國立臺灣大學醫學院微生物學科暨研究所

碩士論文

Graduate Institute of Microbiology
College of Medicine
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Master Thesis

利用次世代定序分析 B 型肝炎病毒在干擾素治療中的演變

Analysis of HBV adaptations to interferon treatment – NGS approach

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本論文係馬元策君（學號 R05445135）在國立臺灣大學微生物學所完成之碩（博）士學位論文，於民國 107 年 7 月 16 日承下列考試委員審查通過及口試及格，特此證明

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Dedication

To all those who suffer from Hepatitis B and to their families.

Acknowledgements

I would like to thank my advisor, Hung-Chih Yang M.D. Ph.D., from the Graduate Institute of Microbiology at National Taiwan University for his invaluable advice and guidance throughout this project. I would also like to thank Su-Ru Lin Ph.D., who processed and sequenced the patient samples. Without your help, this project would not have been possible. I would like to acknowledge the precious feedback I received from Hurng-Yi Wang Ph.D. and Pay-Long Chen M.D. Ph.D. of the thesis committee, who allowed this project to progress to its current level.

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Abstract

The Hepatitis B Virus is a promising candidate to observe the immune response of its host. Because the evolutionary rate of HBV is as high as that of RNA viruses, the virus has the opportunity to change in response to host factors, including the immune pressure. In addition, HBV causes a chronic infection that lasts long enough for the virus to change in response to the immune pressure. We aim to use this opportunity to investigate how measurements of HBV evolution may provide information about the immune response of patients.

Our main hypothesis is that different immune responses cause different evolutionary pressures. As a result, the goal of this research project is to develop a method to monitor the quality of the patient's immune response through analysis of its impact on the evolution of the HBV virus. The final objective is to correlate this information with clinical outcomes to evaluate the potential of this analytical method as a tool that might provide additional indications to help guide HBV treatment.

To conduct this study, we used samples from 23 patients with chronic HBV genotype B infection that underwent 24 weeks of interferon therapy. We analyzed NGS sequences of the virus before treatment, at the halfway point of treatment, at the end of treatment, and 24 weeks after termination of treatment.

All patients were HBeAg-positive at the beginning of the treatment. We studied the differences in HBV evolution that correlated with two clinical outcomes: HBeAg seroconversion and viral load reduction by at least 90%. These two outcomes are the most important immune-related events in the natural course of HBV infection.

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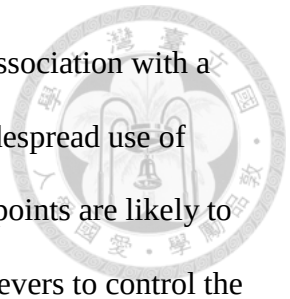

These two outcomes are particularly important because of their association with a decreased probability of mother-to-child transmission. With the now widespread use of vaccination at birth for children born of HBV carriers, these clinical endpoints are likely to become of increased public health relevance as the next best actionable levers to control the HBV pandemic.

We developed quantitative measurements to detect signs of selection against a particular allele (diversifying selection) in the evolution of the virus sequences. This allows for the evaluation of the intensity of the diversifying selection as well as the identification of the precise location of the amino acids that cause the allele to be selected against.

This method can be used to derive general trends in groups of patients as well as to build personal profiles of individual patients. We hope that further advances in the development of applications from the methods described in this document will provide clinicians with additional information for their prognosis and for guiding targeted treatment to improve the clinical outcomes of patients suffering from chronic Hepatitis B.

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中文摘要

B 型次肝炎病毒很適合當作模式來觀察宿主的免疫反應。HBV 的演化速度跟 RNA 病毒一樣快， 因此 HBV 會因爲宿主的因素（包含免疫反應）而改變。再加上，HBV 經常造成慢性的感染，所以該病毒有足夠的時間因爲免疫反應而演化。我們的目標是利用這兩個特色來研究 HBV 演化的衡量方式如何能提供病人免疫反應的更多資訊。

我們主要的假設是不同的免疫反應帶來不同的演化壓力。本論文的研究目的是找到一個方法利用 HBV 演變的分析來評估病人免疫反應的品質。最後的目標是探討從這個分析得到的資訊是否跟臨床病情有關係，且是否可以利用這些資訊來協助病人的治療。

23 位慢性 B 型肝炎病人（基因型 B）經過 24 周的干擾素治療。治療前，所有病人屬於 HBeAg 陽性的狀態。在四個時間點，我們利用次世代定序技術來分析病人的樣本：治療前，治療中點（第 12 周），治療結束，以及治療後第 24 周。

我們研究 HBV 的演變與兩個臨床結果的關係：HBeAg seroconversion 和病毒量達到 90%以上的減少。這兩個臨床結果在 HBV 的自然歷史成爲跟免疫系統有關的最重要的結果，而且對病人死亡率的減少和對母親傳染嬰兒機率的減少都有重要的貢獻。目前嬰兒 HBV 疫苗接種越來越普遍，因此，這兩個臨床結果逐漸成爲預防母親傳染嬰兒的重要工具，利於協助解決 HBV 大流行。

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本研究介紹不同計量衡量方式來分析病毒定序檢測對等位基因不利的演化壓力（多樣化壓力）。這個工具可以辨認多樣化壓力是針對基因裏頭的哪一個位子，以及衡量其多樣化壓力的强度。

這個分析方法可以在不同的病患組顯示多數的趨勢以及建立病患個人的資料. 我們希望未來更多的進步能讓本論文介紹的計量方法提供醫師更多的資訊，協助預後判斷與治療客製化，改善 B 型肝炎病患的治療績效。

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Table of Contents

doi:10.6342/NTU201802768

doi:10.6342/NTU201802768

List of figures and tables

doi:10.6342/NTU201802768

doi:10.6342/NTU201802768

1. Introduction

1.1 The public health burden of HBV

Deaths from viral hepatitis are increasing alarmingly and may overcome TB for the top position among the most lethal infectious diseases. The three major infectious diseases that cause the most significant mortality burden – tuberculosis, human immunodeficiency virus, and malaria – all are on a declining trend of mortality. In contrast, global annual mortality from viral hepatitis is increasing quickly. Worldwide deaths from viral hepatitis increased by 22% from 2000 to 2015, reaching a level of 1.34 million deaths in 2015 [1].

HBV is the leading cause of mortality from viral hepatitis, accounting for two third the annual deaths. The two main complications associated with chronic infection by HBV are liver cirrhosis and hepatocellular carcinoma (HCC). Epidemiologic studies have shown that HBV might be responsible for half of HCC cases and a third of cirrhosis cases on a worldwide basis [2]. According to most recent estimates, approximatively 2,100 patients die every day because of cirrhosis and liver cancer from chronic HBV infection. 778,000 deaths happened in 2015 as a result of HBV-related cirrhosis and HCC. In contrast, acute hepatitis accounts for only less than 10% of HBV-related mortality [1] [3].

HBV is a major public health issue. 257 million people live with chronic HBV around the world, representing 3.5% of the global population [1]. In addition, epidemiologic studies suggest that chronic HBV (CHB) patients have an all-cause mortality rate 70% higher than that of the general population [4].

doi:10.6342/NTU201802768

1.2 The long-term control of the HBV pandemic

A very effective vaccine is available to protect from HBV. In many countries, the HBV vaccine is part of the routine immunization schedule and efforts to increase the vaccine coverage are underway in developing nations with a high HBV burden. As a result, infection after childhood is becoming less common [5].

A growing proportion of new infections occurs in newborns, who cannot benefit from the protective effects of vaccination. In developed countries with a high HBV prevalence where the vaccine is widely available, mother-to-child transmission has become the primary route of infection. To reach the public health goals of HBV control and eradication, prevention of mother-to-child transmission will be the key actionable factor once vaccine coverage has become universal [1] [5].

With the continuous application of current practices that reduce childhood transmission, the prevalence of HBV in successive generations should be ever decreasing. We can expect the incidence and prevalence of HBV to slowly converge to zero over the very long run. This might however require an extended amount of time and effort [5].

To speed up this process, it is particularly important to work on developing new strategies that help understand and prevent mother-to-child transmission.

doi:10.6342/NTU201802768

1.3 Important milestones toward recovery from chronic HBV infection

HBe antigen (HBeAg) seroconversion is important because it lowers the rate of mortality of HBV patients and reduces mother-to-child transmission. In an epidemiologic study, the all-cause mortality rate has been estimated to be reduced by as much as 41% in HBeAg-negative patients compared to that in HBeAg-positive patients [4]. HBe-positive mothers have an approximate 20% risk of transmitting HBV to their child if vaccinated at birth [6]. In contrast, transmission from an HBeAg-negative mother to her child vaccinated at birth is a rare event (< 1%) [7]. This is particularly important because adults chronically infected with HBV include around 65 million women of childbearing age worldwide. Similarly, viral load reduction is important because it also reduces the mortality rate and mother-to-child transmission [1] [4].

As vaccination at birth increases, the mother's HBeAg seroconversion status and her viral load become two of the leading factors determining transmission at birth. Vaccination coverage at birth has increased dramatically over the 2000-2015 period. It has recently reached a high of about 80% in the Western Pacific region, which has a high prevalence (0.9%) in children under 5 years. In contrast, most progress can be made in the African region the prevalence is as high as 3.0% but the vaccination at birth coverage is only approximatively 10%. Other areas that could benefit from increased vaccination include the Eastern Mediterranean region (1.6% prevalence in children, 20% vaccination at birth) and the South-East Asian region (0.7% prevalence in children, 30% vaccination at birth) [1].

doi:10.6342/NTU201802768

1.4 Natural history of chronic HBV infection

CHB patients usually go through three phases over the course of their disease: the immune tolerant phase, the immune clearance phase, and the inactive carrier phase [8].

In the immune tolerant phase, the action of the immune system is thought to be minimal. Accordingly, there is little immunopathology-mediated tissue damage. Viral titers are very high but liver enzymes (ALT and AST) do not show a heightened level of hepatocellular death. This is the initial status of CHB patients and can last for decades [8].

In contrast, the immune clearance phase is characterized by inflammation and active targeting of HBV-infected cells by the host immune system. Liver enzymes are elevated and fluctuating, signaling multiple rounds of liver damage. Viral titers fall drastically. Oftentimes, the patient concomitantly undergoes seroconversion against the envelope antigen. This phase can last multiple years during which the patient typically displays many of the visible symptoms caused by HBV infection [8].

Last, the patient enters the inactive carrier phase. Viral titers are low and liver enzymes remain in the normal range. Most patients have developed antibodies against the envelope antigen of HBV [8].

While in the inactive carrier phase, some patients can experience a reactivation of the infection. In this situation, a rebound in viral load and an elevation of liver enzymes occur. Reactivation is associated with poorer long-term prognosis [8].

On the other hand, if the patient develops antibody against the surface antigen, the viral load drops to undetectable levels and the infection becomes functionally resolved [8].

doi:10.6342/NTU201802768

1.5 Treatment options for Chronic Hepatitis B

Two approved regimens of treatment are available to CHB patients: treatment with interferon, or treatment with nucleoside and nucleotide analogs [9].

Interferon treatment is based on interferon-α or PEGylated interferon-α-2a. This treatment has immunomodulatory effects in addition to its antiviral effects. The typical treatment course is 6 or 12 months and is associated with a ~30% chance of HBeAg seroconversion. There is no emergence of resistance to this treatment. However, it is associated with uncomfortable side effects and is not ideally tolerated by patients. In addition, the treatment must be administered by frequent subcutaneous injections, which further increases the discomfort for patients [9] [10].

Treatment with nucleoside and nucleotide analogs induces stronger direct antiviral effects but seldom causes HBeAg seroconversion. The treatment is not limited in time and must be continued indefinitely until the therapeutic range is reached. Relapse is more common. The main advantage is that this treatment is well-tolerated and is administered orally. The drugs that might be used are lamivudine, entecavir, telbivudine, adefovir, and tenofovir. However, their long-term safety profile has not been entirely elucidated [9] [10].

The ultimate goal of current CHB treatment strategy is to attain what is best described as a “functional cure”. If the patient can develop antibodies against both the E and S antigens, the replication and viral load of HBV genome is almost obliterated. As a result, patients can become asymptomatic with good clinical outcomes [10].

However, a functional cure is not an actual cure because the HBV genome may persist within the hepatocytes, either in integrated or episomal forms [10] [11].

doi:10.6342/NTU201802768

1.6 HBV specificities making it suitable to observe the host immune response

One of challenges in treating CHB patients is that physicians usually cannot know in detail what the immune system of a specific patient is doing, and how this might differ from other patients. We typically only know whether or not the patient is producing a set of antibodies against specific viral targets: the surface (S), core (C), and envelope (E) proteins. All of which are markers of the humoral adaptive immune response.

One important aspect that remains hidden from health care providers is the status of the action of the cell-mediated adaptive immune response. Which epitopes are recognized by T-cells, how many epitopes are recognized, and the strength of the resulting immune reaction are crucial factors that need to be further explored. As a result, we need new tools designed to observe and measure these factors.

Fortunately, HBV is a good candidate to observe the host immune response. The most important reason for this is that the virus has a high mutation rate caused by replication through reverse transcription. This unusual replication mechanism makes HBV able to mutate quickly, as fast as RNA viruses do [12]. In consequence, HBV should be able to adapt promptly to host factors, including the immune pressure.

In addition, HBV has the particularity of causing a chronic type of infection. This allows the virus to stay long enough into the host and evolve in response to the immune system. Current deep-sequencing technology is capable of measuring this evolution. As a result, we find worthy to investigate whether this technology can be used on HBV genome sequences to provide additional information about the state of the immune response within patients.

doi:10.6342/NTU201802768

1.7 Current knowledge on virus host interaction

HBV has been shown to successfully change its viral targets when they come under pressure [13]. For example, HBV has developed several common mutations in its reverse transcriptase domain that helps the virus resist treatment with nucleoside analogs [14] [15]. It is just as plausible that HBV could make changes (immunoediting) in the targets that are under immune pressure. The specificities that allow HBV to adapt to the immune response and the subsequent changes in the immune response represent a special opportunity to observe the coevolution of the virus and the host immune response.

The human immunodeficiency virus (HIV) is another virus that has the same two attributes (high mutation rate and long-term infection). Previous studies have shown that HIV is able coevolve with the host immune response. Specifically, it was shown to adapt to the HLA phenotype of the host [16]. The hepatitis C virus (HCV), a third pathogen meeting these two attributes, has also been proven to adapt to the immune response of its host [17].

The main challenge in HBV research is the lack of suitable animal models that could accurately mimic the human infection course [18]. As a result, experimentation is limited in scope and analysis is restricted to the few types of human samples that can be collected in accordance with ethics and availability. HBV is one the least well understood pathogen of major human significance. New methods must therefore be developed to obtain new insights on this viral disease while working under these restrictive constraints.

So far, the majority of NGS studies in HBV have focused on investigating the role of HBV variants and quasi-subspecies in the development of cirrhosis and HCC. In this study we applied NGS technology toward studying the immunoediting mechanism occurring during interferon treatment, with or without the patients undergoing eSC.

doi:10.6342/NTU201802768

2. Study aims

In this research project, we tried to take advantage of deep-sequencing technology to test hypotheses about the how the viral genome evolves depending on the specificities of the host immune response and apply results to improving patient care.

The first goal of this project is identifying the factors in the HBV genome that influence clinical outcomes in chronic HBV patients. Our hypothesis is that the genome sequence has elements that influences the outcomes of chronic HBV treatment. The objective of this analysis is to improve forecasting of clinical outcomes.

The second goal is developing a method to monitor the quality of the host immune response through analysis of its influence on the evolution of the HBV virus. The hypothesis is that different immune responses cause different evolutionary pressures. We aimed at correlating immune response profiles in patients with clinical outcomes in order to evaluate the clinical impact of the different types of immune responses. The objective is to evaluate the potential of this method as a tool to provide additional help for guiding HBV treatment.

doi:10.6342/NTU201802768

3. Materials and methods

3.1 Patients

This research is based on the NGS data obtained from a retrospective cohort of 23 patients with CHB of genotype B recruited by Hepatitis Research Center at National Taiwan University Hospital. All patients were HBeAg-positive at baseline.

3.2 Treatment

Patients underwent 24 weeks of interferon treatment and were followed for an additional 24 weeks after the end of the treatment (Figure 1).

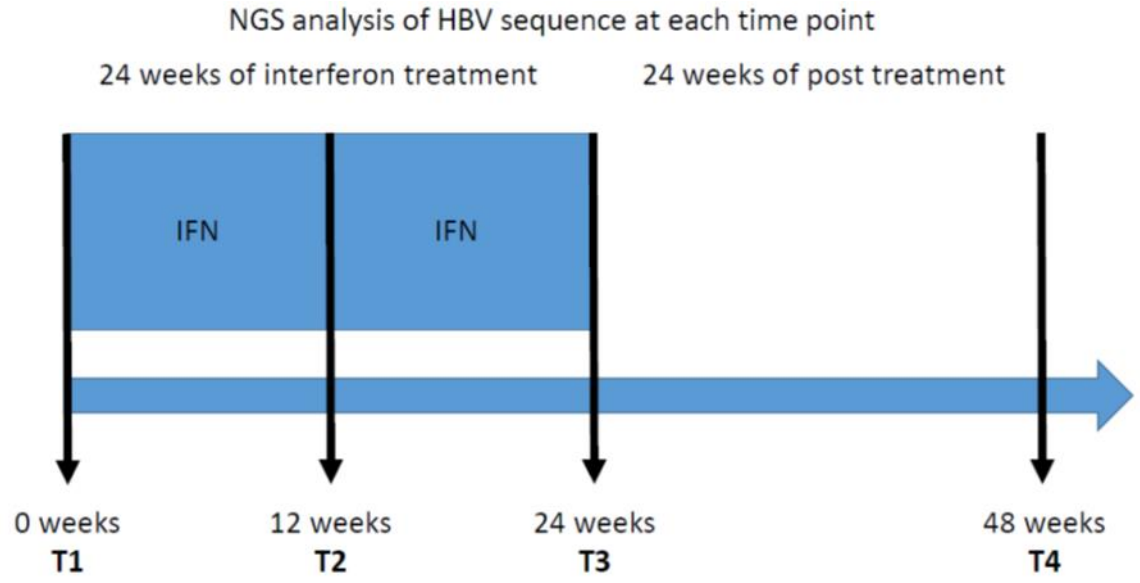

Figure 1: Treatment protocol and definition of time points

doi:10.6342/NTU201802768

3.3 Sample collection and analysis

Samples were collected the beginning, midpoint, and end of the treatment. In addition, samples were taken at the end of the following period. At each time point, deep-sequencing was performed to obtain nucleotide frequencies at each position within the HBV genome.

Expected pattern of viral evolution:

- Before T1: Adaptation to host factors
- T1 to T3: Adaptation to IFN treatment
- T3 to T4: Adaptation to new host status + reversion of adaptation to IFN

To study how HBV adapted to interferon treatment, most analyses in this project focused on the T1 to T3 comparison. Additional analyses of T4 were performed when relevant. Patients with a missing sequence at any collection time were excluded.

3.4 Next-generation sequencing

Sequencing was performed using the Illumina MiSeq platform and the protocol previously described in HC Yang et al. (manuscript unpublished as of 2018/08/08 – this section will be updated upon its publication).

doi:10.6342/NTU201802768

3.5 Software analysis

Scripts to analyze the data obtained from NGS sequencing were written in Python on the Anaconda platform and executed by the Spider interface. The source code of the scripts used in this project is available upon request. As part of the processing of NGS data, nucleotide alleles with a frequency below 5% were removed from the analyzed sequences to reduce the noise from sequencing uncertainty.

doi:10.6342/NTU201802768

4. Clinical outcomes and classification of patients

The outcomes measured when analyzing patients were the HBeAg seroconversion status at T4, viral load reduction from T1 to T4, and ALT reduction from T1 to T4.

35% (8/23) of patients experienced HBeAg seroconversion (eSC). We defined viral load response as a reduction of at least 90% in viral load (1 log). 65% (15/23) of patients were classified as responders by this criteria. We investigated whether or not these two outcomes were dependent. Both the Chi-square independence test (P = 0.47) and Fischer's exact test (P = 0.29) revealed no conclusive evidence of dependence based on our patient outcomes. We therefore proceeded by treating those two outcomes separately as independent treatment endpoints, classifying patients into four groups.

	HBeAg seroconversion	No HBeAg seroconversion	Total
Response	6 (26%)	9 (39%)	15
No response	2 (9%)	6 (26%)	8
Total	8	15	23

E-seroconversion rate:		Response rate:	
▪ Responders	40%	▪ eSC	75%
▪ Non-responders	25%	▪ No eSC	60%

Figure 2: Summary of the principal clinical outcomes

Unfortunately, 9% (2/23) of patients, or 25% (2/8) of HBeAg seroconverters, underwent eSC without experiencing a sustained reduction in viral load. This particular outcome has been linked with a poorer prognosis, recurrent flare-ups, and a higher risk of

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developing cirrhosis. Of interest, this outcome is associated with the emergence of HBeAg-negative mutant HBV. This issue is further discussed in section 5.1.3 (page 16).

We observed a correlation between the change in viral load and the change in ALT of the patients between T1 and T4 (Figure 3). This represents a correlation of 0.76 ($P < 0.0001$). If we separate the patients into eSC and no-eSC groups, the correlations are respectively 0.71 ($P = 0.048$, $n = 8$) and 0.78 ($P = 0.0006$, $n = 15$). Because the ALT level is an indicator of liver damage, this further emphasizes the beneficial impact of a sustained viral load reduction.

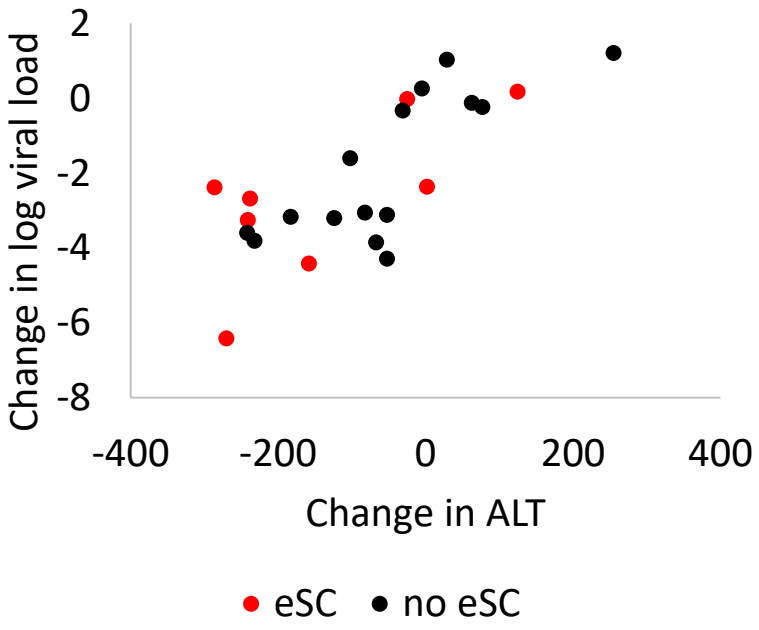

Figure 3: Correlation between changes in viral load and ALT

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5. Identification of factors in the HBV genome that might influence clinical outcomes in chronic HBV patients

Hypothesis: the genome sequence has elements that influence the outcomes of chronic HBV treatment

Objective: to improve forecasting of clinical outcomes

Part1:

Identification of point mutations that could be associated with important adaptation mechanisms

Part 2:

Analysis of the pair-wise distances between viral genomes that lead to similar clinical outcomes

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5.1 Identification of point mutations that could be associated with important adaptation mechanisms

5.1.1 Points with extensive change in amino acid frequencies

First, we wanted to investigate whether our samples could help identify new point mutations within the HBV genome that could be associated with the development of specific clinical outcomes. We thought that such points may indicate the presence of positive selection and optimization of the viral sequence to the new immune status of the host, especially if these mutations displayed a large change in amino acid allele frequency that is shared among several patients.

We listed, on all four genes, the amino acid position for which an amino acid allele frequency changed from < 50% at T1 to > 90% at T4. Unfortunately, we found that these points with extensive change in amino acid allele frequency were not shared among patients and therefore concluded that our data failed to provide enough evidence that these mutations had a particular biological meaning.

Nevertheless, we noticed the presence of abnormal stop codons within our list of points with extensive change. Because of the dramatic biological effects that an abnormal stop codon has on the encoded protein, we decided to further investigate these types of mutations.

doi:10.6342/NTU201802768

5.1.2 Abnormal stop codons

We listed all the amino acid positions that had a stop codon allele with a frequency of at least 5%, excluding normal stop codons at the end of the genes. Next, we looked if these abnormal stop codons were conserved among patients and patient groups (Figure 4 and Figure 5).

5.1.3 Stop codon at position 28 of gene C: pre-core stop mutation

We found that the C28 stop codon, often known as the pre-core stop mutation, was extensively shared among patients. This mutation is known to be associated with the transition to HBeAg-negative CHB by abolition of HBeAg production. In addition, this mutation displayed distinct patterns of prevalence and emergence among different patient groups (Figure 4, upper left).

The C28 stop codon is present at a high frequency even before the beginning of the treatment (T1) in the patients who will eventually develop eSC without experiencing a viral load reduction. Furthermore, its frequency quickly and distinctly increases to near the totality of the analyzed sequences in this particular group only.

This suggests that the emergence of C28 stop codon could be both a cause of HBeAg production loss and its consequence. The biological meaning of the mutation and its presence before the determination of clinical outcome suggests a causal relationship with eSC without viral load response, potentially becoming a measurable risk factor for this unfavorable outcome. The data was also consistent with the mutation being selected by the development of antibodies against HBeAg (Figure 4, upper left).

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We propose that the dramatic and progressive increase in the frequency of this mutation in patients who underwent eSC without viral load reduction could be a mechanism for the virus to escape control by anti-HBeAg antibodies. If the patient mounts an immune responses toward only HBeAg, the C28 stop mutation is effective in allowing HBV to escape the immune response, preventing viral load reduction. In contrast, should the patient also develop an effective immune response toward other HBV targets, the host might become able to successfully reduce HBV viral load. This proposed model would mean that immune targeting of HBeAg is not enough to effectively suppress HBV. Further studies are required to test and refine this hypothesis.

Because all HBV patients develop antibodies against HBcAg early in the infection, the implication of our proposed model is that immune targeting of gene C's two gene products (HBeAg and HBcAg) is not enough to establish an effective immune control of HBV viral load and replication.

5.1.4 Stop codon at position 211 of gene C

The C211 stop codon removes the last two amino acids of the C and E proteins. The biological significance of this mutation is not yet fully understood. This mutation was more frequent in patients that underwent both eSC and viral load response at T1 and at T4 (Figure 4, upper right).

The C-terminal has been characterized as a protamine-like domain that mediates interaction with HBV genetic materials [19]. In addition, some animal models have suggested that deletions of even a few amino acids from the C-terminal end of HBcAg may

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inhibit HBV clearance while maintaining its replication competency. This was shown to be linked with an impairment in both humoral and cell-mediated immune recognition against HBcAg [20].

5.1.5 Stop codon at position 141 of gene X

The X141 stop codon was present only in patients that experienced a viral load response (Figure 4, lower left). C-terminal deletion of the X protein have been associated with an increased risk of developing HCC [21]. In vitro studies have suggested that the C-terminal domain has a pro-apoptotic activity inhibiting the oncogenic properties of the X protein and that its removal promotes hepatocarcinogenesis [21].

5.1.6 Multiple stop codons on the S gene

Notably, several patients had multiple stops codons at seemingly random location across the S gene. The location of these stop codons was not conserved among different patients. To measure this phenomenon, we computed for each patient the cumulative frequency of all the abnormal stop codons found on the S gene. We call this measurement the multiple S stop frequency (Figure 4, lower right). The meaning of the different pattern of the average multiple S stop frequency in patients groups is not clear to us at this point. We think this should be further explored and the existence of a link with the development of occult HBV is worthy to be investigated.

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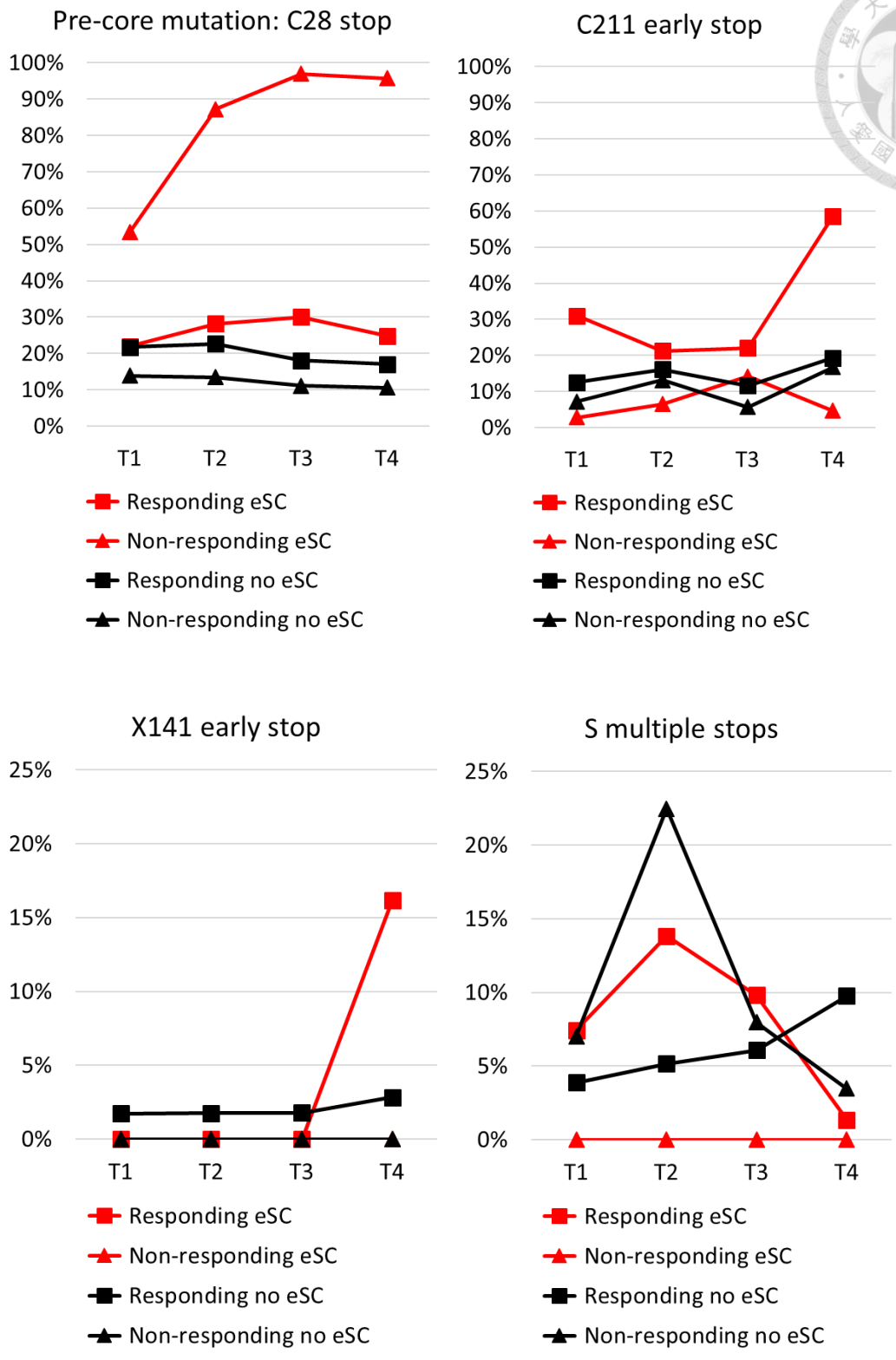

Figure 4: Frequencies of abnormal stop codons – group averages

doi:10.6342/NTU201802768

C28					
Comparison	Versus	T1	T2	T3	T4
HBeAg seroconversion with viral load response	HBeAg seroconversion without viral load response	0.33	**0.003**	**0.001**	**0.0006**
HBeAg seroconversion with viral load response	No HBeAg seroconversion but viral load response	0.99	0.71	0.31	0.45
HBeAg seroconversion with viral load response	No HBeAg seroconversion and no viral load response	0.56	0.27	0.15	0.17
HBeAg seroconversion without viral load response	No HBeAg seroconversion but viral load response	0.32	**0.0002**	**0.0000**	**0.0001**
HBeAg seroconversion without viral load response	No HBeAg seroconversion and no viral load response	0.27	**0.0001**	**0.0001**	**0.0009**
No HBeAg seroconversion but viral load response	No HBeAg seroconversion and no viral load response	0.56	0.45	0.46	0.40
C211					
Comparison	Versus	T1	T2	T3	T4
HBeAg seroconversion with viral load response	HBeAg seroconversion without viral load response	**0.09**	0.30	0.73	**0.035**
HBeAg seroconversion with viral load response	No HBeAg seroconversion but viral load response	0.27	0.71	0.55	**0.099**
HBeAg seroconversion with viral load response	No HBeAg seroconversion and no viral load response	0.15	0.57	0.34	**0.08**
HBeAg seroconversion without viral load response	No HBeAg seroconversion but viral load response	0.28	0.41	0.90	0.24
HBeAg seroconversion without viral load response	No HBeAg seroconversion and no viral load response	0.42	0.56	0.66	0.28
No HBeAg seroconversion but viral load response	No HBeAg seroconversion and no viral load response	0.57	0.80	0.39	0.85
X141					
Comparison	Versus	T1	T2	T3	T4
HBeAg seroconversion with viral load response	HBeAg seroconversion without viral load response	N/A	N/A	N/A	0.11
HBeAg seroconversion with viral load response	No HBeAg seroconversion but viral load response	0.19	0.35	0.35	0.18
HBeAg seroconversion with viral load response	No HBeAg seroconversion and no viral load response	N/A	N/A	N/A	0.11
HBeAg seroconversion without viral load response	No HBeAg seroconversion but viral load response	0.19	0.35	0.35	0.20
HBeAg seroconversion without viral load response	No HBeAg seroconversion and no viral load response	N/A	N/A	N/A	N/A
No HBeAg seroconversion but viral load response	No HBeAg seroconversion and no viral load response	0.19	0.35	0.35	0.20
Multiple S					
Comparison	Versus	T1	T2	T3	T4
HBeAg seroconversion with viral load response	HBeAg seroconversion without viral load response	0.20	0.24	0.24	0.36
HBeAg seroconversion with viral load response	No HBeAg seroconversion but viral load response	0.59	0.46	0.65	0.11
HBeAg seroconversion with viral load response	No HBeAg seroconversion and no viral load response	0.96	0.66	0.85	0.44
HBeAg seroconversion without viral load response	No HBeAg seroconversion but viral load response	0.35	0.25	**0.04**	**0.06**
HBeAg seroconversion without viral load response	No HBeAg seroconversion and no viral load response	0.27	0.22	0.28	0.19
No HBeAg seroconversion but viral load response	No HBeAg seroconversion and no viral load response	0.66	0.35	0.80	0.24

Figure 5: Statistical analysis of abnormal stop codons – p-value table

Red: statistically significant ($P < 0.05$); Green: not statistically significant but $P < 0.10$

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5.2 Analysis of the pair-wise distances between viral genomes that lead to similar clinical outcomes

5.2.1 Estimation of amino acid frequencies from nucleotide frequencies

Our method for sequencing the HBV genome provides us with nucleotide allele frequencies (A, T, C, and G) at each nucleotide position within the HBV genome. However, it does not indicate the frequency of the encoded amino acids alleles. It is therefore crucial to compute the amino acid allele frequencies from NGS nucleotide data. Amino acids are encoded in codons of three nucleotides. Even though we know the frequency of nucleotide alleles in each of the three positions within the codon, we do not always know the frequency of a specific combination of three nucleotide alleles. As a result, a method to estimate these frequencies was implemented as follows.

From nucleotide allele frequencies in a codon, we can estimate amino acid allele frequencies:

- Perfectly if there is no SNP or one SNP in the codon
- Perfectly if there are two SNPs in the codon but one is synonymous
- Imperfectly if there are two SNPs in the codon and both are non-synonymous
- Imperfectly if there are three SNPs in the codon

An SNP is defined as a nucleotide position with at least one minor allele: a nucleotide allele other than the main nucleotide allele with a frequency of at least 5%.

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Summary of the types of codons present in the patient samples:

- 23 patients, 4 NGS sequence per patient, 1613 codons in the HBV genome
- 1613 * 4 * 23 = 148,396 codons
- Codons without SNP: 93.09 %
- Codons with one SNP: 6.28 %
- Codons with two SNPs: 0.60 %
- Codons with three SNPs: 0.02 %
- Codons with perfect knowledge of the associated amino acid frequency: > 99.38 %

Importantly, the location of the few codons for which we have imperfect knowledge of the amino acid alleles encoded is known. In some of the analyses in this study, we will compare the results obtained from analyzing the data with and without including the information from these uncertain codons to obtain a more accurate picture of the phenomena we attempt to investigate.

Example: computation of the amino acid allele frequency for isoleucine

- Three codons encode Isoleucine: ATT, ATC, and ATA.
- F[isoleucine] = (F[1A] * F[2T] * F[3T]) + (F[1A] * F[2T] * F[3C])
  + (F[1A] * F[2A] * F[3A])
- Where F[1A] stands for the frequency of the nucleotide allele A at codon location 1

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5.2.2 Measurement of genetic distance

The Manhattan distance was chosen to measure genetic distances between samples because it is the most unbiased distance measurement for NGS data.

$$Distance_{position} = \frac{\sum(\,|\,\Delta f[a.a.]_{position}\,|\,)}{2}$$

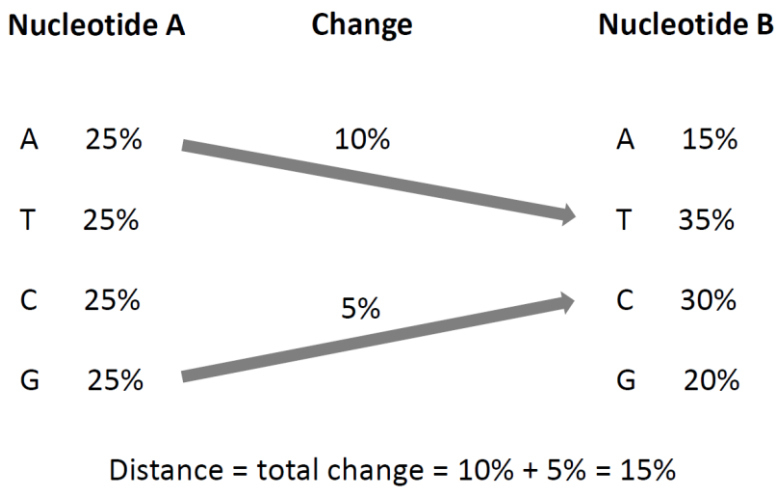

Figure 6: Illustration of genetic distance computation

The Euclidian distance was also considered and computed, but the results were not materially different than what is obtained using the Manhattan distance. Figure 6 depicts the measurement of genetic distance between nucleotides. The same principle was used to compute the genetic distance between amino acids, except that each position had 21 possible alleles (20 amino acids + stop codon) instead of just four (A, T, C, G).

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5.2.3 Pair-wise distances within patient groups for each HBV gene

Pair-wise distances are computed as the average distance between each possible combination of two patients within the patient group.

On gene X, non-responding eSC patients tended to have sequences closest to each other while non-responding no-eSC patients tended to have them farthest. Only responding eSC patients showed a large increase in their pair-wise distance from T1 to T4 (Figure 7).

On gene C, all groups showed a similar pair-wise distance and a moderate increase except for non-responding no-eSC patients, who remained steady at a closer distance.

On genes P and S, all groups showed an increase in pair-wise distances, with responding eSC patients displaying the most dramatic increase. Non-responding eSC patients again tended to have sequences closest to each other (Figure 7).

The fact that groups diverged instead of converging suggests that while patients in the same group may share a similar evolutionary rate, they are not evolving in the same direction. Each seems to go to its own destination by selecting random mutations. This suggests that selection against existing alleles is prevalent over positive selection, further highlighting the role that the immune system must have in driving viral evolution.

5.2.4 Regions in HBV genes that vary in pair-wise distances within patient groups

To further investigate the biological implication of the divergence of HBV sequences over time, we computed the pair-wise distance for each amino acid in HBV genes (Figure 8, Figure 9, Figure 10, and Figure 11). The blue line represents T1 and the red line represents T3. The vertical axis is the average genetic distance at the position.

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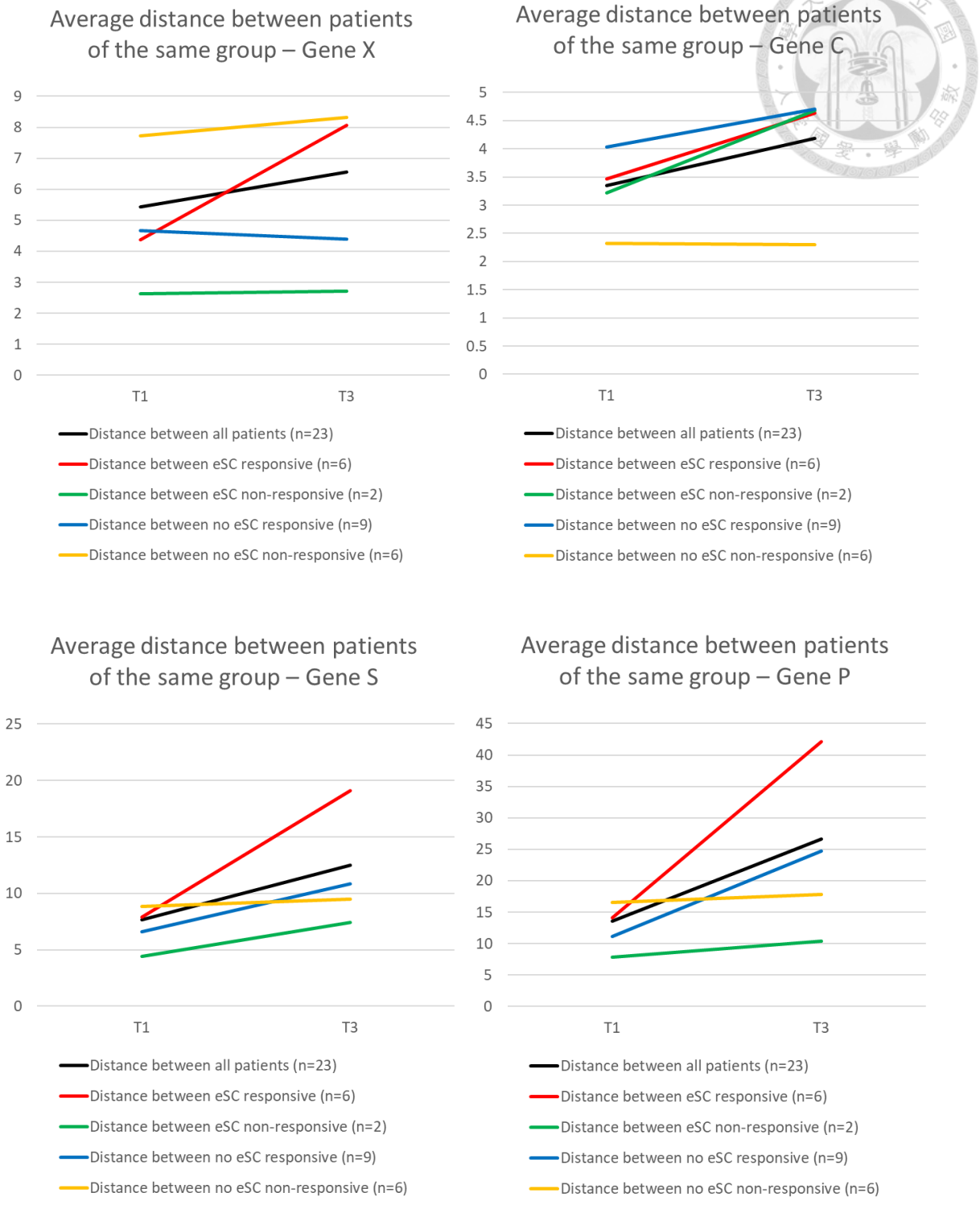

Figure 7: Average distances within groups

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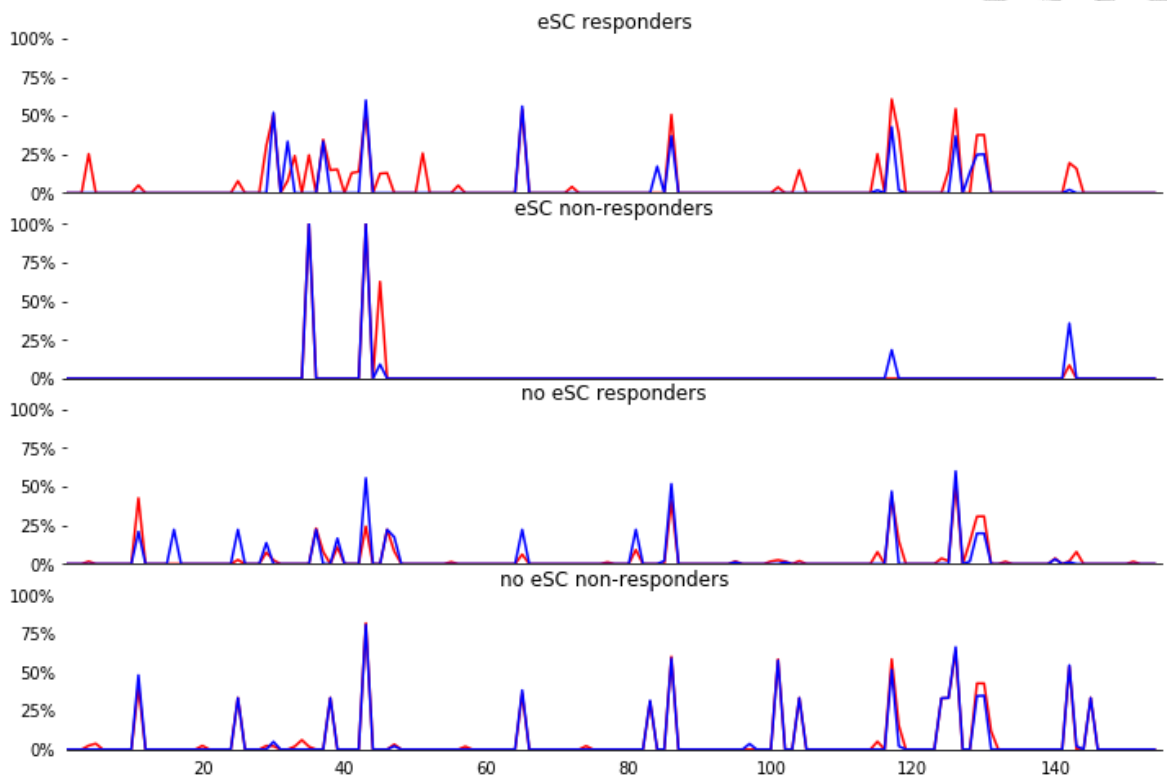

Figure 8: Regional pairwise distances - gene X

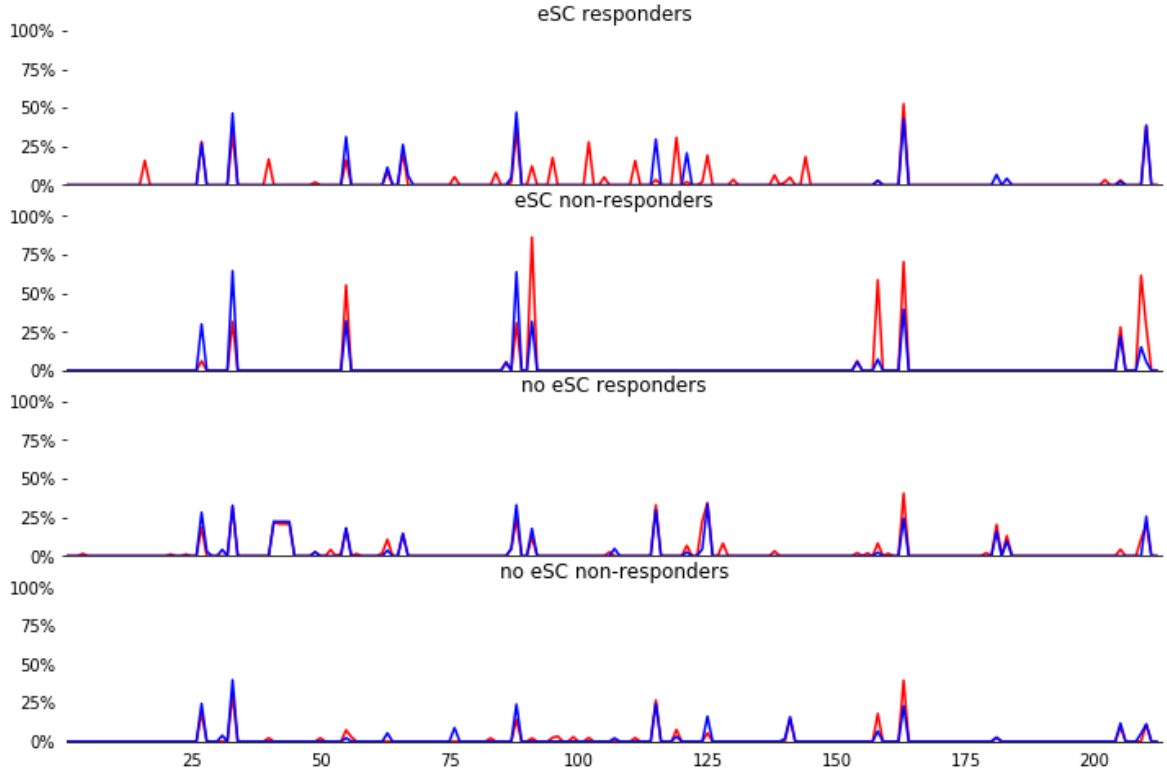

Figure 9: Regional pairwise distances - gene C

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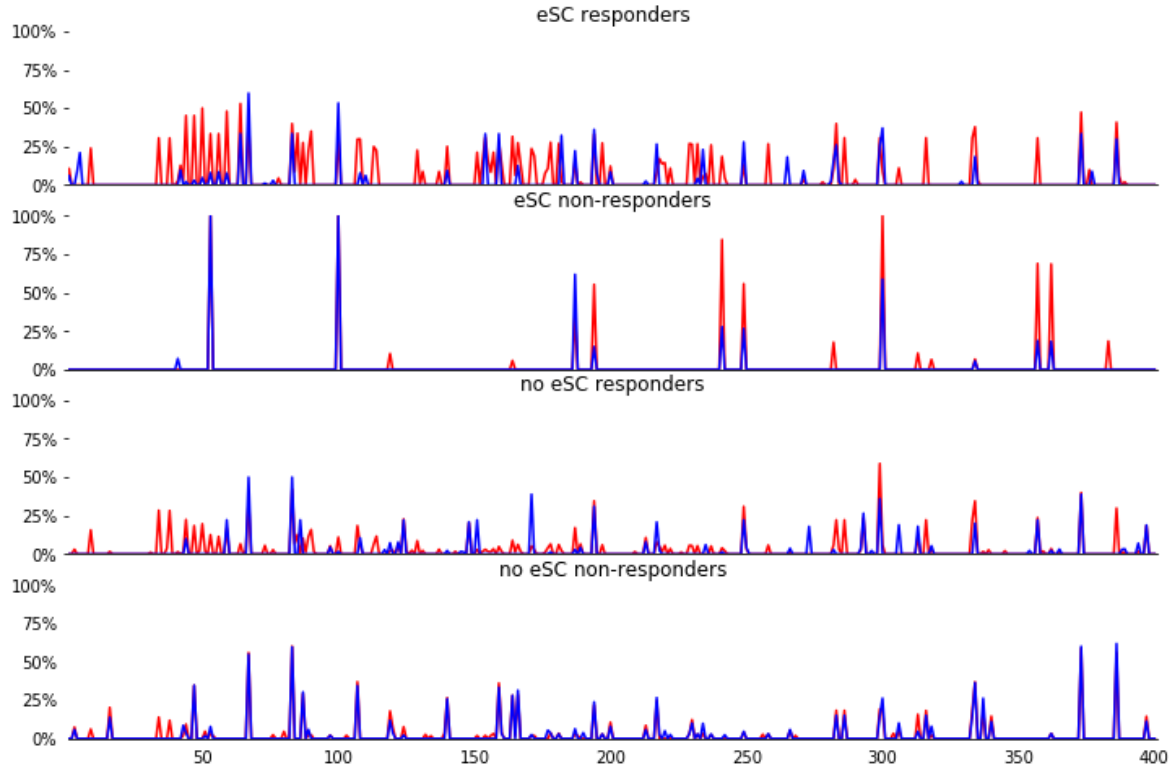

Figure 10: Regional pairwise distances - gene S

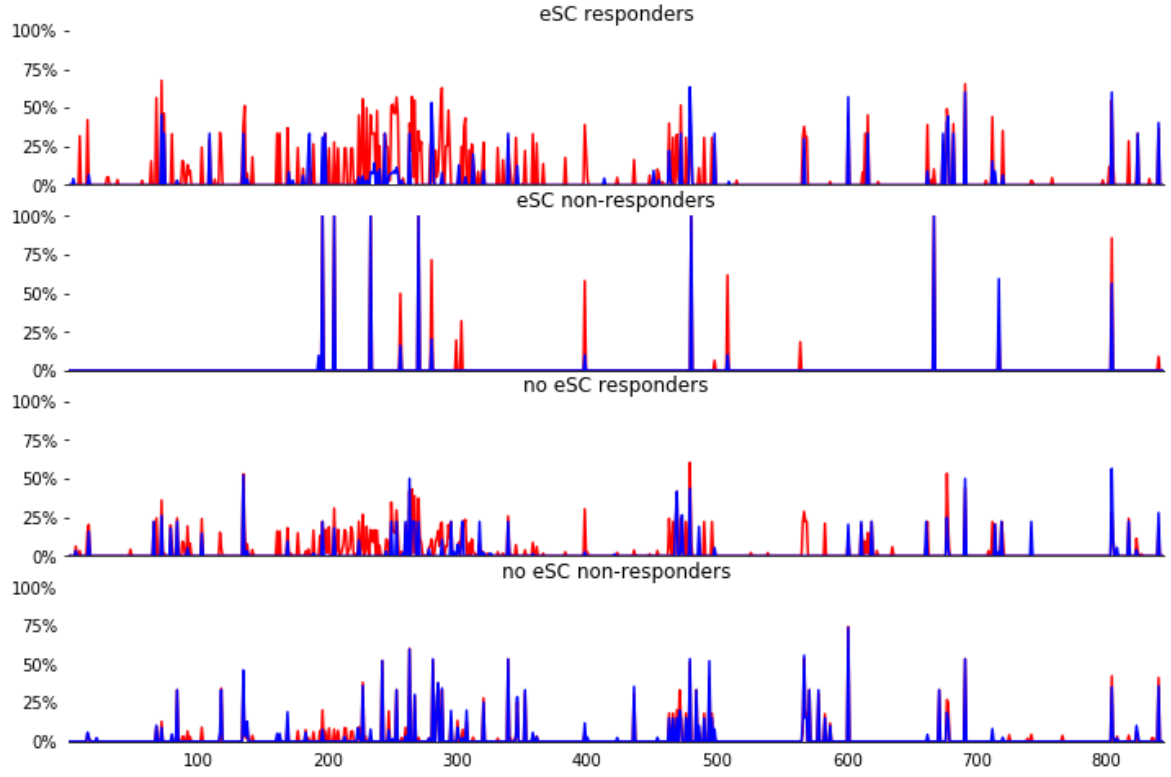

Figure 11: Regional pairwise distances - gene P

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6. Developing a method to evaluate the quality of the host immune response through analysis of its influence on the evolution of the HBV virus

Hypothesis: different immune responses cause different evolutionary pressures

Goal: to correlate immune response profiles in patients with clinical outcomes in order to evaluate the clinical impact of the different types of immune responses

Objective: to evaluate the potential of this method as a tool to provide additional help for guiding HBV treatment

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6.1 Influence of clinical outcomes on HBV evolution

HBV evolution is influenced by the host immune response. We observed that different groups of patients had different patterns of evolutionary rate on HBV genes. Viral load responders all had the similar pattern of evolutionary rate: X > S > P > C.

However, responders who also underwent eSC had a higher magnitude of evolutionary rate overall. In contrast, eSC patients who did not have viral load response displayed the lower overall magnitude of evolution. The C gene seems to have a stable evolutionary rate that might be unaffected by the type of clinical outcome experienced by the patient. This may be linked to a higher level of structural constraints exerted on this gene (Figure 12).

The table in Figure 12 summarizes the p-values associated with the differences in mean evolutionary rate among each pair of patient groups. Evolution on gene X appears to be the most impacted by the different clinical outcomes. The absence of statistical significance on some of the observed differences might be caused by the very small size of samples when patients are categorized into four groups: responding eSC (6 patients) non-responding eSC (2 patients), responding non-eSC (9 patients), and non-responding non-eSC (6 patients).

Each patient group had a different pattern. This is consistent with our hypothesis that different immune responses cause different evolutionary pressures (Figure 12).

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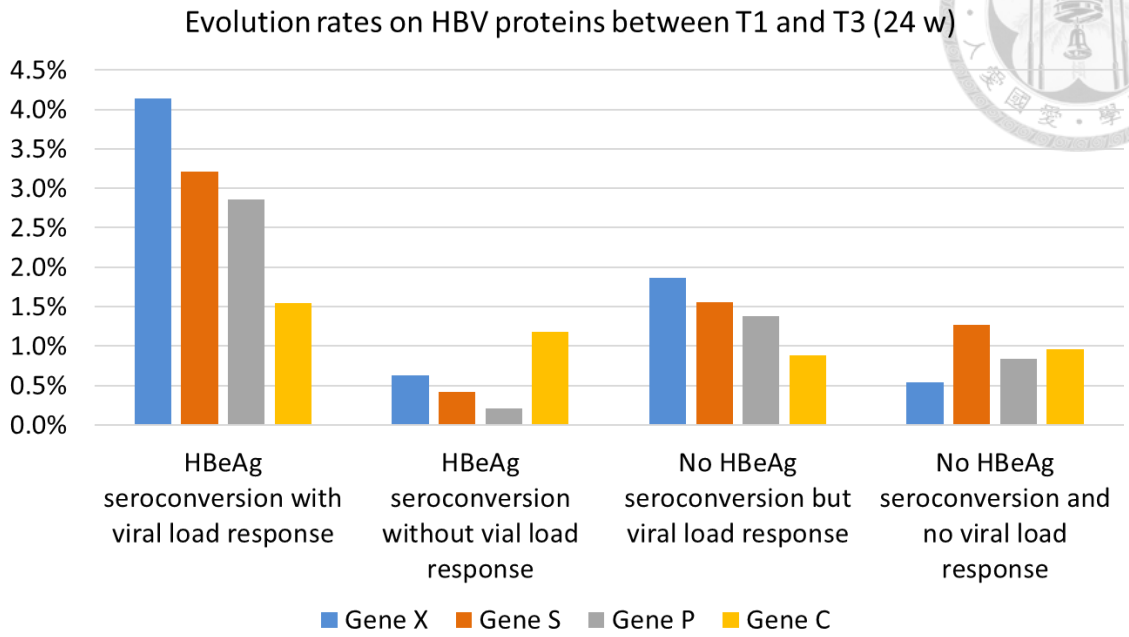

Evolutionary rate (manhatan distance - change from T1 to T3)		p-value			
Comparison	Versus	C	P	S	X
HBeAg seroconversion with viral load response	HBeAg seroconversion without viral load response	0.21	**0.071**	**0.072**	**0.028**
HBeAg seroconversion with viral load response	No HBeAg seroconversion but viral load response	**0.049**	0.27	0.25	0.10
HBeAg seroconversion with viral load response	No HBeAg seroconversion and no viral load response	**0.072**	0.15	0.20	**0.023**
HBeAg seroconversion without viral load response	No HBeAg seroconversion but viral load response	**0.029**	**0.017**	**0.009**	0.11
HBeAg seroconversion without viral load response	No HBeAg seroconversion and no viral load response	0.15	0.15	0.23	0.89
No HBeAg seroconversion but viral load response	No HBeAg seroconversion and no viral load response	0.67	0.34	0.70	**0.041**

Figure 12: Patterns of evolutionary rates associated with clinical outcomes
Red: statistically significant ($P < 0.05$); Green: not statistically significant but $P < 0.10$

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6.2 Flexibility index

6.2.1 Purpose

The purpose of the flexibility index is to detect negative selection from possible T cell epitopes. The strategy behind it is the identification of regions under intense diversifying selection. A signal indicating a high level of diversifying selection may correlate with an epitope-rich region.

6.2.2 Mechanism

Identifying regions where the number of combination of amino acid alleles among the population in sequences of nine amino acids positions might help identify possible sites under widespread T-cell mediated negative selection.

Proposed mechanism for the flexibility index to detect epitope-rich regions:

- Some regions within HBV proteins might be rich in CD8 T cell epitopes
- In many individuals, mutations in that region will be selected
- Because mutations occur randomly from the error-rate of the reverse transcriptase, the sequences that are selected will be different among individuals (within the limits of structural constraints)
- This region will see a large number of different amino acid alleles within the population
- The number of combinations of amino acid alleles among the population in sequence of nine amino acid positions in the HBV protein will be distinctively high in this region

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The flexibility index is probably a good indicator to identify common T cell epitopes because:

- T cells recognize sequential epitopes of amino acids
- HBV has a high mutation rate, allowing it to respond to immune pressure

6.2.3 Computation

Flexibility index at position p :

$$Flexibility\ index_p = n_{p-4} \times n_{p-3} \times n_{p-2} \times n_{p-1} \times n_p \times n_{p+1} \times n_{p+2} \times n_{p+3} \times n_{p+4}$$

Where n represents the number of amino acid minor alleles with a frequency > 5% in the population at the position indicated in subscript.

6.2.4 Interpretation

How to interpret the chart:

- Diversifying selection: flexibility index ↑ ↑
- Neutral selection: flexibility index ↑
- Positive selection: flexibility index ↓
- Structural constraints: flexibility index ↓ ↓

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6.3 Selection index

6.3.1 Purpose

To investigate how the virus responds to immune pressure

6.3.2 Mechanism

Selection pressure favorable to an allele should cause a convergence of frequencies toward this allele. Similarly, selection pressure unfavorable to an allele should cause a divergence of frequencies away from this allele. We attempted to measure this by analyzing the changes in frequency other than the main change occurring.

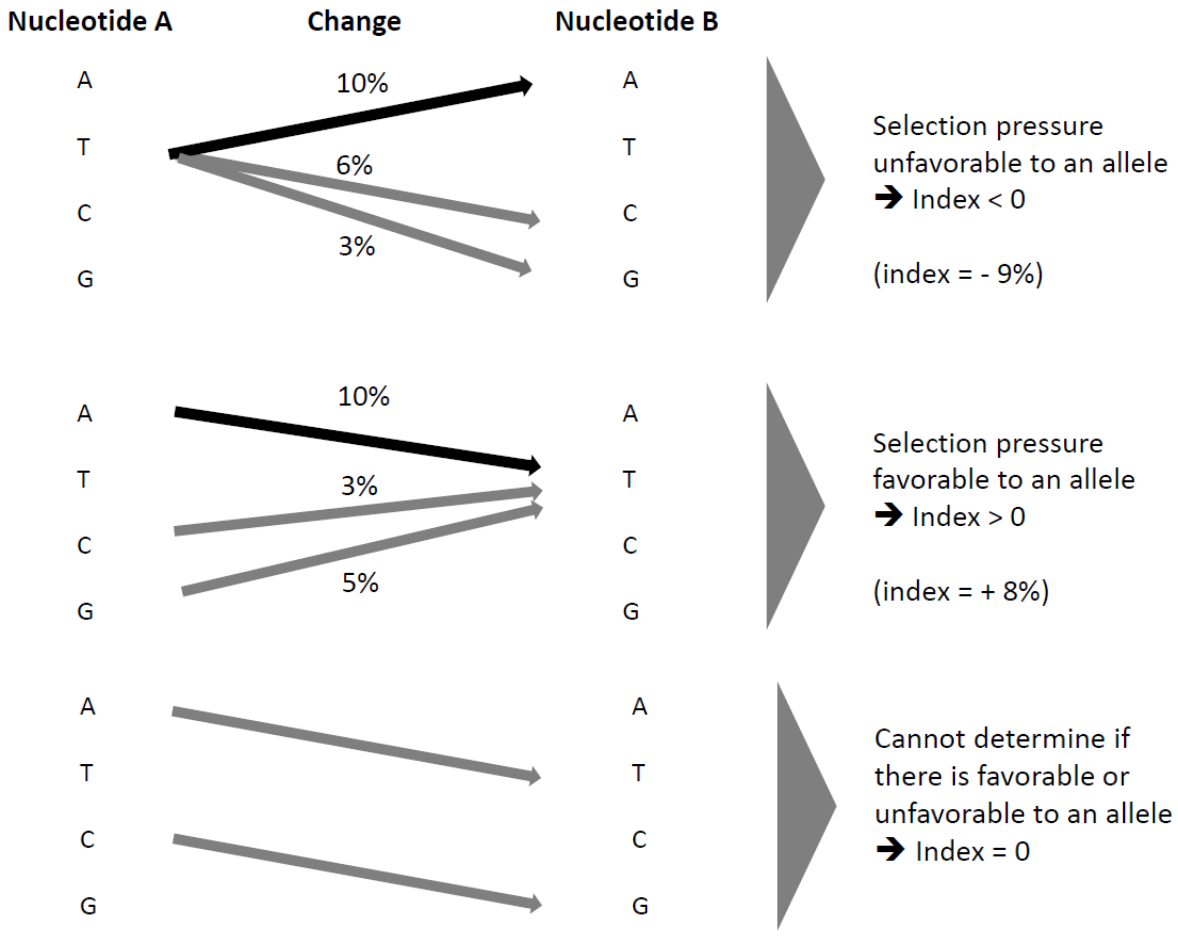

Figure 13: Illustration of the mechanism behind the selection index

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Advantages:

- Can distinguish selection against an allele and structural constraints
- Might be applied to the individual patient to make personalized profiles

One major drawback of this approach is that when most nucleotide positions do not have any minor allele at baseline, the signal is not very sensitive for the detection of selection favorable to an allele. When the baseline frequency is 100% one particular nucleotide allele, it is not possible for the distribution of frequencies between alleles at this position to converge any further. This tool is therefore best use to study selection against an allele (diversifying selection).

The percentage of nucleotide positions with more than one allele was relatively small all over the course experiment, and increased for responders only (Figure 14).

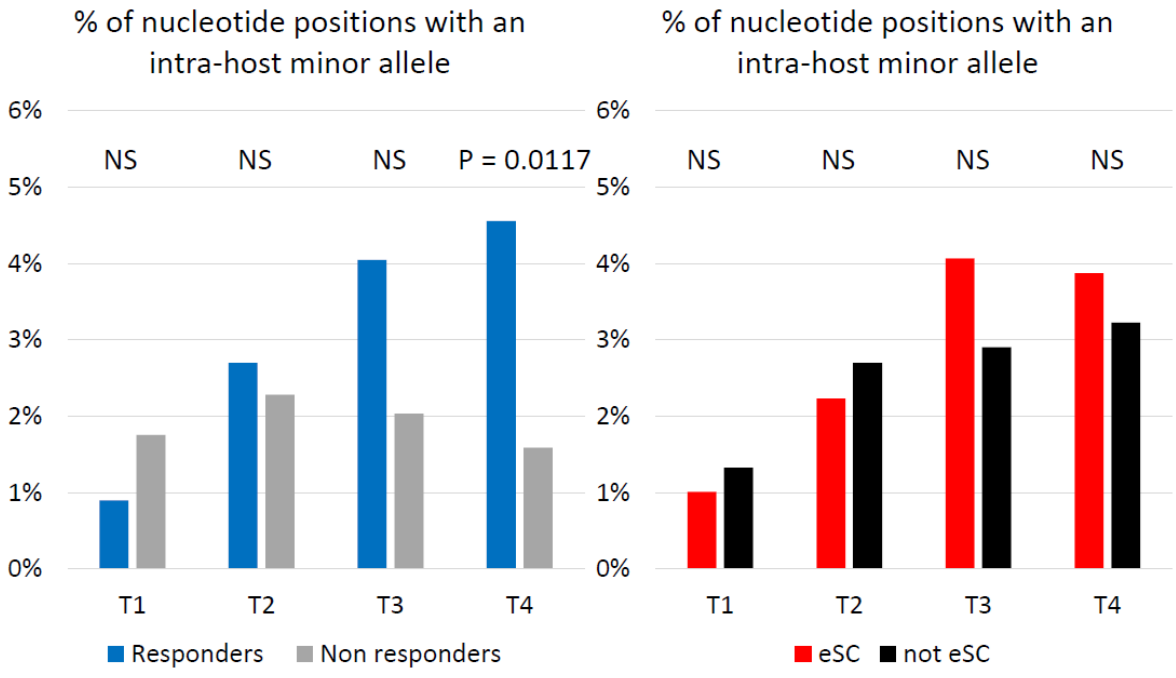

Figure 14: Change in the proportion of nucleotides with a minor allele

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6.3.3 Computation

Divergence:

$$D_{position} = |\min(\Delta f[a.a.]_{position})|$$

Convergence:

$$C_{position} = \max(\Delta f[a.a.]_{position})$$

Selection index = Convergence – Divergence

Figure 13 depicts the computation based on nucleotides. The same principle was used to execute the computation based on amino acids. The process was identical except that each position had 21 possible alleles (20 amino acids + stop codon) instead of just four possible alleles (A, T, C, and G).

6.3.4 Interpretation

How to interpret the chart:

- Selection favorable to an allele: selection index > 0
- Selection unfavorable to an allele: selection index < 0
- Neutral selection: selection index small +/- value
- Structural constraints: selection index close to 0

While the signal measured by the selection index measures convergence and divergence, reflecting selection favorability, it doesn't measure point mutations that are not accompanied by convergence or divergence. The change chart (see section 6.5 Establishing patient profiles) must be used in coordination to obtain a more complete representation of the selection present on the gene under investigation.

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6.4 Susceptibility index

6.4.1 Purpose

To evaluate which regions are more or less likely to have amino acids change in response to mutations in underlying nucleotide base pairs.

6.4.2 Mechanism

Some codons are more or less susceptible to cause a mutation in the encoded amino acid because of the varying percentages of non-synonymous sites in different codons.

6.4.3 Computation

Susceptibility Index at position p:

- Percentage of NS sites in the codons encoding the amino acids
- Scope: from position p - 10 to position p + 10 → total 21 amino acid positions
- For each codon: % NS sites = NS sites / (NS sites + S sites) = NS sites / 3

6.4.4 Interpretation

How to interpret the chart:

- More prone to change amino acids if nucleotides change: > 76.4 %
- Neutrally prone to change amino acids if nucleotides change: = 76.4 %
- Less prone to change amino acids if nucleotides change: < 76.4 %

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6.5 Establishing patient profiles

The selection index and the susceptibility index were used to create patient profiles (Annex I). These profiles also put these signals in relation to the change (genetic distance) that occurred over the gene regions during the time interval analyzed. We propose that these patient profiles can provide more detailed information about the action of the host immune system than traditional serologic monitoring. In addition, it shares the advantage of being minimally invasive: it only requires blood samples from periodic serologic monitoring.

The flexibility index was not further developed in the establishment of patient profiles because it is only applicable for analyzing a population and is unsuitable for studying single individuals.

The selection index was enriched by being computed in two different way. The normal computation is represented as a blue line. In addition, the red line represents the signal that was obtained when information from uncertain codons were removed from the computations.

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7. Discussion

This thesis reports several observations about factors in the HBV genome that might influence the clinical outcomes of patients. These observations must however be evaluated as only the result from a small-scale study and should be validated using data from a larger cohort of patients. The primary purpose of this small-scale study is to identify phenomena of interest and gather preliminary evidence that would help justify the allocation of further resources toward further investigation of these elements in the HBV genome. An important aspect that would benefit from a larger scale study is the analysis KIR and HLA associations in this context to better take into account host immunogenetics.

Next, we worked on the first steps toward the developed a methodology to evaluate the individual characteristics of patient's immune responses. The next logical step in the development of this methodology would be giving formal proof that the observed signals reflect what is actually happening within the patient. But several challenges are in the way of this objective. To scientifically prove or disprove the applicability of the antigen-identification methods described in this study, experimental validation must determine whether or not they are reliable and valid.

If animal models were readily available, it would be easy to perform validation:

- Evaluating the signal magnitude and the probability of a false negative when analyzing a positive control: an animal model engineered for immune recognition of a predetermined epitope
- Evaluating the signal magnitude and the probability of a false positive when analyzing a negative control: an animal model engineered to have no adaptive immune system

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Unfortunately, such animal models are not readily available to study HBV infection. As a result, computational methods must be used to estimate:

- The signal magnitude required to reach statistical significance
- The probability of a false negative
- The probability of a false positive

A computational model of HBV freely replicating and mutating within the host and without any selection effect (random walk) could become a negative control. In contrast, a model of computer-generated selection could become a positive control. Next, it would become possible to test the ability of the selection index to recognize these models and characterize their features accurately. Such research could provide the full statistical characterization of the values measured by the methods presented in this document to better understand the statistical significance and degree of confidence that can be associated with the results of the analyses.

Finally, numerical findings should be triangulated with biological experiments on patient's immune cells to confirm whether immune cells target the same epitopes that appear as signal in patient's individual profiles.

If further research validates the usefulness of patient profiles to establish a clearer picture of the action of the immune system of patient, this application of viral immunogenetics could be used to guide precision medicine treatments, such as therapeutic vaccines, for the purpose of helping patients with unfavorable immune response profiles.

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8. References

[1] World Health Organization, "Global Hepatitis Report," 2017.

[2] J. F. Perz, G. L. Armstrong, L. A. Farrington, Y. J. Hutin and B. P. Bell, "The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide," *Journal of Hepatology*, vol. 45, pp. 529-538, 2006.

[3] World Health Organization, "Monitoring and evaluation for viral hepatitis B and C: recommended indicators and framework - technical report," 2016.

[4] C. Montuclard, S. Hamza, F. Rollot, P. Evrard, J. Faivre, P. Hillon, V. D. Martino and A. Minello, "Causes of death in people with chronic HBV infection," *Journal of Hepatology*, vol. 62, pp. 1265-1271, 2015.

[5] S. Nayagam, M. Thursz, E. Sicuri, L. Conteh, S. Wiktor, D. Low-Beer and T. B. Hallett, "Requirements for global elimination of hepatitis B: a modelling study," *The Lancet Infectious Diseases*, vol. 16, no. 12, pp. 1399-1408, 2016.

[6] C. Lee, Y. Gong, J. Brok, E. H. Boxall and C. Gluud, "Hepatitis B immunisation for newborn infants of hepatitis B surface antigen-positive mothers," *Cochrane Database of Systematic Reviews*, no. 2, 2006.

[7] M. Machaira, V. Papaevangelou, E. K. Vouloumanou, G. S. Tansarli and M. E. Falagas, "Hepatitis B vaccine alone or with hepatitis B immunoglobulin in neonates

doi:10.6342/NTU201802768

of HBsAg+/HBeAg− mothers: a systematic review and meta-analysis," *Journal of Antimicrobial Chemotherapy*, vol. 70, no. 2, p. 396–404, 2015.

[8] Y.-F. Liaw and C.-M. Chu, "Hepatitis B virus infection," *The Lancet*, vol. 373, pp. 582-592, 2009.

[9] T. Santantonio and M. Fasano, "Current Concepts on Management of Chronic Hepatitis B," in *Practical Management of Chronic Viral Hepatitis*, 2013.

[10] C.-L. Lin, H.-C. Yang and J.-H. Kao, "Hepatitis B virus: new therapeutic perspectives," *Liver International*, vol. 36, pp. 85-92, 2016.

[11] M. Nassal, "HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B," *Gut*, vol. 64, pp. 1972-1984, 2015.

[12] E. C. Holmes, "Evolutionary history and phylogeography of human viruses," *Annual Review of Microbiology*, vol. 62, pp. 307-328, 2008.

[13] E. Domingo, J. Sheldon and C. Perales, "Viral Quasispecies Evolution," *Microbiology and Molecular Biology Reviews*, vol. 76, no. 2, pp. 159-216, 2012.

[14] F. Zoulim and S. Locarnini, "Hepatitis B Virus Resistance to Nucleos(t)ide Analogues," *Gastroenterology*, vol. 137, no. 5, pp. 1593-1608, 2009.

[15] H. Thai, D. S. Campo, J. Lara, Z. Dimitrova, S. Ramachandran, G. Xia, L. Ganova-Raeva, C.-G. Teo, A. Lok and Y. Khudyakov, "Convergence and coevolution of Hepatitis B virus drug resistance," *Nature Communications*, vol. 3:789, 2012.

doi:10.6342/NTU201802768

[16] J. M. Carlson, A. Q. Le, A. Shahid and Z. L. Brumme, "HIV-1 adaptation to HLA: a window into virus–host immune interactions," *Trends in Microbiology*, vol. 23, no. 4, pp. 212-224, 2015.

[17] M. A. Ansari, V. Pedergnana, C. Ip, A. Magri, A. V. Delft, D. Bonsall, N. Chaturvedi, I. Bartha, D. Smith, G. Nicholson, G. McVean, A. Trebes, P. Piazza, J. Fellay, G. Cooke, G. R. Foster, STOP-HCV consortium, E. Hudson, J. McLauchlan, P. Simmonds, R. Bowden, P. Klenerman, E. Barnes and C. C. A. Spencer, "Genome-to-genome analysis highlights the impact of the human innate and adaptive immune systems on the hepatitis C virus," *Nature Genetics*, vol. 49, p. 666–673, 2017.

[18] M. B. Zeisel, J. Lucifora, W. S. Mason, C. Sureau, J. Beck, M. Levrero, M. Kann, P. A. Knolle, M. Benkirane, D. Durantel, M.-L. Michel, B. Autran, F.-L. Cosset, H. Strick-Marchand, C. Trépo, J.-H. Kao, F. Carrat, K. Lacombe, R. F. Schinazi, F. Barré-Sinoussi, J.-F. Delfraissy and F. Zoulim, "Towards an HBV cure: state-of-the-art and unresolved questions — report of the ANRS workshop on HBV cure," *Gut*, vol. 64, pp. 1314-1326, 2015.

[19] T.-H. Chu, A.-T. Liou, P.-Y. Su, H.-N. Wu and C. Shih, "Nucleic Acid Chaperone Activity Associated with the Arginine-Rich Domain of Human Hepatitis B Virus Core Protein," *Journal of Virology*, vol. 88, p. 2530–2543, 2014.

[20] Y.-J. Lin, L.-R. Huang, H.-C. Yang, H.-T. Tzeng, P.-N. Hsu, H.-L. Wu, P.-J. Chen and D.-S. Chen, "Hepatitis B virus core antigen determines viral persistence in a

doi:10.6342/NTU201802768

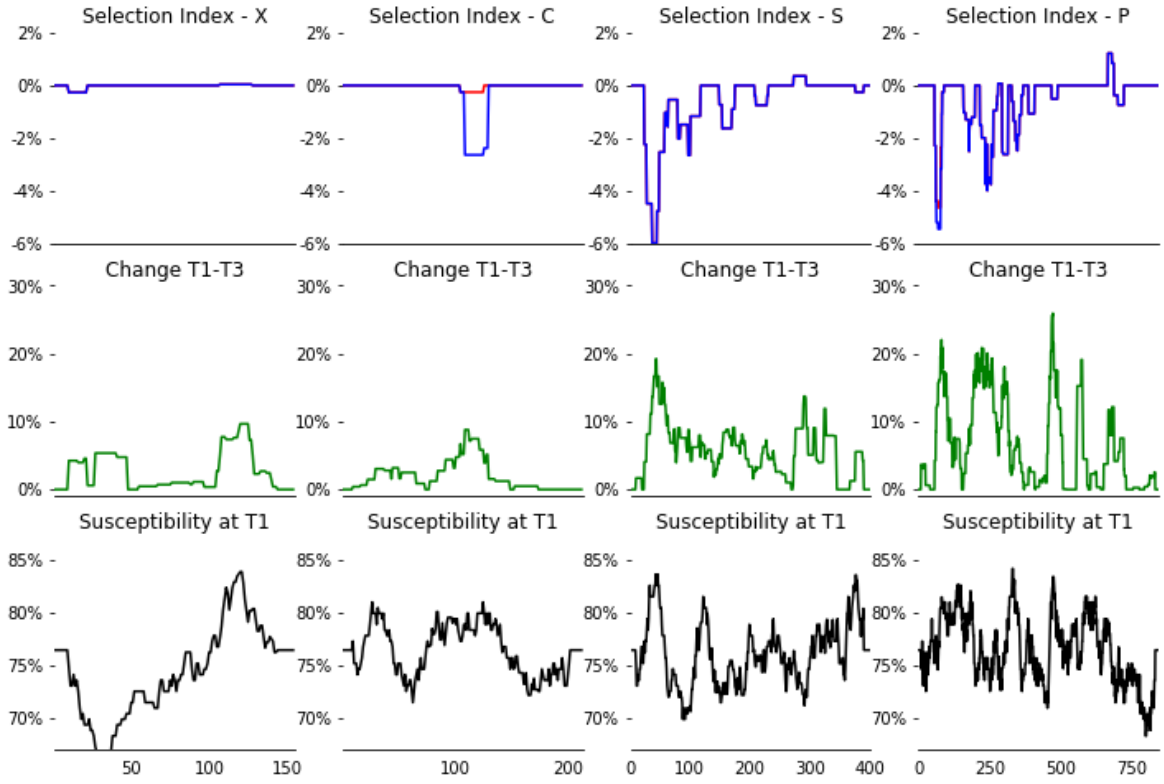
C57BL/6 mouse model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 20, p. 9340–9345, 2010.

[21] N.-F. Ma, S. H. Lau, L. Hu, D. Xie, J. Wu, J. Yang, Y. Wang, M.-C. Wu, J. Fung, X. Bai, C.-H. Tzang, L. Fu, M. Yang, Y. A. Su and X.-Y. Guan, "COOH-terminal truncated HBV X protein plays key role in hepatocarcinogenesis," *Clinical Cancer Research*, vol. 14, pp. 5061-5068, 2008.

doi:10.6342/NTU201802768

Annex: Patient profiles

Figure 15: Patient 1 profile (eSC, responder)



doi:10.6342/NTU201802768

Figure 16: Patient 2 profile (eSC, responder)

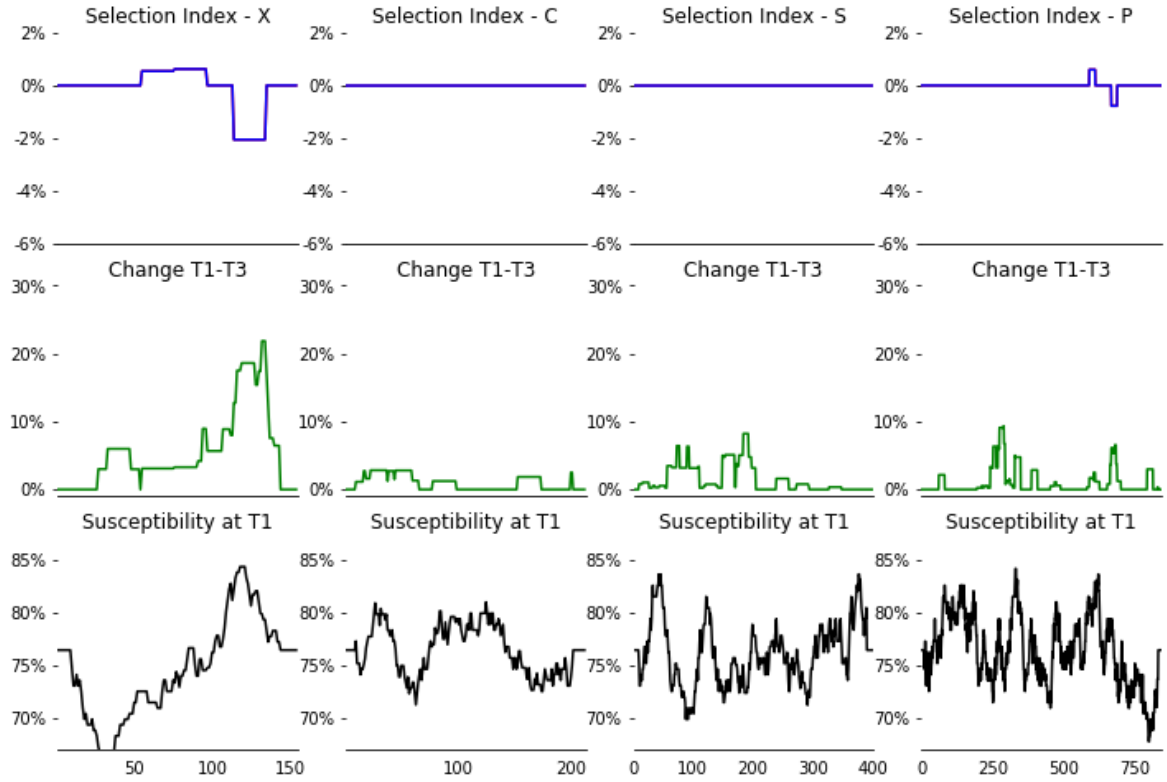
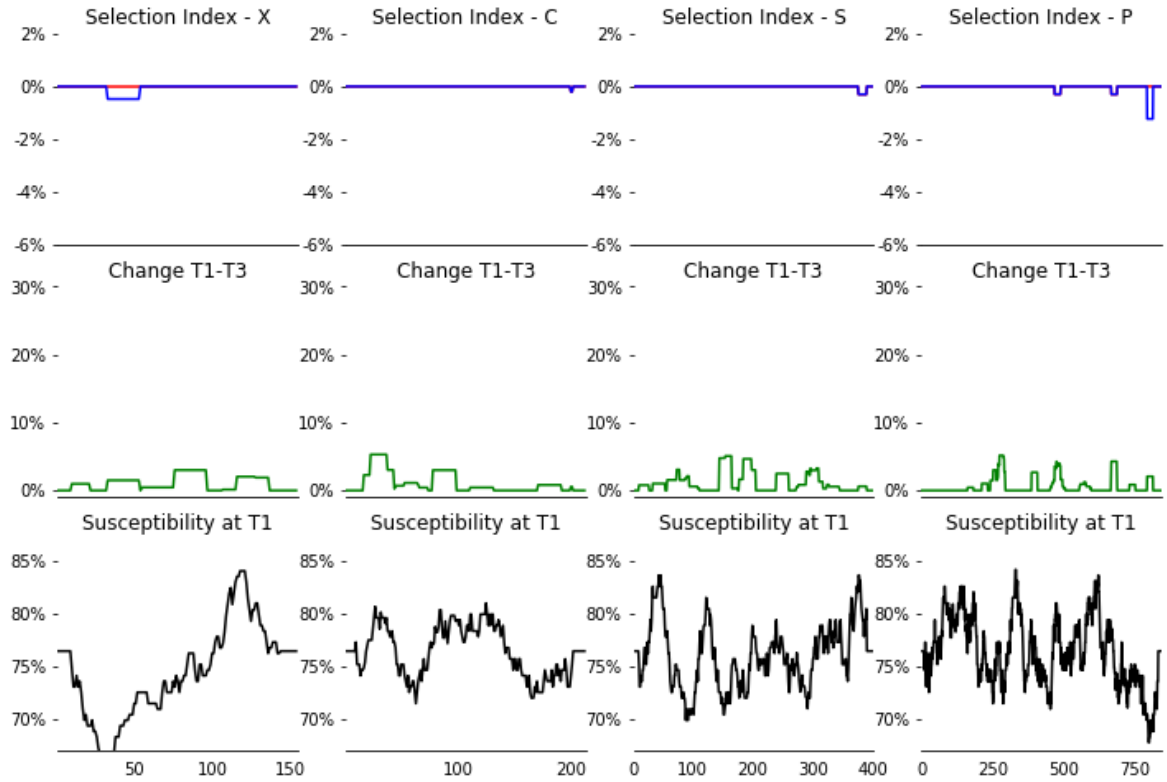


Figure 17: Patient 3 profile (eSC, responder)



doi:10.6342/NTU201802768

Figure 18: Patient 4 profile (eSC, responder)

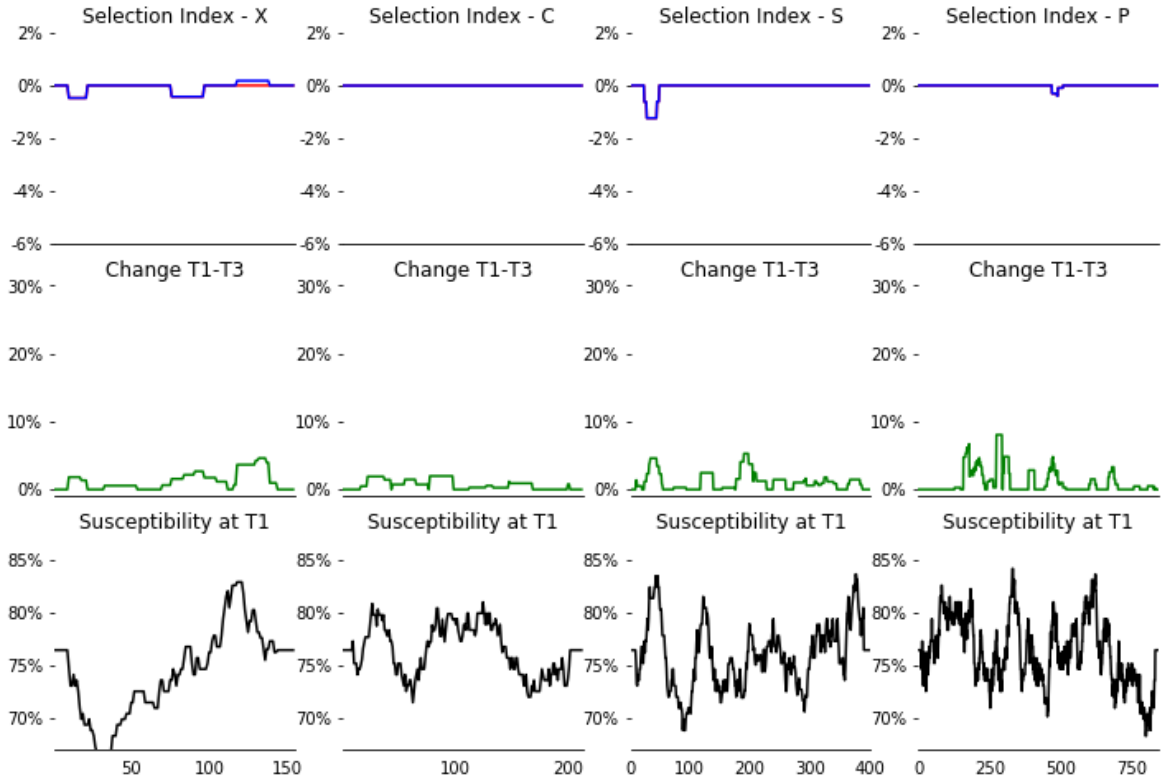
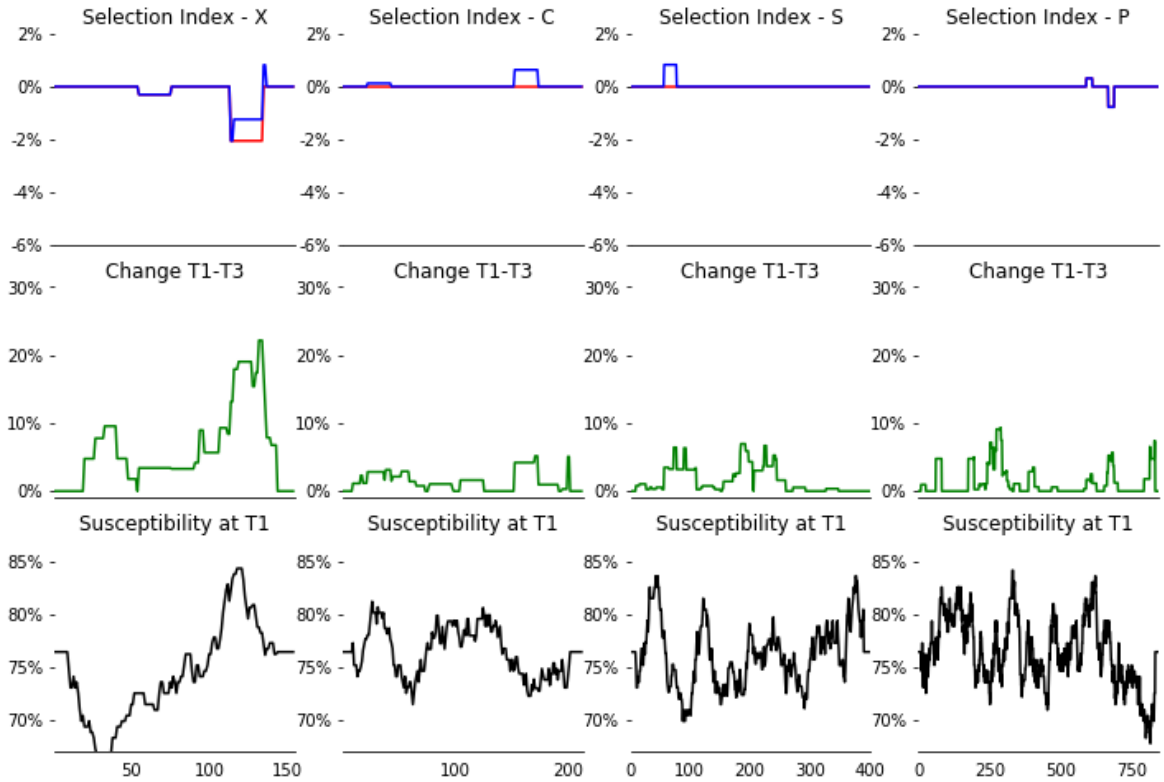


Figure 19: Patient 6 profile (eSC, responder)



doi:10.6342/NTU201802768

Figure 20: Patient 21 profile (eSC, responder)

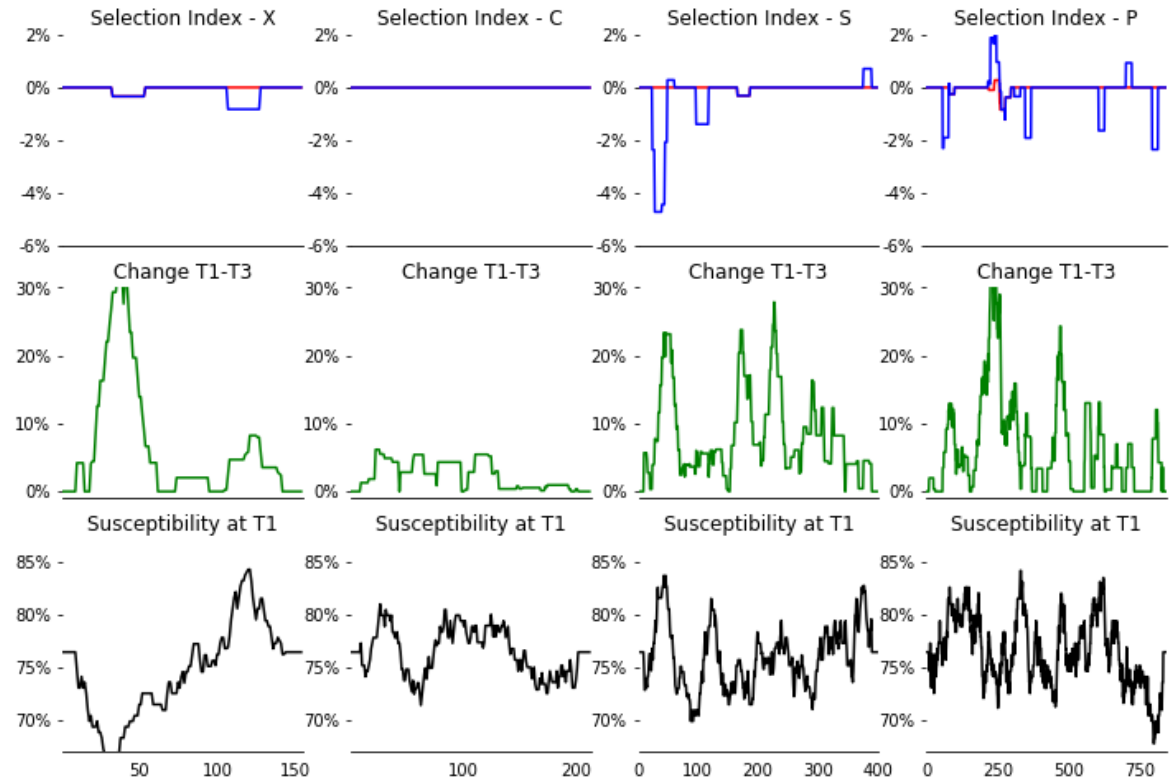


Figure 21: Patient 7 profile (eSC, non-responder)

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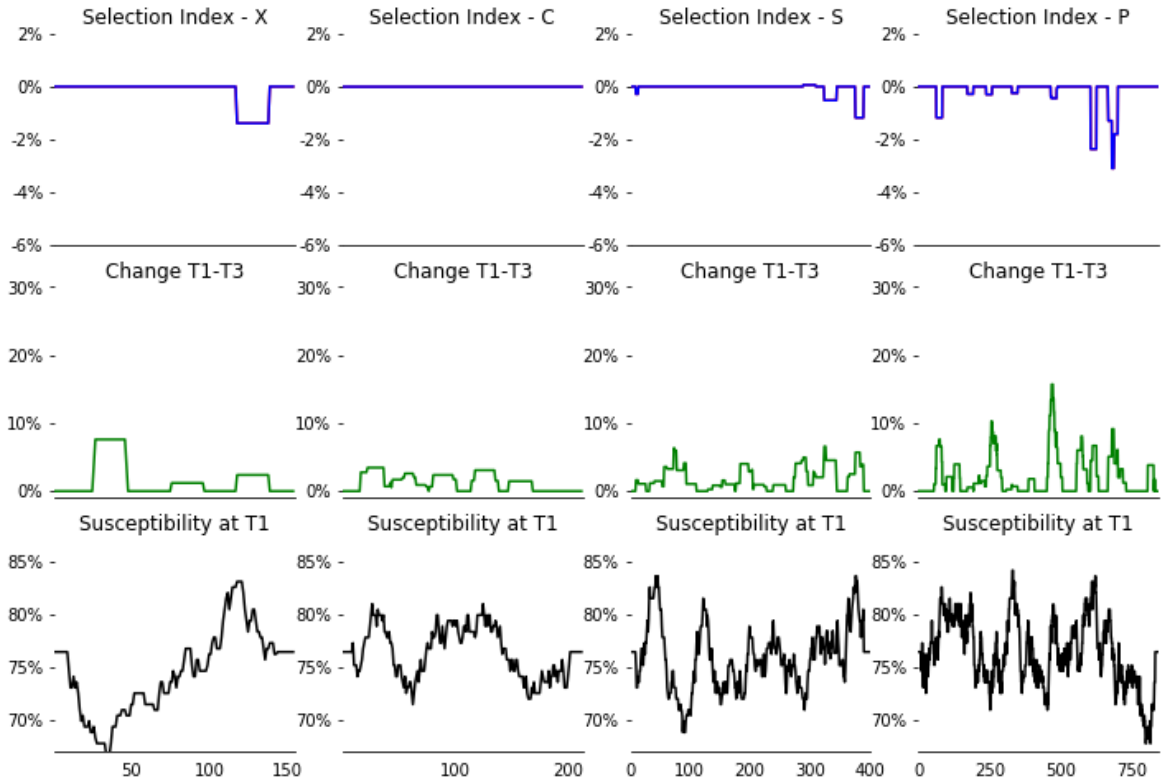
Figure 22: Patient 8 profile (eSC, non-responder)

Figure 23: Patient 11 profile (non-eSC, responder)



doi:10.6342/NTU201802768

Figure 24: Patient 13 profile (non-eSC, responder)

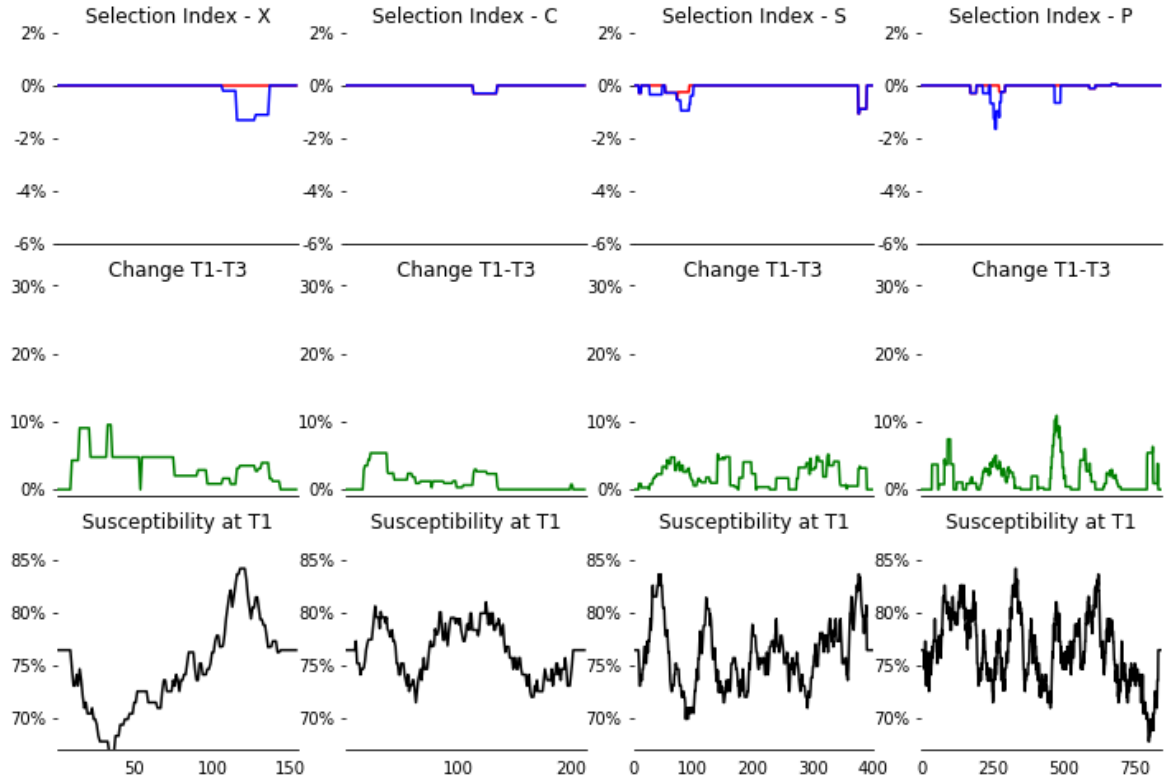
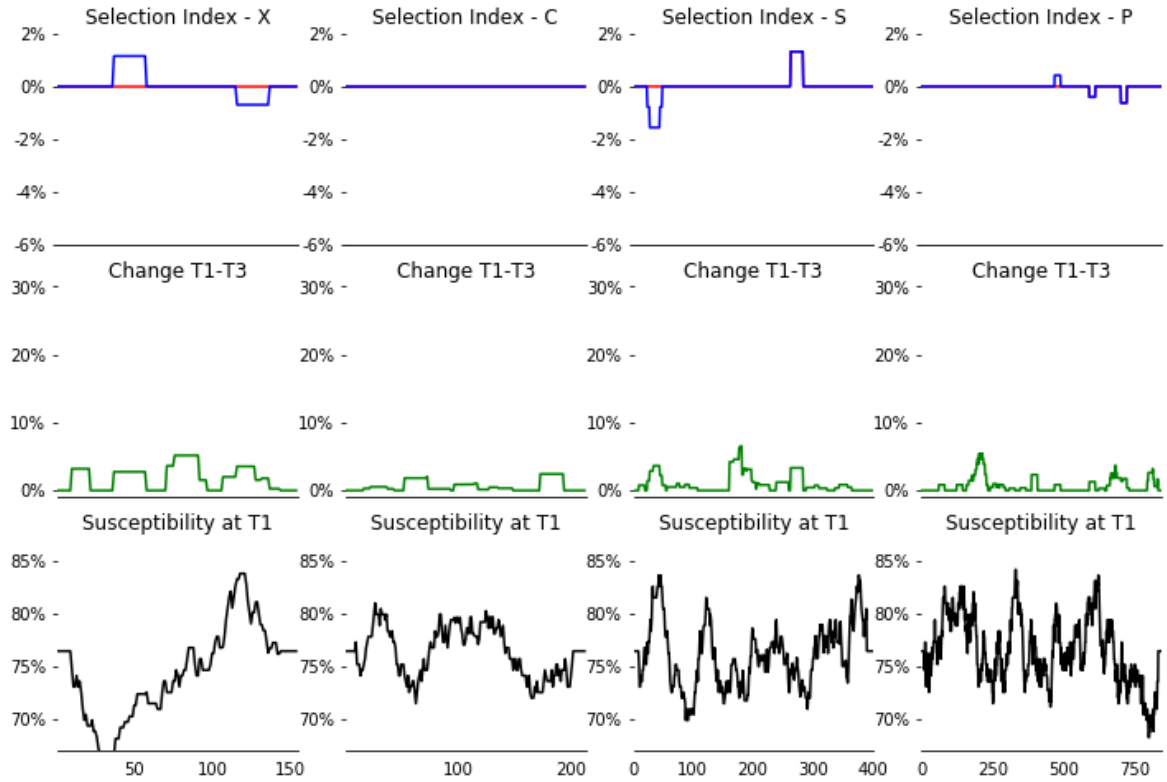


Figure 25: Patient 14 profile (non-eSC, responder)



doi:10.6342/NTU201802768

Figure 26: Patient 16 profile (non-eSC, responder)

Figure 27: Patient 18 profile (non-eSC, responder)

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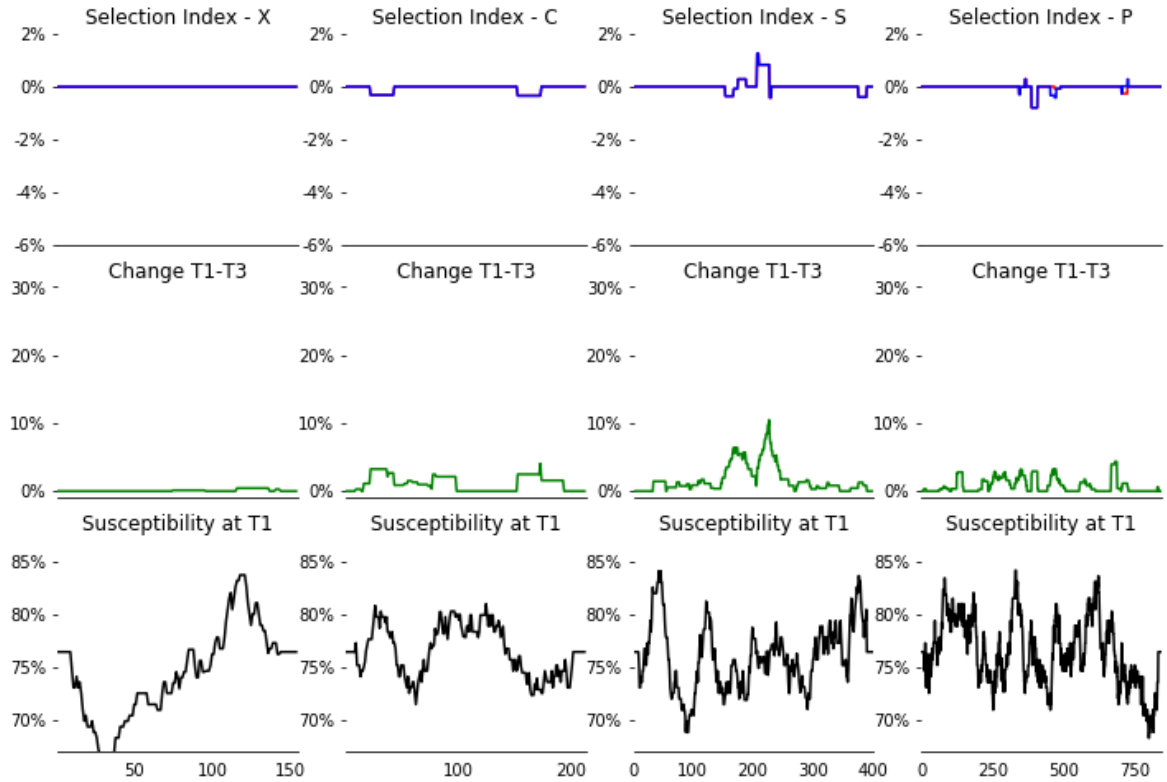
Figure 28: Patient 19 profile (non-eSC, responder)

Figure 29: Patient 20 profile (non-eSC, responder)



doi:10.6342/NTU201802768

Figure 30: Patient 53 profile (non-eSC, responder)

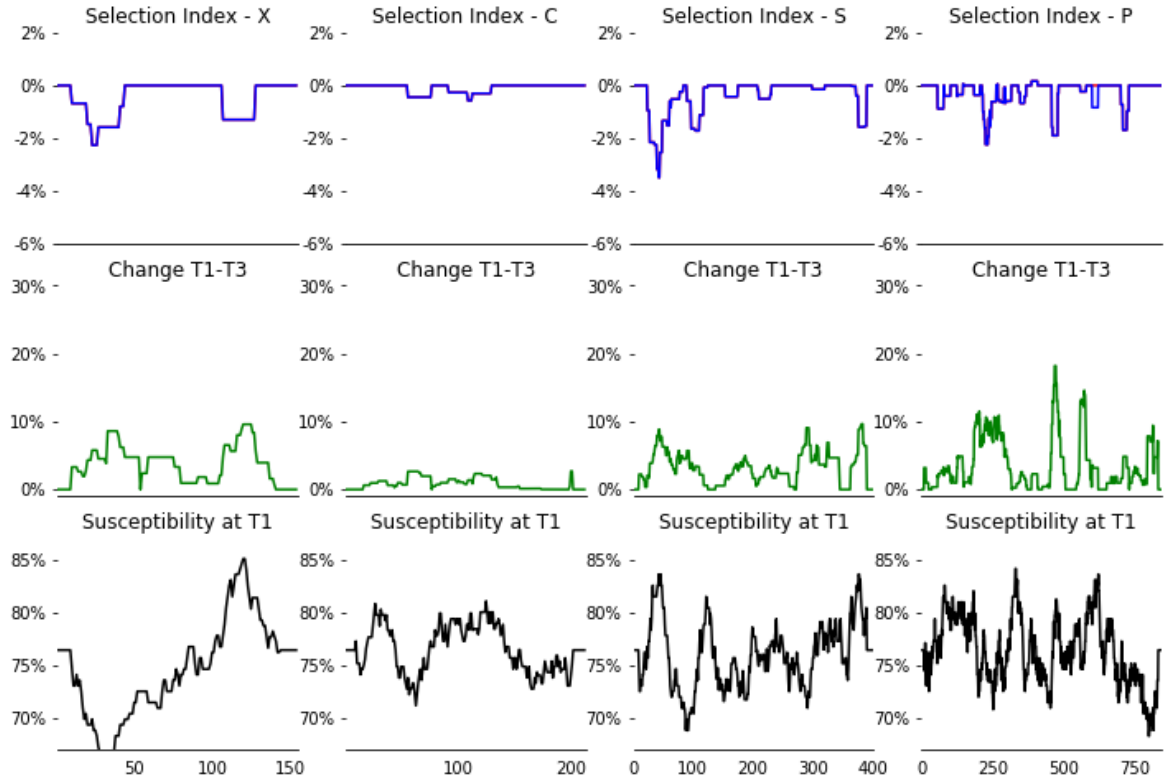
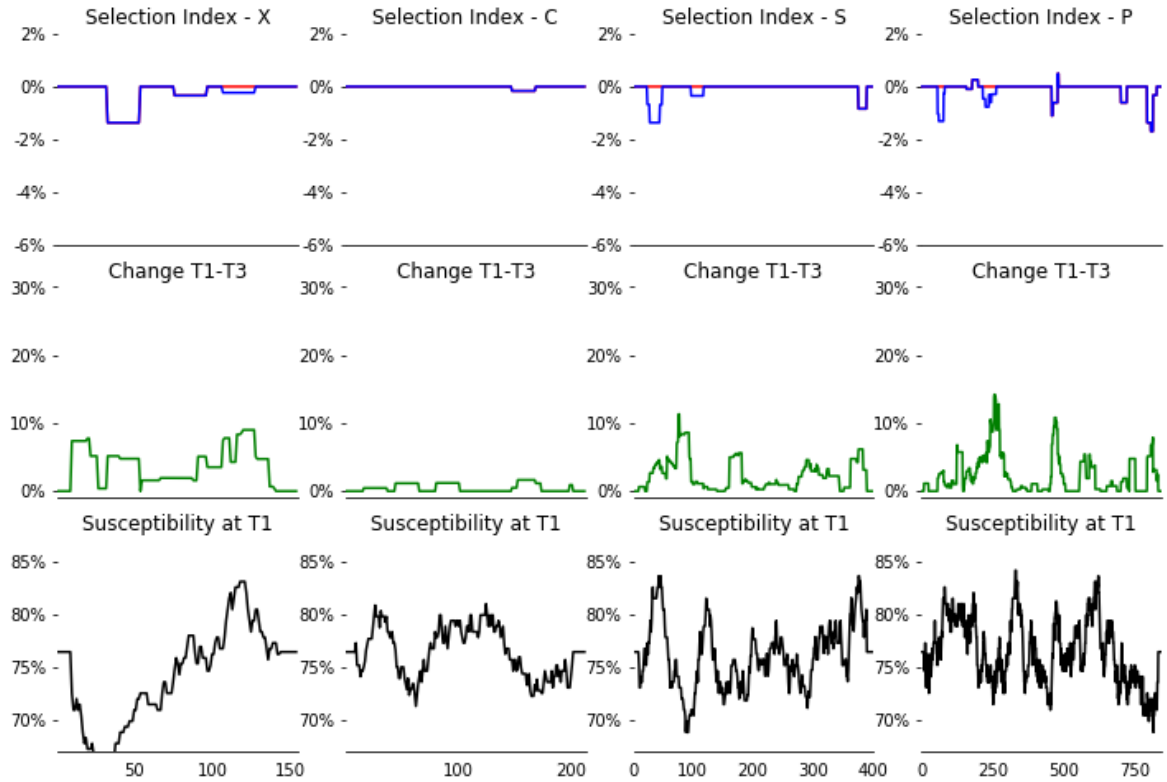


Figure 31: Patient 54 profile (non-eSC, responder)



doi:10.6342/NTU201802768

Figure 32: Patient 12 profile (non-eSC, non-responder)

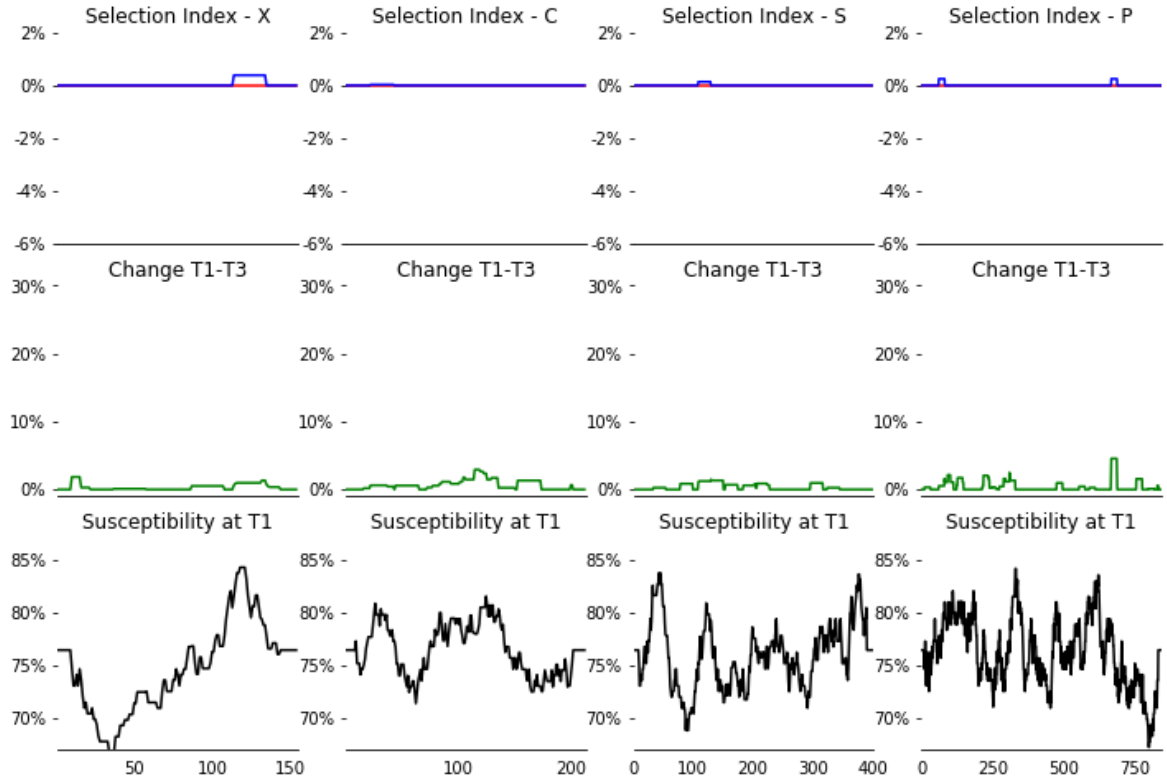
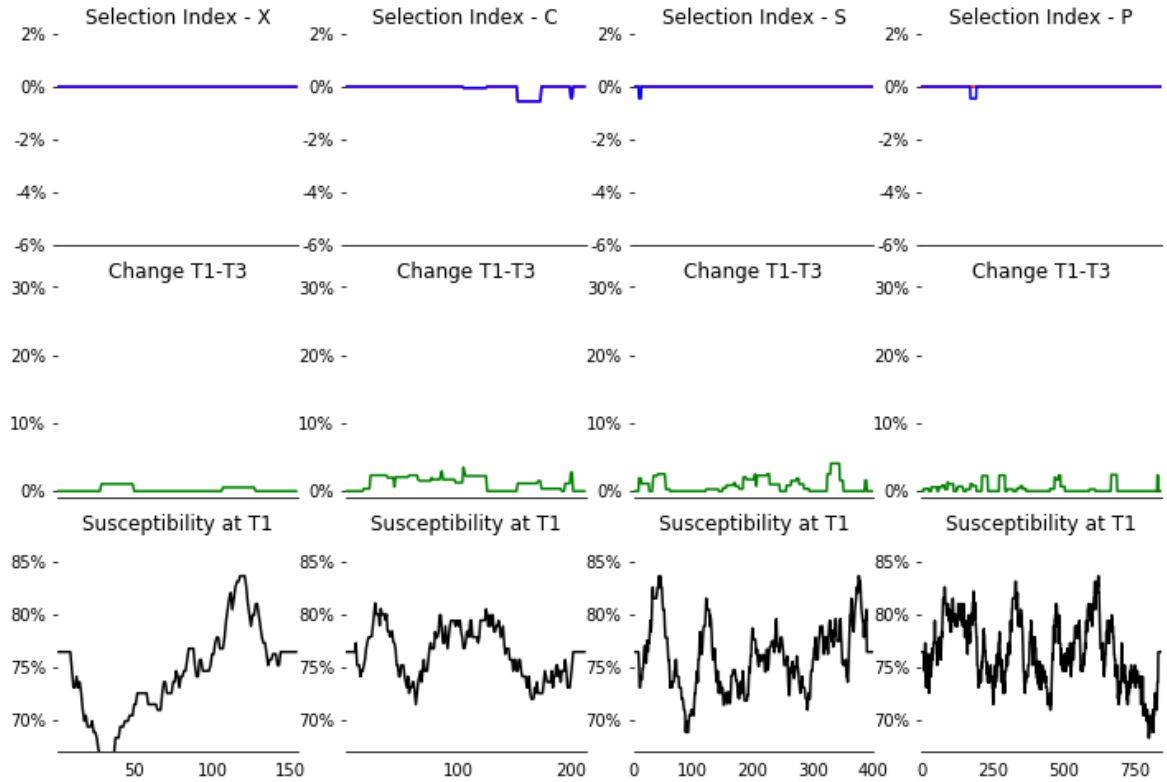


Figure 33: Patient 17 profile (non-eSC, non-responder)



doi:10.6342/NTU201802768

Figure 34: Patient 51 profile (non-eSC, non-responder)

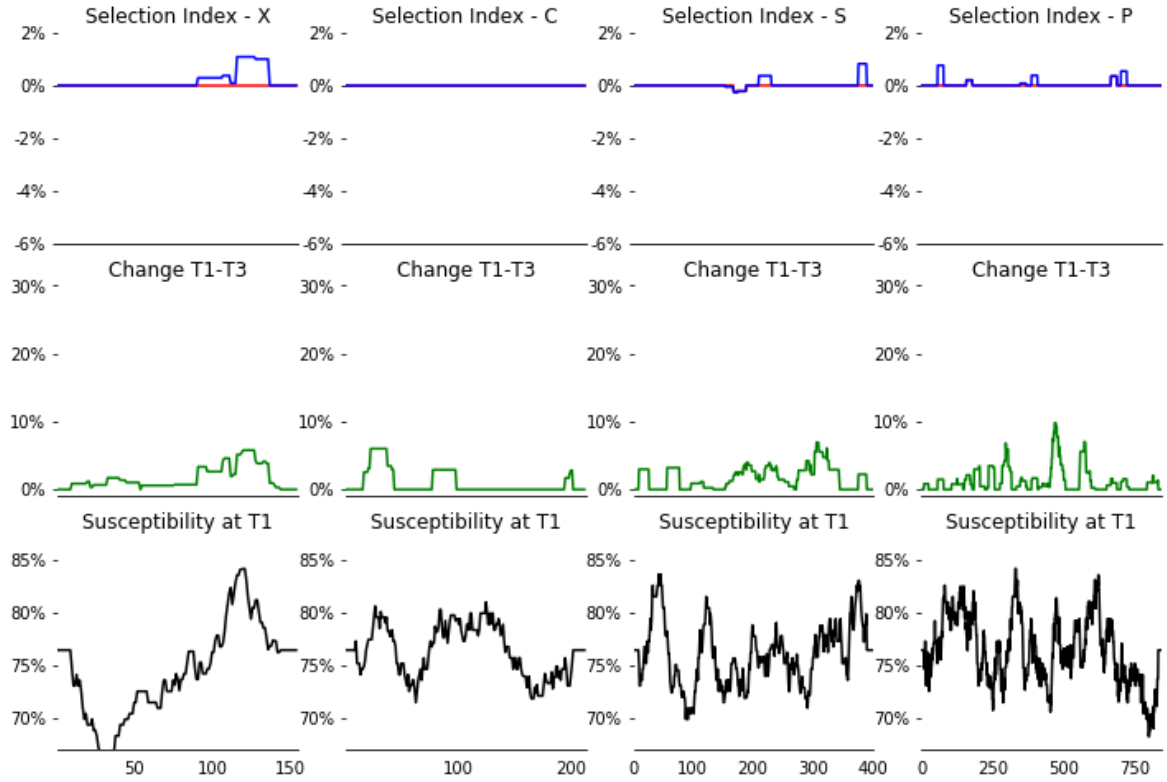


Figure 35: Patient 52 profile (non-eSC, non-responder)

doi:10.6342/NTU201802768

Figure 36: Patient 55 profile (non-eSC, non-responder)

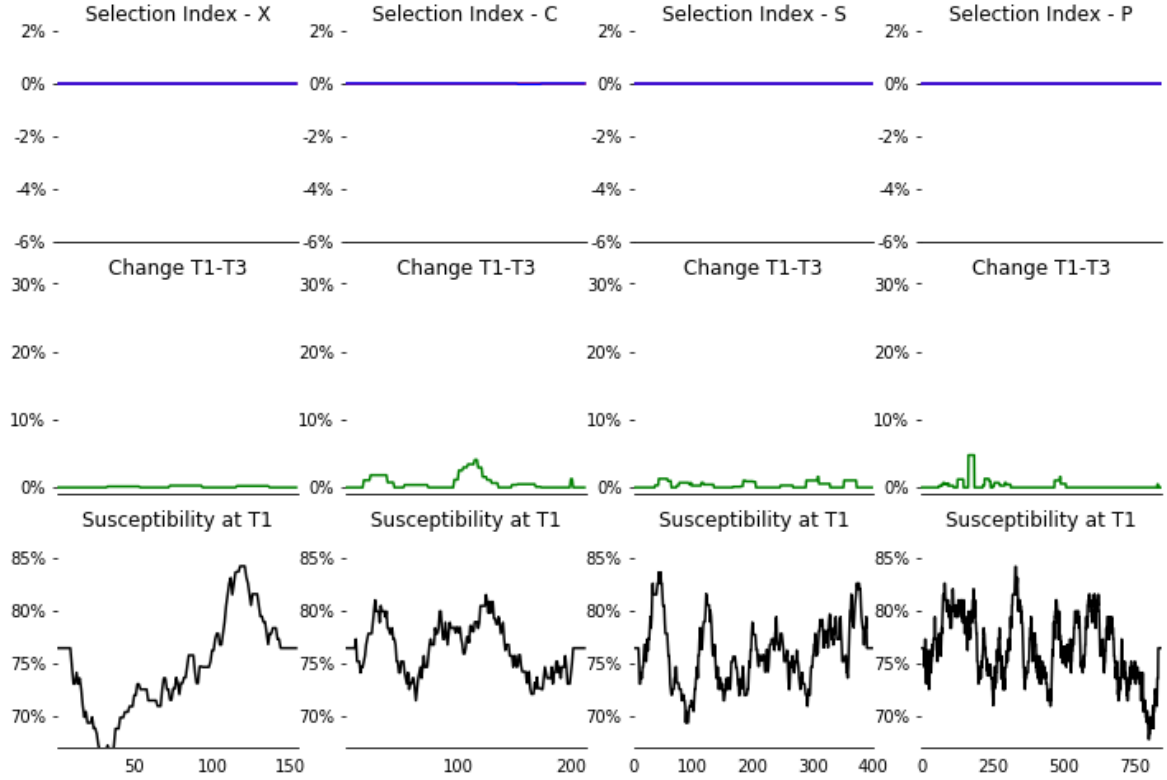
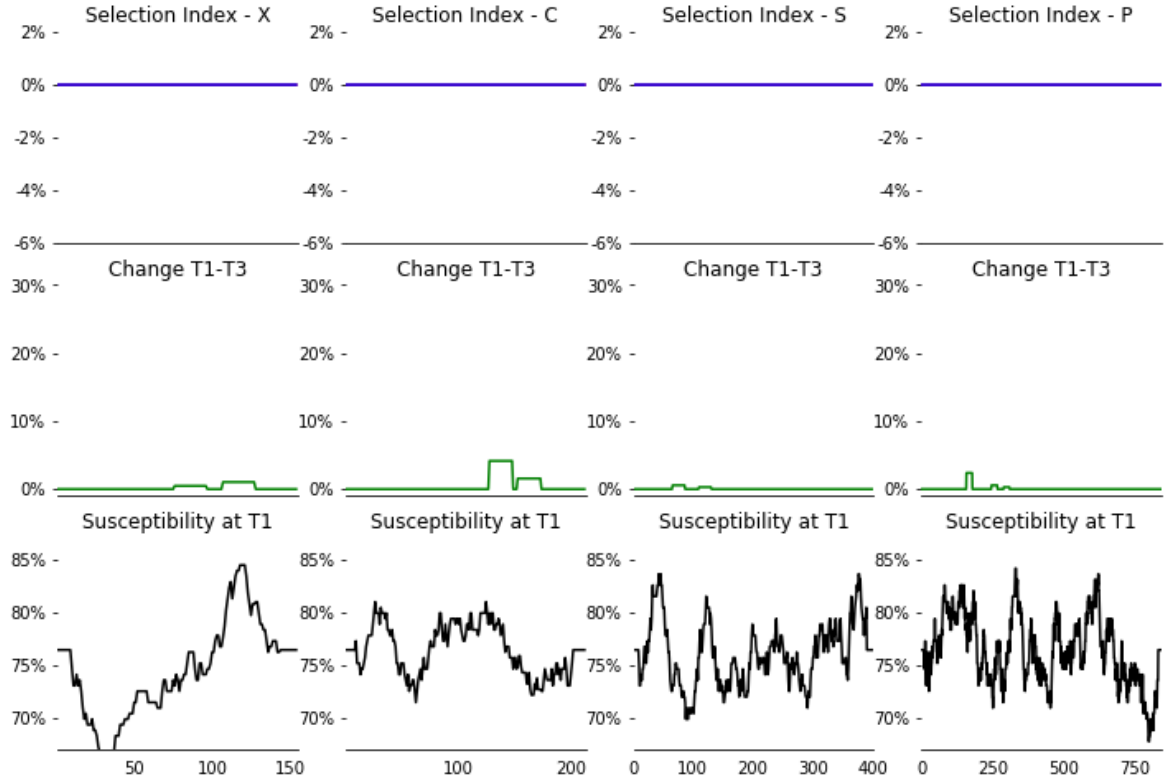


Figure 37: Patient 56 profile (non-eSC, non-responder)



doi:10.6342/NTU201802768