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Role of immunoglobulin 3´ regulatory region polymorphism in somatic hypermutation and vaccination response

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Abstract.

Human immunoglobulin heavy chain 3´ regulatory regions (3´RR1 and 3´RR2) are gene regulatory regions downstream of Igalpha1 and Igalpha2 constant genes, respectively. The 3´RRs include three core enhancers: HS3, HS4 and HS1,2. Enhancer HS1,2 in 3´RR1 is polymorphic with four prevalent alleles. Enhancers HS3 and HS4 are not polymorphic. The 3´RRs has an important role in class switch recombination. Its role in somatic hypermutation is debated. The first aim of this study was to determine if HS1,2 alleles affected somatic hypermutation and, if so, do alleles affect mutation differently.

Different individuals respond to vaccination differently. One factor influencing this is genetics, including polymorphism in genes related to immune response. Thus, the second aim of this study was to determine if 3´RR1 polymorphism affected vaccination response.

A specific reporter genes assay (GFP4 loss assay) was used to evaluate the frequency somatic hypermutation induced by HS1,2 enhancer elements. To estimate the effect of enhancer variants on vaccination response, the Finnish part of BERT study was used. PCR assay was set up to determine HS1,2 alleles for patients. Vaccination response was evaluated by correlating obtained results to the quantity of total IgG, IgG subclasses and IgG avidity.

HS1,2 alleles were found to increase somatic hypermutation, but only weakly. There were also some small differences between alleles. For vaccination response, the results showed no differences in quantity of total IgG amounts or IgG subclasses. The results were not fully conclusive on differences in antibody avidity.

In conclusion, HS1,2 alleles have detectable, yet unlikely physiologically relevant, effect on somatic hypermutation. Additionally, there might be some differences between allelic variants, but these were neglectable. Differences in vaccination responses were not statistically significant between alleles, and investigation of larger populations is needed to establish potential effect of allelic variants.

Key words: 3´ regulatory region, somatic hypermutation, vaccination response, HS1,2 enhancer, polymorphism

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1 INTRODUCTION

1.1 3´ regulatory region

The immunoglobulin heavy chain locus in chromosome 14 in humans is responsible for coding the heavy chain of antibodies. At the 3´ end of this immunoglobulin heavy chain locus, there is a complex regulatory region named 3' regulatory region or 3´RR. Humans have two duplicates of this 3´ regulatory region, 3´ regulatory region 1 and 3´ regulatory region 2 or 3´RR1 and 3´RR2. In humans 3´regulatory region consists of three core enhancers: HS3, HS4 and HS1,2. As humans have two duplicates of the 3´ regulatory region, there are also two duplicates of these core enhancers. For example, in 3´RR1, there is enhancer HS1,2-A and in 3´RR2, there is enhancer HS1,2-B.

The 3´ regulatory region is important for regulating the production of immunoglobulin heavy chain. The 3´ regulatory region plays an important role in regulating transcription and class switch recombination (Pinaud et al 2011). Class switch recombination is a process where B-cells change antibody class. For example, changing between different antibody subclasses like IgG1 and IgG2. Bruzeau et al (2024) have shown that 3´RR core enhancers are necessary for creating the conditions allowing for class switch recombination to happen.

The role of the 3´ regulatory region in somatic hypermutation has been unclear (Rouaud et al 2013). Immunoglobulin somatic hypermutation is a process where mutations are targeted to exons encoding antigen-binding sites of antibodies. Somatic hypermutation allows for the production of antibodies with higher affinity. Rouaud et al (2013) have shown 3´RR to be mandatory for somatic hypermutations using 3´RR-deficient mice.

1.2 HS1,2 polymorphism

As previously stated, the 3´ regulatory region consists of three core enhancers: HS3, HS4 and HS1,2. The enhancer HS1,2 is known to be polymorphic. Humans have four different main alleles for this enhancer. The main alleles have a different number of copies of a repeating element. Thus, the main alleles are named 1A, 2A, 3A and 4A with 1, 2, 3 or 4 repeats of the repeating element for enhancer HS1,2-A, respectively. Enhancer HS1,2-B has alleles 3B and 4B with 3 or 4 repeats of the repeating element. (Giambra et al 2005.)

In addition to these four main alleles, there are different variations of these main alleles. These variations have the same number of the repeating element, but otherwise the sequence

is different. (Jodice et al 2024.) In this study, I refer to these different variants of the same main allele with an asterisk. For example, the variation of allele 2A is named 2A*. These different HS1,2 alleles are described in Figure 1.

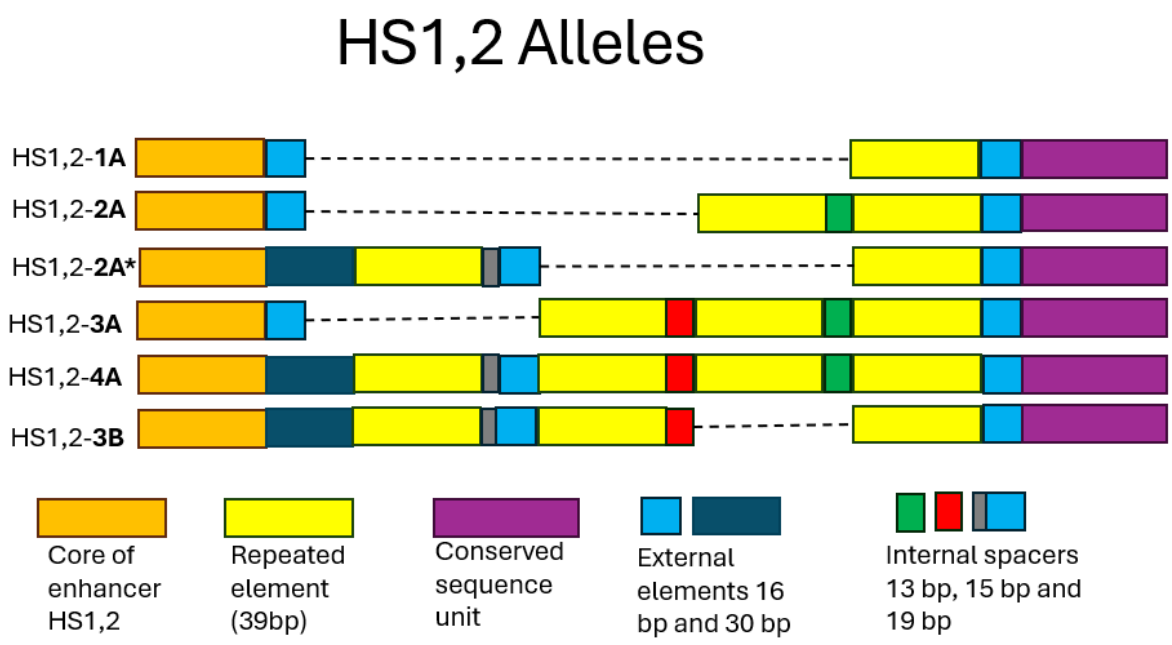

Figure 1 Different HS1,2 alleles. Different variants of HS1,2 allele and what DNA sequences they contain.

Enhancer HS1,2-A allele 1A is the most common one in most populations, with 32-65% of people with allele 1A depending on the population. Still, there is much variation in allele frequencies between populations. Usually, allele 4A is the rarest, with a frequency of 3-22% depending on the population. Frequencies for alleles 2A and 3A are between 1-55% and 1-58% depending on the population. (Giambra et al 2006.) The frequencies of the HS1,2 alleles have not been determined for the Finnish population.

The 3´ regulatory region includes enhancers HS3 and HS4 in addition to the HS1,2 enhancer. However, only enhancer HS1,2 is polymorphic. Enhancers HS3 and HS4 are not polymorphic. (Guglielmi et al 2004.)

The different HS1,2 enhancer alleles have been linked to various diseases. Frezza et al (2004) showed that HS1,2-A enhancer allele 2A increases the risk for coeliac disease. HS1,2 allele 2A also increases the risk for systemic lupus erythematosus and systemic sclerosis, as well as other autoimmune diseases (Frezza et al 2007, Frezza et al 2012). This suggests that HS1,2 polymorphism might have a role in autoimmunity and immune response.

1.3 HS1,2 polymorphism and somatic hypermutation

Somatic hypermutation is targeted by enhancers and enhancer-like sequences in the genome. In human and other mammalian immunoglobulins, many of these enhancers and enhancer-like sequences increase somatic hypermutation. For example, in human immunoglobulin heavy chain, the intronic enhancer (IgHEi) targets somatic hypermutation (Buesrstedde et al 2014). In these enhancer sequences, different transcription factor binding sites (Buerstedde et al 2014) and transcription factors binding to them (Dinesh et al 2020) seem to determine the targeting of somatic hypermutation.

As previously stated, the role of 3´RR in somatic hypermutation is debated, with some results suggesting that it has an important role (Rouaud et al 2013). As enhancers and enhancer-like elements are important for targeting somatic hypermutation (Buerstedde et al 2014), the three core enhancers in 3´RR could have a role in targeting somatic hypermutation.

Part of this study was to determine if enhancer HS1,2 has a role in targeting somatic hypermutation. As the HS1,2 enhancer is polymorphic and has different alleles, another part of this study was to figure out if these alleles affected somatic hypermutation differently (Giambra et al 2005). HS1,2 alleles have a different number of copies of a repeating element and thus a different number of binding sites (Giambra et al 2005). This led to the hypothesis that alleles with more copies of the repeating element and as such, more binding sites, would lead to more somatic hypermutation than those alleles with fewer repeats.

1.4 HS1,2 polymorphism and vaccination response

The second part of this study concerned the antibody response to vaccination. It is known that individual patients respond to vaccination differently. Some achieve a good vaccination response, while some have a poor response. Many factors in many different categories influence vaccination response. One of the factors influencing vaccination response is genetics. It’s known that polymorphism in several different genes related to immune response, such as major histocompatibility complex (MHC) genes and pattern recognition receptor genes like Toll-like receptor (TLR) or RIG-like receptor (RLR) affects vaccination response. (Zimmermann et al 2019.)

HS1,2 enhancer allele 2A may increase a risk to at least some autoimmune diseases and thus HS1,2 polymorphism might contribute to autoimmunity and variations in immune response

(Frezza et al 2004, Frezza et al 2007 and Frezza et al 2012). This would suggest that HS1,2 enhancer polymorphism could affect immune response and as such could also affect vaccination response. As such, part of this study was to determine if polymorphism in HS1,2 enhancer affected vaccination response.

Vaccination response can be evaluated in different ways. In this study, vaccination response was examined in amount of IgG antibodies produced and in the avidity of antibodies produced. Avidity of an antibody reflects how strongly it binds to its antigen. Somatic hypermutation is important to produce of high-avidity antibodies. Therefore, if HS1,2 allele polymorphism affects somatic hypermutation, it could also lead to differences in antibody avidity.

Another aspect of vaccination response is different antibody classes and subclasses. As previously stated, 3´ regulatory region has a crucial role in class switch recombination (Bruzeau et al 2024, Pinaud et al 2011). Thus, it's possible that polymorphism in HS1,2 enhancer has a role as well. This could lead to differences in amounts of specific antibody classes or subclasses. As such, a part of this study was to determine if HS1,2 polymorphism affected class switch recombination by comparing the amounts of different antibody classes in response to vaccination in patients with different alleles.

2 MATERIALS AND METHODS

2.1 GFP4 loss assay for somatic hypermutation

Somatic hypermutation targeting was tested using GFP4 loss assay as described by Buerstedde et al (2014). The rationale of the assay is that a test sequence, in this case HS1,2 allele, is placed near GFP4 gene in B-cells. As cells are cultivated, if the test sequence targets somatic hypermutation, mutations begin to accumulate in the GFP4 gene, leading to premature stop codons and eventually loss of GFP4 fluorescence, which can be measured using flow cytometry.

HS1,2 alleles 1A, 2A, 3A, 4A and 3B were tested. For this purpose, sequences for these alleles were ordered from Eurofins as reported by Giambra et al (2005). Next, allele sequences were added to the GFP4 vector using digestion and ligation reactions. Plasmids with the GFP4 vector and HS1,2 allele sequence were then transfected into DT40 *UNG* $^{-/-}$ *AID* $^{R/puro}$ chicken bursal lymphoma B-cell line using electroporation. The reporter construct will integrate to the deleted AID locus of the cells line. These spontaneously undergo somatic hypermutation and have been modified by deleting *Uracil-DNA glycosylase* to reduce base excision repair of DNA and thus increase in the rate of AID-induced mutations. The cells also have exogenous AID expression construct in place of one AID endogenous allele to allow strong and consistent AID expression as well as *puromycin N-acetyltranferase* expression construct (conferring puromycin resistance) in the other AID locus to allow simple detection of targeted integration of the reporter construct in the deleted AID locus. Correctly targeted clones were screened using puromycin sensitivity testing, as in correctly targeted clones, the GFP4 vector and HS1,2 allele sequence had integrated in place of the puromycin resistance gene. Correctly targeted clones were then analysed with cytometry to determine GFP4 expression.

From these correctly targeted clones, one was selected for every HS1,2 allele, as well as one negative control with only the GFP4 vector and one positive control with human immunoglobulin lambda enhancer. Next, these clones were subcloned and 18 subclones were picked per allele and control. Picked subclones were cultivated for 14 days and after this, GFP4 expression was measured with NovoCyte flow cytometer. DT40 cells were cultured at +40 °C, 5 % CO2, 90 % humidity. Growth media included RPMI 1640 HEPES modification

(Sigma) with 10 % FBS (HyClone), 1 % NCS (Biowest), 1x penicillin-streptomycin antibiotic (Gibco), 1x Glutamax (Gibco), and 50 μM β-mercaptoethanol.

2.2 BERT study for vaccination response

BERT study is short for Booster Pertussis Vaccine study, a clinical study conducted by a team lead by professor Qiushui He at the University of Turku to analyse the adaptive and innate immune response to pertussis booster vaccination. The BERT study was conducted across the UK, Finland and the Netherlands and in this study, patient data from the Finnish part of the BERT study was used to evaluate antibody response. The Finnish part of the BERT study had 124 patients who received booster vaccination against pertussis and vaccination response was measured at three time points: day 0, 28 and 365. Vaccination response was measured with IgG amounts and antibody avidity or how strongly antibodies bind to antigens. Additionally, amounts of different IgG subclasses were measured at day 0 and 28 for part of the study patients. BERT study and its results are described in detail by Anabe et al (2025) and Versteegen et al (2021).

The 124 Finnish patients in the BERT study were from four different age groups: children, adolescents, young adults and older adults. The group children had 7-10 year old patients and the group adolescents had 11-15 year old patients. There were 37 patients in both of these groups. The group young adults had patients between the ages of 20 and 34 and the group older adults had patients between the ages of 60 and 70. There were 25 patients in the younger adults as well as in the older adults. (Anabe at al 2025.)

The effect of HS1,2 enhancer polymorphism on antibody response was evaluated by determining which HS1,2 allele the 124 Finnish BERT patients had and then comparing this to the BERT study data. The idea was to see if patients with a specific allele had different antibody response compared to those with different alleles.

2.3 PCR for identification of HS1,2 alleles

A nested PCR program was used to identify which enhancer HS1,2 allele each BERT patient had. The PCR program was based on the one used by Giambra et al (2005) in their study. First PCR reaction was used to amplify the 3´ regulatory region 1. After this, a second PCR reaction was performed using PCR 1 as a template. This second PCR was used to amplify enhancer HS1,2. Finally, the results were checked using agarose gel electrophoresis. As the

different alleles had different sizes, which allele or alleles a patient had could be determined by the size of the band on the gel. PCR strategy for identification of HS1,2 alleles is also shown in Figure 2.

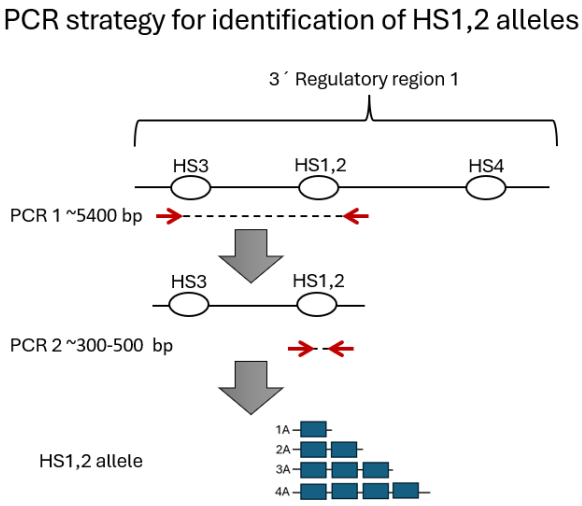

Figure 2 PCR strategy for identification of HS1,2 alleles. Figure describing how nested PCR program was used to amplify HS1,2 alleles.

For PCR reaction, extracted genomic DNA was needed from the BERT patients. For this, the Zymo Research Quick-DNA Miniprep Kit and protocol for whole blood were used. The DNA was extracted from frozen whole blood samples from each patient. DNA concentrations were measured with Qbit HS.

2.3.1 First PCR for amplification of 3'RR1

As previously stated, the first PCR was used to amplify a 5402 bp part of the 3´RR1 region containing enhancer HS1,2. For this, the Platinum Tag DNA polymerase by ThermoFisher Scientific was used. The PCR reaction had a final concentration of 1x High Fidelity Reaction Buffer, 0.2 mM dNTPs, 2 mM MgSO4, 0.2 µM forward and reverse primers, about 50 ng patient DNA as a template and 0.3 µl/reaction of Platinum polymerase. The reaction was filled by H2O. The forward primer used was SA2.5 and the reverse primer was A2R, as also used by Giambra et al (2005). The sequence of the forward primer was "GGATCCCTGTTCCTGATCACTG" and the sequence for the reverse primer was "GCCCTTCCTGCCAACCTG". The actual PCR program used was a touchdown PCR program with the first 2 minutes at 94°C and the next 10 cycles with 30 seconds at 94°C, 30 seconds at 59°C and 5 minutes at 68°C. Following this, 20 cycles with 30 seconds at 94°C, 30 seconds at 57°C and 5 minutes at 68°C. Lastly, final extension at 72°C for 5 minutes.

2.3.2 Second PCR for amplification of HS1,2 enhancer

The second PCR was used for the amplification of enhancer HS1,2. The product from the first PCR was used as a template for this PCR reaction. Q5 High-Fidelity DNA Polymerase by New England Biolabs was used for the second PCR. The PCR reaction had a final concentration of 1x Q5 reaction buffer, 1x High GC Enhancer, 0.2 mM dNTPs, 0.25 µM forward and reverse primers, 2 µl/reaction of PCR 1 product as a template and 0.5 µl/reaction of polymerase. The forward primer used was P3Frw and the reverse primer was D3Rev, the same as used by Giambra et al (2005). These primers also had infusion overhangs that added 2 x 16 bp to the product size. The sequence of the forward primer was "GACTCATTCTGGGCAGACTTG" and the sequence of the reverse primer was "GTCCTGGTCCCAAAGATGG ". The PCR program had first 2 minutes at 98°C and next 30 cycles with 30 seconds at 98°C, 15 seconds at 66°C and 30 seconds at 72°C. Finally, the program had a final extension of 5 minutes at 72°C.

2.3.3 Gel electrophoresis for determining the results

Finally, the results from this two-step PCR were checked using agarose gel electrophoresis. The gel used had 3% agarose from llg-labware, TAE and Midori Green. The PCR product was loaded on the gel with loading dye and the gel was run with 100 V for about an hour. GeneRuler 100 bp DNA Ladder (Thermo Fischer Scientific) was used as a ladder.

After running the gel, the results could be checked. As the HS1,2 alleles are different sizes depending on the number of copies of the repeating element, what allele each patient had could be determined from the size of the band on the gel. The different sizes of the alleles and their sizes on the gel are shown in Table 1. An example of different alleles on a gel is shown in Figure 3.

Table 1: Sizes of HS1,2 alleles on gel

HS1,2 allele	Target allele size	PCR product size (primers add 2 x 16 bp)
1A	287 bp	319 bp
2A	339 bp	371 bp
2A*	359 bp	391 bp
3A	393 bp	425 bp
4A	465 bp	497 bp

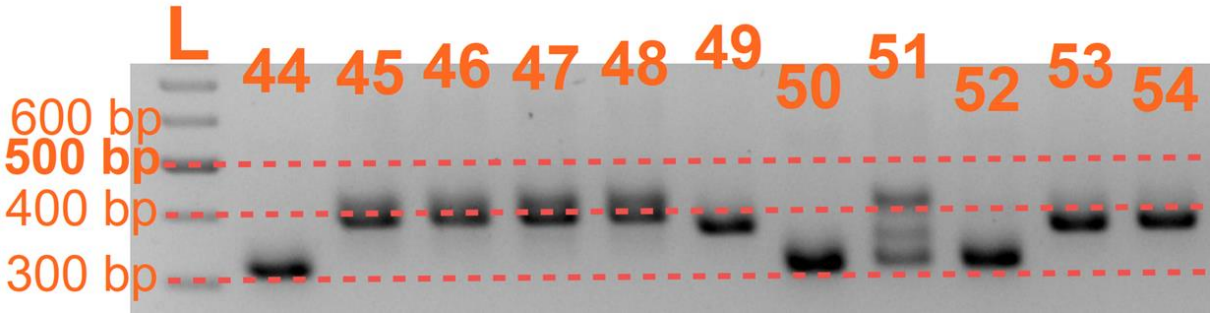

Figure 3: Examples of different HS1,2 alleles on gel. Picture showing different alleles of HS1,2 enhancer on a gel. L = Ladder. 44, 50 and 52: Patients with allele 1A. 49, 53 and 54: Patients with allele 2A. 45, 46, 47 and 48: Patients with allele 2A*. 51: Patient with alleles 1A and 2A*.

To confirm PCR results, some of the PCR products were sequenced to confirm which HS1,2 allele they represented. For this, Eurofins Sanger sequencing services were used. Specifically, PlateSeq supreme for sequencing 96-well plates. For sequencing, PCR products were cloned into Stellar competent cells by Takara Bio using the Zero Blunt TOPO PCR cloning kit. Sequencing results were analysed using SnapGene software.

2.4 Statistical analysis

Statistical analysis for the GFP loss assay for somatic hypermutation was performed using GraphPad Prism version 8.3.1. P-values were calculated using Mann-Whitney test, a nonparametric t-test.

Statistical analysis for GFP expression data was performed using JMP Student Edition version 19.0.1. P-values were calculated using nonparametric multiple comparison with Steel-Dwass method.

For antibody response, statistical analysis was performed using JMP Student Edition version 19.0.1. Data were checked for normality using Shapiro-Wilk test and all non-normal distributed data were log-transformed. P-values were calculated using Tukey HSD multiple comparison.

3 RESULTS

3.1 Somatic hypermutation targeting activity of HS1,2 alleles

GFP loss assay was performed in three replicates to assess if HS1,2 alleles target somatic hypermutation. A reporter with human Ig lambda light chain enhancer (Burstedde et al. 2014) was used as a positive control and a reporter without a test sequence (JMB#20) was used as a negative control. A summary of these 3 assays and their results are in Tables 2-4 and Figures 4-6.

Table 2: Summary of GFP loss assay 1

Tested sequence	Number of values	Median	p-value compared to JMB#20 (negative control)
Allele 1A	17	3.16	**<0.0001**
Allele 2A	17	3.17	**<0.0001**
Allele 3A	15	2.77	**<0.0001**
Allele 3B	17	4.12	**<0.0001**
Allele 4A	18	5.58	**<0.0001**
Pos. control: hIgLE	17	6.89	-
Neg. control: JMB#20	32	1.26	-

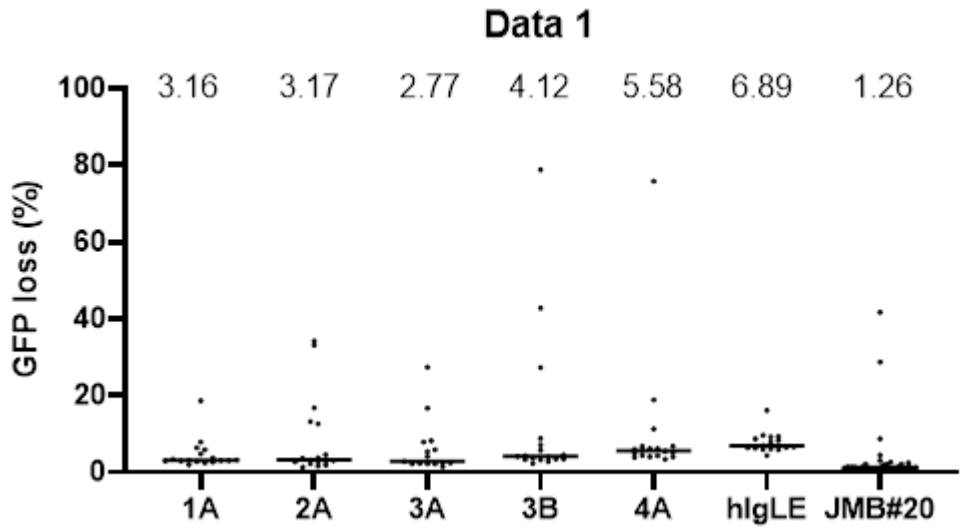

Figure 4: GFP loss assay 1 results. Tested sequence on x-axis: HS1,2 allele or hIgLE for positive control or JMB#20 for negative control. The medians for each tested sequence are at the top of the figure.

Table 3: Summary of GFP loss assay 2

Tested sequence	Number of values	Median	p-value compared to JMB#20 (negative control)
Allele 1A	10	1.395	**0.0004**

Tested sequence	Number of values	Median	p-value compared to JMB#20 (negative control)
Allele 2A	12	2.225	**<0.0001**
Allele 3A	12	1.685	**0.0001**
Allele 3B	8	2.265	**0.0002**
Allele 4A	9	3.35	**<0.0001**
Pos. control: hIgLE	7	54	-
Neg. control: JMB#20	9	0.015	-

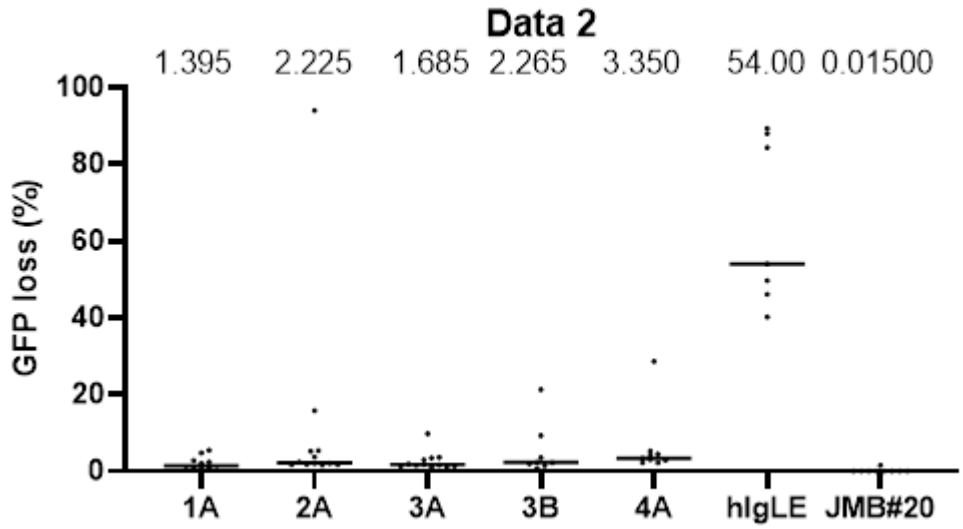

Figure 5: GFP loss assay 2 results. Tested sequence on x-axis: HS1,2 allele or hIgLE for positive control or JMB#20 for negative control. The medians for each tested sequence are at the top of the figure.

Table 4: Summary of GFP loss assay 3

Tested sequence	Number of values	Median	p-value compared to JMB#20 (negative control)
Allele 1A	12	3.02	0.0887
Allele 2A	3	5.89	0.1802
Allele 3A	12	2.495	0.1239
Allele 3B	12	3.645	**0.0249**
Allele 4A	12	3.35	0.1005
Pos. control: hIgLE	4	49.6	-
Neg. control: JMB#20	12	1.5	-

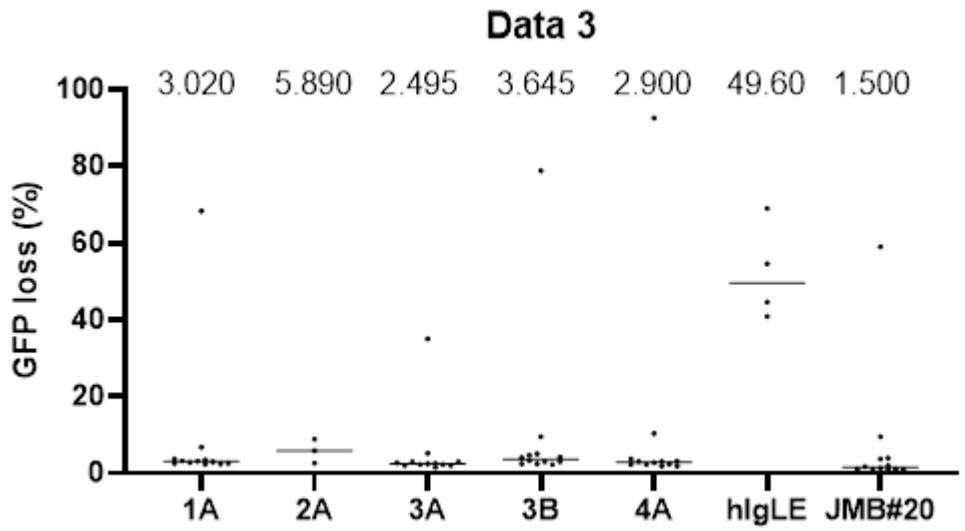

Figure 6: GFP loss assay 3 results. Tested sequence on x-axis: HS1,2 allele or hIgLE for positive control or JMB#20 for negative control. The medians for each tested sequence are at the top of the figure.

After this, results from all three assays were pooled. A summary of these pooled assays and results is in Table 5 and Figure 7.

Table 5: Summary of pooled GFP assay

Tested sequence	Number of values	Median	p-value compared to JMB#20 (negative control)	p-value compared to allele 1A	p-value compared to allele 3A
Allele 1A	39	2.99	0.0002	-	0.2213
Allele 2A	32	3.105	**<0.0001**	0.4959	0.1299
Allele 3A	39	2.48	**<0.0001**	0.2213	-
Allele 3B	37	3.65	**<0.0001**	**0.0379**	**0.006**
Allele 4A	39	4.23	**<0.0001**	**0.0100**	**0.0009**
Pos. control: hIgLE	28	9.265	-	-	-
Neg. control: JMB#20	39	1.27	-	-	-

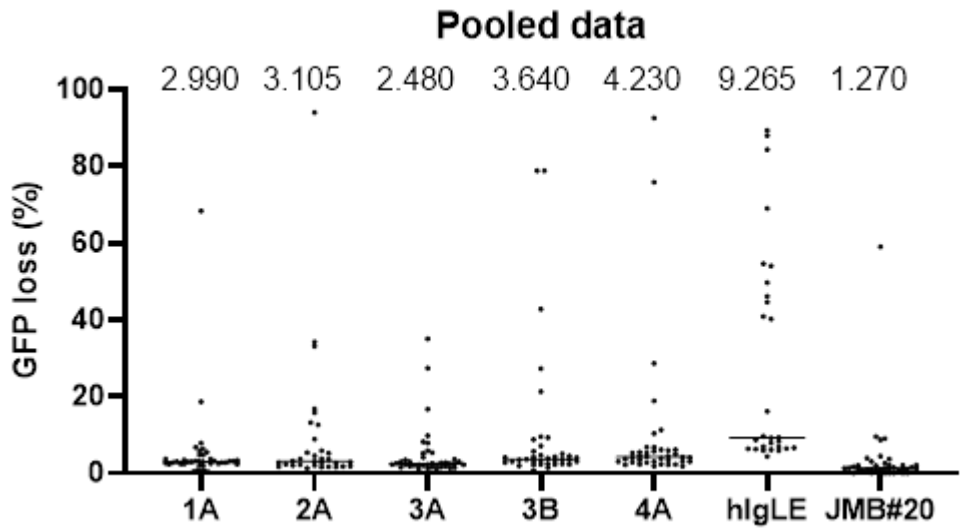

Figure 7: Pooled GFP loss assay results. Tested sequence on x-axis: HS1,2 allele or hIgLE for positive control or JMB#20 for negative control. The medians for each tested sequence are at the top of the figure.

The results show that all tested HS1,2 alleles do target somatic hypermutation. All alleles had a statistically significantly higher GFP loss than the negative control and thus they accumulated more mutations to the GFP gene, indicating recruitment of somatic hypermutation activity. Yet, all HS1,2 alleles had quite small GFP loss compared to the positive control. This indicates that HS1,2 alleles are not as strong or important elements in targeting somatic hypermutation as some others. There are no clear differences between alleles. In the pooled data, alleles 3B and 4A are statistically significantly higher than alleles 1A and 3A. These differences are small and can only be seen in the pooled data. There are no other statistically significant differences between alleles.

3.2 HS1,2 alleles and GFP expression in GFP loss assay

GFP expression was also measured for all alleles. These results for pooled data are shown in Figure 8 and Table 6. GFP expression was similar for all tested sequences. The only statistically significant difference in GFP expression was between alleles 3A and 2A. But still, even this was not a large difference.

Table 6 GFP expression results for pooled data.

Tested sequence	1A	2A	3A	3B	4A	hIgLE (pos control)	JMB#20 (neg control)
Number of values	39	32	39	37	39	49	37

Median	99141	90253,5	96640	94267	97326	90910	95497
p-value to 1A	-	0.3751	0.9965	0.9870	0.4811	0.8224	0.9999
p-value to 2A	0.3751	-	**0.0245**	0.2910	0.5107	0.2782	0.6126
p-value to 3A	0.9965	**0.0245**	-	0.9992	0.7972	0.9949	0.9741
p-value to 3B	0.9870	0.2910	0.9992	-	0.9751	1.0000	1.0000
p-value to 4A	0.4811	0.5107	0.7972	0.9751	-	0.9870	0.9907
p-value to pos control	0.8224	0.2782	0.9949	1.0000	0.9870	-	0.9989
p-value to neg control	0.9999	0.6126	0.9741	1.000	0.9907	0.9989	-

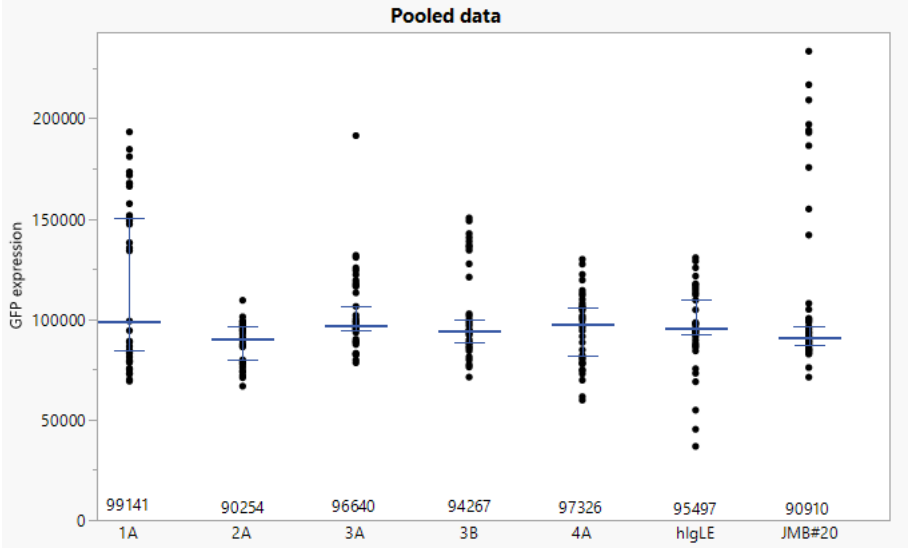

Figure 8 Median of GFP expression for tested sequences. Tested sequence on x-axis: HS1,2 allele or hIgLE for positive control or JMB#20 for negative control. Medians for GFP expression shown by blue lines and confidence intervals shown by error bars. Actual median shown in numbers above x-axis and different individual measurements represented by dots.

3.3 HS1,2 alleles in BERT study patients

Enhancer HS1,2-A allele status was determined for all 124 Finnish BERT study patients. These results can be seen in Table 7. 89 patients or 71.8 % of these BERT patients had one HS1,2-A allele or they were homozygotes. The other 33 patients or 28.2 % had two different alleles or they were heterozygotes. For those with only one allele, allele 1A was the most common one with 60.1 %. Additionally, 15 patients or 16.9 % had allele 2A and 20 patients

or 22.5 % had a variation of allele 2A, allele 2A*. Sequencing showed that this allele 2A* is the same as HS1,2-A allele 2 variant 2B reported by Jodice et al (2024). No patient had alleles 3A or 4A as a homozygote. Results for homozygotes can be seen in Table 8. From those with multiple alleles, most had allele 1A as one of them. These heterozygotes had some patients with alleles 3A and 4A.

Table 7: HS1,2-A alleles in BERT patients

HS1,2-A allele result	Amount	Percent from total
2A + 2A*	1	0.8 %
1A + 2A*	27	21.8 %
2A* + 4A	4	3.2 %
1A + 3A	2	1.6 %
1A + 4A	1	0.8 %
1A	54	43.5 %
2A	15	12.1 %
2A*	20	16.1 %
Total	**124**	**99.9 %**

Table 8: HS1,2-A allele results for only homozygotes

HS1.2-A allele result	Amount	Percent from total
1A	54	60.1 %
2A	15	16.9 %
2A*	20	22.5 %
Total	**89**	**99.5 %**

3.4 HS1,2 alleles and vaccination response

Vaccination response was evaluated in three different categories: antibody amounts, IgG avidity and Ig class switching. Antibody amounts were compared by comparing the amount of IgG antibodies. Class switching was evaluated using amounts of different IgG subtypes from 1 to 4.

Three patient groups were compared to each other. One group was patients with HS1,2 allele 1A, the second group was patients with allele 2A and the final group was patients with allele 2A*. Only those patients who had one allele were included in the analysis. Those patients

with two different alleles were excluded, as with these patients both alleles could contribute to the phenotype and thus the results would be inconclusive.

3.4.1 IgG amounts

IgG amounts were compared using IgG antibodies against three different antigens: pertussis toxin, filamentous haemagglutinin and pertactin. All these are proteins produced by the pertussis bacteria and the aim of vaccination is to produce antibodies against these antigens. In this study, the amounts of IgG antibodies against these three antigens were combined. This combined IgG amount variable was then compared between three groups of patients: those with allele 1A, those with 2A and those with allele 2A*. Summary of these results can be seen in Table 9 and Figure 10 and as they show, there were no statistically significant differences between alleles at any time point. As can be seen, the amount of IgG antibodies increases from day 0 to day 28 after booster vaccination. After this, the amount of IgG antibodies begins to decrease from day 28 to day 365. Still, the amount of IgG antibodies behaves similarly in all three patient groups, indicating that what HS1,2 allele patient has does not seem to affect IgG amounts in response to vaccination.

Table 9: Summary of results for IgG antibody amounts.

Time point	HS1,2 Allele	Number of values	Median	p-value compared to 1A	p-value compared to 2A
Day 0	1A	162	18.405	-	0.9923
Day 0	2A	45	19.725	0.9923	-
Day 0	2A*	60	20.396	0.8161	0.9294
Day 28	1A	162	253.76	-	0.9516
Day 28	2A	45	277.09	0.9516	-
Day 28	2A*	60	230.99	0.9578	0.8852
Day 365	1A	162	79.069	-	0.5963
Day 365	2A	45	113.804	0.5963	-
Day 365	2A*	57	90.236	0.3697	0.9733

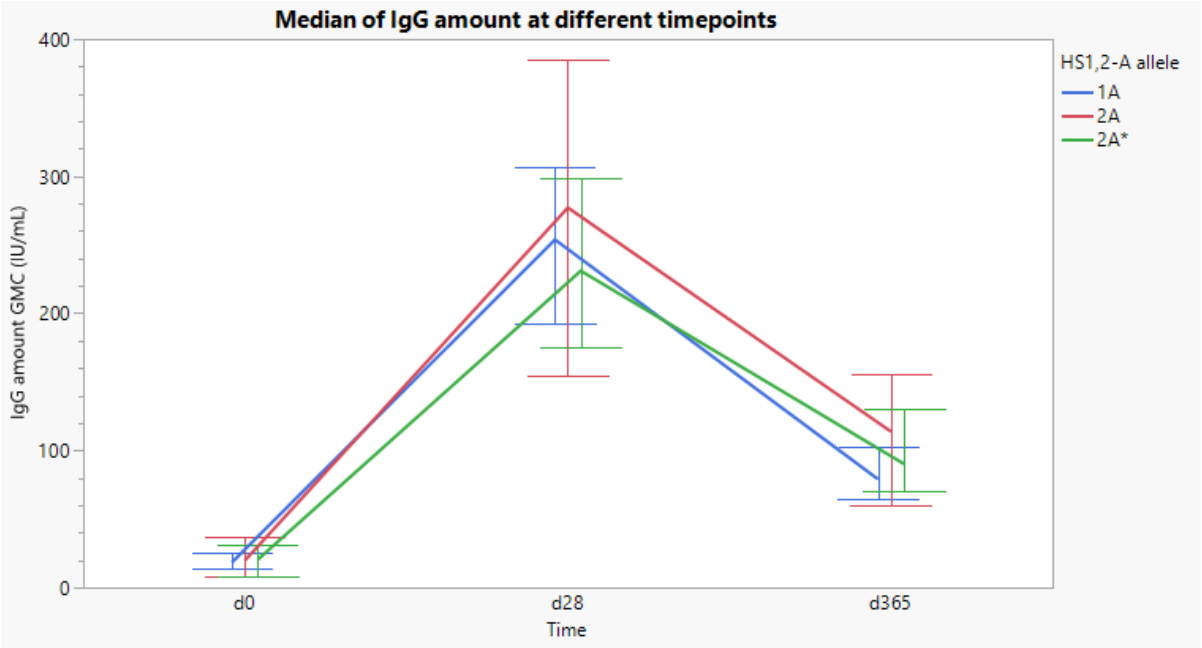

Figure 9: Median for IgG amount at different timepoints by HS1,2 allele. IgG amounts for three patient groups: those with allele 1A, those with 2A and those with allele 2A*. Error bars show a 95% confidence interval for the median.

3.4.2 Antibody avidity

Antibody avidity or how strongly the antibody binds to its antigen, was measured using two different concentrations of urea: 3.5 M and 6.5 M. Urea washes away antibodies that do not bind strongly to their antigens and the higher the concentration of urea, the stronger this effect becomes. As such, the higher the results are, the higher the avidity of the antibodies is. A summary of these results can be seen in Tables 10 and 11, as well as Figures 11 and 12.

Table 10: Summary of results for 3.5 M Avidity.

Time point	HS1,2 Allele	Number of values	Median	p-value compared to 1A	p-value compared to 2A
Day 0	1A	19	59.1	-	0.2256
Day 0	2A	5	82.5	0.2256	-
Day 0	2A*	7	56.8	0.9744	0.2553
Day 28	1A	50	76.75	-	0.7747
Day 28	2A	14	85.05	0.7747	-
Day 28	2A*	17	73	0.7318	0.4815
Day 365	1A	49	59.8	-	0.0541
Day 365	2A	11	63.9	0.0541	-
Day 365	2A*	18	57.95	0.4366	**0.0118**

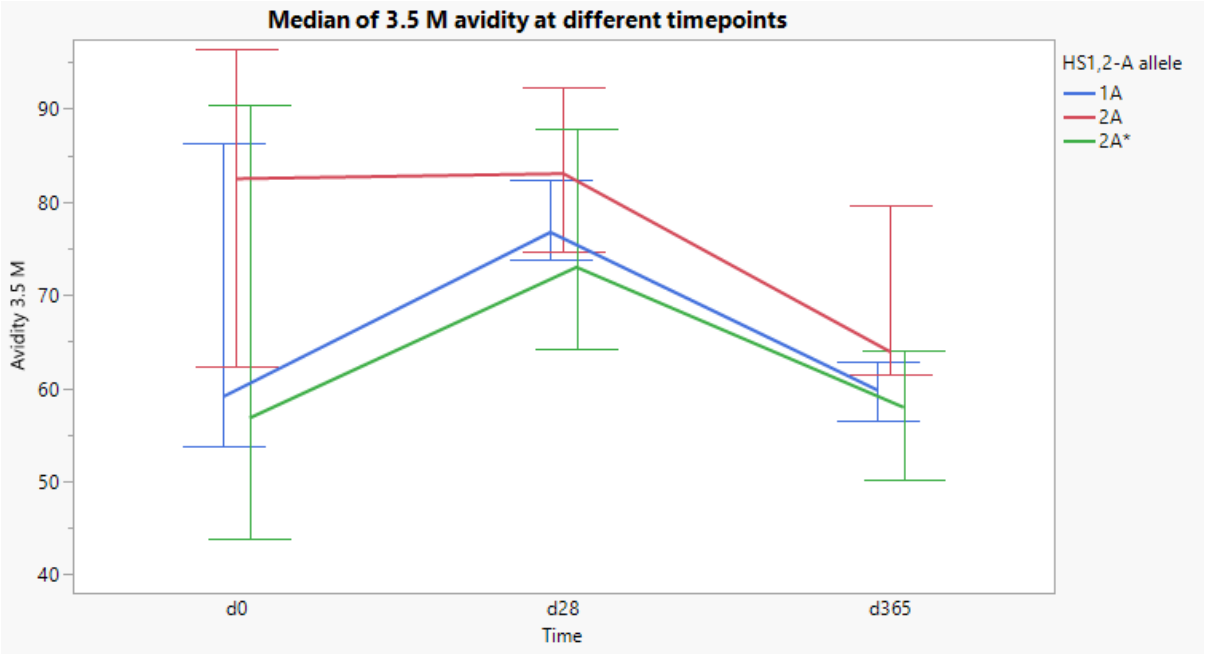

Figure 10: Median for 3.5 M avidity at different timepoints by HS1,2 allele. Antibody avidity for three patient groups: those with allele 1A, those with 2A and those with allele 2A*. Error bars show a 95% confidence interval for the median.

The only statistically significant difference is that patients with allele 2A have higher avidity than patients with allele 2A* at day 365. Still, this can only be seen when the avidity has been measured with 3.5 M urea. When measuring with 6.5 M urea, there are no statistically significant differences between HS1,2 alleles at any time point. Looking at Figures 7 and 8, patients with allele 2A seem to have higher avidity than those with 2A* or 1A at every time point and with both concentrations of urea, but as previously stated, this difference is statistically significant at only one time point and only when measuring with 3.5 M urea.

Table 11: Summary of results for 6.5 M Avidity

Time point	HS1,2 Allele	Number of values	Median	p-value compared to 1A	p-value compared to 2A
Day 0	1A	19	17.6	-	0.3152
Day 0	2A	5	27.1	0.3152	-
Day 0	2A*	7	20.8	0.8557	0.6644
Day 28	1A	50	28.95	-	0.6829
Day 28	2A	14	44.3	0.6829	-
Day 28	2A*	17	29.3	0.7711	0.4367
Day 365	1A	51	21.3	-	0.9788
Day 365	2A	13	20.3	0.9788	-
Day 365	2A*	18	23.4	0.9875	0.9984

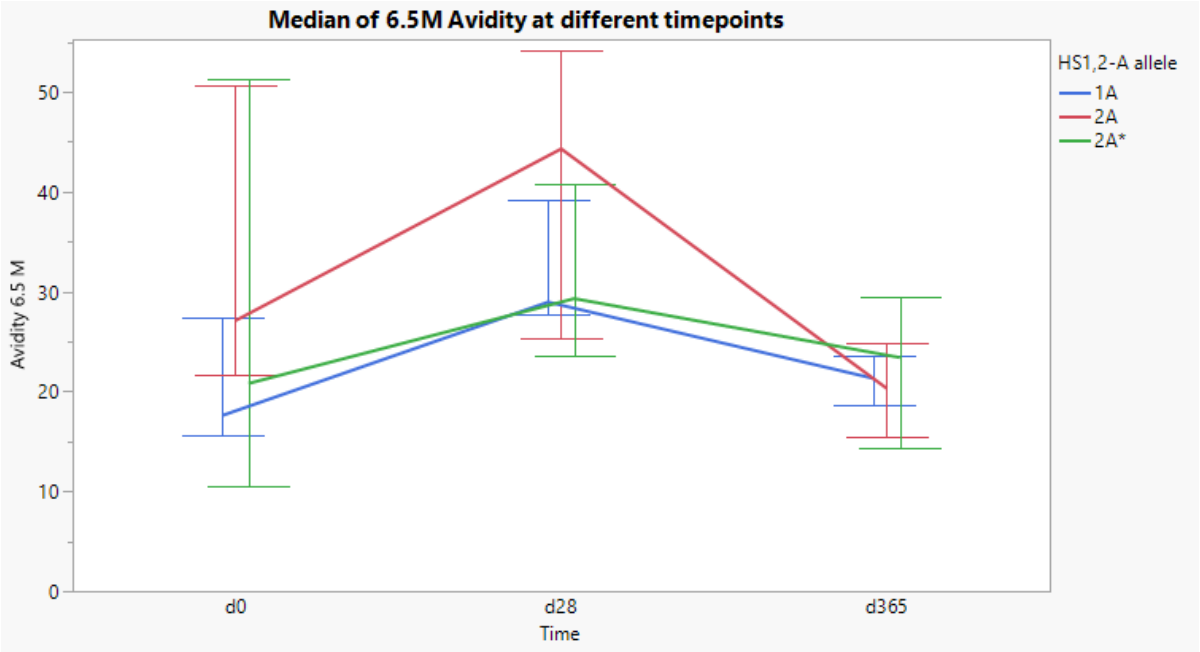

Figure 11: Median for 6.5 M avidity at different timepoints by HS1,2 allele. Antibody avidity for three patient groups: those with allele 1A, those with 2A and those with allele 2A*. Error bars show a 95% confidence interval for the median.

3.4.3 IgG subtypes

Class switch recombination was evaluated using data about different IgG subclasses. Class switch recombination is needed to switch between these classes. The amount of IgG subclasses from 1 to 4 at timepoints day 0 and day 28 was measured for part of the Finnish BERT study patients. More specifically, IgG subclasses were measured for the young adults and older adults. The amount of these different subclasses was compared between the three patient groups with different HS1,2 alleles. A summary of these results can be seen in Table 12 and Figures 13-16.

Table 12: Summary of results for IgG subclasses

Time point	IgG subclass	HS1,2 Allele	Number of values	Median	p-value compared to 1A	p-value compared to 2A
Day 0	IgG 1	1A	19	7.190	-	0.5946
Day 0	IgG 1	2A	6	3.900	0.5946	-
Day 0	IgG 1	2A*	10	4.725	0.7060	0.9566
Day 0	IgG 2	1A	19	2.060	-	0.9863
Day 0	IgG 2	2A	6	1.750	0.9863	-
Day 0	IgG 2	2A*	10	1.830	0.9029	0.9817
Day 0	IgG 3	1A	19	1.240	-	0.9364

Time point	IgG subclass	HS1,2 Allele	Number of values	Median	p-value compared to 1A	p-value compared to 2A
Day 0	IgG 3	2A	6	0.765	0.9364	-
Day 0	IgG 3	2A*	10	0.675	0.4253	0.7977
Day 0	IgG 4	1A	19	0.4	-	0.6167
Day 0	IgG 4	2A	6	0.37	0.6167	-
Day 0	IgG 4	2A*	10	0.2	0.3363	0.9718
Day 28	IgG 1	1A	19	73.53	-	0.5174
Day 28	IgG 1	2A	6	24.79	0.5174	-
Day 28	IgG 1	2A*	10	33.07	0.2976	0.9888
Day 28	IgG 2	1A	19	5.700	-	0.9965
Day 28	IgG 2	2A	6	5.180	0.9965	-
Day 28	IgG 2	2A*	10	3.830	0.8650	0.9463
Day 28	IgG 3	1A	19	3.720	-	0.6200
Day 28	IgG 3	2A	6	2.260	0.6200	-
Day 28	IgG 3	2A*	10	2.690	0.3719	0.9824
Day 28	IgG 4	1A	19	1.170	-	0.9996
Day 28	IgG 4	2A	6	1.190	0.9996	-
Day 28	IgG 4	2A*	10	0.42	0.3071	0.4892

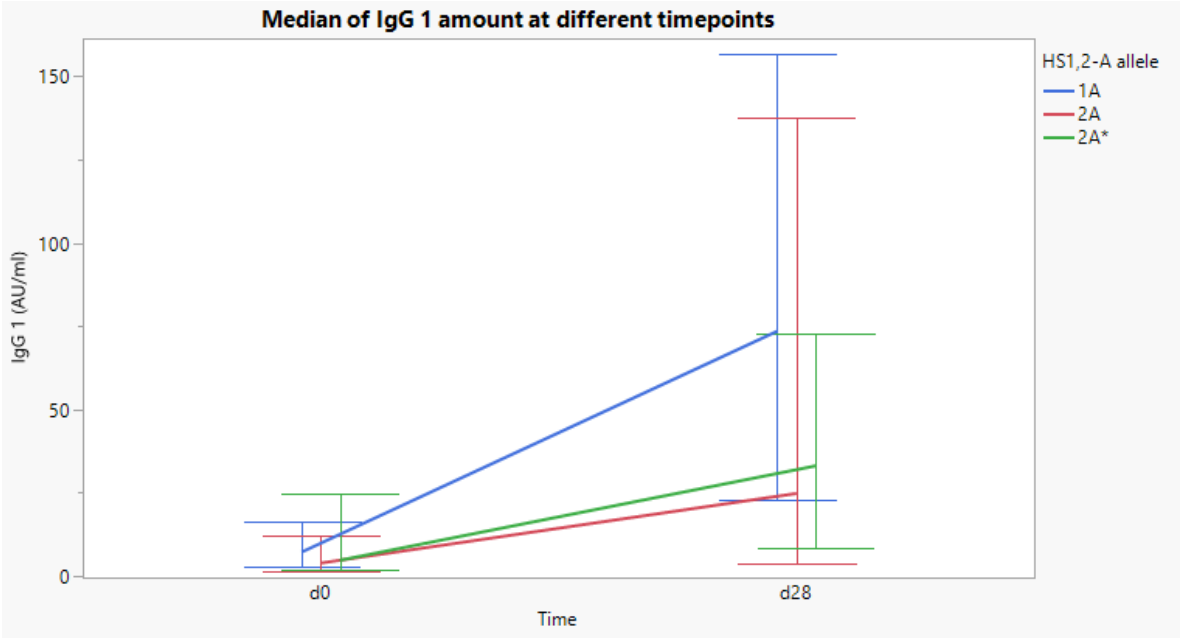

Figure 12: Median for IgG 1 at different timepoints by HS1,2 allele. Amounts of IgG 1 for three patient groups: those with allele 1A, those with 2A and those with allele 2A*. Error bars show a 95% confidence interval for the median.

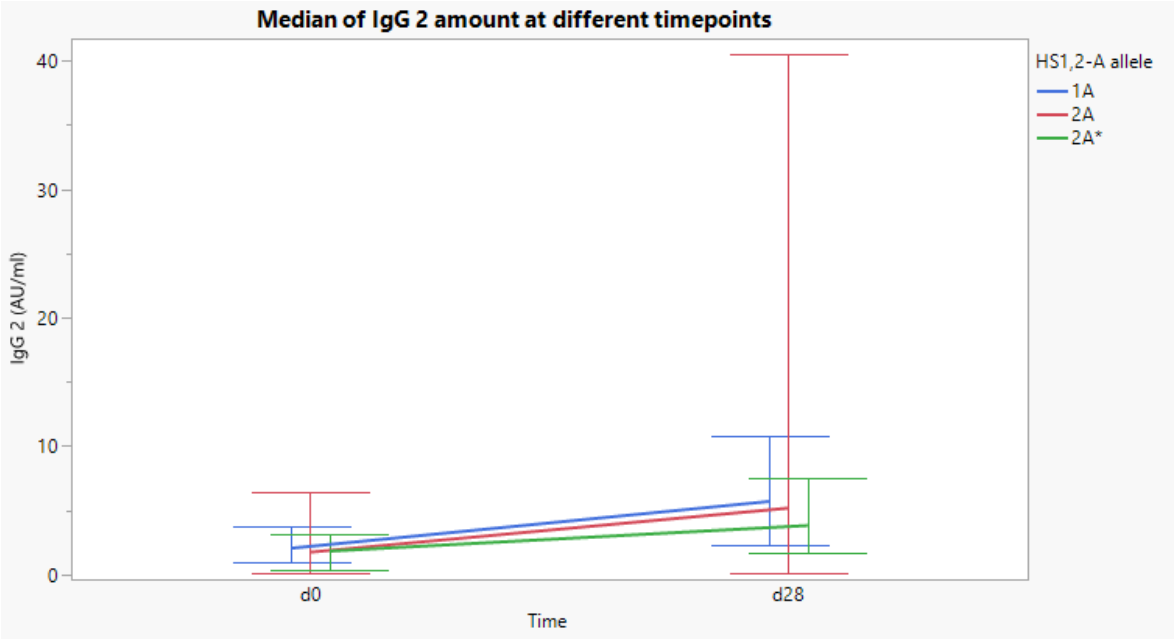

Figure 13: Median for IgG 2 at different timepoints by HS1,2 allele. Amounts of IgG 2 for three patient groups: those with allele 1A, those with 2A and those with allele 2A*. Error bars show a 95% confidence interval for the median.

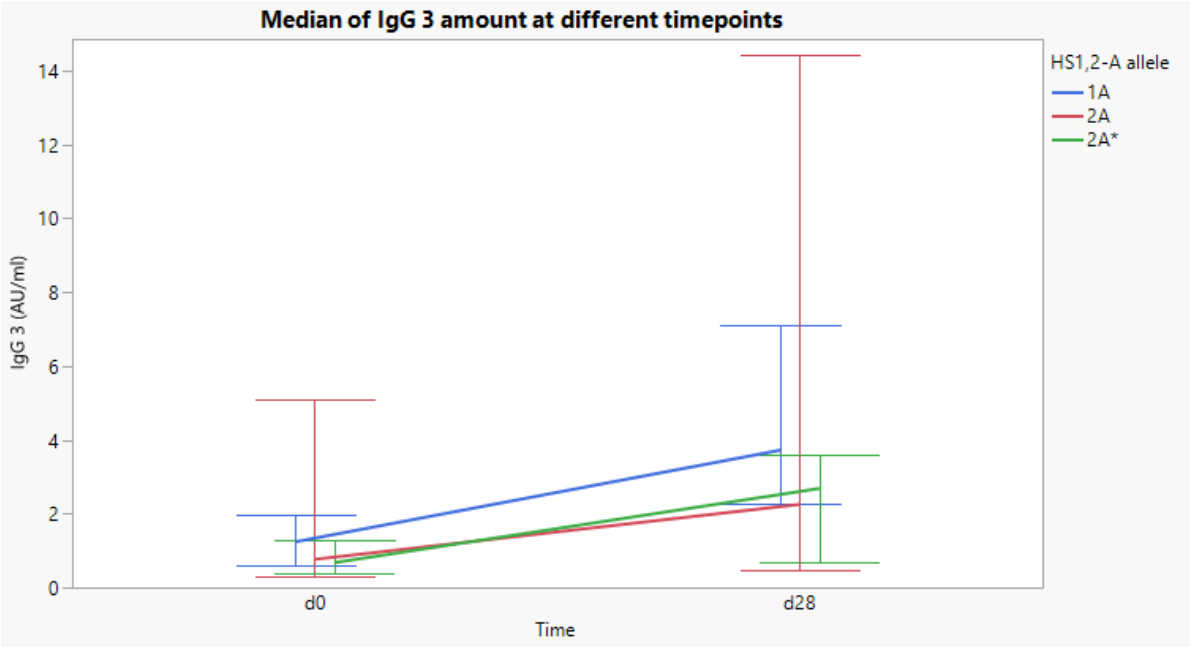

Figure 14: Median for IgG 3 at different timepoints by HS1,2 allele. Amounts of IgG 3 for three patient groups: those with allele 1A, those with 2A and those with allele 2A*. Error bars show a 95% confidence interval for the median.

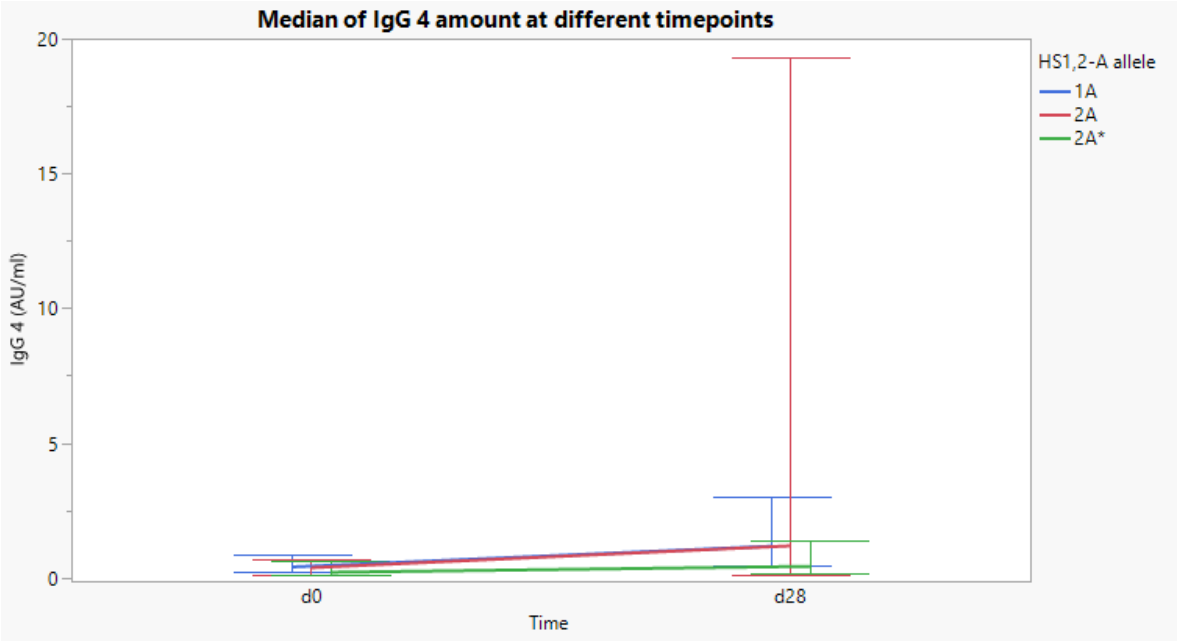

Figure 15: Median for IgG 4 at different timepoints by HS1,2 allele. Amounts of IgG 4 for three patient groups: those with allele 1A, those with 2A and those with allele 2A*. Error bars show a 95% confidence interval for the median.

There were no statistically significant differences between patients with different alleles at any time points. In all patient groups, the amount of all IgG subclasses increased after booster vaccination from day 0 to day 28. All patient groups also had higher amounts of IgG subclass 1 than other subclasses. The amount of different IgG subclasses behaves similarly in all three patient groups, indicating that what HS1,2 allele patient has does not seem to affect IgG subclass amounts in response to vaccination. This would indicate that HS1,2 alleles seem to not affect class switch recombination.

4 DISCUSSION

The first aim of this study was to determine if 3´ regulatory region polymorphism had a role in somatic hypermutation and if different HS1,2 enhancer alleles would differ in this. The second part was to determine if HS1,2 enhancer polymorphism affected antibody response to vaccination and if this polymorphism would explain some of the differences in vaccination response found in patients.

4.1 HS1,2 polymorphism and somatic hypermutation

The results of this study showed that HS1,2 enhancer alleles do target somatic hypermutation, but also that they seem to be quite weak at this. There are other enhancers and enhancer-like sequences that seem to have a much larger role in targeting somatic hypermutation. These results would indicate that enhancer HS1,2 does have a role in somatic hypermutation, but also that likely this role is quite small. Still, as Rouaud et al (2013) showed that 3´ regulatory region seems to have a large role in somatic hypermutation, this raises the question, what sequences in the 3´ regulatory region would explain this large role, as results in this study indicate that it isn't enhancer HS1,2. Of course, 3´ regulatory region also includes enhancers HS3 and HS4. In relation to the 3´ regulatory region's role in somatic hypermutation, the next steps could be to determine if enhancers HS3 and HS4 target somatic hypermutation and if so, would this explain the role of 3´RR in somatic hypermutation? It should also be noted that this method of analysing somatic hypermutation may be unable to show all aspects of regulation of somatic hypermutation. As the tested sequence is close to the target gene, it is possible this method does not include all the physiological regulatory mechanisms especially in relation to chromatin folding and looping that have already been linked to regulation of somatic hypermutation (Schoeberl et al 2025). Therefore, it is possible that enhancer HS1,2 uses these methods to regulate somatic hypermutation in genome where it is far from the target genes and this could explain the differences in results.

Another aim of this study was to determine if HS1,2 and 3´RR polymorphism had a role in somatic hypermutation. The results from this study showed that there are some differences in targeting somatic hypermutation between alleles, as alleles 3B and 4A had higher GFP loss than alleles 1A and 3A. Still, these differences were small and GFP loss was quite low for all alleles. This result does not seem to follow the original hypothesis that the longer the allele, the more it would target somatic hypermutation, as allele 3B had higher GFP loss than 3A

even though they have the same number of copies of the repeating element. Additionally, allele 2A seemed to have higher GFP loss than alleles 1A and 3A, even though this was not statistically significant. These differences are not explained by allele specific differences in GFP expression as GFP expression was similar for all alleles. All in all, there seem to be some minor differences in targeting somatic hypermutation, but these differences are quite small and as all alleles are quite weak at targeting somatic hypermutation, these small differences might not have any effect in practice. Especially given the significant role of the Ig mu intronic enhancer (IgHEi) in increasing the SHM. In comparison, the strongest HS1,2 variant HS1,2 4A (4,23 % GFP4 loss) has more that 14-fold lower SHM recruitment activity than IgHEi (62,1% GFP4 loss, Burstedde et al 2014). So, the results of this study would suggest that 3´ regulatory region polymorphism likely has no role or only a small role in somatic hypermutation. The fact that all alleles seem to be quite weak at targeting somatic hypermutation also leads to the fact that the differences can be hard to see and in the future, differences between alleles could be investigated more thoroughly if there was a more accurate way to measure small differences in somatic hypermutation. Still, as these differences are small and possibly meaningless in practice, it could be pointless to investigate these differences further.

4.2 HS1,2 polymorphism and antibody affinity

Antibody avidity had some signs that there might be differences between alleles, as patients with allele 2A had higher avidity than those with allele 2A* at day 365. Still, this difference could only be seen when avidity was measured with 3.5 M urea and thus the importance of these results is in question. If patients with allele 2A really had higher avidity antibodies, it would be logical that this could also be seen when measuring with 6.5 M urea. As higher concentrations of urea show higher avidity antibodies, it would also be logical if this difference was seen more clearly when measuring with 6.5 M urea. As it is, the result does not reflect this and the difference is seen only when measuring with 3.5 M urea. As such, it has to be questioned if this result is real or the result of chance. This could be solved in the future by investigating the role of HS1,2 polymorphism in antibody avidity with a larger patient population.

Somatic hypermutation is important for the production of high avidity antibodies and as this study showed, HS1,2 alleles do target somatic hypermutation and there possibly some small differences between alleles. This could explain the possible differences in antibody avidity in

response to vaccination. Still, these differences are quite small and as such, larger patient populations would be better for evaluating them. Firstly, for evaluating if there really are differences and secondly, if these differences are large enough to have practical importance.

4.3 HS1,2 polymorphism, GFP expression and antibody amounts

Results from IgG amounts following vaccination were similar in patients with all alleles. This indicates that HS1,2 polymorphism does not affect IgG amounts. There was also no big differences in GFP expression between different alleles as GFP expression had a similar level for all alleles. Only statistically significant results was that allele 2A had slightly lower median than 3A. However, from alleles tested with GFP loss assay, only alleles 2A and 1A were found in homozygote patients and thus were included in vaccination response analysis. This makes comparing results from GFP expression and IgG amounts difficult. Still, alleles 1A and 2A, that were included in both analyses, had no differences in GFP expression or IgG amounts. These results support each other as similar GFP expression indicates no differences in gene transcription.

4.4 HS1,2 polymorphism and class switch recombination

The results from IgG subclass amounts indicated that HS1,2 polymorphism seems to not affect class switch recombination as amounts of different IgG subclasses was similar between patients with different HS1,2 alleles.

In the future, investigating the role of HS1,2 polymorphism in class switch recombination would also benefit from a much larger population, as in this specifically number of patients was specifically low. For example, in this study, of the patients that had the amount of IgG subclasses measured, only 6 patients had allele 2A. This leads to the fact that statistically significant differences were hard to find, and it is possible that there actually are differences between alleles, but that this study population was simply not large enough to show them.

4.5 HS1,2 polymorphism and vaccination response

In conclusion, the results of this study show that vaccination response is similar between patients with different alleles. This would suggest that 3´ regulatory region polymorphism does not have a role in vaccination response. However, it is also possible that HS1,2 polymorphism does have a role, but that this role would only be visible with a larger patient population. The fact that allele 2A specifically is linked to a number of autoimmune diseases

(Frezza et al 2004, Frezza et al 2007 and Frezza et al 2012) would suggest that HS1,2 alleles do have some complex role in antibody production and/or antibody-mediated immunity and thus manifest as differences in vaccination response. The fact that this study did not find large differences suggests that if there are differences, they are small and require a large population of patients to find. Many factors affect vaccination response and as such, the role of HS1,2 polymorphism is likely at most a small part of a larger whole or that there is no role at all.

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