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Research article

The stage- and subgroup-specific impact of non-HLA polymorphisms on preclinical type 1 diabetes progression

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ABSTRACT

Besides variation within the HLA gene complex determining a major part of genetic susceptibility to Type 1 diabetes, genome-wide association studies have identified over 60 non-HLA loci also contributing to disease risk. While individual single nucleotide polymorphisms (SNPs) have limited predictive power, genetic risk scores (GRS) can identify at-risk individuals. However, current models do not fully capture the heterogeneous progression of asymptomatic islet autoimmunity, especially in autoantibody-positive subjects. In this study, we investigated the additional stage-specific impact of 17 non-HLA loci on previously established prediction models in 448 persistently autoantibody-positive first-degree relatives. Cox regression and Kaplan Meier survival analysis were used to assess their influence on progression from single to multiple autoantibody-positivity, and from there to clinical onset. *FUT2* and *CTSH* significantly accelerated progression of single to multiple autoAb-positivity, but only in presence of insulin autoantibodies and HLA-DQ2/DQ8, respectively. At the stage of multiple autoantibody-positivity, progression to clinical onset was impacted by various non-HLA SNPs either as independent predictors (*GLIS3*, *CENPW*, *IL2*, *GSDM*, *MEG3A,* and *NRP-1*) or through interaction with HLA class I alleles (*CLEC16A*, *NRP-1*, *TCF7L2*), maternal diabetes status (*CTSH*), or a high-risk autoantibody-profile (*CD226*). Our data indicate that, unlike for GRS, the weight of distinct non-HLA polymorphisms varies significantly among individuals at risk, depending on disease stage and other stage-specific risk factors. They refine our previous stage-specific prediction models including age, autoantibody-profile, HLA genotype, and other non-HLA SNPs, and emphasize the importance of

Abbreviations: AIC, Akaike information criterion; autoAb, autoantibody; BDR, Belgian Diabetes Registry; CRS, combined risk score; FDRs, first-degree relatives; GADA, glutamate decarboxylase autoantibodies; GRS, genetic risk score; GWAS, genome wide association studies; IAA, insulin autoantibodies; IA-2A, insulinoma-associated protein-2 autoantibodies; SNPs, single nucleotide polymorphisms; T1D, type 1 diabetes; ZnT8A, zinc transporter 8 autoantibodies.

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stratifying accordingly to personalize time-to-event prediction in risk groups, or for preparing or interpreting prevention trials.

1. Introduction

Type 1 diabetes (T1D) is a complex auto-immune disease, and several genetic factors are involved in islet autoimmunity onset and subsequent disease progression. While variations in HLA regions are a major genetic factor, genome-wide association studies (GWAS) have also identified >60 non-HLA loci, each slightly yet significantly influencing the overall risk of developing symptomatic T1D, with typical odds ratios ranging from 0.3 to 2.3 [1–4]. Whereas individual non-HLA single nucleotide polymorphisms (SNPs) have shown limited effectiveness for T1D risk prediction, compounded genetic risk scores (GRS) have been designed that can successfully identify individuals at risk of developing T1D in population screening [5–9]. A risk score developed by Sharp et al. [7], considering both HLA and non-HLA variants, as well as several HLA DR-DQ interaction terms, could select 10 % of the general population comprising >77 % of all future T1D cases. Bonifacio et al. [9] were able to predict multiple autoantibody (autoAb) status in children by the age of 6 using GRS.

In a subsequent study, Ferrat et al. [10] constructed a combined risk score (CRS), comprising GRS, the number of autoAbs present and family history. They showed that GRS can add predictive value to T1D newborn screening when compared to models that only consider autoAbs. However, within autoAb$^+$ subjects, the discriminative power was notably reduced compared to the general population. Specifically, at a 2-year landmark and a 3-year horizon, their CRS showed no significant improvement over a model comprising only autoAbs [10]. This highlights the need for refinement, particularly in the subgroup of autoAb$^+$ first-degree relatives.

While current GRS and CRS do predict susceptibility to T1D, they do not fully capture the interindividual variability in T1D progression in distinct disease stages. We and others demonstrated that non-HLA alleles – alone or through interactions with other risk factors – could exert major effects on the progression rate of asymptomatic islet autoimmunity in a stage- and subgroup-specific way, hereby uncovering large interindividual differences in time-to-event analysis (from single to multiple autoAb-positivity or from that stage to clinical onset) and facilitating more personalized risk estimation [11–15]. For instance, the *ERBB3* rs2292239 GG genotype significantly delayed progression of single to multiple autoAb-positivity exclusively in females [15], while the *CTLA4* rs3087243 GA genotype markedly accelerated progression to stage 1 asymptomatic T1D in both sexes, but only in presence of the HLA-DQ2/DQ8 high-risk genotype [14]. In addition, *CTLA4* GA was an independent predictor of more rapid progression to clinical onset in multivariable Cox regression analysis [14].

Building on these observations we hypothesize that exploration of the effects of a larger panel of non-HLA SNPs can further refine individual risk assessment of progression through a given disease phase in autoAb$^+$ first-degree relatives (FDRs), and that understanding the influence of non-HLA risk factors is crucial for interpreting the outcomes of prevention studies in subclinical islet autoimmunity. To this effect, we tested the stage-specific impact of 17 non-HLA loci (*BACH2*, *BAD*, *CD226*, *CENPW*, *CTSH*, *FUT2*, *GLIS3*, *GSDM*, *IFIH1*, *IL18RAP*, *IL2*, *MEG3A*, *NRP1*, *RGS-1*, *SLC30A8*, *TCF7L2*, *UBASH3A*), in addition to previously studied non-HLA susceptibility loci [14,15], in a cohort of Belgian autoAb$^+$ FDRs under 40 years of age. We studied their impact on progression from single to multiple autoAb-positivity and from there on to clinical onset. Specifically, we investigated interaction effects between non-HLA SNPs and other established stage-specific predictors of subclinical disease progression, including younger age, presence of insulin autoAbs (IAA), HLA class I- and HLA class II-inferred risk, and maternal diabetes status [16,17]. Our long-term goal is to contribute to understanding the role of non-HLA genetic variants in the progression of pre-clinical diabetes, ultimately informing the design and interpretation of future studies focused on diabetes prevention and risk stratification. This knowledge can facilitate the selection of more homogeneous risk (sub)groups in future prevention studies at various disease stages and the interpretation of their outcome.

2. Methods

2.1. Study participants

From March 1989 to December 2015, the Belgian Diabetes Registry (BDR) monitored 461 persistently autoAb$^+$ siblings and offspring aged under 40 years, among 7029 asymptomatic first-degree relatives (FDRs) of T1D patients [17]. Participants were enrolled after written informed consent. If under 18 years of age, written consent was obtained from legal representatives and if deemed age-appropriate, verbally confirmed by the minor. The research received ethical clearance from Universitair Ziekenhuis Brussel's (UZB) institutional review board, year 2019, approval number BUN143201939922. Pseudonymized data encompassing clinical, anthropometric, and biological aspects were supplied by the BDR. Additionally, blood and DNA samples, preserved at −80 °C, were accessible through the IRB UZB's associated biobank (approval number BUN143201524128). The study was conducted in line with the Declaration of Helsinki guidelines [18].

Blood samples were collected at the start of the study (first confirmed autoAb-positivity) and typically on an annual basis thereafter. The participating autoAb$^+$ FDRs in our cohort had a median (interquartile range, IQR) age of 11.6 (6.4–19.3) years and were followed for a median (IQR) duration of 72 (35–129) months. Descriptive statistics of the study population is presented in supplementary data (Suppl. Table 1).

2.2. Analytical procedures

The determination of HLA-DQ and HLA-A and HLA-B haplotypes was conducted using allele-specific oligonucleotide hybridization [16,17]. AutoAbs, including those against insulin (IAA), GAD65 (GADA), insulinoma-associated protein 2 (IA-2A), and zinc transporter 8 (ZnT8A), were identified through liquid-phase radio-binding assays [17]. Genotyping of various SNPs (Suppl. Table 2) was performed using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA) on a QuantStudio™ 12K Flex Real-Time PCR System. Each assay run included controls without DNA, and genotypes were determined using the system's software. We selected SNPs for studying 17 curated non-HLA regions that had previously been associated with 1) T1D through GWAS now grouped under the T1D genetics consortium [19], and 2) progression of autoimmunity or asymptomatic diabetes in independent cohort studies [11,13,20–23].

2.3. Statistical analysis

In cross-tabulation, chi-squared test was used to check whether the observed genotype frequencies followed the Hardy-Weinberg equilibrium, and to compare FDR allele frequencies with the European frequencies (ENSEMBL database). Stage-specific univariable Cox regression analysis was performed to evaluate the individual impact of all SNP genotypes on stage-specific progression rates. Only genotypes with a *p*-value less than 0.100 were included in further analyses. Correction for multiple testing was not performed because this initial univariable screening was considered a preparatory exploration for identifying potential contributors which were to be further tested in multivariable models. Subsequently, multivariable Cox regression models were developed, integrating known predictive factors in addition to the qualifying SNP genotypes. For progression from single to multiple autoAb-positivity, Model 1 included established stage-specific predictors, such as age at first autoAb-positivity, presence of HLA-DQ8, HLA-DQ2/DQ8, *HLA-A*24*, and IAA [16]. Additionally, newly identified interaction effects between examined SNP genotypes and these established predictive factors were incorporated. Model 2 expanded on this by integrating previously reported interaction effects in our dataset: *ERBB3* GG with female sex [15], *CTLA4* GA with HLA-DQ2/DQ8, *SH2B3* TT with HLA-DQ8, and *CLEC16A* AA with IAA [14]. For the progression from multiple autoAb-positivity to T1D onset, Model 1 included stage-specific established predictors, such as a high-risk autoAb-profile (presence of IA-2A and/or ZnT8A), maternal diabetes status, *HLA-B*18*, *HLA-A*24* [16], and *CTLA4* AG [14], as well as independent non-HLA SNP effects. Model 2 further incorporated interaction effects between SNP genotypes and the established predictors. The most pronounced interactions overall were depicted using Kaplan-Meier survival curves and analyzed with the log-rank test for survival.

We verified the proportional hazards assumption in all the Cox models presented, using the cox.zph() function of the survival package in R, which tests for time-dependent variation in Schoenfeld residuals for both the global model and individual covariates. We have used the numDEpi() function of the powerSurvEpi package in R to calculate the numD (=events) or minimal population size required to generate the desired power: numDEpi (X1, X2, power, theta, alpha = 0.05). The theta (hazard ratio) for the SNP is estimated from our data. The power is set at 0.8, while X1 is the binary covariate of interest (SNP genotype), and X2 is the main covariate (binary or non-binary) in the Cox regression. The Akaike information criterion (AIC) was utilized to evaluate the efficacy of these models, with a higher AIC value indicating a less plausible model. Stepwise modelling was executed using SPSS version 27.0 software (IBM, Armonk, NY), applying two-tailed tests, with *P*-values <0.05 considered significant. Multivariate Cox regression was used to adjust for potential confounders. Figures were prepared in GraphPad Prism version 9 for Windows.

Table 1
Univariable Cox regression analysis for the progression from single to multiple autoAb-positivity, and from hereon, to clinical onset of T1D. *P*-Values, hazard ratios (HR), and 95 % confidence intervals are presented for each SNP genotype with $p < 0.100$ in one of the asymptomatic disease stages. Univariable Cox regression analysis data for all studied SNPs including those with $p \geq 0.100$ in both stages can be found in Suppl. Table 3.

Genotype		1 to ≥2 autoAbs			≥2 autoAbs to onset		
		n/n_{tot}[a]	*p*-value	HR (95 % CI)	n/n_{tot}[a]	*p*-value	HR (95 % CI)
CD226	TT	73/260	0.076	0.588 (0.327–1.057)	59/255	0.720	
CENPW	CC	63/251	0.503		62/248	0.024	0.611 (0.398–0.938)
CENPW	GG	70/251	0.970		67/248	0.048	1.438 (1.003–2.063)
CTSH	CC	117/256	0.071	1.553 (0.963–2.504)	106/252	0.246	
FUT2	TT	64/252	0.085	1.566 (0.940–2.610)	83/247	0.939	
FUT2	CT	123/252	0.082	0.650 (0.399–1.056)	108/247	0.273	
GDSM	CT	128/261	0.442		125/256	0.048	0.741 (0.511–0.997)
GLIS3	CC	58/257	0.120		44/252	0.038	0.604 (0.376–0.973)
GLIS3	CG	133/257	0.934		121/252	0.040	1.421 (1.016–1.988)
IL2	AA	105/247	0.509		113/247	0.008	1.581 (1.129–2.214)
IL2	AG	115/247	0.962		105/247	0.005	0.604 (0.425–0.858)
MEG3A	AA	86/252	0.990		80/250	0.059	0.695 (0.477–1.014)
NRP-1	AA	60/256	0.256		71/254	0.066	1.141 (0.978–2.045)
SLC30A8	CT	110/258	0.083	1.525 (0.946–2.457)	111/254	0.919	
SLC30A8	CC	121/258	0.049	0.609 (0.372–0.998)	120/254	0.981	

[a] Number of cases are given for each SNP genotype, along with the total number FDR genotyped for that specific SNP.

3. Results

3.1. SNP genotyping

The risk allele frequencies in 5 out of the 17 examined non-HLA susceptibility loci differed significantly from those in the European population (EUR) in the 1000 Genomes Project [24] with an increased prevalence of the *BAD* A (0.69 vs 0.62, $p = 0.0009$), *FUT2* T (0.53 vs 0.44, $p = 0.0002$), *NRP-1* A (0.48 vs 0.43, $p = 0.02$) and *UBASH3A* A (0.40 vs 0.35, $p = 0.02$) alleles, and a decreased prevalence of the *CENPW* C (0.49 vs 0.54, $p = 0.02$) allele (Suppl. Table 2). All SNPs had call rates >98 % and their genotype distributions did not deviate significantly from the Hardy–Weinberg equilibrium (data not shown). Among our cohort of 461 subjects, 448 could be genotyped for at least one non-HLA SNP. Of these, 261 were single autoAb$^+$ at study entry, with 69 developing an additional autoAb during follow-up. A total of 256 subjects were followed from multiple autoAb$^+$ and 145 progressed towards T1D. An overview of the number of relatives genotyped for each SNP is given in Suppl. Table 2.

3.2. Univariable cox regression analysis

Several SNP genotypes significantly and stage-dependently impacted progression of asymptomatic islet autoimmunity when assessed as individual independent variables in univariable Cox regression analysis. At the stage of single to multiple autoAb-positivity, only *SLC30A8* CC ($p = 0.049$, HR = 0.609) exhibited a significant protective effect on the progression rate. However, borderline effects were observed for *CD226* TT ($p = 0.076$, HR = 0.588), *CTSH* CC ($p = 0.071$, HR = 1.553), *FUT2* TT ($p = 0.085$, HR = 1.556), *FUT2* CT ($p = 0.082$, HR = 0.650), and *SLC30A8* CT ($p = 0.083$, HR = 1.525) (Table 1). At the stage of multiple autoAb-positivity (asymptomatic T1D stage 1), the genotypes *GLIS3* CG ($p = 0.038$, HR = 1.421), *IL2* AA ($p = 0.008$, HR = 1.581), and *CENPW* GG ($p = 0.048$, HR = 1.438) were found to promote progression to clinical onset (stage 3 symptomatic T1D), whereas the genotypes *GLIS3* CC ($p = 0.038$, HR = 0.604), *IL2* AG ($p = 0.005$, HR = 0.604), *GDSM* CT ($p = 0.048$, HR = 0.741), and *CENPW* CC ($p = 0.024$, HR = 0.611) showed a protective effect (Table 1). No statistically significant effects were observed for other polymorphisms (Suppl. Table 3).

3.3. Progression from single to multiple autoAb-positivity

In subsequent multivariable Cox regression analysis, stage-specific models were constructed comprising non-HLA SNPs in combination with previously determined predictors of disease progression [14–16]. At the stage of single to multiple autoAb-positivity, these factors included: age at first autoAb-positivity, *HLA-A*24* genotype, and HLA-DQ2/DQ8 genotype [16]. When attempting to integrate all SNP genotypes with univariate effects as additional independent predictors into the model, none were retained as significant contributors (data not shown). Based on our previous observations on interactions between other non-HLA SNPs and established risk predictors [14,15], we then introduced interaction terms. As a result, our first model revealed a statistically significant interaction between *CTSH* CC and HLA-DQ2/DQ8 ($p = 0.015$, HR = 3.387), and between *FUT2* TT and IAA ($p = 0.005$, HR = 2.811) (Table 2, model 1).

In a second model, we added previously observed interaction effects between non-HLA SNPs and established predictors. Specifically, we introduced the following interaction terms: *CTLA4* AG with HLA-DQ2/DQ8, *SH2B3* TT with HLA-DQ8, *CLEC16A* AA with IAA [14], and *ERBB3* GG with female sex [15]. In the final model the new interaction between *FUT2* TT and IAA persisted as an independent predictor of progression rate ($p = 0.002$, HR = 3.110). However, the interaction between *CTSH* CC and HLA-DQ2/DQ8 was overruled by our previously determined interaction between *CTLA4* AG with HLA-DQ2/DQ8 ($p = 0.010$, HR = 3.861) (Table 2, model 2). We observed a decrease in AIC-values (model 1, AIC = 644; model 2, AIC = 632) when compared to a Cox regression model without non-HLA polymorphisms (AIC = 692).

Kaplan-Meier survival analysis was used to visualize the results from the multivariable Cox analysis and confirmed that the *FUT2*

Table 2
Multivariable Cox regression analysis for the progression from single to multiple autoAb-positivity. *P*-values, hazard ratios (HR), and 95 % confidence intervals are presented.

Variable (0/1[a])	model 1*			model 2**		
	p-value	HR	95 % CI	*p*-value	HR	95 % CI
Age first autoAb$^+$ sample	<0.001	0.919	0.887–0.952	<0.001	0.911	0.878–0.946
*HLA-A*24* (205/56)	0.042	0.462	0.220–0.972	0.048	0.471	0.224–0.994
HLA-DQ2/DQ8 (210/51)	0.390			0.443		
CTSH CC x HLA-DQ2/DQ8 (234/22)	0.015	3.387	1.268–9.044	NM		
FUT2 TT x IAA (237/15)	0.005	2.811	1.359–5.811	0.002	3.110	1.527–6.335
CTLA4 AG x HLA-DQ2/DQ8 (234/24)	NT	NT	NT	0.010	3.861	1.378–10.813
SH2B3 TT x HLA-DQ8 (234/26)	NT	NT	NT	0.015	0.226	0.069–0.745
CLEC16A AA x IAA (225/35)	NT	NT	NT	NM		
ERBB3 GG x female sex (213/46)	NT	NT	NT	0.030	0.359	0.143–0.904

[a] 0/1: number of FDR in cohort negative/positive for genotype or interaction, for progression from single to multiple autoAb-positivity. NM, not retained in conditional forward model. NT, Not tested; AIC-value of model 1 = 644 (*), AIC-value model 2 = 632 (**) as compared to a model not containing any non-HLA polymorphisms (AIC = 692). Total cases/events in the models: 252/68 (model 1*) and 251/68 (model 2**).

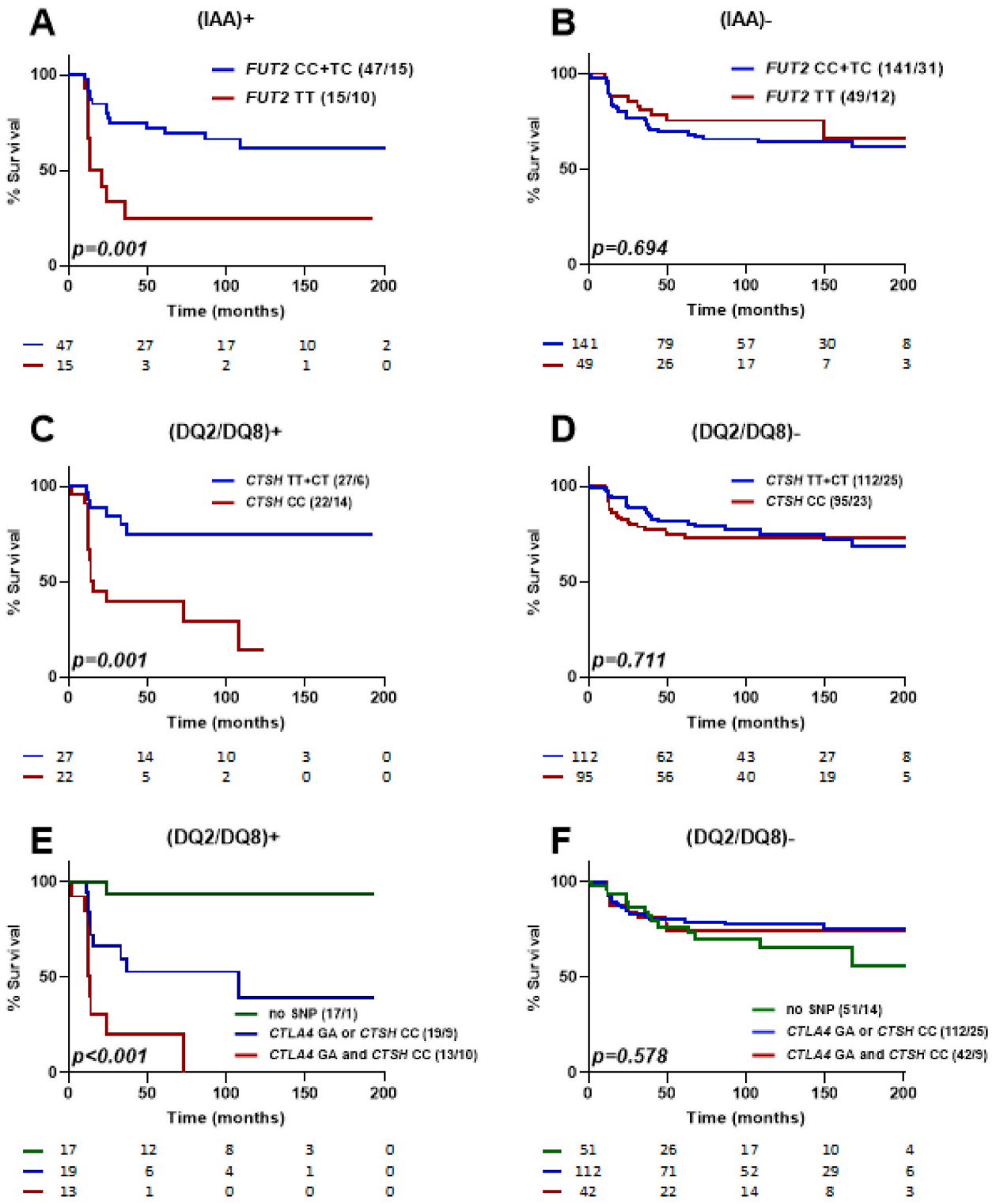

Fig. 1. Interaction effects of non-HLA SNPs on progression of single to multiple autoAb-positivity. Kaplan-Meier survival analysis of progression from single to multiple autoAb-positivity for FDRs with (red) or without (blue) *FUT2* CC according to the presence (A) or absence (B) of IAA as first autoAb; *CTSH* CC according to the presence (C) or absence (D) of HLA-DQ2/DQ8; and the absence of genotypes *CTLA4* AG and *CTSH* CC (green), the presence of only one of these genotypes (blue), and the presence of both genotypes (red), in HLA-DQ2/DQ8+ (E) and HLA-DQ2/DQ8- (F) relatives. *P*-values (*p*) are from the log-rank test on differences in survival. The numbers of individuals at risk are indicated below the time axis. For each arm, the genotype and number (cases/events) are shown in the legend.

TT genotype significantly accelerates progression at this stage, but that the effect is restricted to IAA positive relatives ($p = 0.001$, Fig. 1A and B). Similarly, the *CTSH* CC genotype also conferred risk of more rapid progression, but only in HLA-DQ2/DQ8 positive relatives ($p = 0.001$, Fig. 1C and D). Furthermore, in HLA-DQ2/DQ8 positive relatives, *CTLA4* AG and *CTSH* CC genotypes demonstrated an additive effect. The absence of both non-HLA genotypes was associated with slow progression, while the presence of either one genotype significantly increased the risk, and the highest risk was observed when both genotypes were present (overall $p < 0.001$, with $p \leq 0.007$ for differences between each of the 3 arms; Fig. 1 E). In absence of HLA-DQ2/DQ8 both non-HLA genotypes failed to accelerate progression of single to multiple autoAb-positivity (Fig. 1 F).

3.4. Progression from multiple autoAb-positivity to clinical onset

Several non-HLA SNPs emerged as independent predictors in multivariable Cox regression models of progression from multiple autoAb-positivity to disease onset (Table 3). They were added to a model already comprising previously established predictors, including *HLA-A*24* genotype, *CTLA4* AG genotype, high-risk autoAb-profile and maternal diabetes status [14,16]. A substantial slowing effect was observed for *GSDM* CT ($p = 0.003$, HR = 0.575), *IL2* AG ($p = 0.013$, HR = 0.631), and *MEG3A* AA ($p = 0.016$, HR = 0.613), while *GLIS3* CG ($p = 0.006$, HR = 1.662) and *NRP*-1 AA ($p = 0.043$, HR = 1.515) both demonstrated accelerating effects (Table 3, model 1). Despite not being retained in a conditional forward approach, *CENPW* CC did exhibit a significant effect in Kaplan-Meier analysis ($p = 0.023$, Fig. 2A). Moreover, Kaplan-Meier analysis further substantiated our findings, confirming that the progression rate towards clinical onset was accelerated by the *IL2* AA + GG ($p = 0.004$, Fig. 2B), *GSDM* TT + CC ($p = 0.047$, Fig. 2C), and *GLIS3* CC ($p = 0.036$, Fig. 2D) genotypes, and tended to be promoted by *NRP*-1 AA ($p = 0.064$, Fig. 2E), and *MEG3A* AA ($p = 0.058$, Fig. 2F) genotypes.

We next extended our analysis by constructing multivariable models that accounted for interaction effects at the stage of multiple autoAb-positivity. In our analysis, we initially integrated interactions of each SNP genotype with established variables into the previous model (Table 3, model 1) separately, identifying several significant effects (Suppl. Table 4). Subsequently, we used a conditional forward approach to construct a new model by adding all these significant interactions collectively into the analysis, alongside the initial model 1, for a more comprehensive evaluation. The resulting model (Table 3, model 2) consisted of accelerating effects for interactions between *CLEC16A* AA and *HLA-B*18* (p = 0.001, HR = 5.049), *CTSH* CT and non-diabetic mother (p = 0.043, HR = 1.468), *CD226* CT and high-risk autoAb-profile (p = 0.029, HR = 1.521), *NRP*-1 AA and *HLA-A*24* (p < 0.001, HR = 5.005), and *TCF7L2* CC and *HLA-A*24* (p = 0.016, HR = 2.502). Additionally, *GLIS3* CC, *GSDM* CT and *IL2* AG remained independent predictors (Table 3, model 2). Kaplan-Meier analysis confirmed these findings, highlighting the most pronounced effects: the risk associated with *NRP*-1 AA (p = 0.003) and *TCF7L2* CC (p = 0.059) was indeed restricted to *HLA-A*24* positive FDRs, and the accelerating effect of *CLEC16A* GG (p = 0.060) was confined to *HLA-B*18* positive FDRs (Fig. 3). Again, we observed a decrease in AIC-values for both model 1 (AIC = 1188) and model 2 (AIC = 1168), when compared to the Cox regression model without non-HLA polymorphisms (AIC = 1355) (Table 3).

Table 3
Multivariable Cox regression analysis for the progression from multiple autoAb-positivity to clinical onset of T1D. Model 1 includes previously determined variables (*HLA-A*24* genotype, being offspring of a diabetic mother, high-risk autoAb-profile, and *CTLA4* AG), and non-HLA SNP genotypes that impacted this stage in univariable Cox analysis. *CENPW* CC was not retained in a conditional forward approach. Model 2 includes interactions between SNPs and other risk factors that could be added to model 1. *P*-values, hazard ratios (HR), and 95 % confidence intervals are presented.

Variable (0/1[a])	model 1*			model 2**		
	p-value	HR	95 % CI	*p*-value	HR	95 % CI
*HLA-A*24* (206/51)	<0.001	2.648	1.697–4.132	0.731		
IA-2A and/or ZnT8A[b] (50/207)	0.001	2.705	1.518–4.818	0.044	1.852	1.018–3.370
No Diabetic Mother (37/216)	0.024	2.011	1.095–3.690	0.129		
CTLA4 GA (126/129)	0.012	1.583	1.107–2.264	0.008	1.631	1.137–2.340
GLIS3 CG (131/121)	0.006	1.662	1.159–2.384	0.022	1.522	1.062–2.181
GSDM CT (131/125)	0.003	0.575	0.401–0.823	0.001	0.529	0.366–0.766
IL2 AG (142/105)	0.013	0.631	0.380–0.908	<0.001	0.487	0.333–0.766
MEG3A AA (170/80)	0.016	0.613	0.412–0.911	NM		
NRP-1 AA (183/71)	0.043	1.515	1.014–2.264	NM		
*HLA-B*18* x *CLEC16A* GG (249/7)	NT	NT	NT	0.001	5.049	1.913–13.324
No Diabetic Mother x *CTSH* CT (157/94)	NT	NT	NT	0.043	1.468	1.013–2.128
IA-2A and/or ZnT8A x *CD226* TC (157/98)	NT	NT	NT	0.029	1.521	1.043–2.218
*HLA-A*24* x *NRP*-1 AA (239/15)	NT	NT	NT	<0.001	5.005	2.138–11.717
*HLA-A*24* x *TCF7L2*[c] CC (230/26)	NT	NT	NT	0.016	2.502	1.185–5.284

[a] 0/1: number of FDR in cohort negative/positive for genotype or interaction, for progression from multiple autoAb-positivity to clinical onset.
[b] High-risk autoAb-profile.
[c] *TCF7L2* rs7903146. NM, not retained in conditional forward model. NT, not tested; AIC-value off model 1 = 1188 (*), AIC-value model 2 = 1168 (**) as compared to a model not containing any non-HLA polymorphisms (AIC = 1355). Total cases/events in the models: 229/132 (model 1*) and 228/132 (model 2**).

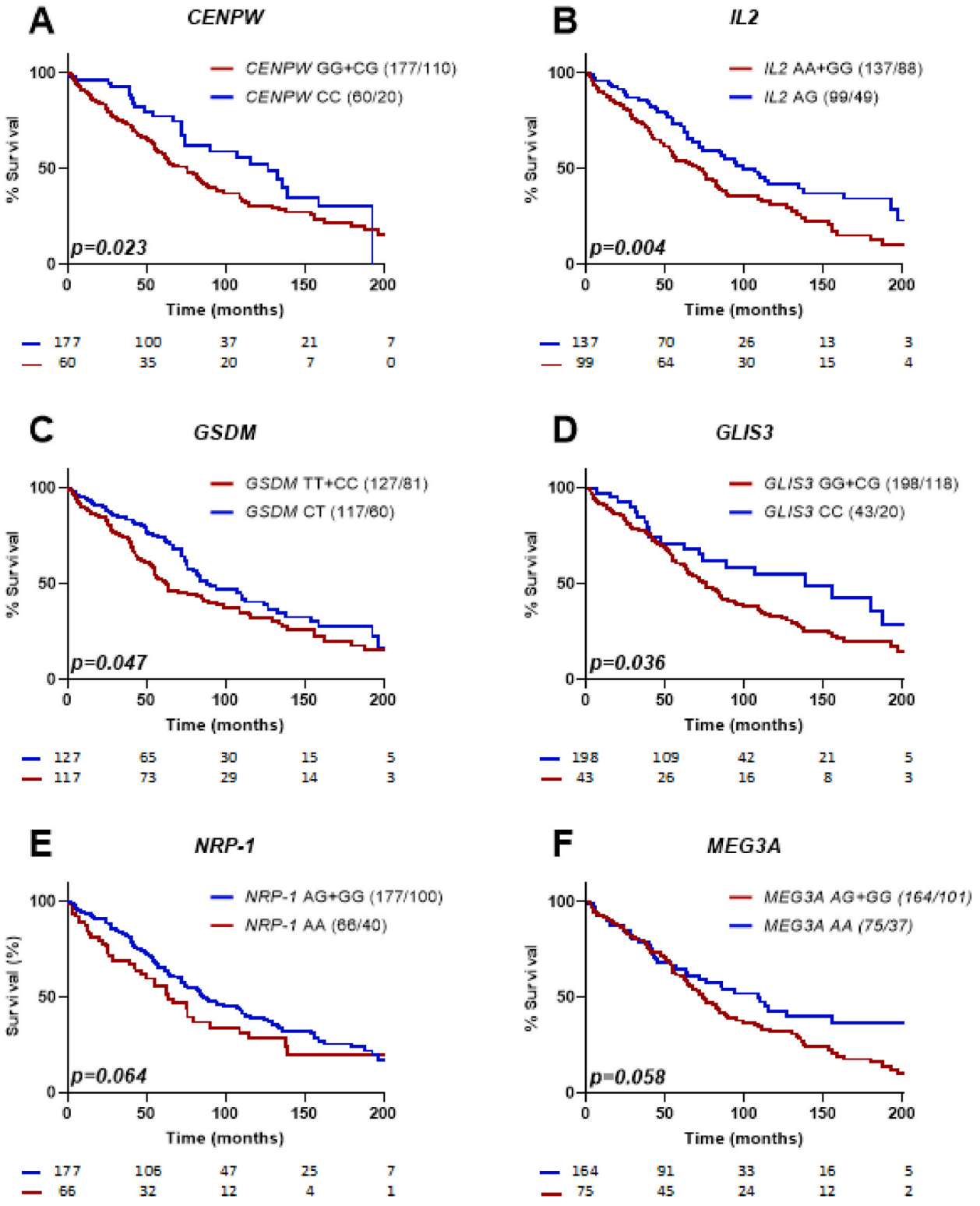

Fig. 2. Independent effects of non-HLA SNPs on progression from multiple autoAb-positivity to clinical onset. Kaplan-Meier survival analysis for FDRs with (red) or without (blue) *CENPW* CC (A), *IL2* AG (B), *GSDM* CT (C), *GLIS3* CC (D), *NRP*-1 AA (E) or *MEG3A* AA (F) genotype. *P*-values (*p*) are from the log-rank test on differences in survival. The numbers of individuals at risk are indicated below the time axis. For each arm, the genotype and number (cases/events) are shown above the graph.

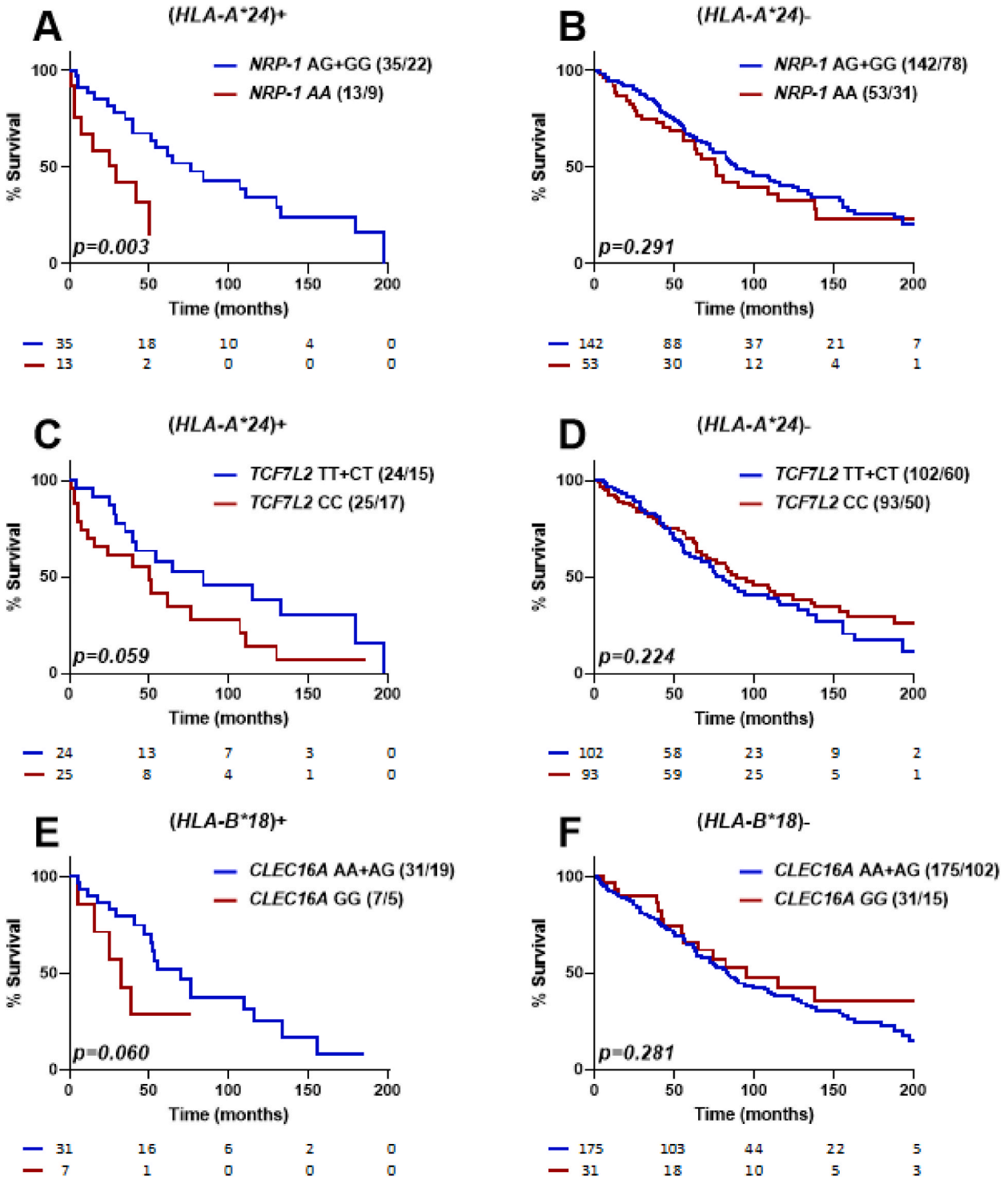

Fig. 3. Interaction effects of non-HLA SNPs on progression from multiple autoAb-positivity to clinical onset. Kaplan-Meier survival analysis for *HLA-A*24* positive (A,C) or negative (B,D) FDRs with (red) or without (blue) *NRP*-1 AA (A,B) or *TCF7L2* CC (C,D) genotype, and for FDRs with (red) or without (blue) *CLEC16A* GG, according to the presence (E) or absence (F) of for *HLA-B*18*. *P*-values (*p*) are from the log-rank test on differences in survival. The numbers of individuals at risk are indicated below the time axis. For each arm, the genotype and number (cases/events) are shown above the graph.

4. Discussion

The present study confirms and extends our previous observations [14,15] in islet autoAb$^+$ FDRs of T1D patients showing that distinct high-risk alleles of non-HLA SNPs significantly impact the progression of asymptomatic islet autoimmunity in a stage- and subgroup-specific way, either in their own right, or through selective interactions with other risk factors. Including more non-HLA SNPs in our previous prediction models [14–17] uncovered hitherto unknown selective strong interactions which help to further refine and personalize estimates of the time to a next pivotal event in the subclinical disease phase. These observations should benefit counselling and monitoring of at-risk individuals in the perspective of preventing diabetes ketoacidosis, and planning and interpreting stage-specific intervention trials.

In our autoAb + cohort we observed a higher allele frequency for *BAD* A, *FUT2* C, *NRP-1* A and *UBASH3A* as compared to the European population, and a decreased prevalence for *CENPW* C. This suggests a potential influence of these SNPs on immune activation in our cohort. While this confirms previous reports on the association of *UBASH3A* [25,26], *NRP-1* [26] and *FUT2* [26] with the development of islet autoAbs, we could not replicate similar claims for *GLIS3* and *IFIH1* SNPs [25]. As far as we know, the *BAD* gene has not been reported to impact islet autoAb development.

In line with our previous studies [14,15], non-HLA SNPs affect the progression of asymptomatic autoimmunity in a stage-specific way. At the stage of single autoAb-positivity, none of the tested SNPs impacted progression towards multiple autoAb-positivity when considered individually. Nevertheless, multivariable analysis unveiled that *CTSH* exerted a strong accelerating effect, however restricted to HLA-DQ2/DQ8 positive relatives, while *FUT2* did the same but only in relatives with IAA as their first autoAb at this stage. The *CTSH* gene encodes cathepsin H, which functions in endolysosomal protein degradation and antigen presentation. It has been associated with early T1D onset [27], and its genetic influence on T1D development has been shown to be mediated by DNA methylation [28,29]. Both *CTSH* and *FUT2* have been associated with a decline in first-phase insulin response in children with multiple autoAbs [30].

When introducing previously determined interaction effects to the above multivariable model for predicting progression of single to multiple autoAb-positivity [14,15], the significance of the *FUT2* interaction persisted, whereas the effect of *CTSH* was overruled by the slightly more robust interaction between *CTLA4* and HLA-DQ2/DQ8 [14]. Subsequent investigation with Kaplan-Meier survival analysis demonstrated an additive effect of *CTSH* and *CTLA4,* with the highest progression rate in presence of risk alleles for both SNPs. Interestingly, *CTLA4* has also been implicated as a locus where DNA methylation may potentially modulate T1D risk [29], suggesting an epigenetic mechanism impacting this stage and subpopulation.

At the stage of multiple autoAb-positivity, multivariable Cox regression analysis identified *GLIS3*, *GSDM*, *IL2*, *MEG3A* and *NRP-1* as independent predictors of accelerated progression towards clinical onset in our cohort, in addition to the previously determined effect of *CTLA4* [14]. Furthermore, *CTSH* impacted progression in subjects without a diabetic mother, *CD226* affected progression in subjects exhibiting the high-risk autoAb-profile (presence of IA-2A or ZnT8A and at least one other autoAb type [16,17], *CLEC16A* accelerated progression in presence of *HLA-B*18*, and both *NRP-1* and *TCF7L2* in presence of *HLA-A*24*. These hitherto unreported interactions with HLA class I susceptibility alleles – particularly the strong accelerating effect of *NRP*-1 AA in presence of *HLA-A*24* – could be confirmed and visualized through Kaplan-Meier analysis.

Power calculations (not shown) considering population characteristics and retrospective knowledge of hazard ratios, suggested that the detection of significant individual SNP-effects (Tables 1 and 2) may have been limited by a modest number of events in the stage from one to multiple autoAbs. However, the cohort proved sufficiently powered to capture SNP-interaction effects with higher hazard ratios (Table 2), and for detecting SNP-effects, either direct or by interaction, in going from multiple autoAb-positivity to diabetes (Table 3). The higher hazard ratios in the interaction models further highlight the potential importance of genetic interactions in influencing survival outcomes.

Our findings partly align with other prominent prospective studies, although there are discrepancies as well. For example, in the TrialNet study, *GLIS3* was shown to influence the onset of T1D in children under 12 years, but not specifically the progression from single to multiple autoAb-positivity [20], like our data suggest. Moreover, *GLIS3* showed a marginally significant impact on islet autoimmunity development in the DAISY cohort [25], and was implicated in a predictive T1D model constructed by Winkler et al. [31] comprising 9 non-HLA SNPs. Interestingly, *BACH2* was also implicated in this model, while our results did not show a significant effect for this SNP [31]. Another risk score by Bonifacio et al. [32] includes *IL2* and *IFIH1* amongst other SNPs to predict progression to T1D within 6 years after seroconversion. While we did observe an accelerating effect of *IL2*, we did not find evidence for the involvement of *IFIH1*. The latter agrees with a previous study showing no impact of *IFIH1* in a Belgian population [33]. In the DIPP study [26], *CD226* emerged as a factor impacting progression from seroconversion to T1D, yet to our knowledge, its interaction with the high-risk autoAb-profile [16,17] has not been previously reported. Notably, none of our identified SNPs emerged as significant factors during this stage in the TEDDY cohort [13,34,35].

Discordant results may be attributable to several factors, including geographical variations in SNP allelic distributions, and differences in the selection of non-HLA SNPs across research groups [11–13]. Unlike in most birth cohorts, participants in our study were all FDRs of a T1D patient, were not preselected for HLA-DQ risk haplotypes, and comprised both children and adults [36]. Although the vast majority of T1D patients have no familial history at clinical onset, prediction models built in FDRs can conceivably be extrapolated to the general population, as previously exemplified [37] in view of the largely similar characteristics of familial and sporadic forms of the disease [38]. The inclusion of both adolescents and adults in our study is to be considered a strength. Indeed, T1D can become clinically overt at any age, and, in spite of the increasing incidence in children [39], is reportedly diagnosed more often after age 15 years than before with age-dependent differences in phenotypical and biological characteristics [40–43]. AutoAb$^+$ adolescents and adults represent an important target group for immune intervention trials. Unlike in children followed from birth, studies monitoring

autoAb status from older ages on, such as conducted by the Belgian Diabetes Registry or TrialNet, may be limited by the uncertainty surrounding the exact time of seroconversion in some participants [44,45]. However, previous investigations conducted by our group have demonstrated that excluding these subjects from the analysis did not significantly change its conclusions [45].

The most striking observations in the progression towards multiple autoAbs were 1) the interaction between *FUT2* and type of first autoAb (IAA) and 2) the interaction between HLA-DQ2/DQ8 and *CTSH* CC and/or *CTLA4* AG. The subsequent progression towards diabetes showed striking impact by SNP genotypes *CTLA4* AG, *GSDM* CT, and *IL2* AG, and additionally a strong impact (HR $\geq$ 5) by interactions between *HLA-B*18* and *CLEC16A* GG, and between *HLA-A*24* and *NRP*-1 AA. It is tempting to speculate that these (interaction) effects reflect underlying biological mechanisms driving disease progression. For instance, *CTSH* is involved in peptide processing and *HLA-DQ* in peptide presentation, and *CTLA4*, *NRP-1* and *IL2* are all involved in regulatory T-cell function [46] which significantly affects disease progression and onset in NOD mice [47,48]. Modelling interaction-effects of genetic biomarkers with non-genetic factors may help to explain the architecture of disease trajectories and lead to refined risk assessment within populations. On the other hand, such interactions may vary across populations with different genetic backgrounds or environmental exposures. This highlights the need for replicating our specific observations in independent cohorts.

GRS based risk assessment is grounded in large datasets and aims at genetic risk evaluation across different populations. We also recognize the potential for CRS for population screening in identifying autoAb-negative children who may be at high risk of early onset, a risk that might otherwise go undetected [10]. Although GRS exist that can predict preclinical progression rates once autoAb-positivity develops in genetically at-risk children [49], current methodologies do not consider stage- and subgroup-specific effects of non-HLA SNPs. As a result, their ability to differentiate between rapid and slow progressors could be improved. Moreover, CRS and GRS have primarily been investigated in cross-sectional studies for their impact on overall T1D development in very young children [7,10]. Our longitudinal dataset, consisting of relatively older subjects (median 11.6 years at autoAb-positivity), followed for a median duration of 72 months, thus offers a unique point of view into the different stages of preclinical diabetes.

In conclusion, both at the stage of single to multiple autoAb-positivity and the stage of multiple autoAb-positivity to T1D, existing predictive models of disease progression could be improved by inclusion of non-HLA polymorphisms, as indicated by a decrease in AIC-values. Our data demonstrate that the impact of a given SNP is both stage- and subgroup-dependent. Consequently, the impact or weight of a specific non-HLA factor on time-to-event varies significantly among individuals at risk, dependent on the presence or absence of other stage-specific risk factors, and the event investigated. This indicates the importance of stratification when analyzing time-to-event data or when preparing prevention trials. More research in other cohorts at familial or genetic risk is still needed to fully capture the interindividual variability of preclinical T1D, but so far, our data indeed suggest that existing risk score calculations may be overly generalized and may lack precision, particularly in addressing the complex interactions underlying subclinical disease progression in autoAb + first-degree relatives.

CRediT authorship contribution statement

Julie Vandewalle: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Aster K. Desouter:** Writing – review & editing, Resources, Data curation. **Bart J. Van der Auwera:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation. **Kaven B. Chapaza:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Frank Nobels:** Writing – review & editing, Resources, Data curation. **Pascale Abrams:** Writing – review & editing, Resources, Data curation. **Marie-Christine Lebrethon:** Writing – review & editing, Resources, Data curation. **Bruno Lapauw:** Writing – review & editing, Resources, Data curation. **Bart Keymeulen:** Writing – review & editing, Resources, Project administration, Funding acquisition, Data curation. **Frans K. Gorus:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mark Van de Casteele:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

The datasets generated and/or analyzed during the present study are not openly available as they were derived from pseudonymized data and samples collected by the BDR, a controlled access repository of sensitive human data. They can be made available upon reasonable request to co-author Prof. Dr. Bart Keymeulen, president of the BDR.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42156.

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