAg després de la retirada.

S'ha de tenir en compte, que hi ha diferències importants entre els estudis de parada de tractament que en fan difícil la seva comparació. D'una banda, la distribució diferent dels genotips del VHB (especialment el genotip D) en les diferents àrees geogràfiques, ja que s'ha relacionat el genotip amb els nivells de l'HBsAg[102] i la probabilitat de pèrdua de l'HBsAg després de la parada[83]. En segon lloc, els estudis asiàtics inclouen pacients amb malaltia hepàtica avançada i cirrosi[98] que no es recomana en la guia europea. I en tercer lloc, ni els estudis asiàtics ni els europeus tenen regles de retractament predefinides i s'ha descrit que el moment del retractament té implicacions importants en les probabilitats de pèrdua de l'HBsAg[103].

El nostre estudi va demostrar una correlació significativa entre la durada del tractament i la disminució de l'HBsAg, ja que la disminució de l'HBsAg va ser més gran a mesura que el tractament era més llarg. També en aquest segon estudi vam observar que la disminució anual de l'HBsAg durant el tractament amb AN va ser molt estable i lenta (0,06 log10IU/mL/any). Vam avaluar si la cinètica als 3 anys de tractament es relacionava amb la pèrdua de l'HBsAg després de la retirada i no vam poder demostrar relació entre el descens de l'HBsAg durant els primers anys de tractament i la pèrdua de l'HBsAg en parar l'AN probablement degut a l'exclusió dels 9 pacients que prèviament havien aconseguit la pèrdua de l'HBsAg (6 durant el primer estudi[73] i 3 durant l'estudi inclòs a l'annex 1 amb addició de peg-IFN[85]). En l'anàlisi univariant, vam veure que els pacients amb una durada més llarga del tractament tenien més probabilitats d'eliminar l'HBsAg durant el primer any després de la interrupció. Una metaanàlisi recent ha demostrat que la durada de la teràpia antiviral pot ser crucial per aconseguir una remissió viral persistent després de la interrupció del tractament en pacients HBeAg negatius[101], però en el nostre estudi la variable independent que es va associar a la

pèrdua de l'HBsAg va ser el seu descens (o cinètica) i no el temps. Tanmateix, tots els pacients que van perdre l'HBsAg havien estat tractats durant més de 9 anys. Per tant, el temps de tractament també és una variable que s'ha de tenir en compte per la seva elevada correlació amb el descens de l'HBsAg.

La cinètica de l'HBsAg durant el tractament es va associar de manera independent amb la pèrdua de l'HBsAg i va demostrar una excel·lent fiabilitat diagnòstica (AUROC=0,935) per predir la pèrdua de l'HBsAg. Un descens de l'HBsAg ≥1 log UI/mL va mostrar un VPP del 50% per a identificar la pèrdua de l'HBsAg a l'any de la retirada del tractament. En canvi, la probabilitat de perdre l'HBsAg en els pacients que no van assolir aquest descens durant el tractament va ser pràcticament nul·la (VPN 97,6%). En la nostra cohort, el 19% (10 de 52) dels pacients va presentar un descens de l'HBsAg ≥1log UI/ml abans de parar el tractament. D'aquests, el 50% va aconseguir la pèrdua de l'HBsAg durant el primer any després de la parada de l'AN, el 40% es va mantenir en resposta sostinguda i nivells molt baixos d'HBsAg (mediana de 103 UI/ml), i en cap pacient va caldre reiniciar el tractament. D'altra banda, tot i que només el 2,4% dels pacients amb una disminució de l'HBsAg < 1 log10 UI/ml van aconseguir la pèrdua d'HBsAg, el 40% d'ells van persistir en resposta sostinguda sense necessitat de tractament.

Un altre punt interessant de la nostra cohort va ser que el 37% dels pacients que van parar el tractament, havien rebut IFN-peg en l'estudi comentat a l'annex 1[85]. Els pacients, havien finalitzat el tractament amb IFN-peg com a mínim dos anys abans de la parada de tractament. Els pacients que havien rebut IFN-peg van presentar un descens més ràpid de l'HBsAg durant el tractament que no va continuar després de parar l'IFN-peg. Per tant, l'estratègia d'afegir IFN-peg a l'AN i parar tot el tractament al finalitzar l'IFN-peg no va poder avaluar-se donat que els pacients van continuar amb AN durant dos anys. De fet, les últimes guies de l'APASL[65] i un consens d'experts publicat recentment [96] proposen que l'addició d'IFN-peg en els pacients que no aconsegueixen

disminuir els nivells d'HBsAg amb el tractament amb AN pot ser una bona estratègia per accelerar la cinètica de l'HBsAg i així poder escurçar la durada del tractament abans de la seva interrupció.

Finalment, un punt important d'aquest segon estudi és que vam observar que després de la interrupció dels AN, la velocitat de descens de l'HBsAg es va accelerar en tots els pacients tractats durant més de 6 anys. Recentment s'ha postulat que la supressió a llarg termini de l'ADN del VHB pot revitalitzar les cèl·lules T CD8+ esgotades i augmentar la funció de les cèl·lules NK, per a restablir el control immunitari contra els hepatòcits infectats després de la seva retirada [84,104–106]. En consonància, el nostre estudi va mostrar que la velocitat de disminució de l'HBsAg després de la interrupció del tractament va ser 3 vegades més ràpida que l'últim any de tractament, no només en els pacients que van perdre l'HBsAg sinó també en pacients que van mantenir l'HBsAg positiu, i per tant, això suggeriria un efecte beneficiós de la retirada del tractament en els pacients tractats durant un període de temps prolongat. Tot i això, es requereixen estudis amb un major número de pacients i amb un seguiment més prolongat per confirmar aquests resultat. La velocitat de descens de l'HBsAg després de parar l'AN en els pacients que havien estat tractats menys de 6 anys es va mantenir igual que durant el tractament antiviral, al menys durant el primer any de retirada del tractament.

7. CONCLUSIONS

1. El descens dels nivells de l'antigen de superfície (HBsAg) en pacients amb hepatitis crònica B antigen e negatiu durant el tractament amb anàlegs de nucleòs(t)ids és molt lent, però una disminució significativa de l'HBsAg al principi del tractament (descens > 0,3 log UI/mL als 3 anys) pot identificar els pacients amb major probabilitat d'aconseguir nivells baixos i la seva negativització durant el tractament.

2. La cinètica de l'antigen de superfície durant el tractament amb anàlegs de nucleòs(t)ids pot predir la probabilitat de perdre l'HBsAg després de la retirada del tractament amb una elevada fiabilitat. La meitat dels pacients amb una disminució significativa de l'HBsAg durant el tractament (descens ≥ 1 log10 UI/ml) poden eliminar-lo durant el primer any després de parar el tractament.

3. Després de la interrupció dels anàlegs de nucleòs(t)ids, la disminució de l'HBsAg durant el primer any és més ràpida que durant el tractament en els pacients que han estat tractats durant més de 6 anys.

8. LINIES DE FUTUR

A partir dels treballs d'aquesta tesi, hem instaurat la determinació de l'HBsAg com un marcador habitual en la pràctica clínica. En els propers anys, seria interessant poder avaluar la cinètica de l'HBsAg després de la parada del tractament a més llarg termini, així com la cinètica en els pacients als que s'ha reiniciat el tractament antiviral i avaluar si una estratègia de tractament intermitent podria ser efectiva en aquests pacients. També serà interessant avaluar la cinètica de l'HBsAg quan hi hagi disponibles nous fàrmacs antivirals i també poder avaluar la cinètica de les diferents proteïnes que conformen l'HBsAg per separat.

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ANNEXOS

10.1. ANNEX 1

Article: Broquetas T, Garcia-Retortillo M, Micó M, Canillas L, Puigvehí M, Cañete N, et al. Hepatitis B surface antigen and hepatitis B core-related antigen kinetics after adding pegylated-interferon to nucleos(t)ids analogues in hepatitis B e antigen-negative patients. World J Hepatol. 2020 Nov 27;12(11):1076–88.

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ORIGINAL ARTICLE

Clinical Trials Study

Hepatitis B surface antigen and hepatitis B core-related antigen kinetics after adding pegylated-interferon to nucleos(t)ids analogues in hepatitis B e antigen-negative patients

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Author contributions: Broquetas T completed statistical analysis and drafting of the manuscript; Broquetas T and Carrión JA analyzed and interpreted the data; Micó M and Hernandez JJ analyzed samples; Carrión JA completed concept, design and supervision of the study; all authors performed the acquisition of data, critical revision of the manuscript.

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Abstract

BACKGROUND

Hepatitis B e antigen-negative chronic hepatitis B patients under nucleos(t)ids analogues (NAs) rarely achieve hepatitis B surface antigen (HBsAg) loss.

AIM

To evaluate if the addition of pegylated interferon (Peg-IFN) could decrease HBsAg and hepatitis B core-related antigen (HBcrAg) levels and increase HBsAg loss rate in patients under NAs therapy.

METHODS

Prospective, non-randomized, open-label trial evaluating the combination of Peg-IFN 180 μg/week plus NAs during forty-eight weeks *vs* NAs in monotherapy. Hepatitis B e antigen-negative non-cirrhotic chronic hepatitis B patients of a tertiary hospital, under NAs therapy for at least 2 years and with undetectable viral load, were eligible. Patients with hepatitis C virus, hepatitis D virus or human immunodeficiency virus co-infection and liver transplanted patients were excluded. HBsAg and HBcrAg levels (log10 U/mL) were measured at baseline and during ninety-six weeks. HBsAg loss rate was evaluated in both groups.

reviewed and approved by the Ethical Committee of our Institution "Comitè Ètic d'Investigació Clínica - Parc de Salut Mar", study reference 2014/5787/I, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Clinical trial registration statement: The study was registered at http://clinicaltrials.gov with the number NCT02743182.

Informed consent statement: Study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Authors declare no conflict-of-interest.

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Adverse events were recorded in both groups. The kinetic of HBsAg for each treatment group was evaluated from baseline to weeks 24 and 48 by the slope of the HBsAg decline (log10 IU/mL/week) using a linear regression model.

RESULTS

Sixty-five patients were enrolled, 61% receiving tenofovir and 33% entecavir. Thirty-six (55%) were included in Peg-IFN-NA group and 29 (44%) in NA group. After matching by age and treatment duration, baseline HBsAg levels were comparable between groups (3.1 *vs* 3.2) (P = 0.25). HBsAg levels at weeks 24, 48 and 96 declined in Peg-IFN-NA group (-0.26, -0.40 and -0.44) and remained stable in NA group (-0.10, -0.10 and -0.10) (P < 0.05). The slope of HBsAg decline in Peg-IFN-NA group (-0.02) was higher than in NA group (-0.00) (P = 0.015). HBcrAg levels did not change. Eight (22%) patients discontinued Peg-IFN due to adverse events. The HBsAg loss was achieved in 3 (8.3%) patients of the Peg-IFN-NA group and 0 (0%) of the NA group.

CONCLUSION

The addition of Peg-IFN to NAs caused a greater and faster decrease of HBsAg levels compared to NA therapy. Side effects of Peg-IFN can limit its use in clinical practice.

Key Words: Chronic hepatitis B; Hepatitis B e antigen-negative; Hepatitis B surface antigen; Hepatitis B core-related antigen; Pegylated-interferon; Nucleos(t)ids analogues

Core Tip: The functional cure of chronic hepatitis B defined as the loss of the hepatitis B surface antigen is the optimal end-point with the currently available therapies. However, it is rarely achieved in hepatitis B e antigen-negative chronic hepatitis B patients under nucleos(t)ids analogues (NAs). In the present study, we report that the addition of pegylated interferon (Peg-IFN) to NAs during forty-eight weeks caused a greater and faster decrease of hepatitis B surface antigen levels compared to NA monotherapy. No changes in hepatitis B core-related antigen were observed. However, the low applicability and poor tolerance of Peg-IFN make difficult its use in clinical practice.

Citation: Broquetas T, Garcia-Retortillo M, Micó M, Canillas L, Puigvehí M, Cañete N, Coll S, Viu A, Hernandez JJ, Bessa X, Carrión JA. Hepatitis B surface antigen and hepatitis B core-related antigen kinetics after adding pegylated-interferon to nucleos(t)ids analogues in hepatitis B e antigen-negative patients. *World J Hepatol* 2020; 12(11): 1076-1088
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INTRODUCTION

Chronic hepatitis B (CHB) affects around 240 million people worldwide[1]. Hepatitis B virus (HBV) cannot be completely eradicated with the available therapies due to the presence of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes[2]. Hepatitis B surface antigen (HBsAg) loss is the optimal treatment endpoint, representing a functional cure of CHB and improving long-term outcome[3].

Although liver biopsy for the quantification of intrahepatic cccDNA and intrahepatic HBV DNA remains the most accurate measurement for viral reservoir, it is limited by its invasive nature and the potential for sampling error. Therefore, noninvasive serological tests are necessary as surrogate markers of intrahepatic viral replicative activity. Serum HBsAg is the glycosylated envelope protein of the mature HBV, which is produced by transcription and translation of the surface genes[4]. On the other hand, the hepatitis B core-related antigen (HBcrAg) combines the antigenic reactivity resulting from denatured hepatitis B e antigen (HBeAg), HBV core antigen and a core-related protein (p22cr), all products of the precore/core gene share an

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identical 149 amino acid sequence[5].

Currently, there are two strategies to treat HBeAg-negative CHB patients, a finite course with pegylated interferon (Peg-IFN) or a long-term therapy with nucleos(t)ids analogues (NAs). Entecavir or tenofovir monotherapy have been shown to achieve the virological response in almost all adherent patients[6]. However, the reduction of HBsAg levels in HBeAg-negative CHB patients under NAs is very slow (-0.1 log IU/mL/yr)[7,8] with HBsAg loss rates < 1% after five years of NAs therapy[7,9] compared to 4% after 48 wk of Peg-IFN[10]. Moreover, it has been suggested that interleukin 28B (IL28B) rs12979860 polymorphism CC could confer a better probability of response to Peg-IFN in HBeAg-negative CHB patients infected by genotype D[11]. On the other hand, differences in Peg-IFN response rates have been demonstrated according to HBV genotype especially in HBeAg-positive patients[12]. Despite NAs are the most used therapy in HBeAg-negative CHB patients because of its safety, long term therapy is needed. In contrast, the addition of the immunomodulatory effect of Peg-IFN could improve HBsAg loss rates[10,13]. However, this strategy has been mostly evaluated in naïve treatment or HBeAg-positive patients being the information about pre-treatment predictors and the kinetics of serological markers (HBsAg and HBcrAg) scarce during the add-on strategy in HBeAg-negative patients.

In the present study, we have prospectively evaluated the levels of HBsAg and HBcrAg in HBeAg-negative non-cirrhotic CHB patients receiving NAs after the addition of Peg-IFN during forty-eight weeks. The primary aim was to compare the HBsAg and HBcrAg kinetics in both treatment strategies (NA group *vs* Peg-IFN-NA group). The secondary aim was to evaluate the proportion of HBsAg loss at week 96.

MATERIALS AND METHODS

Patients and study design

This is a single center, prospective, non-randomized, open-label trial including HBeAg-negative non-cirrhotic CHB patients, receiving NAs for at least 2 years. Recruitment period was from August 2014 to February 2016 in a tertiary center (Hospital del Mar, Barcelona, Spain). Patients were eligible if they received a stable NAs dose with virological response (undetectable HBV-DNA viral load during the last twelve months). Exclusion criteria were as follows: Patients with a previous Peg-IFN treatment, NA treatment for HBV reactivation prophylaxis, patients with human immunodeficiency virus, hepatitis D virus or hepatitis C virus co-infection, and liver transplanted patients. All patients provided written informed consent.

Patients with any malignancy in the last 5 years, those with psychiatric, thyroid or autoimmune disorders, and non-liver transplanted patients were only eligible for NAs monotherapy. Peg-IFN alpha-2a was offered to be added in all eligible patients. Those who accepted it, received 180 μg/week during forty-eight weeks (Peg-IFN-NA group) and all the other participants remained in NAs monotherapy (NA group). At week 48 all the patients continued with NAs in monotherapy and were followed up until week 96 or loss of follow-up. Protocol visits were at weeks 0, 12, 24, 48, 72 and 96. Figure 1 shows the flowchart of patients and study design.

The study protocol was approved by the Ethical Committee of our Institution "Comitè Ètic d'Investigació Clínica-Parc de Salut Mar", study reference 2014/5787/I, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Clinical variables and definitions

Demographic data, liver stiffness measurement (LSM) and polymorphism rs12979860 of IL28B were assessed at baseline. HBV-genotype was collected from electronic data as it had been performed prior to the initiation of NAs therapy. The levels of HBV-DNA, HBsAg and HBcrAg were analyzed at weeks 0, 24, 48, 96. Adverse events were recorded at each protocol visit, following the Common Terminology Criteria for Adverse Events. All the data were collected and tabulated in a database with an access code to ensure patient confidentiality.

LSM was performed at baseline by a single experienced operator (> 5000 examinations), using the FibroScan® 502 Touch (FibroScan® EchosensTM, Paris, France) following the manufacturer's recommendations as previously described[14]. Liver fibrosis was categorized according to previously published cut-offs for LSM considering significant fibrosis for LSM > 7.2 kPa. Patients with LSM > 12 kPa were considered as having cirrhosis and were excluded[15].

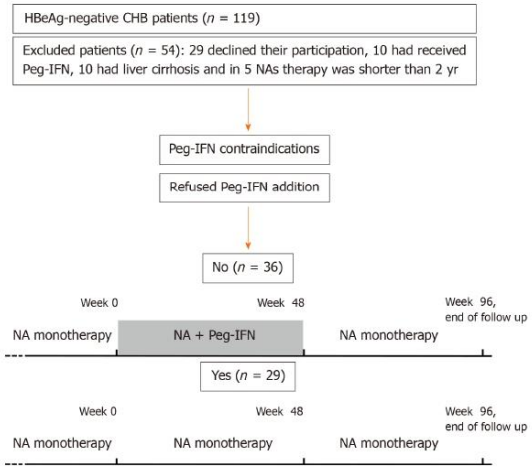

Figure 1 Flowchart of patients and study design. HBeAg: Hepatitis B e antigen; CHB: Chronic hepatitis B; Peg-IFN: Pegylated interferon; NAs: Nucleos(t)ids analogues.

Virological markers

HBV DNA was measured by polymerase chain reaction with a limit of quantification of 10 IU/mL (ABBOTT RealTime HBV m2000®, Abbott Molecular Inc., IL, United States). Serum HBsAg was quantified by Electro-chemiluminescence immunoassay Elecsys® HBsAgII QuantII (Roche Diagnostic, Rotkreuz, Switzerland) according to the manufacturer's instructions. The assay ranged from 0.05 to 117000 IU/mL. In highly concentrated samples above the upper limit, the value of manual dilution was multiplied by the dilution factor. Serum HBcrAg was measured using a quantitative fully automated chemiluminiscent enzyme immunoassay (LUMIPULSE®, Fujirebio Europe, Belgium).

The monoclonal antibodies used in this two-step immunoassay measure simultaneously denatured HBeAg, HBV core antigen and the precore protein p22cr (aa-28 to aa-150). Samples were processed according to the manufacturer's instructions. The lower limit of detection was 2.0 log U/mL, and a linear range of 3.0 log U/mL-7.0 log U/mL (1 kU/mL was equal to 3 log U/mL).

Statistical analysis

Quantitative variables were expressed as medians and ranges. Categorical variables were expressed as proportions. Continuous variables were compared by the Mann–Whitney *U* test or Kruskall-Wallis when appropriate and categorical by the Pearson chi-square test, Fisher test or the Mc Nemar test. Patients were categorized according to antiviral treatment (Peg-IFN-NA group *vs* NA group). Differences between NA and Peg-IFN-NA groups regarding age, sex, IL28B polymorphism, ethnicity, liver function, liver stiffness, treatment duration, viral genotype, HBsAg and HBcrAg levels and HBsAg loss rate were analyzed by univariate analysis. A two-sided P value < 0.05 was considered to indicate statistical significance. The kinetic of HBsAg for each treatment group was evaluated from baseline to weeks 24 and 48 by the slope of the HBsAg decline (log10 IU/mL per week) using a linear regression model (LRM). Statistical analyses were performed with the SPSS® 25.0 (SPSS Inc., Chicago, IL, United States) and LRM with the Prism 7.0 (© 1994-2016 GraphPad Software, Inc.).

RESULTS

Study population and baseline characteristics

From August 2014 to February 2016, 119 HBeAg-negative CHB patients were evaluated. Twenty-nine (24%) patients declined their participation, 10 (8.4%) had

previously received Peg-IFN, 10 (8.4%) had liver cirrhosis and in 5 (4.2%) patients NAs therapy duration was shorter than 2 years. Among the 65 included patients, 5 were only eligible for the NA therapy due to Peg-IFN contraindications and 60 were eligible for both therapies: 36 accepted to receive Peg-IFN and 24 refused the addition of Peg-IFN. Therefore, 36 (55.4%) patients were included in the Peg-IFN-NA group and 29 (44.6%) in the NA group. Two patients in NA group were receiving low doses of corticosteroids (prednisone 2.5 to 5 mg/d) for rheumatoid arthritis and no kidney transplanted patients were included because none of them fulfilled the inclusion criteria.

Figure 1 shows the flowchart and Table 1 the main characteristics of the included patients. Patients in Peg-IFN-NA group compared to NA group were younger (age 45 *vs* 53, $P = 0.01$) and had a shorter previous NA treatment duration (259 *vs* 393 wk, $P = 0.01$), but were comparable in gender, IL28B polymorphism, ethnicity, liver function, liver stiffness, type of NA, HBV genotype and baseline HBcrAg and HBsAg levels. Due to the baseline differences, patients of both treatment groups were individually matched for age and treatment duration. Therefore, pre-treatment predictors and the kinetic of serological markers (HBsAg and HBcrAg) were performed in 48 patients. Table 2 shows the characteristics of matched patients.

HBcrAg kinetics according to baseline variables and treatment group

The median (range) HBcrAg values (log 10 U/mL) was 2.7 (< 2-4.9) in NA group and 2.3 (< 2-3.7) in Peg-IFN-NA group ($P = 0.18$) at baseline. The rate of patients with HBcrAg values below the limit of detection (HBcrAg < 2 log10 U/mL) was 25% and 38%, respectively ($P = 0.39$). The HBcrAg kinetics was described as the delta (Δ) of its levels at weeks 24, 48 and 96. The HBcrAg levels remained stable at weeks 24, 48 and 96 (Table 2). We did not detect differences on HBcrAg levels between both treatment strategies according to the treatment group, the IL28B polymorphism or the HBV genotype. We did not find any correlation between HBcrAg and HBsAg levels nor HBsAg loss rate (data not shown).

HBsAg kinetics according to baseline variables and treatment group

The baseline levels of HBsAg (log 10 IU/mL) were similar in NA and Peg-IFN-NA groups (3.1 *vs* 3.2) ($P = 0.25$). The HBsAg kinetics was described as the delta (Δ) of their levels at weeks 24, 48 and 96. The decline of the HBsAg level was greater in Peg-IFN-NA group (-0.26, -0.40, -0.44) compared to NA group (-0.11, -0.10, -0.12) ($P < 0.05$ in all determinations) (Figure 2).

The HBsAg kinetics was different between treatment arms according to IL28B polymorphism and HBV genotype. In patients with IL28B CC polymorphism ($n = 22$) the decline of HBsAg at weeks 24, 48 and 96 was greater in Peg-IFN-NA group (-0.27, -0.92 and -0.64) than in NA group (-0.11, -0.11 and -0.10) ($P < 0.05$ in all cases) (Figure 3A). In contrast, in patients with IL28B CT/TT ($n = 26$) we did not find differences on HBsAg kinetics at weeks 24, 48 and 96 between Peg-IFN-NA group (-0.09, -0.11 and -0.19) and NA group (-0.10, -0.07 and 0.13) (not significant in all determinations) (Figure 3B). Moreover, the decline of HBsAg were different between NA and Peg-IFN-NA group at weeks 48 and 96 in patients infected by HBV genotype A (-0.07 *vs* -1.05 and -0.08 *vs* -0.53) and genotype D (-0.08 *vs* -0.42 and -0.51 *vs* -0.80) ($P < 0.05$ in all cases) (data not shown).

LRM to recognize different HBsAg kinetics

In order to demonstrate the existence of different HBsAg kinetics for each treatment strategy, we evaluated the slope of the HBsAg decline (log10 IU/mL per week) from baseline to weeks 24 and 48 using a LRM (Figure 4). In patients receiving NA monotherapy, HBsAg levels did not decrease during the forty-eight weeks. The slope of HBsAg kinetics in NA group (-0.00) was similar to zero ($P = 0.6$). On the contrary, in patients receiving Peg-IFN-NA, HBsAg levels significantly decreased during the forty-eight weeks and the slope of HBsAg kinetic (-0.02) was different to zero ($P < 0.001$) and greater than that found in NA group ($P = 0.015$).

Rate of low HBsAg levels and HBsAg loss during follow-up

The proportion of patients reaching low levels of HBsAg (HBsAg < 100 IU/mL) at baseline and at weeks 24, 48 and 96 are depicted in Figure 5. In the NA group the rate of patients with low HBsAg levels was 21% at baseline, but did not change at weeks 24, 48 and 96 (not significant) (Figure 5A). On the contrary, rate of patients with low HBsAg levels in Peg-IFN-NA group was 4.2% at baseline and increased at weeks 24 (16.7%), 48 (29.6%) and 96 (16.7%) ($P = 0.001$) (Figure 5B). The proportion of patients

Table 1 Main characteristics of the included patients

	NA group (*n* = 29)	Peg-IFN-NA group (*n* = 36)	*P* value
Age (yr)	53 (36-70)	45 (26-72)	0.01
Males, *n* (%)	21 (72)	29 (81)	0.44
IL28B polymorphism, *n* (%)			0.16
CC	11 (37.9)	20 (55.6)	
CT/TT	14 (62.1)	16 (44.4)	
Origin (ethnicity), *n* (%)			0.70
Europe	20 (69)	20 (56)	
Asia	12 (33)	12 (33)	
Africa	3 (10)	3 (8)	
AST (IU/mL)	20 (15-59)	22 (12-62)	0.37
ALT (IU/mL)	19 (12-101)	25 (12-91)	0.20
GGT (IU/mL)	19 (9-197)	22 (10-125)	0.33
LSM, *n* (%)			0.91
< 7.2 kPa	28 (97)	34 (97)	
7.2-12 kPa	1 (3)	1 (3)	
NA treatment, *n* (%)			
Tenofovir	20 (69)	22 (61)	0.46
Entecavir	7 (24)	11 (31)	
Others	2 (7)	3 (8)	
NA treatment duration (wk)	393 (113-763)	259 (118-496)	0.01
HBV genotype, *n* (%)			0.99
Non-D	7 (24.1)	16 (44.4)	
D	12 (41.4)	13 (36.1)	
Not available	10 (34.5)	7 (19.4)	
Baseline HBcrAg (log 10 U/mL)	2.65 (< 2-4.9)	2.30 (< 2-3.7)	0.18
Baseline HBsAg (log 10 IU/mL)	2.96 (1.3-4.2)	3.22 (1.6-4.6)	0.07

Quantitative variables are expressed as median (range); qualitative variables are expressed as *n* (%). NA: Nucleos(t)id analogue; Peg-IFN: Pegylated interferon; IL28B: Interleukin 28B; AST: Aspartate aminotransferase; ALT Alanine aminotransferase; GGT: Gamma-glutamyl transferase; LSM: Liver stiffness measurement; HBV: Hepatitis B virus; HBcrAg: Hepatitis B core-related antigen; HBsAg: Hepatitis B surface antigen.

achieving HBsAg loss in the Peg-IFN-NA group (*n* = 3, 12.5%) was higher compared to NA group (*n* = 0, 0%), but the difference did not reach the statistical significance (*P* = 0.07).

Patients with HBsAg loss were male, with low fibrosis stage (F0-F1), and infected by HBV-genotype A (*n* = 1) or B (*n* = 2). Two patients had an IL28B CC polymorphism and the other a CT polymorphism. All of them had been on NAs therapy for more than 5 years before the addition of Peg-IFN. The NAs treatment was entecavir (*n* = 1), tenofovir (*n* = 1) and telbivudine (*n* = 1). Baseline levels of HBsAg (log10 IU/mL) were 4.0, 2.1 and 1.6, and baseline levels of HBcrAg (log10 U/mL) were 2.7, < 2 and 3.4, respectively. All of them received Peg-IFN during forty-eight weeks. Two patients lost HBsAg during therapy (week 24 and 36) and one at week 24 after Peg-IFN discontinuation (week 72).

Safety

No serious adverse events were observed during treatment and follow-up. However, 8 (22%) patients did not complete Peg-IFN treatment. The reasons for Peg-IFN discontinuation were flu-like symptoms and asthenia (*n* = 3), DNA flare (*n* = 3),

Table 2 Characteristics of matched patients in each treatment group

	NA group (*n* = 24)	Peg-IFN-NA group (*n* = 24)	*P* value
Age (yr)	54 (36-60)	45 (26-63)	0.07
Male sex, *n* (%)	18 (75)	22 (91)	0.12
IL28B polymorphism, *n* (%)			0.25
CC	9 (38)	13 (54)	
CT/CT	15 (62)	11 (46)	
Origin (ethnicity), *n* (%)			0.20
European	17 (70)	12 (50)	
Asia	3 (12)	9 (38)	
Africa	2 (8)	3 (12)	
AST (IU/mL)	20 (15-59)	22 (15-38)	0.69
ALT (IU/mL)	20 (12-101)	23 (15-50)	0.41
GGT (IU/mL)	23 (9-197)	22 (11-125)	0.44
LSM, *n* (%)			0.32
< 7.2 kPa	23 (96)	24 (100)	
7.2-12 kPa	1 (4)	0 (0)	
NA treatment, *n* (%)			0.32
Tenofovir	16 (67)	12 (50)	
Entecavir	6 (25)	9 (38)	
Others	2 (8)	3 (12)	
NA treatment duration (wk)	378 (113-763)	272 (139-495)	0.06
HBV genotype, *n* (%)			0.43
A	5 (21)	4 (17)	
B	1 (4)	3 (12)	
C	0 (0)	2 (8)	
D	10 (42)	8 (33)	
E	1 (4)	2 (8)	
F	0 (0)	1 (4)	
Not available	7 (29)	4 (18)	
Baseline HBcrAg (log 10 U/mL)	2.7 (< 2-4.9)	2.3 (< 2-3.7)	0.18
Baseline HBcrAg (log10 U/mL), *n* (%)			0.39
< 2	6 (25)	9 (38)	
2-2.5	4 (17)	6 (25)	
2.5-3	6 (25)	3 (12)	
3-3.5	2 (8)	3 (12)	
3.5-4	3 (13)	3 (12)	
> 4	3 (13)	0 (0)	
Baseline HBsAg (log10 IU/mL)	3.1 (1.3-4.2)	3.2 (1.6-4.4)	0.25
Baseline HBsAg (IU/mL), *n* (%)			0.22
> 1000	12 (50)	14 (48)	
100-1000	7 (29)	9 (38)	
< 100	5 (21)	1 (4)	

HBcrAg decline (log10 U/mL)			
Δ Week 24	0.00 (-1.10-1.21)	0.00 (-0.71-0.30)	0.96
Δ Week 48	0.00 (-1.00-0.30)	0.00 (-1.31-1.10)	0.25
Δ Week 96	0.00 (-1.00-0.10)	0.00 (-0.71-0.71)	0.12
HBsAg decline (log10 IU/mL)			
Δ Week 24	-0.11 (-0.04-0.00)	-0.26 (-3.8-0.1)	0.01
Δ Week 48	-0.10 (-1.17-0.04)	-0.40 (-4-0.02)	0.00
Δ Week 96	-0.12 (-1.39-0.96)	-0.44 (-4-0.01)	0.00
HBsAg Loss; *n* (%)	0 (0)	3 (12.5)	0.07

Quantitative variables are expressed as median (range); qualitative variables are expressed as *n* (%). NA: Nucleos(t)id analogue; Peg-IFN: Pegylated interferon; IL28B: Interleukin28B; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; LSM: Liver stiffness measurement; HBV: Hepatitis B virus; HBcrAg: Hepatitis B core related antigen; HBsAg: Hepatitis B surface antigen.

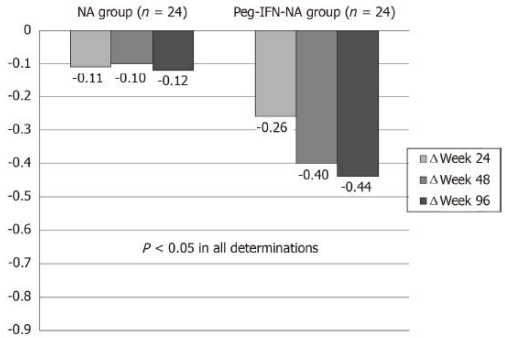

Figure 2 Hepatitis B surface antigen delta (Δ) (log10 IU/mL) at wk 24, 48 and 96 according to treatment group. Peg-IFN: Pegylated interferon; NA: Nucleos(t)ids analogue.

polyarthritis (*n* = 1) and Graves' thyroiditis (*n* = 1). No patients discontinued antiviral treatment in NA group.

DISCUSSION

In this controlled trial of HBeAg-negative CHB non-cirrhotic patients under NAs treatment and with undetectable DNA, the addition of 48 wk of Peg-IFN alfa-2a reduced HBsAg levels further and faster than continuing with NAs monotherapy. However, the proportion of patients with HBsAg loss during the first ninety-six weeks did not reach the statistical significance with this add-on strategy.

HBsAg kinetics has been shown as one of the best predictors of treatment response[6,16,17]. However, patients of our Peg-IFN-NA group were younger and had a shorter previous NA treatment duration compared to NA group. According to previously published studies showing a decrease of HBsAg levels with NA therapy[18] and a higher probability to HBsAg clearance in aged populations[19] we decided to match the included patients for age and treatment duration.

The present study prospectively confirms our previously published results[7] regarding the slow decline of HBsAg levels in HBeAg-negative CHB patients receiving NAs therapy. The current study has demonstrated a very low decline (-0.12 log10 IU/mL at week 96) and very slow change (-0.00 log10 IU/mL per week) of HBsAg levels in patients receiving NAs. As a consequence, the rate of patients with low HBsAg levels (< 100 IU/mL) did not change at weeks 24, 48 and 96, and no patient achieved HBsAg loss. On the contrary, the addition of Peg-IFN clearly increased the

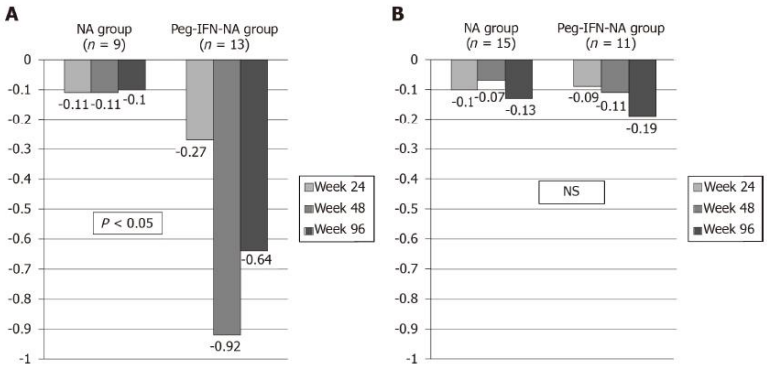

Figure 3 Hepatitis B surface antigen delta (Δ) (log10 IU/mL) according to interleukin 28B polymorphism and treatment group. A: Hepatitis B surface antigen delta (Δ) in interleukin 28B CC patients (n = 22); B: Hepatitis B surface antigen delta (Δ) in interleukin 28B CT/TT patients (n = 26). NS: Not significant; NA: Nucleos(t)ids analogue; Peg-IFN: Pegylated interferon.

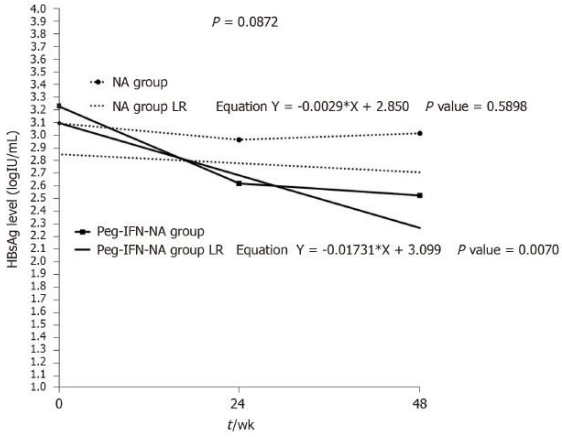

Figure 4 Linear regression model of hepatitis B surface antigen levels according to treatment group. NA: Nucleos(t)ids analogue; Peg-IFN: Pegylated interferon; LR: Linear regression.

decline (-0.44 log10 IU/mL at week 96) and accelerate the decrease (-0.02 log10 IU/mL per week) of HBsAg levels compared to NA group. Therefore, in the Peg-IFN-NA group the rate of patients with low HBsAg levels was higher at weeks 24 (16.7%) and 48 (29.6%) and the rate of HBsAg loss increased (n = 3, 12.5%) compared to NA group (n = 0, 0%).

We also analyzed the HBcrAg levels during the study in both treatment strategies. However, levels of HBcrAg remained stable during the 96 wk without differences between both treatment strategies and without correlation with HBsAg levels or HBsAg loss rate. This could be explained by the fact that baseline levels of HBcrAg in our cohort of HBeAg negative patients, receiving NAs during a long time period before inclusion, were already low. As described before, the rate of patients with a baseline HBcrAg value below the limit of detection (< 2 log10 U/mL) was high in both treatment groups (25% and 38%). Recent studies have shown that HBcrAg can reflect cccDNA transcriptional activity in the different phases of HBV infection[20,21]. However as HBeAg is included in HBcrAg, this could explain the low baseline HBcrAg levels in our cohort of HBeAg-negative patients. Moreover, recent studies, have described that

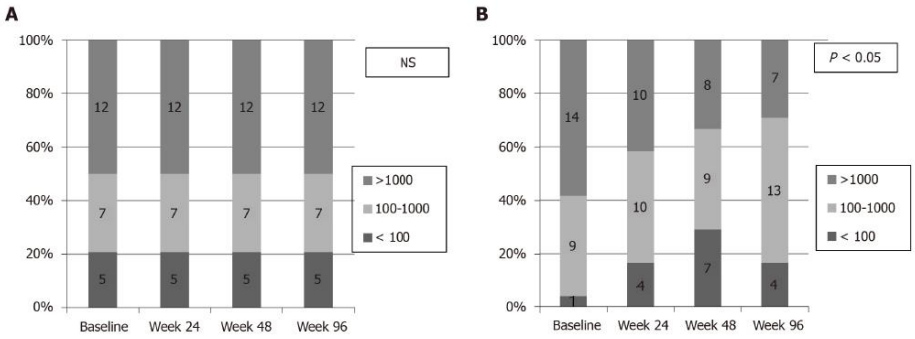

Figure 5 Rate of patients with low hepatitis B surface antigen levels (Hepatitis B surface antigen < 100 IU/mL and 100-1000 IU/mL) according to treatment group. A: NA group; B: Peg-IFN-NA group. NS: Not significant; NA: Nucleos(t)ids analogue; Peg-IFN: Pegylated interferon.

HBcrAg levels can decline over the time in patients undergoing NAs therapy, especially in HBeAg-negative patients[22,23]. Thus, according to our results, we have not found that HBcrAg determination could be a useful serum marker in clinical practice for monitoring treatment response in HBeAg-negative patients receiving NAs or Peg-IFN-NAs.

It has been suggested that low levels of HBsAg are related to higher rates of HBsAg loss after NA discontinuation, being advisable to achieve low levels of HBsAg before stopping NA therapy[24,25]. Our study showed that the rate of patients with HBsAg < 100 IU/mL increased in the Peg-IFN-NA group from 4.2% at baseline to 29.6% at 48 wk (P = 0.001). The NAs have shown to restore partly adaptive immunity, whereas Peg-IFN boosts innate immunity and depletes the ccc-DNA, which leads to a major HBsAg loss[26-29]. The analysis performed in matched patients by age and treatment duration showed that the proportion of HBsAg loss during the first 96 wk was higher in the Peg-IFN-NA group compared to the NA group. However, this difference did not reach the statistical significance probably due to the limited number of included patients and the short follow-up time of our study. Nevertheless, our results are in accordance with smaller studies previously published[30,31] and in line with the results published by Bourlière *et al*[32] during the execution of the current study.

Previous studies have linked the presence of IL28B CC polymorphisms with the HBsAg loss in HBeAg-negative CHB patients receiving Peg-IFN. It has been shown that CC polymorphism could confer a better response profile to Peg-IFN therapy than CT/TT polymorphisms, especially in patients infected by HBV genotype D[11,33]. We analyzed the HBsAg kinetics according to IL28B polymorphism, and we found that patients with CC polymorphism showed a higher HBsAg decline in Peg-IFN-NA group compared to NA group. On the contrary, HBsAg kinetics was similar in both treatment strategies in CT/TT patients. Therefore, the add-on strategy should not be recommended in patients with IL28B CT or TT polymorphism.

Our study has several limitations. First, the treatment assignment was not randomized. However, patients on both treatment strategies were individually matched for age and treatment duration to make the cohort comparable. Second, the acceptance of the add-on strategy was low and only 40% of eligible patients with a previous (well-tolerated) NA therapy accepted the addition of Peg-IFN due to its potential toxicity. Third, the frequent adverse events of Peg-IFN (22% of discontinuations) caused a low number of patients completing 48 wk of therapy making this therapeutic strategy difficult to be introduced in clinical practice. However, this applicability and tolerability are in line with previous published data[32]. Fourth, the treatment duration of Peg-IFN was limited to 48 wk and the follow-up period to 96 wk. Therefore, patients with a rapid HBsAg decline could have taken advantage of a longer therapy or longer follow-up. Finally, the low rate of HBsAg loss did not allow to identify predictors associated with HBsAg loss. However, the LRM demonstrated different HBsAg kinetics after adding Peg-IFN.

CONCLUSION

In conclusion, our prospective, non-randomized, open-label clinical trial has demonstrated that the addition of Peg-IFN to NAs decreased HBsAg levels further and faster compared to NA monotherapy. The HBcrAg levels remained stable. Despite the low applicability and poor tolerance of Peg-IFN making difficult its use in clinical practice, it could be considered in selected patients with favorable HBV genotype and IL28B polymorphism.

ARTICLE HIGHLIGHTS

Research background

Functional cure of chronic hepatitis B (CHB), defined as the loss of hepatitis B surface antigen (HBsAg), is very unusual with current antiviral treatments in hepatitis B e antigen (HBeAg)-negative patients. HBsAg levels decline very slow in patients receiving nucleos(t)ids analogues (NAs). Therefore, they need long-term antiviral treatment.

Research motivation

The hypothesis that we wanted to answer with our study was that the addition of pegylated-interferon (Peg-IFN) could accelerate the decline of HBsAg levels in patients that were receiving NAs and that this therapeutic strategy could increase the HBsAg loss rate.

Research objectives

In our study we wanted to evaluate in patients under NAs therapy if the addition of Peg-IFN could decrease HBsAg and hepatitis B core-related antigen (HBcrAg) levels, and increase HBsAg loss rate. If HBeAg-negative patients could achieve low levels of HBsAg it could be a good strategy to shorten the antiviral treatment.

Research methods

We have performed a prospective, non-randomized, open-label trial evaluating the combination of Peg-IFN 180 μg/wk plus NAs during forty-eight weeks *vs* NAs in monotherapy, in HBeAg-negative non-cirrhotic CHB patients after a minimum of two years of NA therapy and with virological response.

Research results

We have shown that the addition of Peg-IFN 180 μg/wk during forty-eight weeks to NAs caused a greater and faster decrease of HBsAg levels compared to NA therapy alone, especially in those patients with interleukin 28B polymorphism CC. However, the HBcrAg levels remained stable after adding Peg-IFN to NAs. We have also shown that, the low acceptance by the patients of this therapeutic strategy and the side effects of Peg-IFN can limit its use in clinical practice.

Research conclusions

This study shows that the addition of Peg-IFN to NA therapy accelerates the decline of HBsAg, especially in patients with interleukin 28B polymorphism CC. Therefore, even Peg-IFN has several side effects, this treatment strategy could be offered to some selected patients in order to achieve the functional cure of CHB. On the other hand, our study shows that HBcrAg levels do not seem useful to monitor this kind of treatment, neither as a predictor of HBsAg loss.

Research perspectives

It is well known that patients with HBeAg-negative CHB usually need a long-term therapy with NAs, even lifelong, to achieve HBsAg loss. However, it has been suggested that low levels of HBsAg are related to higher rates of HBsAg loss after NA discontinuation, being advisable to achieve low levels of HBsAg before stopping NA therapy.

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10.2. ANNEX 2:

Aquesta tesi ha estat elaborada amb el suport econòmic de la beca del Instituto de Salud Carlos III, Ministerio de Economía y Competitividad (PI14/00540), co-finançada pel Fons Europeu de Desenvolupament Regional (FEDER), Unión Europea, Una manera de hacer Europa.