1 Transmembrane helices are an overlooked and
 2 evolutionarily conserved source of major
 3 histocompatibility complex class I and II epitopes

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Abstract

Cytolytic T cell responses are predicted to be biased towards membrane proteins. The peptide-binding grooves of most haplotypes of histocompatibility complex class I (MHC-I) are relatively hydrophobic, therefore peptide fragments derived from human transmembrane helices (TMHs) are predicted to be presented more often as would be expected based on their abundance in the proteome. However, the physiological reason of why membrane proteins might be over-presented is unclear. In this study, we show that the over-presentation of TMH-derived peptides is general, as it is predicted for bacteria and viruses and for both MHC-I and MHC-II. Moreover, we show that TMHs are evolutionarily more conserved, because single nucleotide polymorphisms (SNPs) are present relatively less frequently in TMH-coding chromosomal regions compared to regions coding for extracellular and cytoplasmic protein regions. Thus, our findings suggest that both cytolytic and helper T cells respond more to membrane proteins, because these are evolutionary more conserved. We speculate that TMHs therefore are less prone to escape mutations that enable pathogens to evade T cell responses.

Keywords: antigen presentation, membrane proteins, bioinformatics, adaptive immunity, transmembrane domain, transmembrane helix, epitopes, T lymphocyte, MHC-I, MHC-II, evolutionary conservation

Abbreviations

Abbreviation	Full
MAP	Membrane-associated protein
TMH	Transmembrane helix
TMP	Transmembrane protein

1 Introduction

Our immune system fights diseases and infections from pathogens, such as fungi, bacteria or viruses. An important part of the acquired immune response, that develops specialized and more specific recognition of pathogens than the innate immune response, are T cells which recognize peptides, called epitopes, derived from antigenic proteins presented on Major Histocompatibility Complexes (MHC) class I and II on the cell surface.

The MHC proteins are heterodimeric complexes encoded by the HLA (Human Leukocyte Antigens) genes. In humans, the peptide binding groove of MHC-I is made by only the alpha subunit. There are three classical forms of MHC-I, hallmarked by a highly polymorphic alpha chain called HLA-A, HLA-B and HLA-C, that all present epitopes to cytolytic T cells. For MHC-II, both the alpha and the beta chains contribute to the peptide binding groove. There are three classical forms of MHC-II as well, called HLA-DR, HLA-DQ and HLA-DP, that all present epitopes to helper T cells. Each MHC complex can present a subset of all possible peptides. For example, HLA-A and HLA-B have no overlap in which epitopes they bind [1]. Moreover, the HLA genes of humans are highly polymorphic, with hundreds to thousands of different alleles, and each different HLA allele is called an MHC haplotype and presents a different subset of peptides [2].

Humans mostly express two haplotypes per MHC form, one from the parental and one from the maternal chromosome, and therefore an individual's immune system detects only a fraction of all possible peptide fragments. However, at the population level, the coverage of pathogenic peptides that are detected is very high, because of the highly polymorphic MHC genes. It is therefore believed that MHC polymorphism improves immunity at the population level, as mutations in a protein that disrupt a particular MHC presentation at the in-

dividual level, so-called escape mutations, will not affect MHC presentation for all haplotypes present in the population [3].

Many studies are aimed at identifying the repertoire of epitopes that are presented in any MHC haplotype and determining which epitopes will result in an immune response, as this will for instance aid the design of vaccines. These studies have led to the development of prediction algorithms that allow for very reliable *in silico* predictions of the binding affinities of peptides [4, 5, 6]. For example, [6] found that, of the 432 peptides that were predicted to bind to MHC, 86% were experimentally confirmed to do so.

Using these prediction algorithms, we recently predicted that peptides derived from transmembrane helices (TMHs) will be more frequently presented by MHC-I than expected based on their abundance [7]. Moreover, we showed that some well-known immunodominant peptides stem from TMHs. This overpresentation is attributed to the fact that the peptide-binding groove of most MHC-I haplotypes is relatively hydrophobic, and therefore hydrophobic TMH-derived peptides have a higher affinity to bind than their soluble hydrophilic counterparts.

TMHs are hydrophobic as they need to span the hydrophobic lipid bilayer of cellular membranes. They consist of an alpha helix of, on average, 23 amino acids in length. TMHs can also be predicted with high accuracy from a protein sequence by bioinformatics approaches [8, 9, 10, 11, 12, 13], for example, [11] found that, from 184 transmembrane proteins (TMPs) with known topology, 80% of the TMH predictions replicated this finding.

TMHs are common structures in the proteins of humans and microbes. Different TMH prediction tools estimate that 15-39% of all proteins in the human proteome contain at least one TMH [14]. However, the physiological reason why peptides derived from TMHs would be presented more often than peptides

stemming from soluble (i.e., extracellular or cytoplasmic) protein regions is unknown. We hypothesized that the presentation of TMH residues is evolutionary selected for, because TMHs are less prone to undergo escape mutations. One reason to expect such a reduced variability (and hence evolutionary conservation) in TMHs, is that these are restricted in their evolution by the functional requirement to span a lipid bilayer. Due to this requirement, many of the amino acids genuinely present in TMHs are limited to the ones with hydrophobic side chains [15, 16]. Therefore, we speculated that the TMHs of pathogens might have a lower chance to develop escape mutations, as many mutations will result in a dysfunctional TMH and render the protein inactive.

This study had two objectives. First, we aimed to generalize our findings by predicting the presentation of peptides from different kingdoms of life and for both MHC-I and -II. From these *in silico* predictions, we conclude that TMH-derived epitopes are presented more often than expected by chance, in a human, viral and bacterial proteome, and for most haplotypes of both MHC-I and II. We confirmed the presentation of TMH-derived peptides by re-analysis of peptide elution studies. Second, we tested our hypothesis that TMHs are more evolutionary conserved than soluble protein regions. Our analysis of human single nucleotide polymorphisms (SNPs) showed that random point mutations are indeed less likely to occur within TMHs. These findings strengthen the emerging notion that TMHs are important for the T cell branch of the adaptive immune system, and hence are of overlooked importance in vaccine development.

106 2 Methods

107 2.1 Predicting TMH epitopes

108 To predict how frequently epitopes overlapping with TMHs are presented, a
109 similar analysis strategy was applied as described in [7] for several haplotypes
110 of both MHC-I and MHC-II, and for a human, viral and bacterial proteome.
111 To summarize, for each proteome, all possible 9-mers (for MHC-I) or 14-mers
112 (MHC-II) were derived. For each of these peptides, we determined if it over-
113 lapped with a predicted TMH and if it was predicted to bind to each haplotype.

114 For MHC-I, 9-mers were used, as this is the length most frequently pre-
115 sented in MHC-I and was used in our earlier study [7]. For MHC-II, 14-
116 mers were used, as these are the most frequently occurring epitope length [17].
117 A human (UniProt ID UP000005640.9606), viral (SARS-CoV-2, UniProt ID
118 UP000464024) and bacterial (*Mycobacterium tuberculosis*, UniProt ID UP000001584)
119 reference proteome was used. TMHMM [8] was used to predict the topology of the
120 proteins within these proteomes. To predict the affinity of an epitope to a cer-
121 tain MHC haplotype, **EpitopePrediction** [7] for MHC-I and **MHCnuggets** [18]
122 for MHC-II was used. The 13 MHC-I haplotypes used in this study are the same
123 as used in the previous study [7]. For MHC-II, haplotypes were selected with a
124 phenotypic frequency of at least 14% in the human population [19], resulting in
125 21 MHC-II haplotypes.

126 In previous work, it was found that the over-presentation of TMH-derived
127 peptides can be explained from the hydrophobicity of the MHC-I binding cleft
128 [7]. Here, a similar analysis was applied, by correlating the percentage of pre-
129 dicted TMH-derived epitopes versus the mean hydrophobicity of all peptides.

130 This study differs in one important aspect from our previous work [7]. The
131 definition of a binder differs from [7]: in the current study, a peptide is called a
132 binder if, for a certain haplotype, any of its 9-mer or 14-mer peptides have an

IC50 value in the lowest 2% of all peptides within a *proteome* (see supplementary Tables 4 and 5 for values), whereas the previous study defined a binder as having an IC50 in the lowest 2% of the peptides within a *protein*. This revised definition precludes bias of proteins that give rise to no or only very few MHC epitopes. To verify that the results are similar, a side by side comparison was performed shown in the supplementary materials.

2.2 Peptide elution studies

To obtain experimental evidence that epitopes derived from TMHs are presented in MHC, peptide elution studies for MHC-I [20] and MHC-II [17] were reanalyzed. For each of the detected epitopes, its possible location(s) in a human reference proteome, with UniProt ID UP000005640.9606, was mapped. For the epitopes that were present in the proteome exactly once, the topology of the proteins in which these epitopes were located was predicted using both TMHMM [8] and PureseqTM [13]. From this topology, we determined if the epitope overlapped with a TMH.

The full analysis can be found at https://github.com/richelbilderbeek/bbbq_article_issue_157.

2.2.1 Evolutionary conservation of TMHs

To determine the evolutionary conservation of TMHs, human single nucleotide polymorphisms (SNPs) were first collected that resulted in a single amino acid substitution, and we then determined if this substitution occurred within a predicted TMH or not.

As a data source, multiple NCBI (<https://www.ncbi.nlm.nih.gov/>) databases were used: the *dbSNP* [21] database, which contains 650 million catalogued non-redundant humane variations (called RefSNPs, <https://www.ncbi.nlm.nih.gov/>).

158 `gov/snp/docs/RefSNP_about/`), and the databases *gene* (for gene names, [22])
159 and *protein* (for proteins sequences, [23]).

160 The first query was a call to the *gene* database for the term 'membrane
161 protein' (in all fields) for the organism *Homo sapiens*. This resulted in 1,077
162 gene IDs (on December 2020). The next query was a call to the *gene* database
163 to obtain the gene names from the gene IDs. Per gene name, the *dbSNP* NCBI
164 database was queried for variations associated with the gene name. As the
165 NCBI API constrains its users to three calls per second (to assure fair use), we
166 had to limit the extent of our analysis.

167 The number of SNPs was limited to the first 250 variations per gene, resulting
168 in ≈ 61 k variations. Only variations that result in a SNP for a single amino acid
169 substitution were analyzed, resulting in ≈ 38 k SNPs. The exact amounts can be
170 found in the supplementary materials, Tables 9 and 10.

171 SNPs were picked based on ID number, which is linked to their discovery
172 date. To verify that these ID numbers are unrelated to SNP positions, the
173 relative positions of all analyzed SNPs in a protein were determined. This
174 analysis showed no positional bias of the SNPs, as shown in supplementary
175 figure 15.

176 Per SNP, the *protein* NCBI database was queried for the protein sequence.
177 For each protein sequence, the protein topology was determined using **Pureseq**TM.
178 Using these predicted protein topologies, the SNPs were scored to be located
179 within or outside TMHs.

180 **3 Results**

181 **3.1 TMH-derived peptides are predicted to be over-presented** 182 **in MHC-I**

183 Figure 1A shows the predicted presentation of TMH-derived peptides in MHC-
184 I, for a human, viral and bacterial proteome. Per MHC-I haplotype, it shows
185 the percentage of binders that overlap with a TMH with at least one residue.
186 The horizontal line shows the expected percentage of TMH-derived epitopes
187 that would be presented, if TMH-derived epitopes would be presented just as
188 likely as epitopes derived from soluble regions. For 11 out of 13 MHC-I hap-
189 lotypes, TMH-derived epitopes are predicted to be presented more often than
190 the null expectation, for a human and bacterial proteome. For the viral pro-
191 teome, 12 out of 13 haplotypes present TMH-derived epitopes more often than
192 expected by chance. The extent of the over-presentation between the different
193 haplotypes is similar for the probed proteomes, which strengthens our previous
194 conclusion [7] that the hydrophobicity of the MHC-binding groove is the main
195 factor responsible for the predicted over-presentation of TMH-derived peptides.

196 **3.2 TMH-derived peptides are predicted to be over-presented** 197 **in MHC-II**

198 We next wondered if the over-representation of TMH-derived peptides would
199 also be present for MHC-II. Figure 1A shows the percentages of MHC-II epitopes
200 predicted to be overlapping with TMHs for our human, viral and bacterial
201 proteomes. We found that TMH-derived peptides are over-presented in all of
202 the 21 MHC-II haplotypes, for a human, bacterial and viral proteome, except
203 for HLA-DRB3*0101 in *M. tuberculosis*. See supplementary Table 8 for the exact
204 TMH and epitope counts.

Figure 1: Over-presentation of TMH-derived epitopes on most MHC-I and -II haplotypes (A) The percentage of epitopes for MHC-I and -II haplotypes that are predicted to overlap with TMHs for the proteomes of SARS-CoV-2 (top row), human (middle row) and *M. tuberculosis* (bottom row). The red lines indicate the percentages as expected by chance. See supplementary Tables 7 and 8 for the exact TMH and epitope counts. (B-C) Correlation between the percentages of predicted TMH-derived epitopes and the hydrophobicity score of all predicted epitopes for MHC-I (B) and MHC-II haplotypes (C). Red curve: linear regression analysis. Labels are shorthand for the HLA haplotypes, see the supplementary Table 6 for the names.

205 3.3 The over-presentation of TMH-derived peptides is caused 206 by the hydrophobicity of the MHC peptide binding 207 groove

208 For MHC-I, we previously showed that the over-presentation of TMH-derived
209 peptides is caused by the hydrophobicity of the peptide binding grooves [7]. Fig-
210 ures 1B and 1C show the extent of over-presentation of TMH-derived epitopes
211 as a function of the hydrophobicity preference score for the different haplo-
212 types. An assumed linear correlation explains 88% of the variability in MHC-
213 I. For MHC-II, 62% of the variability is explained by hydrophobicity. This
214 strengthens our previous finding [7] and indicates that TMH-derived peptides
215 are over-presented because the peptide binding grooves of most MHC-I and -II
216 haplotypes are relatively hydrophobic.

217 3.4 Experimental validation of presentation of TMH-derived 218 peptides

Figure 2: **Robust prediction that TMH epitopes are presented *in vivo*.** Bars show the percentage of peptides obtained from elution studies that is derived from a TMH, for MHC-I and -II, for two TMH prediction tools.

219 To obtain experimental confirmation that peptides stemming from TMHs are
 220 presented in MHC-I and MHC-II, two peptide elution studies were reanalyzed:
 221 For MHC-I, peptides presented *in vivo* by the (humane) haplotypes HLA-A
 222 and B were sequenced [20], for MHC-II these were haplotypes DQ2.5, DQ2.2,
 223 and DQ7.5 [17]. Figure 2 shows the percentages of epitopes derived from TMHs
 224 found in the MHC-I and MHC-II elution studies, for the two topology prediction
 225 tools TMHMM [8] and PureseqTM [13]. Regardless of the prediction tool, at least
 226 100 epitopes were predicted to be derived from a TMH for each condition.
 227 From these findings, it is robustly predicted that epitopes derived from TMHs
 228 are presented in both MHC-I and MHC-II. See the supplementary Table 3 for
 229 the exact values.

230 3.5 Human TMHs are evolutionarily conserved

231 We addressed the question whether there is an evolutionary advantage in pre-
 232 senting TMHs. We determined the conservation of TMHs by comparing the
 233 occurrences of SNPs located in TMHs or soluble protein regions for the genes
 234 coding for membrane proteins. We obtained 911 unique gene names associated
 235 with the phrase 'membrane protein', which are genes coding for both membrane-
 236 associated proteins (MAPs, which have no TMH) and transmembrane proteins
 237 (TMPs, which have at least one TMH). These genes are linked to 4,780 pro-
 238 tein isoforms, of which 2,553 are predicted to be TMPs and 2,237 proteins are
 239 predicted to be MAPs. We obtained 37,630 unique variations, of which 9,621
 240 are SNPs that resulted in a straightforward amino acids substitution, of which
 241 6,062 were located in predicted TMPs. See supplementary Tables 9 and 10 for
 242 the detailed numbers and distributions of SNPs.

243 Per protein, we calculated two percentages: (1) the percentage of the total
 244 protein predicted to be TMHs, and (2) the percentage of SNPs located within

Figure 3: **Evolutionary conservation of human TMHs.** (A) The number of SNPs in TMHs as expected by chance (left bar) and found in the dbSNP database (right bar). Percentages show the relative conservation of SNPs in TMHs found. (B) Percentage of SNPs found in TMHs. Each point shows for one protein the predicted percentage of TMH (x -axis) and the observed occurrence of SNPs being located within a TMH (y -axis). The dashed diagonal line shows the line of equality (i.e., equal conservation of TMHs and soluble protein regions). The red line indicates a linear fit, the gray area its 95% confidence interval. (C) Distribution of the percentages of TMH in the TMPs used in this study.

Figure 4: Membrane proteins with multiple TMHs are evolutionary more conserved than proteins with only a single TMH. (A) The number of SNPs in TMHs as expected by chance and observed in the dbSNP database, for TMPs with one TMH (single-spanners) and multiple TMHs (multi-spanners). Percentages show the relative conservation of SNPs in TMHs found. (B) Distribution of the proportion of amino acids residing in the plasma membrane. (C) Percentage of SNPs found in TMPs predicted to have only a single (left) or multiple (right) TMHs. Each point shows for one protein the predicted percentage of TMH (x -axis) and the observed occurrence of SNPs being located within a TMH (y -axis). The dashed diagonal lines show the line of equality (i.e., equal conservation of TMHs and soluble protein regions). The red line indicates a linear fit, the gray area its 95% confidence interval.

these predicted TMHs. Each percentage pair was plotted in figure 3B. The proportion of SNPs found in TMHs varied from none (i.e. all SNPs were in soluble regions) to all. To determine if SNPs were randomly distributed over the protein, we performed a linear regression analysis, and added a 95% confidence interval on this regression. This linear fit nearly goes through the origin and has a slope below the line of equality, which shows that less SNPs are found in TMHs than expected by chance.

We determined the probability to find the observed amount of SNPs in TMHs by chance, i.e., when assuming SNPs occur just as likely in soluble domains as in TMHs. We used a binomial Poisson distribution, where the number of trials (n) equals the number of SNPs, which is 21,208. The probability of success for the i th TMP (p_i), is the percentage of residues within a TMH per TMP. These percentages are shown as a histogram in figure 3C. The expected number of SNPs expected to be found in TMHs by chance equals $\sum p \approx 4,141$. As we observed 3,803 SNPs in TMHs, we calculated the probability of having that amount or less successes. We used the type I error cut-off value of $\alpha = 2.5\%$. The chance to find, within TMHs, this amount or less SNPs equals $6.8208 \cdot 10^{-11}$. We determined the relevance of this finding, by calculating how much less SNPs are found in TMHs, when compared to soluble regions, which is the ratio between the number of SNPs found in TMHs versus the number of SNPs as expected by chance. In effect, per 1000 SNPs found in soluble protein domains, one finds 918 SNPs in TMHs, as depicted in figure 3A.

We split this analysis for TMPs containing only a single TMH (so-called single-membrane spanners) and TMPs containing multiple TMHs (multi-membrane spanners). We hypothesized that single-membrane spanners are less conserved than multi-membrane spanners, because multi-membrane spanners might have protein-protein interactions between their TMHs, for example to accommodate

active sites, and thus might have additional structural constraints. From the split data, we did the same analysis as for the total TMPs. Figure 4C shows the percentages of TMHs for individual proteins as a function of the percentage of SNPs located in TMHs. For both single- and multi-spanners, a linear regression shows that less SNPs are found in TMHs, than expected by chance.

We also determined the probability to find the observed amount of SNPs by chance in single- and multi-spanners. For single-spanners, we found 452 SNPs in TMH, where ≈ 462 were expected by chance. The chance to observe this or a lower number by chance is 0.319. As this chance was higher than our $\alpha = 0.025$, we consider this no significant effect. For the multi-spanners, we found 3,351 SNPs in TMH, where $\approx 3,678$ were expected by chance. The chance to observe this or a lower number by chance is $8.315841 \cdot 10^{-12}$, which means this number is significantly less as explained by variation.

Also, for single- and multi-spanners, we determined the relevance of this finding by calculating how much less SNPs are found in TMHs when compared to soluble regions, as depicted in figure 4A. In effect, per 1,000 SNPs found in soluble protein domains, one finds 978 SNPs in TMHs of single-spanners and 911 SNPs in TMHs of multi-spanners.

4 Discussion

Epitope prediction is important to understand the immune system and for the design of vaccines. In this study, we provide evidence that epitopes derived from TMHs are a major but overlooked source of MHC epitopes. Our bioinformatics predictions indicate that TMH-derived epitopes are presented to both cytolytic and helper T cells more often than expected by chance, regardless of the organism. Moreover, reanalysis of peptide elution studies confirmed the presentation of TMH-derived epitopes. Finally, our SNP analysis shows that

298 TMHs are evolutionary more conserved than solvent-exposed protein regions.

299 **4.1 Mechanism of MHC presentation of TMH-derived epi-** 300 **topes**

301 Although our data show that TMH-derived epitopes are presented in MHC-I
302 and MHC-II, the molecular mechanisms of how integral membrane proteins are
303 processed for MHC presentation are largely unknown [7]. Most prominently, the
304 fundamental principles of how TMHs are extracted from their hydrophobic lipid
305 environments into the aqueous vacuolar lumen, and their prior or subsequent
306 proteolytic processing are unresolved.

307 A first possibility is that the extraction of TMPs from the membrane is
308 mediated by the ER-associated degradation (ERAD) machinery. For MHC class
309 I (MHC-I) antigen presentation of soluble proteins, the loading of the epitope
310 primarily occurs at the endoplasmic reticulum (ER). The chaperones tapasin
311 (TAPBP), ERp57 (PDIA3), and calreticulin (CALR) [24] first assemble and
312 stabilize the heavy and light chains of MHC-I. Later, this complex binds to the
313 transporter associated with antigen processing (TAP) leading to the formation of
314 the so-called peptide-loading complex (PLC). The PLC drives import of peptides
315 into the ER and mediates their subsequent loading into the peptide-binding
316 groove of MHC-I [25]. Membrane proteins first will have to be extracted from
317 the membrane before they become amenable to this MHC-I loading by the
318 PLC. In the ER, this process can be orchestrated by the ERAD machinery,
319 consisting of several chaperones that recognize TMPs, ubiquitinate them, and
320 extract them from the ER membrane into the cytosol (retrotranslocation) for
321 proteasomal degradation [26, 27]. Similar to the peptides generated from soluble
322 proteins, the TMP-derived peptides might then be re-imported by TAP into the
323 ER for MHC-I loading. This ERAD-driven antigen retrotranslocation might be

324 facilitated by lipid bodies (LBs) [28], since LBs can serve as cytosolic sites for
325 ubiquitination of ER-derived cargo [29].

326 A second possibility is that TMPs are proteolytically processed by intramem-
327 brane proteases that cleave TMHs while they are still membrane embedded.
328 Supporting this hypothesis is the well established notion that peptides gener-
329 ated by signal peptide peptidases (SPPs), an important class of intramembrane
330 proteases that cleave TMH-like signal sequences, are presented on a specialized
331 class of MHC-I called HLA-E [30]. The loading of peptides generated by SPP
332 onto MHC-I does not depend on the proteasome and TAP, possibly because
333 the peptides are directly released into the lumen of the ER [30]. However, this
334 mechanism would not explain how multspanner polytopic membrane proteins
335 can be processed for antigen presentation, because SPPs only cleave TMH-like
336 signal sequences at the N-terminus of a protein. Nevertheless, the presenta-
337 tion of peptides with a high hydrophobicity index was shown to be independent
338 of TAP as well [31], suggesting the TMH peptides might perhaps be released
339 directly in the ER lumen by other intramembrane proteases.

340 A third possibility is that peptide processing and MHC-loading occur in
341 multivesicular bodies (MVBs) [30]. TMPs can be routed from the plasma mem-
342 brane and other organelles by vesicular trafficking to endosomes. Eventually,
343 these TMPs can be sorted by the endosomal sorting complexes required for
344 transport (ESCRT) pathway into luminal invaginations that pinch off from the
345 limiting membrane and form intraluminal vesicles. This thus results in MVBs
346 where the membrane proteins destined for degradation are located in intralumi-
347 nal vesicles. Upon the fusion of MVBs with lysosomes, the entire intraluminal
348 vesicles including the TMPs are degraded [32]. Via this mechanism, TMPs
349 might well be processed for antigen presentation, particularly since the loading
350 of MHC class II molecules is well understood to occur in MVBs [33, 34, 35].

351 However, such processing of membrane proteins in MVBs for antigen presenta-
 352 tion poses a problem, because complexes of HLA-DR with its antigen-loading
 353 chaperon HLA-DM were only observed on intraluminal vesicles, but not on the
 354 limiting membranes of MVBs [35], indicating that epitope loading of MHC-II
 355 also occurs at intraluminal vesicles. This observation hence raises the question
 356 how the intraluminal vesicles carrying the TMPs destined for antigen presen-
 357 tation can be selectively degraded, while the intraluminal vesicles carrying the
 358 MHC-II remain intact. A second problem is that phagosomes carrying inter-
 359 nalized microbes lack intraluminal vesicles, and it is hence unclear how TMPs
 360 from these microbes would be routed to MVBs for MHC-II loading [35].

361 Alternatively to the enzymatic degradation of lipids in MVBs by lipases
 362 [36, 37], they might be oxidatively degraded by reactions with radical oxygen
 363 species (ROS) produced by the NADPH oxidase NOX2 [38]. This oxidation can
 364 result in a destabilization and disruption of membranes [38] and might thereby
 365 lead to the extraction of TMPs. Due to the hydrophobic nature of TMHs,
 366 however, the extracted proteins will likely aggregate and it is unclear how these
 367 aggregates would be processed further for MHC loading.

368 **4.2 T cells recognize different protein regions than B cells**

369 An important implication from the over-presentation of TMH-derived epitopes
 370 is that T cells will largely recognize different protein regions than B cells. Pre-
 371 sentation of antigens by MHC-II is important for the activation of naive B cells
 372 by helper T cells. For this activation, B cells first ingest antigen that is bound
 373 to their B cell receptor, and subsequently present peptides derived from this
 374 antigen in MHC-II to helper T cells. Following their activation by the T cells, B
 375 cells mature into plasma cells and release antibodies which recognize the same
 376 part of the antigen as the original B cell receptor. B cell receptors and antibod-

ies will thus recognize solvent-exposed regions of antigens that are accessible for binding to the B cell receptor. However, the results from our study predict that most MHC-II haplotypes present relatively hydrophobic peptides, which are less likely to be solvent-exposed. It is unknown why B and T cells seem to predominantly recognize different protein regions, but one possibility might be that this lowers the chance of B cell mediated autoimmune diseases, because auto-reactive B and T cells recognizing different parts of the same antigen would need to be present for breakage of B cell tolerance.

4.3 Evolutionary conservation of TMHs

In general, one might expect that evolutionary selection results in an immune system that is most attentive for protein regions that are essential for the survival, proliferation and/or virulence of pathogenic microbes, as these will be most conserved. In SARS-CoV-2, for example, there is preliminary evidence that the strongest selection pressure is upon residues that change its virulence [39]. These regions, however, may only account for a small part of a pathogen's proteome. Additionally, the structure and function of these essential regions might differ widely between different pathogenic proteins. Because of this scarcity and variance in targets, one can imagine that it will be mostly unfeasible to provide innate immune responses against such rare essential protein regions, as suggested in a study on influenza [40], where it was found that the selection pressure exerted by the immune system was either weak or absent.

Evolutionary selection of pathogens by a host's immune system, however, is likelier to occur for proteomic patterns that are general, over patterns that are rare. While essential catalytic sites in a pathogenic proteome might be relatively rare, TMHs are common and thus might be a more feasible target for evolution to respond to. Indeed, we have found the signature of evolution when both

403 factors, that is, TMHs and catalytic sites are likely to co-occur, which is in TMPs
 404 that span the membrane at least twice. In contrast to single-spanners, where
 405 we found no significant evolutionary conservation, the TMHs of multi-spanners
 406 are more evolutionary conserved than soluble protein regions. Likely, the TMHs
 407 in many multi-spanners need to interact with each other for correct protein
 408 structure and function and they might hence be more structurally constrained
 409 compared to the TMHs of single-spanners. Thus, we speculate that the human
 410 immune system is more attentive towards TMHs in multi-spanners, as these are
 411 evolutionarily more conserved.

412 There have been more efforts to assess the conservation of TMHs, using
 413 different methodologies. One such example is [41], in which aligned protein
 414 sequence data was used. Also this study found that TMHs are evolutionarily
 415 more conserved, as the mean amino acid substitution rate in TMHs is about ten
 416 percent lower, which is a similar value as we found. Another example is a study
 417 that estimated the conservation scores for TMHs and soluble regions based on
 418 alignments of evolutionary related proteins, and also found that TMHs are more
 419 conserved, with a conservation score that was 17% higher in TMHs [42]. Note
 420 that the last study also found that mutations in human TMHs are likelier to
 421 cause a disease, in line with our conclusion that TMHs are more conserved.

422 Together, from this study, two important conclusions can be drawn. First,
 423 the MHC over-presentation of TMHs is likely a general feature and predicted
 424 to occur for most haplotypes of both MHC-I and -II and for humans as well as
 425 bacterial and viral pathogens. Second, TMHs are genuinely more evolutionary
 426 conserved than soluble protein motifs, at least in the human proteome.

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439 6 Data Accessibility

440 All code is archived at <http://github.com/richelbilderbeek/someplace>,
441 with DOI <https://doi.org/12.3456/zenodo.1234567>.

442 7 Authors’ contributions

443 RJCB and FB conceived the idea for this research. MVB helped with the
444 proteome analysis of *M. tuberculosis*. RJCB wrote the code. RJCB, MB, GvdB
445 and FB wrote the article.

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653 A Supplementary materials

654 A.1 Differences with Bianchi et al., 2017

655 A part of this study does the same analysis as Bianchi et al., 2017. mainly
656 concern the use of different software and a different definition of what an MHC
657 binder is.

658 The earlier study defined a peptide an MHC binder if *within the protein* in
659 which it was found, is was among the peptides with the 2% lowest IC50 val-
660 ues. This can be seen at [https://github.com/richelbilderbeek/bianchi_](https://github.com/richelbilderbeek/bianchi_et_al_2017/blob/master/predict-binders.R)
661 [et_al_2017/blob/master/predict-binders.R](https://github.com/richelbilderbeek/bianchi_et_al_2017/blob/master/predict-binders.R), where the binders are written
662 to file.

663 However, in this study, an MHC binder is defined as a peptide within a
664 *proteome* in which it is found, that is among the peptides with the 2% lowest
665 IC50 values. Subsection A.8 shows the IC50 values for a binder per haplotype.
666 We believe that our revised definition is more correct, as it overcomes bias from
667 proteins with very low numbers of peptides and/or MHC-predicted binders.

668 Our previous study used the TMHMM web server to predict TMHs. The
669 desktop version of TMHMM, however, gives an error message on the 25 seleno-
670 proteins found in the human reference proteome. For the sake of reproducible
671 research, we used the desktop version (as we can call it from scripts) and, due
672 to this, we removed the selenoproteins from this analysis.

673 To verify if the previous and the current method give rise to notable dif-
674 ference, we show a side-by-side comparison in figures 5A and 5B. The figures
675 that haplotypes that over-present or under-present TMH-derived epitopes, do
676 so in both studies. The extent to which TMH-derived epitopes are presented,
677 however, is more extreme in our current setup.

Figure 5: **(A)** Results for [7]. Red lines denotes the coincidence interval. **(B)** Results for this study. Red line denotes the percentage as expected by chance.

Goal	Tool	Reference
Predict topology	TMHMM	[8]
Predict topology	PureseqTM	[13]
Predict epitopes MHC-I	epitope-prediction	[7]
Predict epitopes MHC-II	NetMHCIIpan	[43, 44]
Call TMHMM from R	tmhmm	[45]
Call PureseqTM from R	pureseqtmr	[46]
Call NetMHCIIpan from R	netmhc2pan	[47]
Combine all	bbbq	[48]

Table 1: Overview of all software used in this research.

678 A.2 Prediction software used

679 For this research, we needed software to predict protein topology, as well as the
680 MHC-I and MHC-II binding affinities of epitopes. We selected our software, by
681 searching the scientific literature to identify the most recent free and open source
682 (FOSS) prediction software. This was done by searching for papers that (1) cite
683 older prediction software, and (2) present a novel method to make predictions.
684 As a starting point, per type of prediction software, a review paper was used
685 ([49] for protein topology, [50] for MHC-I binding affinities and [51] for MHC-II

686 binding affinities).

687 There are multiple computational tools developed to predict which parts of
688 a protein forms a TMH. In 2001, multiple of such prediction tools have been
689 compared [49], of which TMHMM [8] turned out to be the most accurate, as
690 is used in the previous study [7]. However, TMHMM has a restrictive software
691 license and is nearly two decades old. Therefore, PureseqTM [13], was also used
692 in this study, which has been more recently developed and has a free software
693 license.

694 For MHC-I, there are multiple computational tools developed to predict epi-
695 topes. According to [50], at that time, NetMHCcons [52] gave the best predic-
696 tions. We used the same tool as used in our earlier study, `epitope-prediction`
697 [7],

698 Also for MHC-II, there are multiple computational tools developed to pre-
699 dict epitopes, such as using a trained neural network [51] or a Gibbs sam-
700 pling approach [53]. According to [50], in 2011, from a set of multiple tools,
701 NetMHCIpan [43, 44] made the most accurate predictions. The most recent
702 FOSS tool available now appears to be MHCnuggets [18], which can do both
703 MHC-I and MHC-II predictions. As we already use `epitope-prediction` [7]
704 for MHC-I predictions, we use MHCnuggets only for MHC-II predictions.

705 To retrieve the data from the NCBI databases the `rentrez` R package [54]
706 was used that calls the NCBI website’s API. To provide for a stable user expe-
707 rience for all users, this API limits the user to 3 calls per second. Additionally,
708 the API splits the result of a bigger query into multiple pages, each of which
709 needs one API call. We wrote the `sprentrez` package [55] to provide for bigger
710 queries of multiple (and delayed) API calls.

711 A.3 Prediction software written

712 The R programming language is used for the complete experiment, including the
713 analysis. The complete experiment is bundled in the 'bbq' R package, which
714 is dependent on 'tmhmm', 'pureseqtmr', 'epitope-prediction' and 'mhc-nuggetsr'
715 as described below.

716 The R package 'tmhmm' was developed to do the similar topology predic-
717 tions as our earlier study (that used 'TMHMM'), yet in an automated way.
718 'TMHMM' has a restrictive software license [8] and allows a user to download a
719 pre-compiled executable after confirmation that he/she is in academia. The R
720 package respects this restriction and allows the user to install and use TMHMM
721 from within R, as done in this study. 'tmhmm' has been submitted to and is
722 accepted by the Comprehensive R Archive Network (CRAN).

723 To be able to call, from R, the TMH prediction software 'PureseqTM' [13],
724 which is written in C, the package 'pureseqtmr' has been developed. 'purese-
725 qtmr' allows to install 'PureseqTM' and use most of its features. 'pureseqtmr'
726 has been submitted to and is accepted by CRAN.

727 MHCnuggets is a free and open-source Python package to predict epitope
728 affinity for many MHC-I and MHC-II variants [18]. The R package 'mhc-
729 nuggetsr' allows one to install and use MHCnuggets from within R. Also 'mhc-
730 nuggetsr' has been submitted to and is accepted by CRAN.

731 To reproduce the full experiment presented in this paper, the functions
732 needed are bundled in the 'bbq' R package. This package is too specific to
733 be submitted to CRAN.

Table 2: Percentage of spots and spots that overlap with a TMH

target	mhc_class	n_spots	n_spots_tmh	f_tmh
covid	1	14207	1124	7.91
covid	2	14137	1245	8.81
human	1	11220940	598391	5.33
human	2	11118448	672273	6.05
myco	1	1299707	98613	7.59
myco	2	1279742	108419	8.47

734 A.4 Prediction of percentage of epitopes overlapping with 735 a TMH

736 Supplementary Table 2 shows an overview of the findings, where a target speci-
737 fies the source of the proteome, where `covid` denotes SARS-CoV-2 and `myco` de-
738 notes *Mycobacterium tuberculosis*. `mhc_class` denotes the MHC class, `n_spots`
739 the number of possible 9-mers (for MHC-I) or 14-mers (for MHC-II) possible.
740 `n_spots_tmh` the number of epitopes that overlapped with a TMH that were
741 binders. `f_tmh` the percentage of peptides that had at least 1 residue overlapping
742 with a TMH.

743 A.5 Minor methods

744 These are details that are removed from the 'Methods' section.

745 PureseqTM does not predict the topology of proteins that have less than
746 three amino acids. The TRDD1 ('T cell receptor delta diversity 1') protein,
747 however, is two amino acids long. The R package `pureseqtmr`, however, predicts
748 that mono- and di-peptides are cytosolic.

749 A.6 Minor discussion

750 These are details that are removed from the 'Discussion' section.

751 In this experiment we predicted epitopes that overlap with TMHs from a

human, bacterial and viral proteome, would these proteins be expressed in a human host. Bacteria, however have different cell membranes and cell walls, hence different structural requirements for a TMH. Both topology prediction tools were trained to recognize human TMHs, thus we cannot be sure that the transmembrane regions predicted in bacterial proteins are actually part of a TMH. For the purpose of this study, we assume the error in topology predictions to be unbiased way towards topology. In other words: that a bacterial TMH is incorrectly predicted to be absent just as often as it is incorrectly predicted to be present elsewhere.

Regarding the evolutionary conservation of TMHs using SNPs, again, it is estimated that approximately ten percent of SNPs is a false positive that result from the methods to determine a SNP. One example is that sequence variations are incorrectly detected due to highly similar duplicated sequences [56]. We assume that these duplications occur as often in TMHs as in regions around these, hence we expect this not to affect our results.

In our evolutionary experiment, we removed variations that were synonymous mutations (i.e. resulted in the same amino acid, from a different genetic code) from our analysis. There is evidence, however, that these synonymous mutations do have an effect and may even be evolutionary selected for [57]. As the possible effect of synonymous mutations is ignored by our topology prediction software, we do so as well.

A.7 Elution studies

A.8 IC50 values of binders per haplotype

Per target proteome (i.e. human, SARS-CoV-2, *M tuberculosis*), we collected all 9-mers (for MHC-I) and 14-mers (for MHC-II), after removing the selenoproteins and proteins that are shorter than the epitope length. From these epitopes, per

MHC class	Tool	n
I	PureseqTM	1.38% (109/7897)
I	TMHMM	1.43% (113/7897)
II	PureseqTM	3.92% (498/12712)
II	TMHMM	3.96% (504/12712)

Table 3: Percentage of epitopes derived from a TMH found in the two elution studies, for the two different kind of topology prediction tools. The values between braces show the the number of epitopes that were predicted to overlapping with a TMH per all epitopes that could be uniquely mapped to the representative human reference proteome.

Table 4: IC50 values (in nM) per haplotype below which a peptide is considered a binder. percentage used: 2

haplotype	covid	human	myco
HLA-A*01:01	1470.5912	2545.9537	2812.1714
HLA-A*02:01	118.9596	218.7274	186.7565
HLA-A*03:01	537.0144	804.7455	1544.1073
HLA-A*24:02	984.8147	1590.0623	1971.8258
HLA-A*26:01	1095.2591	1771.6924	1526.1101
HLA-B*07:02	1215.7734	705.6514	435.5361
HLA-B*08:01	886.5661	883.0951	1023.2213
HLA-B*18:01	921.4157	1063.2215	1319.0445
HLA-B*27:05	1186.0963	689.8815	475.6130
HLA-B*39:01	437.3506	484.3843	399.3873
HLA-B*40:02	585.6308	541.2392	600.1688
HLA-B*58:01	435.4693	591.0526	538.9063
HLA-B*15:01	281.9129	440.6541	482.8369

MHC haplotype, we predicted the IC50 (in nM) using `epitope-prediction` (for MHC-I) and `MHCnuggets` (for MHC-II). Here, we show the IC50 value per haplotype that is used to determine if a peptide binds to the haplotype's MHC for MHC-I (see supplementary Table 4) and MHC-II (see supplementary Table 5).

A.9 Presentation of TMH-derived epitopes

Supplementary Table 6 shows the shorthand notation for the HLA haplotypes. Supplementary Tables 7 and 8 show the exact number of binders, binders

Table 5: IC50 values (in nM) per haplotype below which a peptide is considered a binder. percentage used: 2

haplotype	covid	human	myco
HLA-DRB1*0101	7.3896	9.72	9.9600
HLA-DRB1*0301	121.8420	198.40	164.4900
HLA-DRB1*0401	59.8780	74.92	84.3112
HLA-DRB1*0405	46.2324	51.88	66.7100
HLA-DRB1*0701	17.7464	22.40	28.1700
HLA-DRB1*0802	99.7592	137.16	67.9900
HLA-DRB1*0901	42.3464	53.52	41.5400
HLA-DRB1*1101	35.9988	39.01	48.9200
HLA-DRB1*1201	194.4408	248.72	289.7300
HLA-DRB1*1302	21.1084	40.59	35.4100
HLA-DRB1*1501	32.6196	40.69	46.6700
HLA-DRB3*0101	175.2984	298.94	218.7300
HLA-DRB3*0202	176.8168	291.95	405.8724
HLA-DRB4*0101	47.6384	51.04	62.7800
HLA-DRB5*0101	32.8872	43.52	60.2312
HLA-DQA1*0501/DQB1*0201	193.1108	209.89	174.2124
HLA-DQA1*0501/DQB1*0301	51.2028	43.47	20.3200
HLA-DQA1*0301/DQB1*0302	361.8180	365.96	296.4712
HLA-DQA1*0401/DQB1*0402	214.1932	242.68	199.8912
HLA-DQA1*0101/DQB1*0501	550.4488	674.95	930.9612
HLA-DQA1*0102/DQB1*0602	157.4480	174.82	114.3512

that overlap with TMHs and the percentage of binders that overlap with TMHs,
as visualized by figure 1A.

A.10 Relative presentation of TMH-derived epitopes

To compare the over-presentation of TMH-derived epitopes between the different proteomes, we normalized this percentages in such a way that 1.0 is the percentage of TMH-derived epitopes that would be expected by chance. Figure 6 and 7 show these normalized values for the MHC-I and MHC-II haplotypes respectively.

To determine the additional over-presentation of TMH-derived epitopes in MHC-II (as compared to MHC-I), we normalized the data to enable a side-by-side comparison. The percentage of TMH-derived epitopes presented was normalized to the expected percentage of TMH-derived epitopes, where 1.0 denotes that the percentage of presented TMH-derived epitopes matches the values as expected by chance. The normalized values per haplotype are shown in figure 8. To compare the TMH-derived over-presentation per MHC class, we grouped the normalized values per haplotype, and plot the mean and standard error, as shown in figure 9.

A.11 Evolutionary conservation

See supplementary Tables 9 and 10 for an overview of all amounts. In supplementary Table 9 there are multiple instances where the amounts are expected to add up, yet don't, as one SNP can work on multiple isoforms. For example, there are 9,621 unique SNPs found in all proteins, of which 4,219 around found in MAPs and 6,026 in TMPs. Apparently, 624 SNPs work on a set of isoforms that contains both MAPs and TMPs.

Figure 10 shows the distribution of the number of SNPs per gene name, at

Figure 6: Normalized proportion of MHC-I epitopes overlapping with TMHs for human, viral and bacterial proteomes. Legend: covid = SARS-CoV-2, human = *Homo sapiens*, myco = *Mycobacterium tuberculosis*

Figure 7: Normalized proportion of MHC-II epitopes overlapping with TMHs for human, viral and bacterial proteomes. Legend: covid = SARS-CoV-2, human = *Homo sapiens*, myco = *Mycobacterium tuberculosis*

Figure 8: Normalized proportion of MHC-I and MHC-II epitopes overlapping with TMHs, for the different haplotypes and proteomes

Figure 9: Normalized proportion of MHC-I and MHC-II epitopes overlapping with TMHs, for the different MHC classes and proteomes. Error bars denote the standard error.

811 the date we started the experiment, at December 14th 2020.

Figure 10: Distribution of the number of SNPs per gene name in the NCBI database.

812 To verify if SNPs were sampled uniformly over proteins, we show the dis-
813 tribution of the relative position in figure 15. We find no clear evidence of a
814 bias.

815 Supplementary Table 11 shows the statistics for all SNPs, where supplemen-
816 tary Tables 12 and 13 show the statistics for only single-spanners and multi-
817 spanners respectively.

Figure 11: Distribution of the number of protein variations and SNPs per gene name processed.

Figure 12: Histogram of the number of proteins found per gene name. Most often, a gene name is associated with one proteins.

Figure 13: Histogram of the number of SNPs per trans-membrane protein.
Dashed vertical line: average number of SNPs per TMP

Figure 14: Histogram of the number of TMHs predicted per protein, for the trans-membrane proteins used.

Figure 15: Distribution of the relative position of the SNPs used, where a relative position of zero denotes the first amino acid at the N-terminus, where a relative position of one indicates the last residue at the C-terminus.

index	haplotype_name
1	HLA-A*01:01
2	HLA-A*02:01
3	HLA-A*03:01
4	HLA-A*24:02
5	HLA-A*26:01
6	HLA-B*07:02
7	HLA-B*08:01
8	HLA-B*18:01
9	HLA-B*27:05
10	HLA-B*39:01
11	HLA-B*40:02
12	HLA-B*58:01
13	HLA-B*15:01
1	HLA-DRB1*0101
2	HLA-DRB1*0301
3	HLA-DRB1*0401
4	HLA-DRB1*0405
5	HLA-DRB1*0701
6	HLA-DRB1*0802
7	HLA-DRB1*0901
8	HLA-DRB1*1101
9	HLA-DRB1*1201
10	HLA-DRB1*1302
11	HLA-DRB1*1501
12	HLA-DRB3*0101
13	HLA-DRB3*0202
14	HLA-DRB4*0101
15	HLA-DRB5*0101
16	HLA-DQA1*0501/DQB1*0201
17	HLA-DQA1*0501/DQB1*0301
18	HLA-DQA1*0301/DQB1*0302
19	HLA-DQA1*0401/DQB1*0402
20	HLA-DQA1*0101/DQB1*0501
21	HLA-DQA1*0102/DQB1*0602

Table 6: Abbreviations of the haplotype names

Table 7: Percentage of MHC-I 9-mers overlapping with TMH. Values in brackets show the number of binders that have at least one residue overlapping with a TMH (first value) as well as the number of binders (second value). percentage used: 2

haplotype	covid	human	myco
HLA-A*01:01	15.603 (44/282)	12.600 (28377/225209)	11.424 (2947/25797)
HLA-A*02:01	34.155 (97/284)	28.441 (63994/225003)	29.749 (7646/25702)
HLA-A*03:01	9.122 (27/296)	6.606 (14851/224796)	9.972 (2565/25721)
HLA-A*24:02	39.223 (111/283)	22.297 (50313/225648)	22.346 (5752/25741)
HLA-A*26:01	21.739 (65/299)	14.287 (32232/225598)	13.950 (3598/25793)
HLA-B*07:02	9.712 (27/278)	5.347 (11893/222429)	8.899 (2291/25744)
HLA-B*08:01	15.248 (43/282)	8.935 (19981/223616)	10.714 (2750/25667)
HLA-B*15:01	24.324 (72/296)	15.228 (34498/226542)	17.600 (4547/25835)
HLA-B*18:01	11.724 (34/290)	5.993 (13409/223745)	5.960 (1536/25773)
HLA-B*27:05	10.227 (27/264)	4.854 (10882/224178)	8.031 (2063/25688)
HLA-B*39:01	18.182 (50/275)	11.468 (25621/223419)	14.682 (3787/25793)
HLA-B*40:02	4.594 (13/283)	3.647 (8147/223408)	4.264 (1097/25729)
HLA-B*58:01	24.731 (69/279)	16.245 (36409/224119)	20.558 (5292/25742)

Table 8: Percentage of MHC-II 14-mers overlapping with TMH. Values in brackets show the number of binders that have at least one residue overlapping with a TMH (first value) as well as the number of binders (second value). percentage used: 2

haplotype	covid	human	myco
HLA-DQA1*0101/DQB1*0501	40.433 (112/277)	31.214 (69752/223464)	32.158 (8187/25459)
HLA-DQA1*0102/DQB1*0602	22.910 (74/323)	16.167 (35753/221147)	17.950 (4608/25671)
HLA-DQA1*0301/DQB1*0302	10.381 (30/289)	10.179 (22623/222248)	11.144 (2842/25502)
HLA-DQA1*0401/DQB1*0402	11.111 (32/288)	13.135 (29319/223219)	9.890 (2524/25522)
HLA-DQA1*0501/DQB1*0201	20.430 (57/279)	16.240 (36186/222820)	14.999 (3823/25489)
HLA-DQA1*0501/DQB1*0301	15.808 (46/291)	14.106 (31046/220089)	18.969 (4878/25715)
HLA-DRB1*0101	27.119 (80/295)	19.774 (43968/222349)	22.293 (5692/25533)
HLA-DRB1*0301	14.676 (43/293)	9.801 (21831/222752)	7.956 (2025/25451)
HLA-DRB1*0401	19.231 (55/286)	15.325 (34011/221930)	18.113 (4641/25623)
HLA-DRB1*0405	12.996 (36/277)	13.684 (30380/222012)	15.837 (4036/25484)
HLA-DRB1*0701	32.877 (96/292)	21.512 (47856/222465)	29.304 (7471/25495)
HLA-DRB1*0802	23.132 (65/281)	19.339 (42859/221623)	28.805 (7358/25544)
HLA-DRB1*0901	11.565 (34/294)	13.111 (29043/221520)	16.798 (4301/25605)
HLA-DRB1*1101	25.197 (64/254)	11.924 (26582/222928)	16.103 (4101/25467)
HLA-DRB1*1201	36.897 (107/290)	15.482 (34596/223464)	20.018 (5098/25467)
HLA-DRB1*1302	13.962 (37/265)	20.121 (44798/222646)	23.141 (5935/25647)
HLA-DRB1*1501	35.206 (94/267)	21.836 (48671/222893)	25.891 (6584/25430)
HLA-DRB3*0101	9.158 (25/273)	8.496 (18884/222274)	6.819 (1740/25517)
HLA-DRB3*0202	18.657 (50/268)	13.832 (30687/221859)	15.843 (4059/25620)
HLA-DRB4*0101	23.529 (68/289)	12.749 (28376/222568)	16.221 (4131/25467)
HLA-DRB5*0101	23.776 (68/286)	11.235 (24993/222464)	14.648 (3732/25478)

Table 9: Amounts. raw = all variations, including DNA variations. all_proteins = all proteins. map = membrane associated protein. tmp = transmembrane protein. in_tmh = in transmembrane helix of TMP. in_sol = in soluble region of TMP.

what	raw	all_proteins	map	tmp	in_tmh	in_sol
Number of variations	60931	37831	16623	21208	3803	17405
Number of unique variations	60544	37630	16606	21024	3789	17235
Number of unique SNPs	NA	9621	4219	6026	1140	4936
Number of unique gene names	953	911	457	605	325	590
Number of unique protein names	5163	4780	2227	2553	1280	2467
Percentage TMH	NA	10	0	19	26	18

Table 10: Amounts. single_in_tmh = in transmembrane helix of single-spanner. single_in_sol = in soluble region of single-spanner. multi_in_tmh = in transmembrane helix of multi-spanner. multi_in_sol = in soluble region of multi-spanner.

what	single_in_tmh	single_in_sol	multi_in_tmh	multi_in_sol
Number of variations	452	7734	3351	9671
Number of unique variations	451	7733	3338	9502
Number of unique SNPs	160	2393	994	2762
Number of unique gene names	96	282	243	344
Number of unique protein names	304	1032	976	1435
Percentage TMH	11	5	35	26

Table 11: Statistics for all TMPs. p = p value. n = number of SNPs. n_success = number of SNPs found in TMHs (dashed blue line). E(n_success) = expected number of SNPs to be found in TMHs (dashed red line).

parameter	value
p	6.820823e-11
n	21208
n_success	3803
E(n_success)	4140.56

Table 12: Statistics for the single-spanners. p = p value. n = number of SNPs in single-spanners. n_success = number of SNPs found in TMHs of single-spanners (dashed blue line). E(n_success) = expected number of SNPs to be found in TMHs of single-spanners (dashed red line).

parameter	value
p	0.3189532
n	8186
n_success	452
E(n_success)	462.1535

Table 13: Statistics for the multi-spanners. p = p value. n = number of SNPs in multi-spanners. n_success = number of SNPs found in TMHs of multi-spanners (dashed blue line). E(n_success) = expected number of SNPs to be found in TMHs of multi-spanners (dashed red line).

parameter	value
p	8.315841e-12
n	13022
n_success	3351
E(n_success)	3678.406