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BTN2A1 and *BTN3A1* as Novel Coeliac Disease Risk Loci: An *in silico* Analysis

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Abstract

Coeliac disease (CeD) is a gastrointestinal enteropathy triggered by the consumption of gluten in predisposed individuals. Only around 50% of CeD genetic risk is understood, with the majority of risk attributed to the *HLA* loci. We investigated the butyrophilin family of immunomodulators as novel CeD risk loci. We sequenced the butyrophilin loci of 48 CeD and 46 control patients and carried out gene-based burden testing on the captured single nucleotide polymorphisms (SNPs). We found significantly increased *BTN2A1* gene burden in CeD patients. To validate these results, the SNP data of 3094 CeD patients and 29 762 control participants from the UK Biobank database were subjected to single variant analyses. Fourteen *BTN2A1*, 10 *BTN3A1*, and 13 *BTN3A2* SNPs were significantly associated with CeD status. Twenty of the 37 SNPs above were associated with CeD status independent of the risk associated *HLA* genotypes. All twenty of these SNPs, alongside a novel SNP not included in the above SNPs were associated with CeD in *HLA-DQ2.5*-matched case-control groups. This study reaffirmed the association of the *BTN3A2* locus with CeD risk, and identified *BTN2A1* and *BTN3A1* as putative novel CeD risk loci.

Keywords: coeliac disease; butyrophilin family; hypervariable region 4 (HV4); UK Biobank

1. Introduction

Coeliac disease (CeD) is a T-cell mediated autoimmune enteropathy triggered by the consumption of gluten, a protein found in wheat, rye, and barley [1]. During active CeD, individuals with underlying genetic risk suffer from small intestinal inflammation after the consumption of dietary gluten [2]. This chronic inflammation causes villous atrophy, that can lead to symptoms including abdominal pain, diarrhoea, malabsorption, and malnutrition [3]. Currently the only treatment for CeD is eliminating gluten from the diet of patients with CeD predisposition [4].

The genetic background of CeD predisposition is still not fully understood, as only 50% of the genetic risk has been explored [1]. The most well established CeD risk loci is the human leukocyte antigen (*HLA)* complex [1,5–10]. The HLA-DQ2.5, HLA-DQ2.2, and HLA-DQ8 heterodimers are present in more than 80% of CeD patients [11–15]. In contrast, about 20-30% of healthy controls have the CeD associated risk *HLA* genotypes [11,12,16]. These *HLA* genotypes were estimated to explain

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about 30-40% of the total CeD genetic risk [17,18]. Recent evidence has shown the butyrophilin family of genes to be non-*HLA* CeD risk loci of interest [19–21].

The butyrophilin proteins are a family of immunoglobulin-like cell surface receptors that have been shown to regulate both innate and adaptive immunity, including the activity of dendritic cells (DC), natural killer (NK) cells, αβ T cells, and γδ T cells [22–26]. Members of the butyrophilin family were found to maintain local γδ T-cell compartments in the blood and epithelia of both mice and humans (Table 1) [27–33]. Hayday and Vantourout [34] hypothesised that butyrophilin proteins serve as a steady state signal that maintains the local γδ T-cell population in a quiescent or inactive state. In the duodenum, the BTNL3/BTNL8 heterodimers act as the ligand for Vγ4+/Vδ1+ γδ intraepithelial lymphocytes (IELs) (Figure 1) [21,27,28]. Specifically, the BTNL3/BTNL8 heterodimer binds the germline-encoded hypervariable region 4 (HV4) of T cell receptor gamma (TCR-γ), when the variable (V) gene segment encoding that TCR-γ is the *TRGV4* gene.

Table 1. Butyrophilins maintain and activate the γδ T-cell compartments of mice and humans. Human butyrophilin family members are shortened with all letters capitalised, while only the first letter of mouse butyrophilins is capitalised [24].

	Butyrophilins	γδ T-cell subset	Role of butyrophilins	References
Peripheral blood	Mouse unidentified	Unidentified	Unidentified	NA
	Alpaca BTN3	Vγ9Vδ2+ T cells	No interaction has been identified	[35,36]
	Human BTN3A homodimers/ heterodimers and BTN2A1 homodimer	Vγ9Vδ2+ T cells	Phosphoantigen mediated, CDR3-independent γδ T-cell activation	[30,33,37,38]
Skin	Mouse Skint1 and Skint2	Vγ5Vδ1+ DETC	Thymic selection, tissue homing of dendritic epidermal T cells to the skin	[27,28,32]
	Human ?	Vδ1+ T cells	Unidentified, unknown if there is butyrophilin involvement	[39]
Intestinal epithelium	Mouse Btnl1 and Btnl6	Vγ7+ IEL	Phenotypic maintenance of the intestinal IEL compartment	[27,28]
	Human BTNL3 and BTNL8	Vγ4Vδ1+ IEL	Phenotypic maintenance of the intestinal IEL compartment	[21,27,29]

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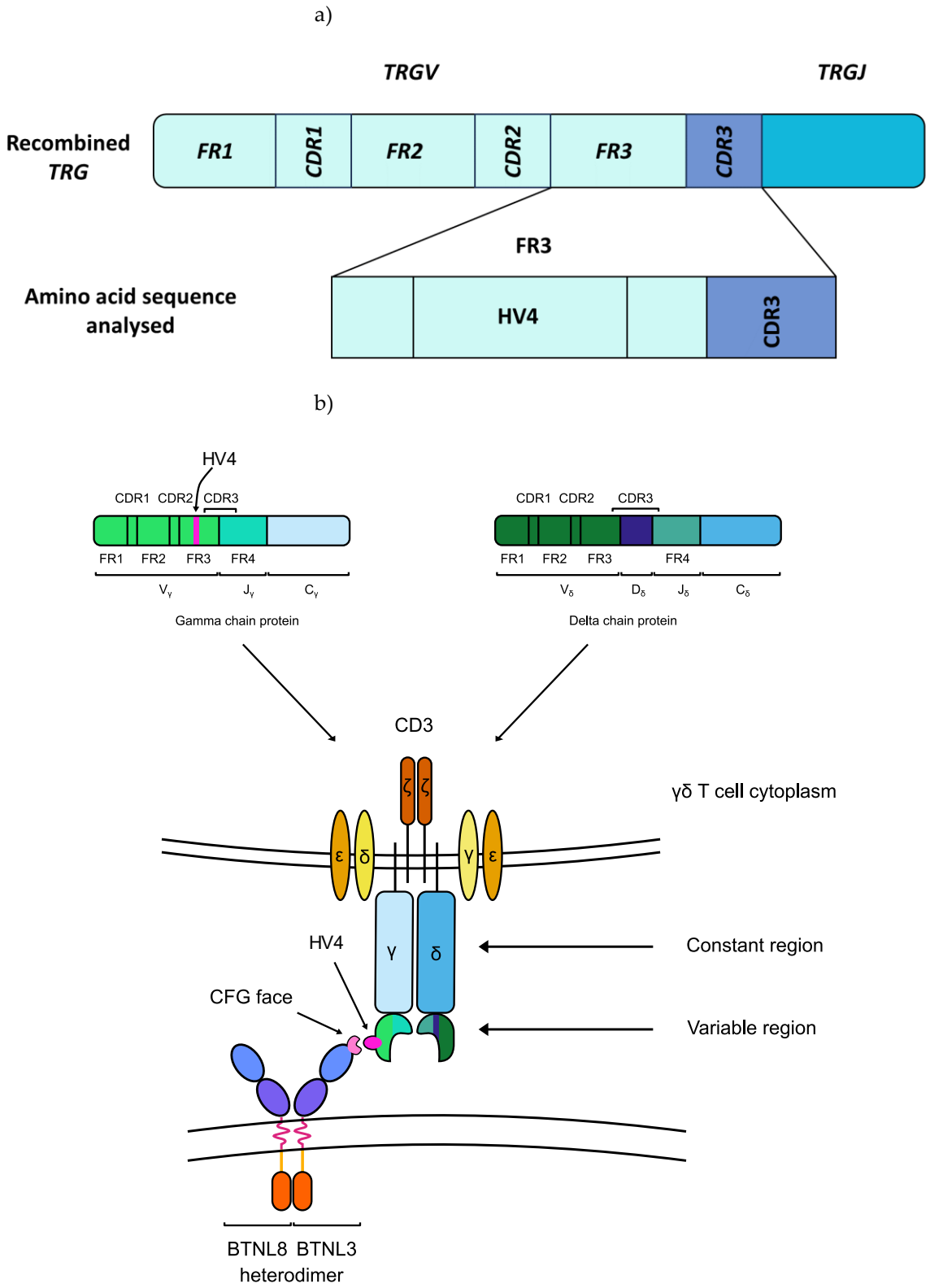

Figure 1. The germline-encoded HV4 loop of the T cell receptor (TCR) of Vγ4+ γδ IELs directly binds to BTNL3. a) HV4 is located at amino acid positions 10-25 in the FR3 of the TRGV4 segment [31]. b) The HV4 of Vγ4+ γδ T cells binds to the C, C″, F, and G canonical immunoglobulin-fold β-strands (CFG face) of the BTNL3 protein [29]. Abbreviations: CDR: complementarity-determining region; FR: framework region; HV4: hypervariable region 4; TRGJ: T cell receptor γ joining region; TRGV: T cell receptor γ variable region.

During active CeD, these γδ T cells, alongside CD4+ and CD8+ αβ IELs are activated by dietary gluten [40]. Mayassi, *et al.* [21] showed the loss of interaction between BTNL3/BTNL8 heterodimers and the duodenal Vγ4+ γδ T cells as a characteristic of active CeD in a study of 62 active CeD, 57

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gluten-free diet (GFD) treated CeD, and 99 control participants. During chronic inflammation induced by dietary gluten, the expression of the BTNL3/BTNL8 heterodimer was lost in the small intestine of patients having CeD predisposition. This was accompanied by the permanent loss of BTNL3/BTNL8-reactive Vγ4+/Vδ1+ γδ T cells. The chronic inflammation only subsided when patients followed a GFD. Although the BTNL3/BTNL8 expression recovered, the local γδ TCR repertoire was permanently reshaped: the innate-like Vγ4+/Vδ1+ γδ T cells and T cell receptor γ variable region 4 (*TRGV4)* gene transcripts were significantly decreased [21].

Recently, a common *BTNL8*BTNL3* 56-kb deletion copy number variant (CNV) was described by Aigner, *et al.* [41] in a cohort of more than 4000 samples. The *BTNL3* and *BTNL8* loci are segmental duplications and share a high sequence similarity. During meiosis, highly identical sequences are prone to recombination, which can give rise to CNVs. Aigner, *et al.* [41] reported 58.4% of their 346 samples of European ancestry had at least one *BTNL8*BTNL3* deletion allele (Table 2). This CNV has been shown to encode a BTNL8*3 fusion protein, which consists of the transmembrane domain, the extracellular IgV and IgC domains of BTNL8, and the intracellular signalling domain of BTNL3. As the BTNL3-IgV domain is missing in the fusion protein, it is plausible that the BTNL8*3 fusion protein has an impaired ability to bind to the Vγ4Vδ1+ T cells in the small intestine [31]. As Mayassi, *et al.* [21] observed a permanent shift in the duodenal γδ TCR repertoire when the interaction between the T cells and the BTNL3/BTNL8 heterodimer was disrupted, this fusion protein could predispose carriers to CeD.

Table 2. The *BTNL8*BTNL3* copy number variation is present in 58.4% of individuals of European ancestry, as first described by Aigner, *et al.* [41]. Carriers are defined as individuals with at least one *BTNL8*BTNL3* deletion allele. Abbreviations: CEU: Utah residents with Northern and Western European ancestry; HapMap: International HapMap Project; het.: heterozygous; HGDP: Human Genome Diversity Panel; hom.: homozygous; N: number.

	Population	Hom. for deletion	Het. for deletion	Hom. for full sequences	Deletion allele N	Deletion allele frequency	Group N	Carriers N	Carriers %
HapMap	CEU	17	56	68	90	0.319	141	73	51.8
	Toskani, Italia	9	45	34	63	0.358	88	54	61.4
HGDP	France	7	28	17	42	0.404	52	35	67.3
	Italy	5	18	13	28	0.389	36	23	63.9
	Italy (Bergamo)	3	2	9	8	0.286	14	5	35.7
	Orkney Islands	1	11	3	13	0.433	15	12	80.0
Total	**European ancestry**	**42**	**160**	**144**	**244**	**0.353**	**346**	**202**	**58.4**

Alongside *BTNL3* and *BTNL8*, *BTNL2* and *BTN3A1* were also implicated in CeD risk. Goudey, *et al.* [19] have identified 14 SNPs associated with CeD, independent of the known CeD risk *HLA* loci in a study of 763 CeD and 1420 control samples. One of the SNPs was located in the proximity of *BTNL2*, a gene harbouring among the highest density of GWAS hits in autoimmune and inflammatory diseases from the butyrophilin family [42–48]. Goudey, *et al.* [19] showed that this SNP was marked as being an expression quantitative trait locus (eQTL) for the *BTNL2* gene in RegulomeDB, a database annotating the function of non-coding SNPs [49,50]. Furthermore, RegulomeDB also reported high evidence for transcription factor binding for this eQTL [19].

In a separate a paediatric study of 26 active CeD, 5 treated CeD and 25 control subjects, *BTN3A1* expression was associated with active CeD in children [20]. The study examined the differential

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expression of more than 25 defence-related genes in the three subject groups, demonstrating the upregulation of *BTN3A1* mRNA and protein expression in the intestinal epithelial cells of children with active CeD. This is an intriguing finding, as BTN3A1 is required for the phosphoantigen (pAg)-induced activation of Vγ9Vδ2+ T cells in peripheral blood, a subset of γδ T cells not previously been implicated in CeD. These two studies indicate that the full functions and roles of the butyrophilin family of proteins in immunomodulation remain to be explored.

These findings raise a previously unexplored question about CeD risk. Do certain individuals co-inherit polymorphisms in their *TRGV4* gene segments and their butyrophilin family genes that predispose them to CeD. In this study, using a 94 patient discovery cohort and the UK Biobank's genome-wide genotyping dataset of 25 192 participants for validation, we show that 14 *BTN2A1*, 10 *BTN3A1*, and 13 *BTN3A2* SNPs are significantly associated with CeD status.

2. Results

2.1. BTN2A1 SNPs Were Significantly Associated with CeD Risk in a Study of 94 Samples

To investigate the association between butyrophilin genes and CeD risk, a cohort of 48 CeD and 46 control patients was examined. The *HLA* loci and selected butyrophilin genes of interests were sequenced from patient samples. The following butyrophilin genes were selected as putative CeD risk loci based on their gene expression profile in the duodenum, small intestines and immune cells (Table E1-E2) and their role in immunomodulation: *BTN2A1*, *BTN2A2*, *BTN3A1*, *BTN3A2*, *BTN3A3*, *BTNL2*, *BTNL3*, *BTNL8*, *ERMAP*, and *MOG*.

2.1.1. Risk Associated HLA Genotypes Were Significantly More Frequent in CeD Patients

First, by way of data quality control, the *HLA* genotypes of the samples were examined. In accordance with previous literature, the majority of the CeD patients had CeD risk associated *HLA* genotypes (Table 3a) [11,13,51]. The percentage of individuals identified as having one or more *HLA* risk genotypes from the CeD group was 95.8% (46/48). Only two CeD patients had *HLA* genotypes other than the CeD risk associated ones (4.17%) (Table 3b). In contrast, non-risk *HLA* genotypes were the most frequent in the control group (54.3%, 25/46) (Figure A1, Fisher's exact test, p = 5.5E-10). To summarise, CeD risk associated *HLA* genotypes were significantly more frequent in CeD patients than in the controls of this dataset.

Table 3. CeD associated *HLA* genotypes were found in 95.8% of CeD patients (n = 48) and 45.7% of controls (n = 46). a) The CeD risk associated *HLA* genotypes were called using HLA-HD [52]. b) The *HLA-DQA1* and *HLA-DQB1* alleles of the two CeD patients, who did not have CeD risk associated *HLA* genotypes. The *HLA-DQA1* allele of sample CD1 could not be typed by HLA-HD. CeD patients possessed a significantly higher proportion of risk *HLA* genotypes, when compared with controls in this dataset (Figure A1, Fisher's exact test, p = 5.5e-10).

a)

***HLA* genotypes**	***HLA-DQ2.5***	***HLA-DQ8***	***HLA-DQ2.2***	***HLA-DQ2.5 & DQ8***	***HLA-DQ2.5 & DQ2.2***	***HLA-DQ8 & DQ2.2***	**Other**
CeD patients	22	3	2	6	13	0	2
Control participants	10	2	6	0	1	2	25

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b)

Sample	*HLA-DQA1* allele	*HLA-DQB1* allele	Potential HLA-DQ type
CB26	*HLA-DQA1*01:01:01, HLA-DQA1*02:01*	*HLA-DQB1*02:01:01, HLA-DQB1*05:01:01*	HLA-DQ5.1
CD1	Not typed	*HLA-DQB1*02:01:01, HLA-DQB1*06:02:01*	Unknown

2.1.2. The BTNL8*BTNL3 Copy Number Variant Was not Associated with CeD

Next, the *BTNL8-BTNL3* loci were examined for the presence of the deletion CNV. This CNV encodes a truncated fusion protein lacking the BTNL3-IgV extracellular domain required for maintaining the duodenal Vγ4+ γδ T cells, which we hypothesised could increase CeD risk [21,29,31,41].

The presence of the CNV was determined using a surrogate SNP. Dart, *et al.* [53] identified the rs72494581 minor allele to be associated with the presence of the deletion variant. Using this method, 58.3% (28/48) of the CeD patients and 47.8% (22/46) of the control participants were found to possess at least one deletion variant, which is in accordance with the findings of Aigner, *et al.* [41] (Table 4). Interestingly, 10.9% (5/46) of controls were homozygous for the *BTNL8*BTNL3* deletion compared to only 4.2% (2/48) of CeD patients, but this did not reach statistical significance (Figure A2, Fisher's exact test, $p = 0.2144$).

Table 4. At least 47% of CeD patients (n = 48) and controls (n = 46) had the *BTNL8*BTNL3* deletion variant using the rs72494581 surrogate SNP. The *BTNL8*BTNL3* deletion variant encodes a truncated BTNL8-BTNL3 fusion protein [41]. Dart, *et al.* [53] identified rs72494581 in the intronic region of *BTNL3*, which serves as a surrogate SNP and the alleles are associated with the *BTNL8*BTNL3* copy number variant. The major allele, the *T* allele, is associated with the full length *BTNL3* and *BTNL8* genes, while the minor allele, the *C* allele, is associated with the *BTNL8*BTNL3* deletion. The differences between the frequencies in CeD and control individuals failed to reach statistical significance (Figure A2, Fisher's exact test, $p = 0.2144$).

rs72494581 genotype	***TT***	***CT***	***CC***
***BTNL8-BTNL3* genes**	Homozygous for full length sequence	Heterozygous for *BTNL8*BTNL3* deletion	Homozygous for *BTNL8*BTNL3* deletion
Coeliac disease patients	20	26	2
Control participants	24	17	5

2.1.3. BTN2A1 Gene Burden was Significantly Higher in CeD Patients

To determine whether any of the butyrophilin family variants were associated with CeD risk, gene-based burden testing, using the TRAPD program [54], was carried out on the non-synonymous coding variants identified in the CeD patients of the cohort. The variants identified in CeD samples were burden tested against variants present in the control samples.

The analysis was carried out on the qualifying variants that passed read depth filtering. Of the 108 and 58 non-synonymous coding variants discovered in the CeD and control samples respectively, only 5 bi-allelic SNPs shared by both the CeD and control groups qualified for burden testing (Table 5, Table A1-A2). Only *BTN2A1* variants were significantly associated with CeD risk gene burden in both the dominant ($p = 1.46E-05$) and the recessive ($p = 3.70E-08$) models, indicating that the presence of a single qualifying *BTN2A1* SNP significantly increased CeD risk (Table 5b). *BTN2A1* variants were more frequent in CeD patients, as 45.8% (22/48) of CeD participants had at least one qualifying *BTN2A1* variant compared to 10.9% (5/46) of controls. To summarise, the gene burden analysis of butyrophilin genes in CeD patients compared with controls showed a significant association between *BTN2A1* gene burden and CeD risk.

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Table 5. Gene-based burden testing of butyrophilin family non-synonymous coding variants in CeD patients (n = 48) against controls (n = 46) showed significant differences in the disease burden of *BTN2A1* variants.

a)

Gene	Qual. SNPs	CeD N(≥1 HET)	CeD N(≥2 HET)	CeD N(HOM ALT)	CeD total allele count	Control N(≥1 HET)	Control N(≥2 HET)	Control N(HOM ALT)	Control total allele count	Dominant model p-value	Recessive model p-value
BTN2A1	3	22	21	3	81	5	4	0	13	**1.46E-05**	**3.70E-08**
BTN3A2	1	5	0	1	7	9	0	1	11	0.929	0.946
ERMAP	1	21	0	8	37	20	0	7	34	0.516	0.988

b) *BTN2A1* variants significantly associated with CeD risk

Position (GRCh38)	rsID	Variation	Impact	HET CeD	HOM ALT CeD	HET control	HOM ALT control	Alt allele in CeD	Alt allele in controls
6:26463432	rs13195509	G>A	Missense variant, Val>Met	43.8% (21)	6.3% (3)	8.7% (4)	0.0% (0)	27.1% (26)	4.3% (4)
6:26468098	rs3734542	G>A	Missense variant, Arg>Gln	45.8% (22)	6.3% (3)	10.9% (5)	0.0% (0)	29.2% (28)	5.4% (5)
6:26468317	rs3734543	G>C	Missense variant, Gly>Ala	43.8% (21)	6.3% (3)	8.7% (4)	0.0% (0)	28.1% (27)	4.3% (4)

Non-synonymous coding variants that were predicted to be pathogenic or had low minor allele frequencies were considered qualifying variants for burden testing. a) Burden tests were carried out using the TRAPD program [54] on butyrophilin family qualifying variants in CeD patients (n = 48) against controls (n = 46). Multiallelic sites were separated into bi-allelic SNPs, as required by the TRAPD documentation [55]. Only sites where more than 90% of samples had a read depth coverage of >10 were selected.

The dominant model defines carriers for gene burden as individuals with at least one qualifying variant within a gene, while the recessive model requires at least two or more qualifying variants. Significant results were highlighted in bold. A version of table a) with the percentage of individuals and alleles within the CeD and the control groups can be found in Table A2.

b) shows the *BTN2A1* qualifying SNPs having significant burden in CeD samples. Count data of individuals and alleles are in parentheses after the percentage value in columns 6-9 and columns 10-11 respectively. The percentage and count data were calculated from the per sample genotypes found in Table A3.

Abbreviations: Alt allele: alternative or minor allele; CeD: coeliac disease; GRCh38: Genome Reference Consortium Human Build 38; HET: heterozygous, HOM ALT: homozygous for alternative allele; N(≥1 HET): number of individuals carrying at least one heterozygous qualifying variant within the gene; N(≥2 HET): number of individuals carrying at least two heterozygous qualifying variant within the gene; N(HOM ALT): number of individuals carrying at least one homozygous qualifying variant within the gene; qual: qualifying; SNP: single nucleotide polymorphism

Although these results were promising, due to the *BTN2A1* gene being part of the extended MHC region and its close proximity (~4Mb) to the classical MHC region (6p21.3), we could not exclude the possibility that this significant association could be secondary to the risk associated *HLA* genotypes of the CeD patients [56,57]. Therefore, these results were validated in a larger cohort using

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the UK Biobank dataset, by single variant testing *BTN3A1*, *BTN3A2*, *BTN2A1*, *BTNL3*, and *BTNL8* SNPs present in the 500,000 genome-wide genotyping dataset.

2.2. BTN3A1, BTN3A2, and BTN2A1 Genes Were significantly Associated with CeD in HLA-DQ2.5-Matched Participants of the UK Biobank Database

The UK Biobank dataset was used to validate the association between *BTN2A1* and CeD risk, and to investigate the association between CeD and butyrophilin SNPs in potentially CeD-relevant genes. After removing participants with missing HLA imputation or genotype data, the final cohort consisted of 3094 CeD patients and 29 762 control participants (Appendix B).

2.2.1. Risk Associated HLA Genotypes Were Significantly More frequent In CeD Patients of the UK Biobank

First, as a means of quality control for CeD diagnosis, the *HLA* genotypes of the CeD and control participants of the UK Biobank were examined. The majority of participants selected from the 500,000 genome-wide genotyping dataset had CeD risk *HLA* genotypes regardless of their CeD status (Table 6, Figure C1). Risk *HLA* genotypes were found in 92.4% (2860/3094) of CeD patients and 57.6% (17 144/29 762) of controls. In both control and CeD participants, *HLA-DQ2.5* was the most frequent *HLA* genotype at 21.6% (6416/29 762) and 53.4% (1652/3094) respectively. Interestingly, *HLA-DQ8* was the second most frequent risk genotype in controls at 14.1% (4203/29 762). Meanwhile individuals heterozygous for HLA-DQ2.5/HLA-DQ8 were the second most frequent in the CeD group, with 19.6% (606/3094) of participants possessing that risk *HLA* genotype.

Table 6. CeD associated *HLA* genotypes were found in 92.4% of CeD (n = 3094) and 57.6% of control (n = 29 762) participants from the UK Biobank's 500,000 genome-wide genotyping dataset. The *HLA* genotype of selected participants from the 500,000 genome-wide genotyping dataset were called using the HLA imputation values provided by the UK Biobank. CeD risk *HLA* genotypes were significantly more frequent in CeD patients compared to control participants (X-squared = 4062.5, df = 6, $p < 2.2E-16$).

***HLA* genotypes**	***HLA-DQ2.5***	***HLA-DQ8***	***HLA-DQ2.2***	***HLA-DQ2.5 & DQ8***	***HLA-DQ2.5 & DQ2.2***	***HLA-DQ8 & DQ2.2***	**Other**
CeD participants	1652	171	199	606	182	50	234
Control participants	6416	4203	4154	895	886	590	12618

To compare the proportion of CeD risk associated *HLA* genotypes in CeD and control participants in the 500,000 genome-wide genotyping dataset, a chi-square test of independence was used. Similar to the results from the targeted butyrophilin and *HLA* sequencing dataset, the CeD participants had significantly higher proportions of CeD risk *HLA* genotypes compared with controls (X-squared = 4062.5, df = 6, $p < 2.2E-16$).

Indeed, when the association between the CeD risk *HLA* genotypes and CeD status was investigated using a binomial regression model in the UK Biobank dataset, the association between the risk *HLA* genotypes and CeD status was confirmed. Interestingly in the regression analysis, all risk *HLA* genotypes were significantly associated with CeD ($p \leq 5.13E-04$, Table C1) except the *HLA-DQ8* genotype ($p = 0.125$).

2.2.2. BTN2A1, BTN3A1, and BTN3A2 SNPs Were Significantly Associated with CeD Status in the UK Biobank

Single variant analyses were carried out to test the association between CeD status and SNPs from the *BTN3A1*, *BTN3A2*, *BTNL3*, and *BTNL8* genes in the UK Biobank [58]. Due to the genotyping array used by the UK Biobank, the genetic information of only a limited number of SNPs from each

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gene was available. A total of 101 butyrophilin SNPs were selected for analysis (Table 7). As the *HLA* loci were significantly associated with CeD risk [1], and the *BTN3A1* and *BTN3A2* loci are in close proximity [22,24], the CeD risk *HLA* genotypes were also taken into account for the single variant analyses by including the risk *HLA* genotypes in the binomial models, and analysing the association between butyrophilin SNPs and CeD status in HLA-matched case-control groups as well. The genetic associations were tested by building binomial regression models, where the association between each variable and CeD status was examined.

Table 7. SNPs of selected butyrophilin genes present in the UK Biobank.

Gene	SNPs in NCBI	Unique SNPs in NCBI	SNPs in UK Biobank
BTN2A1	7912	7605	30
BTN3A1	5348	5164	27
BTN3A2	5905	5611	21
BTNL3	6164	5929	10
BTNL8	18 889	18 197	13

Each of the 101 selected SNPs were individually tested for association with CeD status in the UK Biobank. Thirty-seven SNPs were significantly associated with CeD status in the UK Biobank: 14 *BTN2A1*, 10 *BTN3A1*, and 13 *BTN3A2* SNPs (adjusted p-value ≤ 0.05, Table 8, Table C2-C3). Most of the significant SNPs were non-coding, with 25 of the 37 SNPs being located in intronic regions. Only one *BTN3A1* (rs41266839), and 3 *BTN2A1* (rs13195509, rs3734542, rs3734543) SNPs were missense variants and one *BTN2A1* (rs13195402) SNP encoded a STOP codon. Of the 37 SNPs, the reference alleles of 30 SNPs were associated with a decreased CeD risk. No *BTNL3* nor *BTNL8* SNPs were significant in predicting CeD status in the UK Biobank dataset, after Bonferroni correction.

Table 8. SNPs from *BTN2A1, BTN3A1,* and *BTN3A2* genes were significantly associated with CeD status in the UK Biobank. The name of the SNPs in the UK Biobank database is a combination of the reference SNP ID (rsID) from the SNP database (dbSNP) and the reference allele. All *BTN2A1*, *BTN3A1*, *BTN3A2*, *BTNL3*, and *BTNL8* SNPs in the UK Biobank were subjected to single variant testing to examine their association with CeD. Due to multiple testing, Bonferroni correction was applied. SNPs with a negative ln(OR) are associated with lower CeD risk in this binomial model, meaning that the reference allele is less frequent in CeD patients. SNPs in bold remained significantly associated with CeD in the binomial regression models that also took the *HLA* genotype into account. SNP count and allele count data for the significant SNPs is found in Table C3.

Position (GRCh38)	SNP, reference allele	Gene	SNP consequence	CeD allele freq	Control allele freq	Total allele freq	ln(OR)	CeD risk	adjusted p-value
6:26463347	**rs13195402**	***BTN2A1***	**STOP gained**	**0.768**	**0.892**	**0.880**	**-0.924**	**decrease**	**4.67E-158**
6:26463432	**rs13195509**	***BTN2A1***	**missense**	**0.754**	**0.879**	**0.867**	**-0.857**	**decrease**	**1.61E-151**
6:26475927	rs1407045	*BTN2A1*	intronic	0.584	0.516	0.522	0.273	increase	6.07E-22
6:26465807	rs2273558	*BTN2A1*	intronic	0.583	0.677	0.667	-0.396	decrease	1.69E-41
6:26460493	rs2893856	*BTN2A1*	intronic	0.113	0.131	0.130	-0.175	decrease	3.23E-03
6:26468098	**rs3734542**	***BTN2A1***	**missense**	**0.753**	**0.878**	**0.867**	**-0.855**	**decrease**	**8.59E-151**
6:26468317	**rs3734543**	***BTN2A1***	**missense**	**0.760**	**0.879**	**0.868**	**-0.844**	**decrease**	**1.59E-140**
6:26466954	rs3799380	*BTN2A1*	intronic	0.683	0.790	0.780	-0.549	decrease	8.59E-77
6:26474343	**rs56296968**	***BTN2A1***	**intronic**	**0.696**	**0.807**	**0.796**	**-0.604**	**decrease**	**9.70E-89**
6:26456215	rs6456724	*BTN2A1*	2kb upstream	0.113	0.131	0.130	-0.176	decrease	2.87E-03
6:26458037	rs6929846	*BTN2A1*	5′ UTR	0.146	0.174	0.172	-0.206	decrease	3.60E-06
6:26473816	rs7773938	*BTN2A1*	intronic	0.696	0.806	0.796	-0.600	decrease	1.15E-87
6:26469647	**rs9358944**	***BTN2A1***	**intronic**	**0.695**	**0.806**	**0.796**	**-0.604**	**decrease**	**1.55E-89**

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6:26471886	**rs9358945**	***BTN2A1***	**intronic**	**0.694**	**0.806**	**0.796**	**-0.606**	**decrease**	**4.37E-90**
6:26404730	rs10456045	*BTN3A1*	intronic	0.596	0.698	0.688	-0.448	decrease	2.97E-57
6:26410572	rs1796520	*BTN3A1*	intronic	0.405	0.474	0.467	-0.276	decrease	2.40E-22
6:26404146	**rs3799378**	***BTN3A1***	**intronic**	**0.653**	**0.762**	**0.752**	**-0.535**	**decrease**	**2.92E-75**
6:26405825	rs3857549	*BTN3A1*	intronic	0.948	0.935	0.936	0.221	increase	1.53E-02
6:26409662	**rs41266839**	***BTN3A1***	**missense**	**0.764**	**0.892**	**0.880**	**-0.924**	**decrease**	**2.12E-168**
6:26407180	rs4609015	*BTN3A1*	intronic	0.871	0.854	0.855	0.141	increase	3.82E-02
6:26412860	rs6900725	*BTN3A1*	intronic	0.870	0.853	0.855	0.139	increase	4.33E-02
6:26401210	rs6912853	*BTN3A1*	2kb upstream	0.863	0.844	0.846	0.153	increase	7.85E-03
6:26413007	rs6920986	*BTN3A1*	intronic	0.870	0.854	0.856	0.138	increase	4.99E-02
6:26415409	rs742090	*BTN3A1*	500b downstream	0.406	0.474	0.468	-0.276	decrease	3.58E-22
6:26374321	rs11758089	*BTN3A2*	intronic	0.866	0.844	0.846	0.176	increase	6.30E-04
6:26372558	**rs12176317**	***BTN3A2***	**intronic**	**0.744**	**0.867**	**0.856**	**-0.809**	**decrease**	**6.72E-140**
6:26366990	**rs12199613**	***BTN3A2***	**intronic**	**0.514**	**0.612**	**0.602**	**-0.400**	**decrease**	**1.76E-47**
6:26377318	**rs1977**	***BTN3A2***	**3′ UTR**	**0.740**	**0.864**	**0.853**	**-0.808**	**decrease**	**1.17E-136**
6:26377363	**rs1979**	***BTN3A2***	**3′ UTR**	**0.743**	**0.867**	**0.855**	**-0.809**	**decrease**	**8.23E-140**
6:26375933	**rs1985732**	***BTN3A2***	**intronic**	**0.595**	**0.698**	**0.688**	**-0.457**	**decrease**	**2.87E-59**
6:26374430	rs2073526	*BTN3A2*	intronic	0.370	0.442	0.435	-0.295	decrease	9.15E-25
6:26363527	**rs9358934**	***BTN3A2***	**2kb upstream**	**0.744**	**0.866**	**0.855**	**-0.803**	**decrease**	**2.34E-137**
6:26364702	**rs9379855**	***BTN3A2***	**2kb upstream**	**0.743**	**0.866**	**0.855**	**-0.804**	**decrease**	**8.85E-138**
6:26367461	**rs9379858**	***BTN3A2***	**intronic**	**0.743**	**0.866**	**0.855**	**-0.802**	**decrease**	**3.19E-137**
6:26369321	**rs9379859**	***BTN3A2***	**intronic**	**0.744**	**0.867**	**0.855**	**-0.803**	**decrease**	**5.37E-137**
6:26373450	**rs9393713**	***BTN3A2***	**intronic**	**0.743**	**0.868**	**0.856**	**-0.814**	**decrease**	**1.07E-141**
6:26373512	**rs9393714**	***BTN3A2***	**intronic**	**0.743**	**0.868**	**0.856**	**-0.813**	**decrease**	**6.99E-141**

Abbreviations: CeD: coeliac disease; freq: frequency; GRCh38 Genome Reference Consortium Human Build 38; kb: kilobase, ln(OR): natural logarithm of the odds ratio; SNP: single nucleotide polymorphism; UTR: untranslated region.

2.2.3. Twenty Butyrophilin SNPs from the UK Biobank Remained Significantly Associated with CeD Status When the Participants' risk HLA Genotypes Were Taken into Account

To investigate whether the butyrophilin SNPs in the UK Biobank remained significantly associated with CeD status when taking the *HLA* loci into account, a second set of binomial regression models was produced. Single variant models were built for each of the 101 SNPs of interest, that included the risk *HLA* genotypes of the UK Biobank participants as an additional predictor variable (Table C4). Only 7 *BTN2A1*, 2 *BTN3A1*, and 11 *BTN3A2* SNPs remained significantly associated with CeD status after applying Bonferroni correction (adjusted $p \leq 0.05$, Table 9). Similarly to the previous model, the majority of the significant SNPs were non-coding with the exception of a STOP gained SNP (rs13195402) and 3 missense SNPs (rs13195509, rs3734542, rs3734543) in the *BTN2A1* gene. Out of the 17 non-coding SNPs, 11 SNPs were located in intronic regions. The reference alleles for all 20 SNPs were associated with a decreased CeD risk, meaning that the alternate alleles were more frequent in CeD patients. As the *HLA* loci were taken into account, these SNPs are likely to be real associations with CeD status, instead of being caused by linkage disequilibrium (LD) due to the proximity of the *BTN* and *HLA* loci on chromosome 6.

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Table 9. Twenty SNPs from *BTN2A1*, *BTN3A1*, and *BTN3A2* genes were significantly associated with CeD status in the UK Biobank when *HLA* genotypes were included in the single variant testing models. *BTN2A1*, *BTN3A2*, *BTNL3*, and *BTNL8* SNPs in the UK Biobank were subjected to single variant testing to examine their association with CeD. Due to multiple testing, Bonferroni correction was applied. SNPs with a negative ln(OR) are associated with lower CeD risk in this binomial model.

SNP, reference allele	Gene	SNP consequence	ln(OR)	CeD risk	adjusted p-value
rs13195402	*BTN2A1*	STOP gained	-0.20727	decrease	8.15E-06
rs13195509	*BTN2A1*	missense	-0.19239	decrease	1.62E-05
rs3734542	*BTN2A1*	missense	-0.18831	decrease	2.94E-05
rs3734543	*BTN2A1*	missense	-0.16744	decrease	8.23E-04
rs56296968	*BTN2A1*	intronic	-0.11753	decrease	4.20E-02
rs9358944	*BTN2A1*	intronic	-0.11786	decrease	3.83E-02
rs9358945	*BTN2A1*	intronic	-0.12018	decrease	2.91E-02
rs3799378	*BTN3A1*	intronic	-0.14327	decrease	7.04E-04
rs41266839	*BTN3A1*	missense	-0.21469	decrease	1.06E-06
rs12176317	*BTN3A2*	intronic	-0.1974	decrease	3.50E-06
rs12199613	*BTN3A2*	intronic	-0.12296	decrease	3.31E-03
rs1977	*BTN3A2*	3′ UTR	-0.20238	decrease	2.06E-06
rs1979	*BTN3A2*	3′ UTR	-0.19756	decrease	3.40E-06
rs1985732	*BTN3A2*	intronic	-0.10975	decrease	3.35E-02
rs9358934	*BTN3A2*	2kb upstream	-0.19286	decrease	7.53E-06
rs9379855	*BTN3A2*	2kb upstream	-0.19406	decrease	6.04E-06
rs9379858	*BTN3A2*	intronic	-0.19156	decrease	8.99E-06
rs9379859	*BTN3A2*	intronic	-0.19261	decrease	8.10E-06
rs9393713	*BTN3A2*	intronic	-0.2056	decrease	9.27E-07
rs9393714	*BTN3A2*	intronic	-0.20087	decrease	2.08E-06

Abbreviations: CeD: coeliac disease; ln(OR): natural logarithm of the odds ratio; SNP: single nucleotide polymorphism.

2.2.4. Twenty-One Butyrophilin SNPs Were Significantly Associated with CeD Status in HLA-DQ2.5-Matched Case-Control Groups of UK Biobank Participants

The final set of analyses were carried out to investigate whether the butyrophilin SNPs were significantly associated with CeD status in all of the CeD risk *HLA* genotype patients. Therefore, the UK Biobank participants were separated into risk *HLA*-matched CeD and control groups (Table 10). All 101 butyrophilin SNPs were single variant tested for their association to CeD status in the *HLA*-matched groups.

Table 10. Single variant testing in *HLA*-matched groups from the UK Biobank dataset only identified significant SNPs associated with CeD status in individuals with *HLA-DQ2.5* genotypes. The CeD and control participants of the UK Biobank dataset were divided into *HLA*-matched case-control groups for single variant testing. The association between *BTN2A1*, *BTN3A1*, *BTN3A2*, *BTNL3*, and *BTNL8* SNPs and CeD status was investigated. Significant association between the SNPs and CeD status was only present in *HLA-DQ2.5*-matched individuals (in bold).

HLA genotype of individuals in model	Number of CeD participants	Number of controls	Number of significant SNPs
HLA-DQ2.2	199	4154	0
HLA-DQ2.5	**1652**	**6416**	**21**
HLA-DQ8	171	4203	0
HLA-DQ2.2, HLA-DQ2.5	606	895	0

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HLA-DQ2.2, HLA-DQ8	50	590	0
HLA-DQ2.5, HLA-DQ8	182	886	0
Other	234	12618	0

HLA-DQ2.5 was the most common risk *HLA* genotype in CeD patients both in the UK Biobank, as well as in previous studies [11,12]. Interestingly, significant association between butyrophilin SNPs and CeD status was only present in the *HLA-DQ2.5*-matched UK Biobank participants. The *BTN2A1*, *BTN3A1*, and *BTN3A2* SNPs significantly associated with CeD status in the *HLA* single variant testing models remained significant in the *HLA-DQ2.5*-matched tests as well (Table 11, Table C5). Interestingly, rs7773938, an intronic *BTN2A1* SNP, is a novel SNP that was only significantly associated with CeD status in UK Biobank participants with the *HLA-DQ2.5* genotype. The reference alleles of all 21 significant SNPs were more frequent in controls compared to CeD individuals, meaning that having the alternate allele at these loci significantly increases an individual's CeD risk. These results imply that butyrophilin SNPs could only explain additional CeD risk in HLA-DQ2.5-matched individuals of the UK Biobank. As the presence of these reference alleles remained significantly associated with decreased CeD risk even after *HLA*-matching, the association with these SNPs was not likely to be caused by LD to the *HLA* loci. Therefore, the 21 butyrophilin SNPs identified were significantly associated with CeD status and contributed to further CeD risk in UK Biobank participants possessing the *HLA-DQ2.5* genotype.

Table 11. Butyrophilin SNPs only remained significantly associated with CeD status in the *HLA-DQ2.5* restricted UK Biobank analysis. The name of the SNPs in the UK Biobank database are a combination of the SNP name and the reference allele. All *BTN2A1*, *BTN3A1*, *BTN3A2*, *BTNL3*, and *BTNL8* SNPs in the UK Biobank were subjected to single variant testing to examine their association with CeD. Due to multiple testing, Bonferroni correction was applied. SNPs with a negative ln(OR) are associated with lower CeD risk in this binomial model. The SNP in bold was a novel SNP significantly associated with CeD unique to the *HLA-DQ2.5* model, while the other SNPs were also significant in the non-*HLA* and the *HLA* models.

SNP, reference allele	Gene	SNP consequence	CeD allele freq	Control allele freq	Total allele freq	ln(OR)	CeD risk	adjusted p-value
rs13195402	*BTN2A1*	STOP gained	0.704	0.751	0.741	-0.27812	decrease	5.10E-07
rs13195509	*BTN2A1*	missense	0.687	0.734	0.724	-0.25542	decrease	1.75E-06
rs3734542	*BTN2A1*	missense	0.687	0.733	0.723	-0.25235	decrease	2.52E-06
rs3734543	*BTN2A1*	missense	0.695	0.736	0.728	-0.23279	decrease	5.43E-05
rs56296968	*BTN2A1*	intronic	0.640	0.673	0.666	-0.15928	decrease	2.33E-02
rs7773938	***BTN2A1***	**intronic**	**0.640**	**0.672**	**0.666**	**-0.15755**	**decrease**	**2.66E-02**
rs9358944	*BTN2A1*	intronic	0.638	0.671	0.664	-0.16238	decrease	1.61E-02
rs9358945	*BTN2A1*	intronic	0.638	0.671	0.665	-0.16499	decrease	1.26E-02
rs3799378	*BTN3A1*	intronic	0.596	0.637	0.628	-0.18712	decrease	8.10E-04
rs41266839	*BTN3A1*	missense	0.697	0.747	0.737	-0.28267	decrease	7.25E-08
rs12176317	*BTN3A2*	intronic	0.679	0.729	0.719	-0.26575	decrease	2.82E-07
rs12199613	*BTN3A2*	intronic	0.459	0.500	0.491	-0.1717	decrease	2.06E-03
rs1977	*BTN3A2*	3′ UTR	0.676	0.726	0.716	-0.268	decrease	2.99E-07
rs1979	*BTN3A2*	3′ UTR	0.679	0.728	0.718	-0.26376	decrease	3.63E-07
rs1985732	*BTN3A2*	intronic	0.539	0.574	0.567	-0.15063	decrease	2.35E-02
rs9358934	*BTN3A2*	2kb upstream	0.680	0.729	0.719	-0.25825	decrease	8.85E-07
rs9379855	*BTN3A2*	2kb upstream	0.680	0.728	0.718	-0.25967	decrease	6.76E-07
rs9379858	*BTN3A2*	intronic	0.680	0.728	0.718	-0.25566	decrease	1.18E-06

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rs9379859	*BTN3A2*	intronic	0.681	0.729	0.719	-0.26105	decrease	6.31E-07
rs9393713	*BTN3A2*	intronic	0.678	0.729	0.719	-0.27313	decrease	1.06E-07
rs9393714	*BTN3A2*	intronic	0.679	0.729	0.719	-0.26778	decrease	2.25E-07

Abbreviations: CeD: coeliac disease; freq: frequency; ln(OR): natural logarithm of the odds ratio; SNP: single nucleotide polymorphism.

2.3. HV4 Variation was Not Significantly Associated with CeD Risk in a Study of 379 Samples

2.3.1. TRGV Usage Was not Significantly Different Between CeD and Control Samples

Previous evidence by our group showed that the γδ T-cell repertoire is permanently altered in the duodenum of CeD patients [59]. Mayassi, *et al.* [21] also showed that the *TRGV4* transcripts are lost and the γδ TCR repertoire is permanently reconfigured in the duodenum after active CeD.

To verify these results, the duodenal TRG repertoires of 108 healthy controls and 45 CeD patients were examined for potential differences in TRGV usage, which was defined as the proportion of TRGV segment read counts present within the TCR repertoire of a tissue sample, using data derived from DNA extracted from formalin fixed, paraffin embedded (FFPE) duodenal samples and sequenced using the TRG Lymphotrack kit (Invivoscribe, Inc.) and a MiSeq platform (Illumina Inc.) (Table 14). The proportions of the *TRGV* genes present in duodenum samples from neither healthy controls nor CeD patients showed a normal distribution (Figure D1). The *TRGV10*, *TRGV4*, and *TRGV2* variable (V) gene segments were the most frequent in this dataset. The mean usage proportion of the TRGV4 segment, which is capable of binding the BTNL3/BTNL8 heterodimer was 18.50% and 18.06% of the TRG repertoire in CeD and healthy control samples, respectively. When the proportion of TRGV segments was compared between the combined CeD and healthy control samples, no significant differences were found (Figure 2, Table D1).

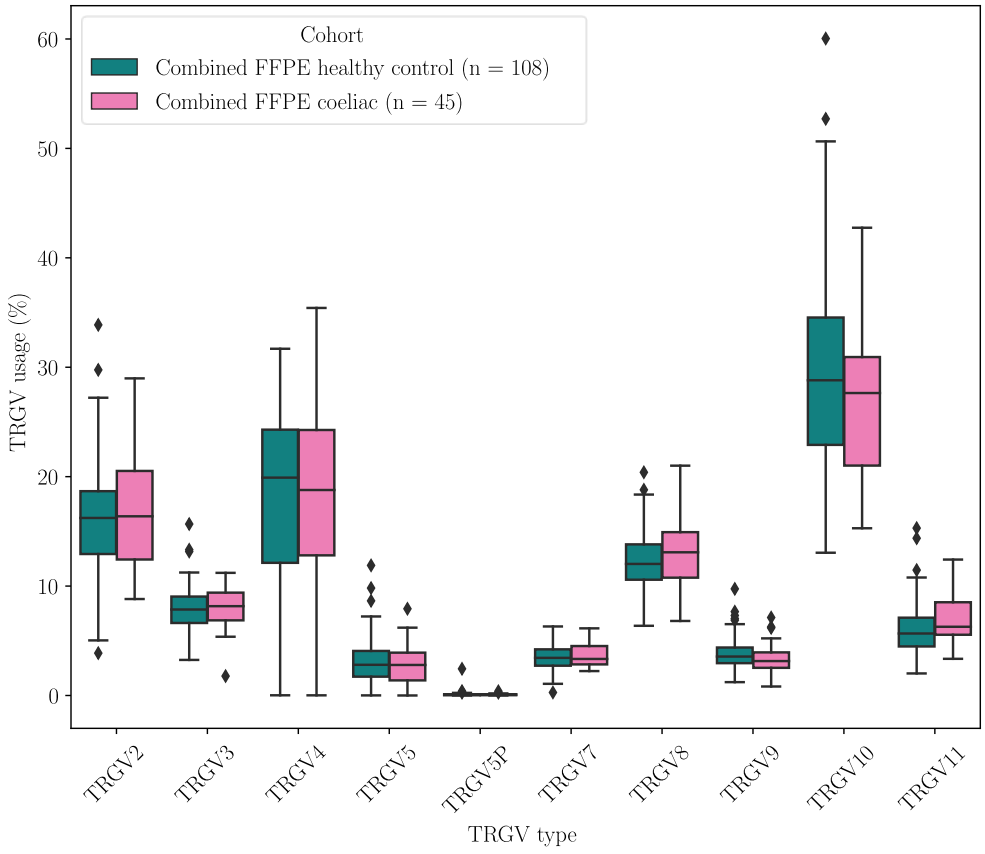

Figure 2. There were no significant differences in the TRGV usage of CeD (n = 45) and healthy control (n = 108) duodenal samples.

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2.3.2. HV4 Sequence Variation Was not Significantly Associated with CeD Risk

Next, the TRGV4-HV4 amino acid sequences were examined in 141 CeD and 238 healthy control samples (Table 14). The *HV4* is germline-encoded, and is located in the *FR3* of the *TRGV4* gene. Neither the *FR3* nor the *HV4* undergo somatic recombination in T cells. As demonstrated by Melandri, *et al.* [29] and Willcox, *et al.* [31], only HV4 loops with the wild type (reference) KYDTYGSTRKNLRMILR amino acid sequence could directly bind BTNL3. Substitutions in the amino acids underlined (KY**D**TY**G**STRKNLRMILR) were found to disrupt this direct binding between BTNL3 and Vγ4+ T cells, while substitutions in the following underlined amino acids (KYDTYGSTR**K**NLR**M**ILR) only caused a marginal reduction in binding [31]. As the HV4 is germline-encoded and does not undergo recombination, we hypothesised that variations in the germline-encoded TRGV4-HV4 amino acid sequence could alter the binding of the Vγ4+ γδ T cells to BTNL3 protein in the duodenum, predisposing to CeD.

Seven unique HV4 amino acid sequences were identified in the dataset (Table 12). The reference HV4 sequence KYDTYGSTRKNLRMILR capable of binding the BTNL3 protein was the most frequent in both healthy control (95.8%, 228/238) and CeD (97.9%, 138/141) samples. Approximately 84.9% (202/238) of healthy control samples and 82.3% (116/141) of CeD were homozygous for the WT HV4 sequence. There were no significant differences in the HV4 amino acid sequence distribution between CeD and healthy control samples (Fisher's exact test, $p = 0.26$, Figure 3, Table D2).

Table 12. More than 95% of participants possessed at least one reference HV4 loop regardless of their CeD status. The dataset consisted of 238 healthy control and 141 CeD samples. a) Seven unique HV4 amino acid sequences were identified in the dataset. b) The homozygous WT HV4 phenotype was the most frequent in both healthy control and CeD groups.

a)

HV4 amino acid sequence	Amino acid change	Effect	Freq. in healthy control samples (n = 238)	Freq. in CeD samples (n = 141)	Predicted change in binding [31]
KYDTYGSTRKNLRMILR (WT)	-	-	430/476 = 0.903	254/282 = 0.901	-
KYDTYGSTR<u>Q</u>NLRMILR	Lysine > Glutamine	Positive charge > polar uncharged	41/476 = 0.086	23/282 = 0.082	Marginal reduction in binding
KYDTYGSTRK<u>S</u>LRMILR	Asparagine > Serine	Polar uncharged > polar uncharged	4/476 = 0.008	2/282 = 0.007	Unknown
KYDTYGSTR<u>_ELENDTA</u>	Lysine > frameshift	Positive charge > different sequence	0	1/282 = 0.003	Unknown
KY<u>N</u>TYGSTRKNLRMILR	Aspartic acid > Asparagine	Negative charge > polar uncharged	0	1/282 = 0.003	Disrupted binding
KYDTYG<u>N</u>TRKNLRMILR	Serine > Asparagine	Polar uncharged > polar uncharged	1/476 = 0.002	0	Unknown
KYDTYGS<u>I</u>RKNLRMILR	Threonine > Isoleucine	Polar uncharged > apolar	0	1/282 = 0.003	Unknown

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b)

Phenotype	Combined healthy control samples (n = 238)	Combined CeD samples (n = 141)
WT	202	116
WT, KYDTYGSTRQNLRMILR	23	18
KYDTYGSTRQNLRMILR	9	2
WT, KYDTYGSTRKSLRMILR	2	2
KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA	0	1
WT, KYNTYGSTRKNLRMILR	0	1
WT, KYDTYGNTRKNLRMILR	1	0
KYDTYGSTRKSLRMILR	1	0
WT, KYDTYGSIRKNLRMILR	0	1

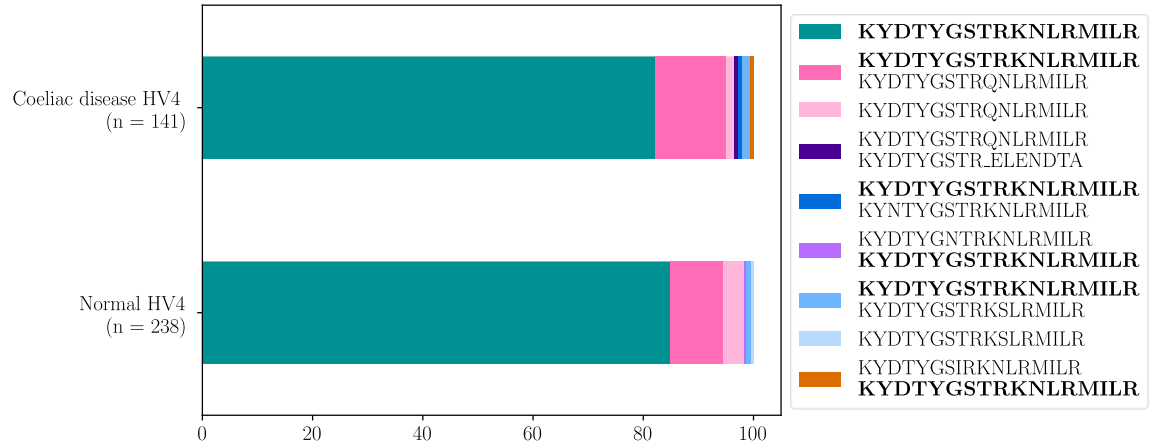

Figure 3. More than 82% of both healthy control and CeD samples were homozygous for the reference HV4 amino acid sequence in the combined cohort. The HV4 analysis was carried out on a cohort of 238 healthy control and 141 CeD samples. The homozygous, reference HV4 sequence was the most common phenotype in both CeD and healthy control samples. Only 10 healthy control and 3 CeD samples did not have any WT HV4 sequences.

To summarise, TRGV usage and HV4 amino acid sequence variation could not explain CeD risk in a dataset of 379 samples.

3. Discussion

Coeliac disease (CeD) is a T-cell mediated enteropathy triggered by the consumption of gluten, which is the collective name of a group of proteins found in wheat, rye, and barley [1]. The genetic risk for CeD is not fully understood; an estimated 50% of genetic predisposition is still unexplored [60]. Around 30% of genetic risk can be explained by the *HLA* risk genotypes *HLA-DQ2.5*, *HLA-DQ8*, and *HLA-DQ2.2*, which were first connected to CeD risk in 1972 [17,18,61,62].

Recently, the butyrophilin family of genes were proposed as non-*HLA* CeD risk loci [19–21]. These genes encode transmembrane proteins that were implicated in regulating the activity of innate and adaptive immune cells, alongside maintaining characteristic epithelial γδ T-cell populations in mice and humans [22,24]. Prior to this study, four genes were associated with CeD: *BTN3A1*, *BTNL2*, *BTNL3*, and *BTNL8* [19–21].

Therefore, we investigated the association of butyrophilin family gene variation to CeD risk via targeted sequencing of 46 healthy control and 49 CeD samples. The non-synonymous coding butyrophilin SNP data of the 94 samples were subjected to gene-based burden testing. Comparing the CeD and control SNPs, *BTN2A1* gene burden was significantly higher in CeD patients in both the dominant (p = 1.46E-05) and the recessive models (p = 3.70E-08).

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The significant association between *BTN2A1* SNPs and CeD risk was validated using the UK Biobank 500,000 genome-wide genotyping dataset. Fourteen *BTN2A1*, 10 *BTN3A1,* and 13 *BTN3A2* SNPs were significantly associated with CeD, the majority of which were non-coding variants. When the risk associated *HLA* genotypes of these participants were taken into account, only 7 *BTN2A1*, 2 *BTN3A1,* and 11 *BTN3A2* SNPs remained significant, showing *HLA* independent associations with CeD risk. Finally, butyrophilin SNPs were single variant tested in CeD risk *HLA*-matched groups. The 20 SNPs above alongside a novel intronic *BTN2A1* SNP were significant in predicting CeD status in 1652 CeD and 6416 control participants with the *HLA-DQ2.5* genotype. UK Biobank validation indicated that the *BTN3A1* and *BTN3A2* non-synonymous coding SNPs were not significantly associated with CeD risk in the hybridization capture cohort, likely due to the majority of significantly associated SNPs being non-coding, and thus excluded from the burden testing analysis.

Due to the association between butyrophilin family members and CeD status, and the involvement of butyrophilin heterodimers in shaping γδ T-cell repertoires, CeD risk was also examined from the angle of the duodenal Vγ4+ γδ T cells [21,27,28]. To investigate any likely effects of polymorphisms in the TCR γ V segment, TRGV4, on the interaction between Vγ4+ γδ T cells and the BTNL3/BTNL8 heterodimer, the TRGV4-HV4 amino acid sequences of 379 samples were examined. Mutations in the reference HV4 sequence, KY**D**TY**G**STR**K**NLR**M**ILR, specifically in the DGKM residues *in vitro* were previously shown to decrease the direct binding between BTNL3 and Vγ4+ γδ T cells [31]. The aim of this analysis was to identify germline-encoded HV4 amino acid sequence variations, that were more frequent in CeD patients. The hypothesis was that individuals having HV4 variations, especially ones that affected the DGKM residues, had higher CeD risk due to the decreased BTNL3-HV4 binding affinity. There were no significant differences in the HV4 amino acid sequence variation between CeD and healthy control samples ($p = 0.2648$). It is possible that the HV4 sequence is conserved via stabilising selection due to its interaction with BTNL3, which serves as the maintenance signal for the Vγ4+ γδ T cells in the duodenum [21].

We thus identified *BTN2A1* and *BTN3A2* as novel CeD risk loci and corroborated *BTN3A1* as a CeD risk locus. The association between *BTN3A1* and CeD is in accordance with evidence showed by Pietz, *et al.* [20], who hypothesised that pAg presentation by intestinal epithelial cells in active CeD may contribute to IFN-γ production and T cell proliferation. Indeed, all three of these butyrophilin genes are involved in the pAg-mediated, innate-like activation of peripheral blood γδ T cells [37,38,63,64]. Taking these results together, we provide a new hypothesis for the role of butyrophilins in CeD (Figure 4).

Firstly, these results could imply that BTN2A1 and BTN3A2 could also act on the duodenal Vγ4+ γδ T cells, which is a novel hypothesis as these proteins have only been implicated in activating peripheral blood Vγ9Vδ2+ γδ T cells (Figure 4a). The nature of this interaction is unexplored, and whether these proteins are responsible for pAg-dependent activation of Vγ4Vδ1+ T cells requires further research. This hypothesis could explain why the *BTNL8*BTNL3* deletion variant, which encodes a BTNL8*3 fusion protein but no full length BTNL3 or BTNL8 proteins, was not significantly associated with CeD risk in the cohort of 94 samples. Participants who are homozygous for the deletion can only express the BTNL8*3 fusion protein, which is not hypothesised to bind the Vγ4+ TCR. If BTN2A1, BTN3A1, or BTN3A2 could provide a survival signal to the Vγ4Vδ1+ IELs in the healthy small intestine, this could explain why controls could be homozygous for the BTNL3/BTNL8 deletion variant without having CeD.

Secondly, the findings of this project could suggest that BTN2A1 and BTN3 regulate duodenal immune cell activity in the context of CeD. BTN2A1 was identified as a novel ligand for the receptors of DCs, which are important cells in displaying the gluten antigen to CD4+ αβ T cells to induce CeD pathogenesis [24,25]. Although the function of this interaction was not explored, taken together with the results of this project, it could explain the association between BTN2A1 and CeD risk, as the protein could regulate the autoimmune response in CeD indirectly via DC activity (Figure 4b). Additionally, previous evidence has shown that BTN3 proteins can provide co-stimulatory signals to αβ T cells, increasing their production of interferon-γ (IFN-γ), a proinflammatory cytokine [26]. This

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same study showed the dual effect of butyrophilins on NK cell activity: BTN3A1 upregulated, while BTN3A2 downregulated IFN-γ production and NK cell activation (Figure 4c).

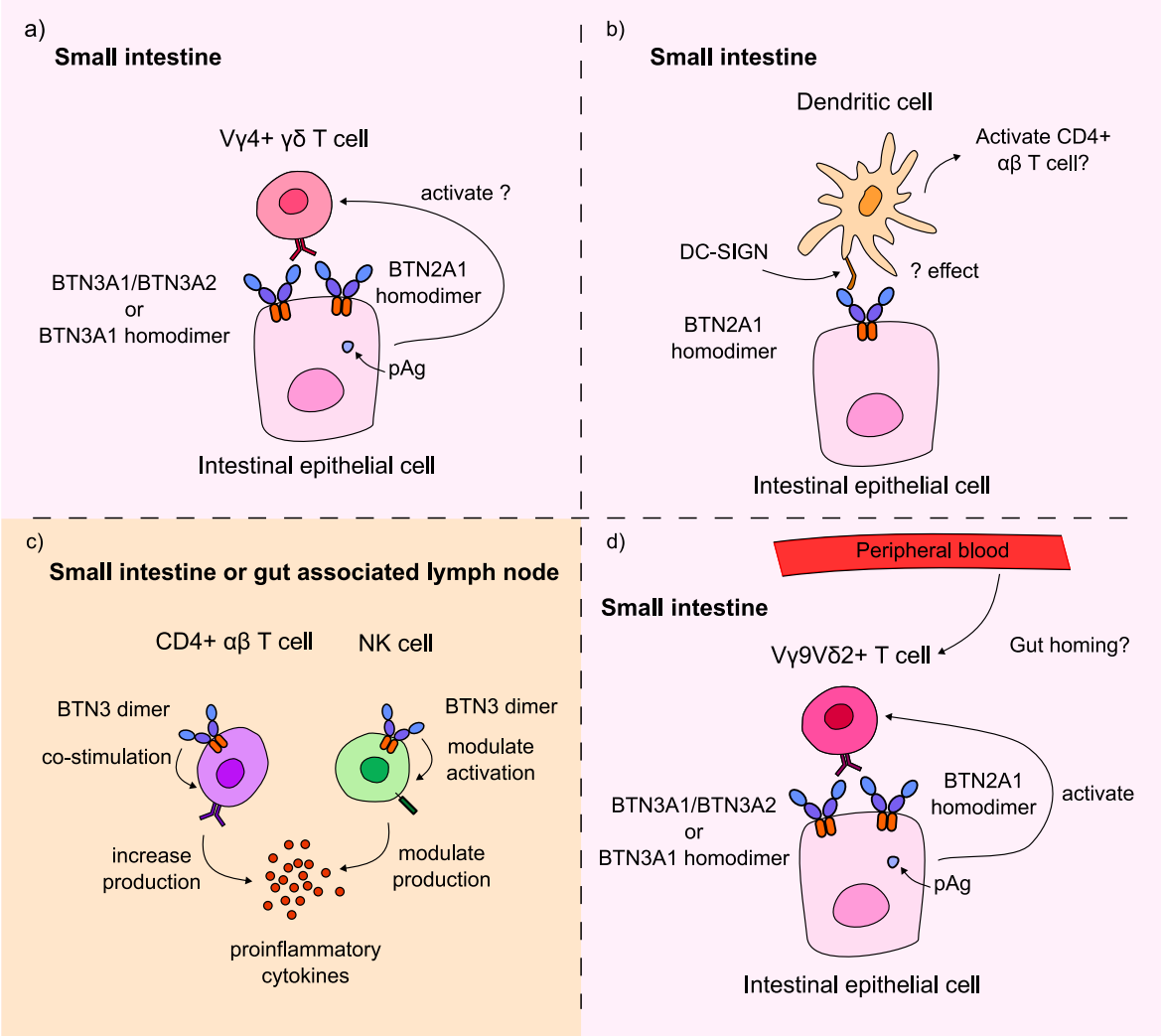

Figure 4. BTN2A1, BTN3A1 and BTN3A2 may be involved in CeD pathogenesis by modulating T cell and innate immune cell activity. *BTN2A1* gene burden was significantly higher in CeD patients in a cohort of 94 samples. Meanwhile, *BTN2A1*, *BTN3A1,* and *BTN3A2* SNPs were significantly associated with CeD status in the UK Biobank database. Based on our results and evidence on the immunomodulatory role of butyrophilins on innate and adaptive immune cells, butyrophilins could contribute to CeD pathogenesis in multiple potential manners [25,26,37,38,63]. a) Via the novel, hypothesised pAg-dependent activation of Vγ4+ γδ T cells; b) via the interaction of BTN2A1 with dendritic cells through the DC-SIGN receptor on the DC cell surface; c) by increasing the co-stimulation and IFN-γ production of CD4+ αβ T cells, or by modulating the activity and IFN-γ production of NK cells depending on whether BTN3A1 or BTN3A2 is expressed predominantly on the surface of the NK cell; or d) via the pAg-dependent activation of potentially gut-homing Vγ9Vδ2+ γδ T cells in the small intestine.

Thirdly, the results of this project could indicate the involvement of peripheral blood Vγ9Vδ2+ T cells in CeD (Figure 4d). Whether these T cells are recruited to infiltrate the small intestine from the peripheral blood or they contribute to CeD pathogenesis in a different way remains unclear. Interestingly, in the analysis of our cohort of 108 healthy control and 45 CeD duodenal samples, only 3-4% of γδ T cells were Vγ9+ T cells (Figure 2, Table 14,). There were no significant differences in the proportion of Vγ9+ T cells in CeD and healthy controls (p = 0.728). Further research is required to

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investigate the activity of these cells, and whether they interact with butyrophilins in the context of CeD.

In conclusion, the butyrophilin family of genes are promising immunomodulators involved in connecting the adaptive and innate immunity [24]. Our results provide evidence that the butyrophilin genes *BTN2A1*, *BTN3A1*, and *BTN3A2* may be putative CeD risk loci. Due to their important roles in the maintenance, activation, and regulation of γδ T cells, the butyrophilins may be involved in the pathogenesis of other autoimmune and inflammatory disorders. Our work provides a clear rationale for further research into the role of the butyrophilin family of genes in CeD.

4. Materials and Methods

4.1. Participant Selection Criteria

All patient samples used in the HV4 analysis and the targeted sequencing cohort were collected, stored and fully anonymised in line with the Human Tissue Act (England and Wales), 2004, with full ethical approval by the Soilleux group (IRAS project ID: 162057, REC reference: 04/Q1604/21, PI: Prof. E. Soilleux).

CeD patient samples were selected using hospital records, while control samples were selected to exclude suspected CeD patients.

Control exclusion criteria:

- Has CeD diagnosis
- Malabsorption
- Anaemia
- Lymphocytosis
- On a GFD
- Diarrhoea

4.1.1. Participant Selection for the Butyrophilin Family Gene Sequencing

A total of 48 CeD samples and 46 control samples were sequenced for the butyrophilin family variance analysis (Table 13).

Table 13. 94 blood and duodenal samples were sequenced to investigate CeD risk loci. a) Samples were collected from diagnostic surplus samples. b) Both blood and FFPE duodenal samples were collected and sequenced.

a)

	Healthy control sample	**Coeliac disease**
Blood sample	Cambridge Haematopathology and Oncology Diagnostic Service bone marrow donor blood samples, surplus to diagnostic requirements	Cambridge University Hospitals NHS Foundation Trust Department of Haematology surplus to diagnostic requirements
Formalin fixed, paraffin embedded (FFPE) duodenal sample	Human Research Tissue Bank of Cambridge University Hospitals NHS Foundation Trust surplus to diagnostic requirements	

b)

	Blood sample	**FFPE duodenal sample**
Coeliac disease	40	8
Control	38	8

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4.1.2. Participant Selection for the UK Biobank Single Variant Analysis

For the validation cohort, CeD patients and controls were selected from the anonymised UK Biobank online database using the online Research Analysis Platform (RAP, https://ukbiobank.dnanexus.com/, application ID: 18532). Participants' sociodemographic, lifestyle, hospital record information, HLA imputation and genome-wide genotyping data were available from the UK Biobank online resource centre (https://biobank.ndph.ox.ac.uk/).

Control and CeD participants were identified using the Cohort Browser program on the RAP, which allowed the selection of participants using a graphical user interface. Control and CeD participants were selected based on their responses to the CeD online questionnaire (data-field 21068, https://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=21086), the dietary web questionnaire (data-field 20086, https://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=20086), and their health records, specifically, their hospital inpatient record (category 2000, https://biobank.ctsu.ox.ac.uk/crystal/label.cgi?id=2000) and their death record (category 100093, https://biobank.ctsu.ox.ac.uk/crystal/label.cgi?id=100093). All clinical data of participants were classified using the World Health Organisation's International Classification of Diseases (ICD) system and made available by the UK Biobank [65]. Although most of the hospital inpatient data were coded in ICD-10, some of the participants, whose data was collected in Scotland before 1997 were described using ICD-9, as outlined in resource 138483 (https://biobank.ndph.ox.ac.uk/ukb/refer.cgi?id=138483).

Control exclusion criteria:

- Filled out CeD online questionnaire
- CeD mentioned in hospital inpatient record or death record
- Malabsorption
- Anaemia
- Lymphocytosis
- On a GFD
- Diarrhoea

Coeliac disease inclusion criteria:

- Hospital diagnosis record includes coeliac disease: ICD9 (5790), ICD10 (K90.0)
- Cause of death includes coeliac disease: ICD10 (K90.0)

After removing individuals with missing data, the finalised UK Biobank cohort consisted of 3094 CeD patients and 29 762 control participants.

4.1.3. Samples Selected for the HV4 Analysis

The sequencing data from three different datasets were used that were selected using the same criteria. A total of 141 CeD and 238 healthy control tissue samples were selected for the HV4 analysis (Table 14).

Table 14. The coeliac disease and healthy control patient TRG datasets analysed for TRGV usage and HV4 sequence variations. FFPE: formalin fixed, paraffin embedded.

	Coeliac disease	Healthy control	Sequencing method
Dataset 1	34 FFPE, 12 fresh frozen duodenal	97 FFPE duodenal	Illumina Miseq micro
Dataset 2	11 FFPE duodenal	11 FFPE duodenal	Illumina Miseq
Dataset 3	84 blood	130 blood	Illumina NextSeq
Combined	84 blood, 48 FFPE duodenal, 12 fresh frozen duodenal	130 blood, 108 FFPE duodenal	NA

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4.2. Analysis of Butyrophilin Family Variation in the Targeted Sequencing Cohort

4.2.1. Gene Selection and Hybridization Probe Design

The Human Protein Atlas (HPA), alongside previous evidence was used to select the butyrophilin family genes of interest to investigate their potential association with CeD predisposition. The expression profiles of the 15 butyrophilin family members outlined by Rhodes, *et al.* [24] were examined in the HPA in tissues and immune cells that could be involved in CeD pathology. The protein expression in the duodenum, small intestine, rectum, and colon were accessed, or their mRNA expression profiles for gene entries where only the latter was available at the time of analysis (Appendix E, Table E1). The mRNA expression of each butyrophilin gene was also accessed from the HPA in the following immune cell types: T cells, DCs, NK cells, macrophages, regulatory T-cells and γδ T cells (Table E2).

The reliability scores provided by the HPA for each entry was also considered, which is a value based on the reliability between the RNA sequencing and antibody staining data provided by the HPA.

The finalised list of 10 genes of interest were the following: BTN2A1, BTN2A2, BTN3A1, BTN3A2, BTN3A3, BTNL2, BTNL3, BTNL8, ERMAP, MOG.

The Genome Reference Consortium Human Build 38 patch release 12 (GRCh38.p12) genomic position of the 10 butyrophilin genes of interest were determined using the NCBI database [66]. The regions targeted by the hybridization probes included the non-coding and coding regions of the gene, as described by their NCBI entry at the time of creating the hybridization panel. The regions of interest were uploaded to the online Nonacus probe design platform (panel id: 890, Table 15) [67]. To ensure high coverage, 2x tiling was selected, so that each base was covered by 2 probes [68]. Gap fill was selected to cover any repetitive regions in the genes of interest. The submitted custom panel was generated by the best in class integrated algorithms of Nonacus Ltd. These algorithms created probe panels that maximised coverage of the target regions, while avoiding under or over sequencing any regions [69]. The finalised uniform coverage probe panel was approved and ordered for hybridization capture.

Table 15. The GRCh38.p12 genomic location of the selected butyrophilin genes.

Gene of interest	Location (GRCh38.p12)
BTN2A1	chr6:26,457,955-26,476,622
BTN2A2	chr6:26,382,893-26,394,874
BTN3A1	chr6:26,402,269-26,415,216
BTN3A2	chr6:26,365,170-26,378,320
BTN3A3	chr6:26,440,504-26,453,415
BTNL2	chr6:32,393,339-32,408,879
BTNL3	chr5:180,988,846-181,006,727
BTNL8	chr5:180,899,097-180,952,166
ERMAP	chr1:42,817,122-42,844,991
MOG	chr6:29,657,092-29,672,365

4.2.2. Library Capture and Sequencing of HLA Loci and Selected Butyrophilin Genes

Hybridization capture of healthy control and CeD DNA samples were modified from the Nonacus Cell3 Target Hybridisation & Capture Kit (Nonacus) version (b) protocol (Figure 5, Appendix E.2-E.3). *HLA* hybridization probes were designed and provided by Nonacus Ltd. The captured butyrophilin and *HLA* loci library were sent to the Department of Biochemistry, University

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of Cambridge for sequencing using the Illumina MiSeq system. The targeted genome sequencing data of CeD and control samples are available here: https://zenodo.org/records/15203243.

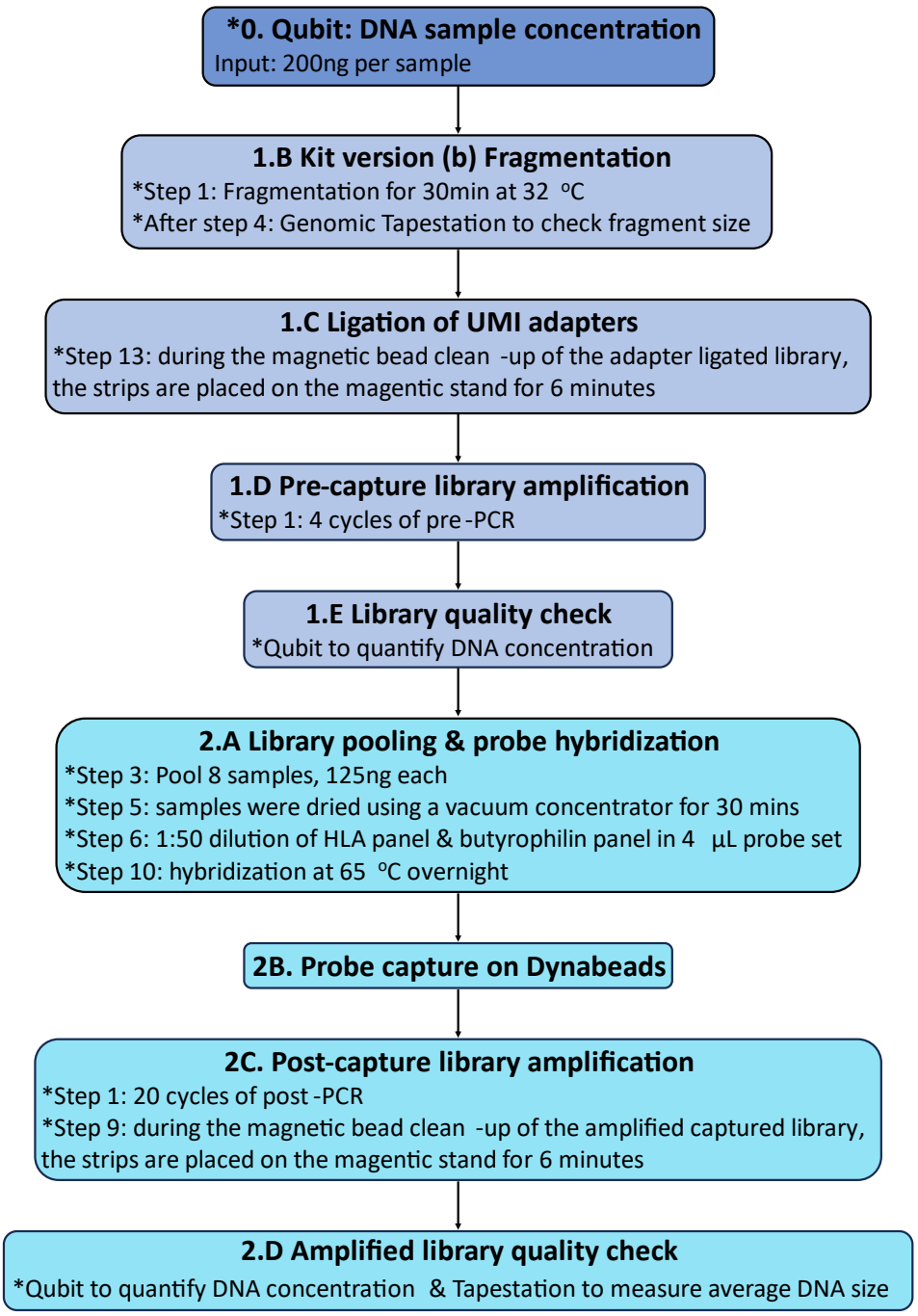

Figure 5. The Nonacus Cell3 capture hybridization capture method was modified for the HLA and butyrophilin sequencing panel. All modifications to the manufacturer's protocol were noted with *. The HLA probes were provided by Nonacus Ltd.

4.2.3. Germline Short Variant Discovery and HLA Typing

The quality of the sequencing files was assessed using the default FastQC v0.11.9 settings, and the Illumina adapters were removed using Trimmomatic v0.39 [70,71].

The variant call pipeline was built by adapting the GATK best practices for germline short variant discovery [72], the analysis pipelines of Zhao, *et al.* [73], the Du group [74,75], and Matthews

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[76] (Appendix F). The code for the pipeline calling SNPs from the raw unmapped FASTQ sequencing files is available here: https://gitlab.developers.cam.ac.uk/path/soilleux/soilleux-group/ced_butyrophilin_phd/-/tree/dropbox/nonacus_miseq_analysis/variant_call

HLA typing of the sequencing data was carried out separately from the GATK pipeline using HLA-HD version 1.7.0. The adapter trimmed FASTQ files were used as input files, and the default settings were retained for each sample, as outlined in the documentation of the program [77]. The program called the alleles at each *HLA* locus, and wrote the results for each patient in a text file. Afterwards, the CeD risk associated *HLA* genotypes were called from the alleles. The code for the risk *HLA* genotyping is available here: https://gitlab.developers.cam.ac.uk/path/soilleux/soilleux-group/ced_butyrophilin_phd/-/tree/dropbox/nonacus_miseq_analysis/hla_typing

4.2.4. Copy Number Variation (CNV) Analysis of the BTNL8-BTNL3 Loci

The presence of the 56-kb deletion variant in the *BTNL8-BTNL3* loci (chr5:180948027-181003596, GRCh38) was analysed by using a surrogate SNP. Dart, *et al.* [53] were the first to identify the *T>C* rs72494581 (chr5:181003797, GRCh38) *BTNL3* intronic SNP to be associated with the CNV (Table 16). The study proposed that the major allele, the *T* allele, is associated with the full length *BTNL3* and *BTNL8* genes, while the minor allele, the *C* allele, is associated with the *BTNL8*BTNL3* deletion. Fisher's exact test was performed to investigate any significant differences in the presence of the *BTNL8-BTNL3* CNV between the CeD and control cohorts of the hybridization capture dataset.

Table 16. The rs72494581 surrogate SNP was used to infer the CNV at the *BTNL8-BTNL3* region of chromosome 5.

	rs72494581 genotypes	**Associated CNV at *BTNL8-BTNL3* region of chromosome 5**
TT	Homozygous for reference allele	Full length *BTNL8-BTNL3* region on both copies of chromosome 5
CT	Heterozygous	1 copy has full length *BTNL8-BTNL3* region 1 copy has BTNL8*BTNL3 deletion
CC	Homozygous for alternative allele	*BTNL8*BTNL3* deletion on both copies of chromosome 5

4.2.5. Burden Testing Analysis

The TRAPD program was used for burden testing the variants found in selected butyrophilin genes in samples of the targeted sequencing cohort [54]. To summarise, qualifying variants within a gene were selected, that had a low minor allele frequency or were predicted to be pathogenic. Any qualifying SNPs with more than two alleles, called multi-allelic sites, were split into SNPs with two alleles for the analysis: the reference allele and one of the alternative alleles. These variants are termed bi-allelic variants [55]. The disease risk burden, or the number of minor alleles, in the control and CeD cohorts was counted and compared. The burden testing was performed using dominant models and recessive models in TRAPD. The dominant model considers individuals as carriers for gene burden, if they have at least one qualifying variant from the selected sites within the gene, while the recessive model requires the presence of two or more variants to be labelled a carrier [55]. As gene burden is an additive value, the zygosity of the qualifying sites do not matter, only the number of qualifying variants. For example, in a gene with 3 qualifying sites, an individual who is homozygous for the alternate allele for one qualifying site carries the same amount of gene burden as an individual who is heterozygous for two of the qualifying sites. The analysis was modified from the one described by Guo [55], to adapt it to the this cohort, as the original pipeline used an external control dataset.

The GATK processed sequences were subjected to further preprocessing before being burden tested with TRAPD, as recommended by Guo [55]. First, multi-allelic variants were separated using BCFtools version 1.16, as required by the TRAPD manual [78]. Next, the control and CeD cohort sequencing files were annotated using Ensembl Variant Effect Predictor (VEP) 109.3, and the SNPs

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were filtered to contain only non-synonymous coding variants [79]. The hybridization capture sequencing files were then analysed after read depth filtering (Figure 6).

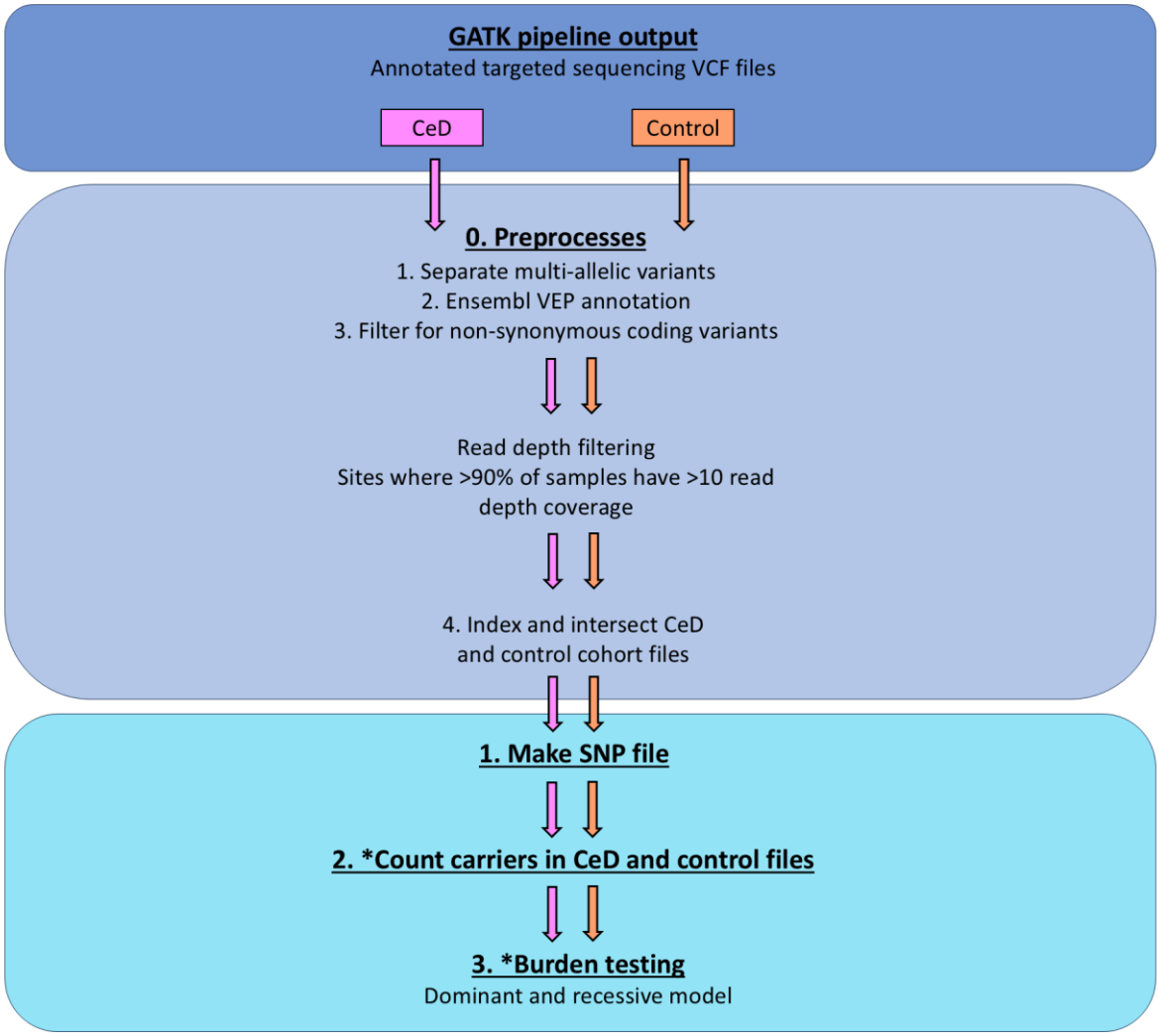

Figure 6. The variants in the CeD cohort (n = 48) were burden tested via the controls (n = 46) using the TRAPD program. Test Rare vAriants with Public Data (TRAPD) was used to burden test the variants in the hybridization capture CeD cohort (n = 48) against the control cohort (n = 46) [54]. The annotated CeD and control files from the GATK pipeline were preprocessed as recommended by the manual [55]. The variants were burden tested after read depth filtering. Steps, in which the code was modified is marked with *.

The cohort files were read depth filtered using VEP to select sites, where more than 90% of samples had a read depth coverage of >10. The final step of the preprocessing was to index and intersect the CeD and control sequencing files, to get the common SNPs between the two groups.

Following the preprocessing, the TRAPD code was applied using python 2.7 to create the SNP file from the CeD and the control cohort sequencing files using `make_snp_file.py`, which contains the qualifying variants from each gene. Carriers of the qualifying SNPs from both the control and the CeD files were counted using the `count_cases.py` file. The `burden.R` code was modified to adapt it to the targeted sequencing cohort, as the original pipeline used an external database as the control, while this analysis uses the control sequences from the same cohort. The variants in the CeD and control groups were burden tested using both the recessive and the dominant models.

The burden testing analysis codes are available here: https://gitlab.developers.cam.ac.uk/path/soilleux/soilleux-group/ced_butyrophilin_phd/-/tree/dropbox/nonacus_miseq_analysis/burden_testing/Code

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4.3. Single Variant Testing Butyrophilin Family Variance in the UK Biobank Database

4.3.1. CeD Risk Associated HLA Genotyping Using the HLA Imputation Values of the UK Biobank 500,000 Genome-Wide Genotyping Cohort

The HLA typing code using the UK Biobank HLA imputation data is available here: https://gitlab.developers.cam.ac.uk/path/soilleux/soilleux-group/ced_butyrophilin_phd/-/tree/dropbox/ukbiobank_hla_typing/hla_imputation_only

To summarise, the code used the HLA imputation values from data-field 22182. These values describe the likelihood of each *HLA* genotype, of which 14 were *HLA-DQA1* alleles and 18 were *HLA-DQB1* alleles. The *HLA* alleles were imputed by the UK Biobank from SNP data using the HLA*IMP:02 program [80]. In resource 182 (https://biobank.ctsu.ox.ac.uk/crystal/refer.cgi?id=182), the UK Biobank suggested using a threshold value of 0.7. If any *HLA* allele had an imputation value below 0.7, it was treated as an absent allele. The code applied this posterior threshold on the HLA imputation data for each participant, and the output was a list of *HLA* alleles that each participant had.

Afterwards, the CeD risk associated *HLA* genotypes were called from the *HLA* allele data. The code calling CeD risk genotypes from the HLA imputation derived alleles is available here: https://gitlab.developers.cam.ac.uk/path/soilleux/soilleux-group/ced_butyrophilin_phd/-/blob/dropbox/ukbiobank_hla_typing/hla_imputation_only/ukbhla_fullcohort.ipynb.

To identify if a participant had CeD risk genotypes, the code looked for the presence of the risk alleles at the *HLA-DQA1* and *HLA-DQB1* loci (Table 17a). Participants who did not have alleles present at either locus were removed from the analysis. The participant was determined to have a CeD associated *HLA* risk genotype if at least one copy of the risk *HLA-DQA1* and the *HLA-DQB1* alleles were present. If there were alleles present for more than one HLA risk genotype, the participant was typed as possessing both *HLA* risk genotypes (Table 17b).

Table 17. CeD risk associated *HLA* genotypes were called from the *HLA-DQA1* and *HLA-DQB1* alleles of UK Biobank participants. a) The CeD risk heterodimers and the *HLA-DQA1* and *HLA-DQB1* allele combinations encoding them. b) The UK Biobank participants' potential *HLA* genotypes depending on their *HLA-DQA1* and *HLA-DQB1* allele combinations. In the table, P and Q stand for any other allele at the mentioned *HLA-DQA1* and *HLA-DQB1* loci respectively, where the P/Q alleles together do not encode any CeD risk *HLA* genotypes.

a)

CeD risk heterodimer	*HLA* risk haplotype(s)	*HLA-DQA1* risk allele	*HLA-DQB1* risk allele
HLA-DQ2.2	*DR7-DQ2*	*HLA-DQA1*02:01*	*HLA-DQB1*02:02*
HLA-DQ2.5	*DR3-DQ2* or *DR5-DQ7/DR7-DQ2*	*HLA-DQA1*05:01*	*HLA-DQB1*02:01*
HLA-DQ8	*DR4-DQ8*	*HLA-DQA1*03:01*	*HLA-DQB1*03:02*

b)

UK Biobank participant's *HLA* genotype	Genotypes at the *HLA-DQA1* locus	Genotypes at the *HLA-DQB1* locus
HLA-DQ2.2	*DQA1*02:01/ DQA1*02:01* Or *DQA1*02:01/ P*	*DQB1*02:02/ DQB1*02:02* Or *DQB1*02:02/ Q*
HLA-DQ2.5	*DQA1*05:01/ DQA1*05:01* Or *DQA1*05:01/ P*	*DQB1*02:01/ DQB1*02:01* Or *DQB1*02:01/ Q*
HLA-DQ8	*DQA1*03:01/ DQA1*03:01* Or *DQA1*03:01/ P*	*DQB1*03:02/ DQB1*03:02* Or *DQB1*03:02/ Q*
HLA-DQ2.2, HLA-DQ2.5	*DQA1*02:01/ DQA1*05:01*	*DQB1*02:02/ DQB1*02:01*
HLA-DQ2.2, HLA-DQ8	*DQA1*02:01/ DQA1*03:01*	*DQB1*02:02/ DQB1*03:02*
HLA-DQ2.5, HLA-DQ8	*DQA1*05:01/ DQA1*03:01*	*DQB1*02:01/ DQB1*03:02*

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Other	Any other allele combination	Any other allele combination

4.3.2. Single Variant Testing Using Binomial Regression Models

The single variant testing model was built in R version 4.2.1 by adapting the UK Biobank analysis of Yu, *et al.* [81]. The code for investigating the association between butyrophilin family SNPs and CeD risk in the UK Biobank is available here:

https://gitlab.developers.cam.ac.uk/path/soilleux/soilleux-group/ced_butyrophilin_phd/-/tree/dropbox/ukbiobank_butyrophilin_snp/Butyrophilin_SNP_analysis?ref_type=heads

Of note, the UK Biobank individual SNP data was annotated using the reference SNP IDs (rsIDs) from the SNP database (dbSNP) and the reference allele for these SNPs from the Genome Reference Consortium Human Build 37 (GRCh37) [58]. As the SNPs were annotated using the dbSNP instead of their genomic position, and the dbSNP was updated to GRCh38 data at the time of this analysis, the SNP data could be used without further modification.

The individual SNP data in the UK Biobank was provided as the number of dbSNP reference alleles at each site, where 2 indicates that the individual is homozygous for the reference allele, while 1 indicates heterozygosity. If a participant had 0 reference alleles at a site, this could indicate homozygosity for the alternate allele or heterozygosity for two of the alternate alleles, depending on the number of potential alternate alleles. However, at the time of the analysis, the dataset did not provide information on which alternate allele was present.

To summarise the butyrophilin variant association analysis, firstly, the UK Biobank genome-wide genotyping dataset was curated and pre-processed for analysis. The downloaded per-chromosome UK Biobank genotyping data was loaded into R as a BEDMatrix object using the BGData R package [82]. Afterwards, the genotype and phenotype data for the selected UK Biobank participants were merged. Next, data for all SNPs recorded in the *BTN2A1*, *BTN3A1*, *BTN3A2*, *BTNL3*, *BTNL8* human genes were obtained from the National Centre for Biotechnology Information (NCBI) SNP database [66,83]. These SNPs were intersected with the genome-wide genotyping data, to identify the butyrophilin SNPs present in the UK Biobank dataset, which were 27 *BTN3A1*, 21 *BTN3A2*, 10 *BTNL3*, 13 *BTNL8*, and 30 *BTN2A1* SNPs. The final butyrophilin genotyping data in the UK Biobank dataset were provided as count data for the number of reference alleles at each SNP, identified by their rsIDs. Due to multiple testing, the resulting p-values were adjusted using Bonferroni correction.

Secondly, the association between butyrophilin variants and CeD risk was tested using binomial regression models, or binomial generalised linear models. In all of the linear models, the response variable was CeD status (CeD or no CeD, Table 18). The assumptions of the tests were that predictor variables were independent of each other. In the first test, the association between CeD risk *HLA* genotypes and CeD status was tested using one binomial model. In the second group of tests, individual binomial models were used to analyse the association between each butyrophilin family SNP and CeD risk. In the third group of tests, iterative binomial models were used that analysed the combined effect of butyrophilin family SNPs and *HLA* risk genotypes on CeD risk. In the fourth group of tests, the association between butyrophilin SNPs and CeD risk were analysed in *HLA*-matched groups.

Table 18. The binomial models tested the association between *HLA* risk genotypes and/or the individual butyrophilin family SNPs. Due to multiple testing, the resulting p-values were adjusted using Bonferroni correction. Abbreviations: CeD: coeliac disease; HLA: human leukocyte antigen; SNP: single nucleotide polymorphism.

Test/group number	Association being tested	Predictor variable(s)	Response variable
First test	Association between *HLA* risk genotypes and CeD risk	*HLA* risk genotype: See Table 17	CeD status: CeD or control

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Second group of tests (101 models)	Association between individual butyrophilin SNPs and CeD risk	Butyrophilin SNP: 2 reference alleles 1 reference allele 0 reference allele	CeD status: CeD or control
Third group of tests (101 models)	Association between the combined effect of *HLA* genotypes and butyrophilin SNPs and CeD risk	*HLA* risk genotype: See Table 17 Butyrophilin SNP: 2 reference alleles 1 reference allele 0 reference allele	CeD status: CeD or control
Fourth group of tests	Association between butyrophilin SNPs and CeD risk in *HLA*-matched groups	Butyrophilin SNP: 2 reference alleles 1 reference allele 0 reference allele	CeD status: CeD or control

Thirdly, the risk ratio or odds ratio (OR), the p-value, and the 95% confidence intervals were calculated for each binomial model assessing the association between butyrophilin SNPs and CeD risk. The direction of each SNP was calculated from the natural logarithm of the OR values (ln(OR)). SNPs where ln(OR) < 1 indicated that the SNP decreased CeD risk. SNPs where ln(OR) >1 indicated that the SNP increased CeD risk. Due to multiple testing, Bonferroni correction was applied for each group of tests.

Finally, the rsnps R package was used to annotate the butyrophilin family SNPs in the UK Biobank significantly associated with CeD using the NCBI database [84].

4.4. Analysis of TRGV Usage and HV4 Variation in CeD and Control Samples

4.4.1. Processing Samples and TCR Sequencing

The methods of DNA extraction, bulk amplification, and sequencing of the TCR repertoires in dataset 1 and dataset 2 were described in Foers, *et al.* [59] (Table 14). The DNA from FFPE duodenal samples and from fresh frozen duodenal samples were extracted using the QiaAmp FFPE DNA kit (Qiagen) and the DNeasy Blood & Tissue Kit (Qiagen) respectively, according to manufacturer's instructions.

Hybridization capture probes were designed for the targeted sequencing of the TCR repertoires of dataset 3. Capture probes were designed against the 3′ end of all productive V segments and the 5′ end of all productive J segments available on IMGT, according to their genomic position in the GRCh38.p13 reference genome [85]. Four capture probes (120 bp long) were designed for each productive segment, with the first probe to anneal 10 bp away from the junctional end, with subsequent probes 6 bp away from the previous one. The designed custom probe panel was sent for manufacturing to Nonacus Ltd.

Samples were prepared for hybridization capture using the Cell3 Target Library Preparation Kit (b) (Nonacus Ltd) according to the manufacturer's instructions. DNA was extracted from the blood samples using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions. Hybridization capture of the pooled libraries were carried out in two stages using the Target Hybridisation & Capture Kit protocol (Nonacus Ltd). First, the J probes were incubated with the pooled library (95^{0}C for 30 s, 65^{0}C for 4 hours), followed by bead cleanup according to manufacturer's instructions. The sequences were subjected to 20 cycles of PCR amplification, after which the DNA was quantified using the Qubit 2.0 fluorometer (Invitrogen). Next, 500 ng of the pooled library was captured using the V probes, subjected to bead clean up and further 10 cycles of PCR. The captured TCR repertoire libraries were Illumina MiSeq platforms according to standard protocols.

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4.4.2. TRGV and HV4 Analysis Pipeline

All the sequencing files were subjected to the same TRGV and HV4 analysis pipeline. Due to the gene rearrangement of the TCR, each mature T cell was assumed to carry one unique TCR combination, encoded by a unique pairing of *TRGV* and *TRGJ* genes in that T cell's genome. In the first step of the analysis pipeline, the paired-end FASTQ files containing the TRG sequencing data were analysed using MiXCR v4.0.0 (Figure 7). MiXCR is an immune profiling software that analyses raw T or B cell receptor repertoire NGS data [86,87]. At the time of analysis, the software had a built-in reference library, and could identify the clonotypes and gene segments used in the repertoire. The `analyze amplicon` was a one-step command that aligned the sequencing data, assembled and exported the clonotypes found in the TCR repertoire. For the purposes of analysing the germline HV4 sequence, the region of interest was set to include the *FR3* of the *TRGV* genes (`--region-of-interest {FR3Begin:CDR3End}`). The resulting text file contained the nucleotide sequence (`targetSequences`), amino acid sequence (`aaSeq`), clone count (`cloneCount`), V and J segment usage (`allVHitsWithScore`, `allJHitsWithScore`) for each unique TRG sequence. The results from the MiXCR output were analysed using the pipeline available here:

https://gitlab.developers.cam.ac.uk/path/soilleux/soilleux-group/ced_butyrophilin_phd/-/tree/dropbox/trgv_hv4_analysis

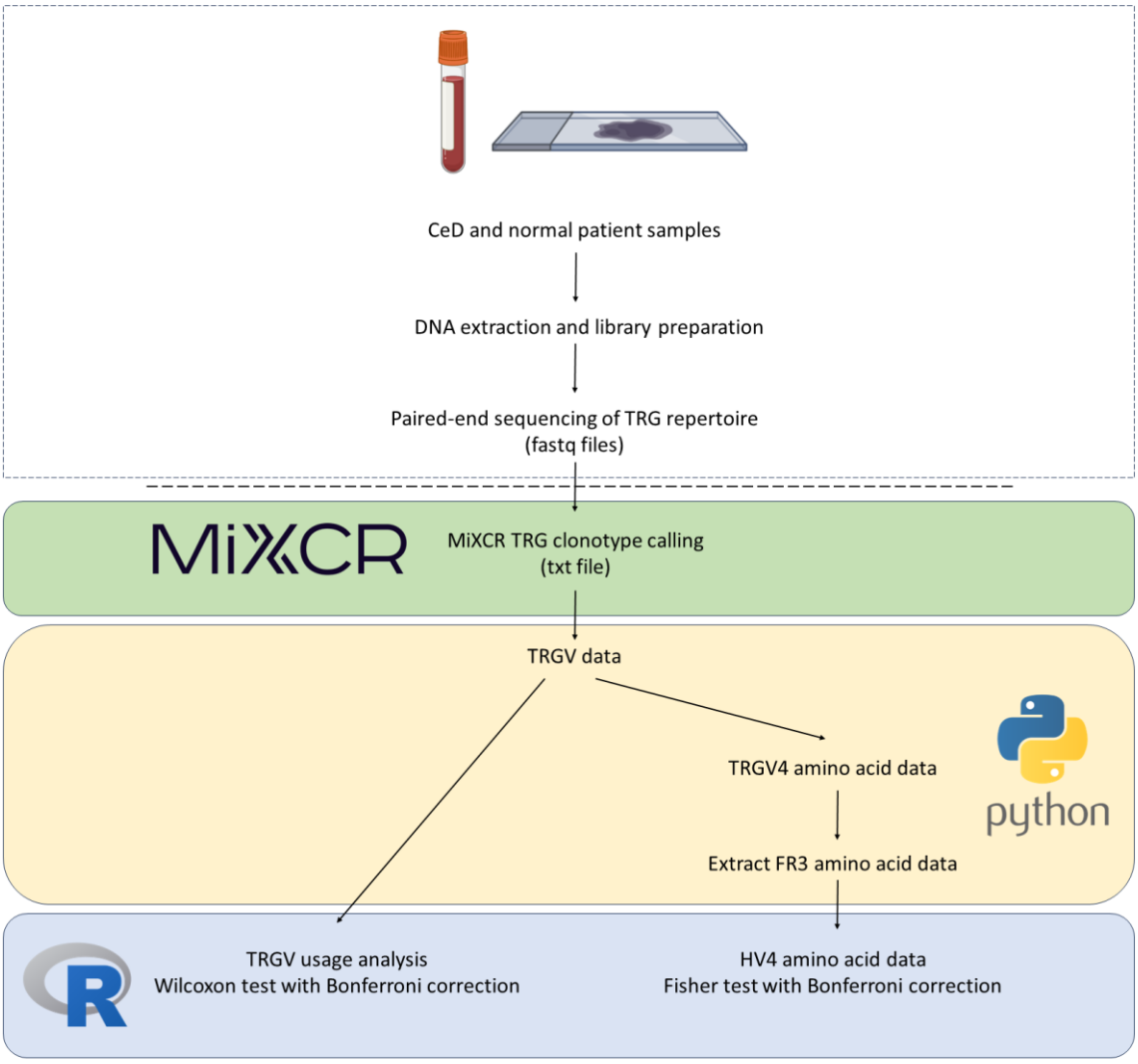

Figure 7. CeD and healthy control patient TRG sequencing data from three cohorts were used to analyse differences in TRGV usage and germline HV4 sequences.

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To identify the differences in the TRGV usage of the duodenal TRG repertoire, the read count of the TRGV section (`allVHitsWithScore`) from the MiXCR output was processed using Python 3. Only samples from the duodenum were analysed for TRGV usage. Therefore, TRGV data from blood samples were not subjected to TRGV usage analysis.

To examine if the TRGV usage was significantly different between duodenal sample types, pairwise Mann-Whitney U tests were carried out for each of the 10 TRGV segments. To eliminate any false positives due to multiple testing, Bonferroni correction was applied to the p-values. For each test, the proportion of the specific TRGV segment was compared between the different sample types.

The germline HV4 analysis was carried out using Python 3, by identifying variations in the amino acid sequence that directly binds BTNL3 [29,31]. The HV4 reference amino acid sequence "KYDTYGSTRKNLRMILR" was demonstrated to be capable of binding BTNL3 [29,31]. For brevity, the reference sequence was named the WT sequence. To summarise the HV4 analysis, clonotypes with TRGV4 segments were selected in each participant's sequencing data. Next, the amino acid sequence of the germline FR3 was isolated. The HV4 was described as the amino acids 10-25 in the FR3 by Willcox, *et al.* [31], so these positions were used for further HV4 analysis. As the HV4 is encoded by the germline *TRGV4* gene, each patient has two *HV4* alleles. Therefore patients can be homozygous or heterozygous for the amino acid sequence of the HV4 loop. For this reason, CeD patient samples were grouped together regardless of their sample type (FFPE duodenum, or fresh frozen duodenum, or blood), as the source of the samples did not affect the germline-encoded *HV4* alleles. The top two unique HV4 amino acid sequences were selected with the highest read counts in each sample. In some patients, there were more than two HV4 amino acid sequences present. This could be due to library preparation and sequencing errors. To avoid including artefacts in the analysis, the percentage of each HV4 sequence was calculated, and 10% was used as a cutoff percentage for heterozygosity. If the HV4 sequence with the second highest read count had less than 10% frequency, the sequence was excluded, and the patient was assigned as homozygous for the most frequent HV4 sequence.

Fisher's exact test was applied to analyse if there were significant differences in the proportion of germline-encoded HV4 amino acid sequences between CeD and healthy control samples. As the sample size for all datasets were less than 1000, Fisher's test was used instead of the chi-square test [88]. As this analysis required germline sequences, the tissue of origin of the samples did not matter. Therefore all samples could be subjected to HV4 analysis. Bonferroni correction was applied to eliminate false positives due to multiple testing.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Appendix A: Supplementary materials for Results section 2.1; Appendix B: Demographic data of the selected UK Biobank participants; Appendix C: Supplementary materials for Results section 2.2; Appendix D: Supplementary materials for Results section 2.3; Appendix E: Selecting and sequencing the butyrophilin genes of interest; Appendix F: Detailed germline short variant discovery protocol.

Author Contributions: Conceptualization, E.J.S.; methodology, K.N.L.H., S.E., H.K., K.D., L.S., K.P., A.F.; software, K.N.L.H., T.W., H.K., A.F.; validation, K.N.L.H., A.F. and E.J.S.; formal analysis, K.N.L.H., A.F.; investigation, K.N.L.H., K.D., S.E.; resources, E.J.S., A.F.; data curation, K.N.L.H., S.E., K.D.; writing—original draft preparation, K.N.L.H..; writing—review and editing, T.W., E.J.S.; visualization, K.N.L.H.; supervision, E.J.S.; project administration, E.J.S.; funding acquisition, E.J.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Oxfordshire Research ethics Committee A (IRAS project ID: 162057, REC reference: 04/Q1604/21, Principal Investigator: Professor E. Soilleux).

Informed Consent Statement: Study-specific patient consent was not required under the terms of our ethical approval.

Data Availability Statement: We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. Targeted sequencing data of selected butyrophilin family genes in a cohort of 94 samples (2.1): https://zenodo.org/records/1520324 γδ TCR sequencing data of 46 CeD and 97 healthy control duodenal samples (Dataset 1, Table 14): https://dataview.ncbi.nlm.nih.gov/object/PRJNA1330789?reviewer=sp7hjkohgpbo6mt3qv7thd57fp γδ TCR sequencing data of 11 CeD and 11 healthy control duodenal samples (Dataset 2, Table 14): https://dataview.ncbi.nlm.nih.gov/object/PRJNA1330746?reviewer=dcl0ipo3ftc3m83b637i0hjmpd γδ TCR sequencing data of 84 CeD and 130 healthy control blood samples (Dataset 3, Table 14): https://dataview.ncbi.nlm.nih.gov/object/PRJNA1330754?reviewer=uoqvavqemvh35apdc0ifn99585

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Conflicts of Interest: L.S. and K.P. are employees of Nonacus Ltd. The other authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BTN/BTNL	Butyrophilin/butyrophilin-like
CeD	Coeliac disease
CNV	Copy number variation
DC	Dendritic cell
GFD	Gluten-free diet
FFPE	Formalin fixed, paraffin embedded
HLA	Human leukocyte antigen
HPA	Human Protein Atlas
HV4	Hypervariable region 4
IEL	Intraepithelial lymphocyte
NK cell	Natural Killer cell
TRGV	T cell receptor γ variable region
SNP/SNV	Single nucleotide polymorphism/variation
WT	Wild type

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Appendix A – Supplementary Materials for Results Section 2.1

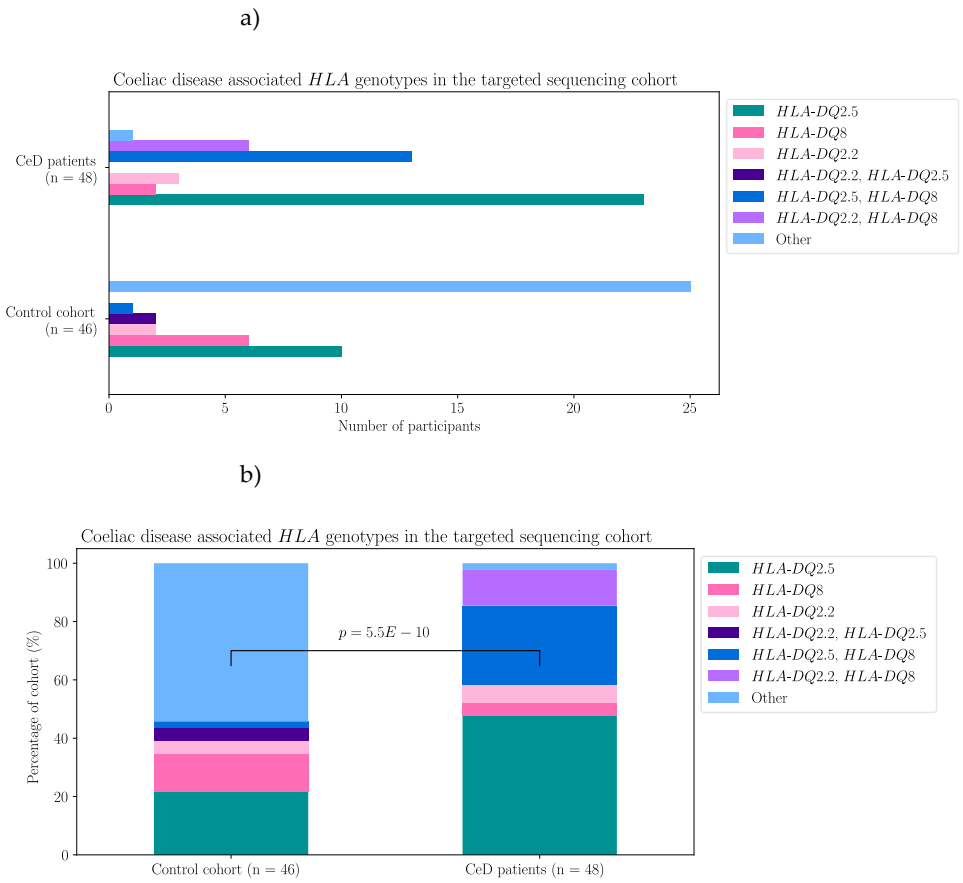

Figure A1. CeD patients (n = 46) had significantly higher proportions of CeD risk *HLA* genotypes compared to controls (n = 48). The a) number and b) percentage of individuals with CeD risk associated *HLA* genotypes were significantly higher in CeD patients compared to controls in this dataset (Fisher's exact test, p = 5.5e-10). The *HLA* genotypes for participants were called using HLA-HD [52].

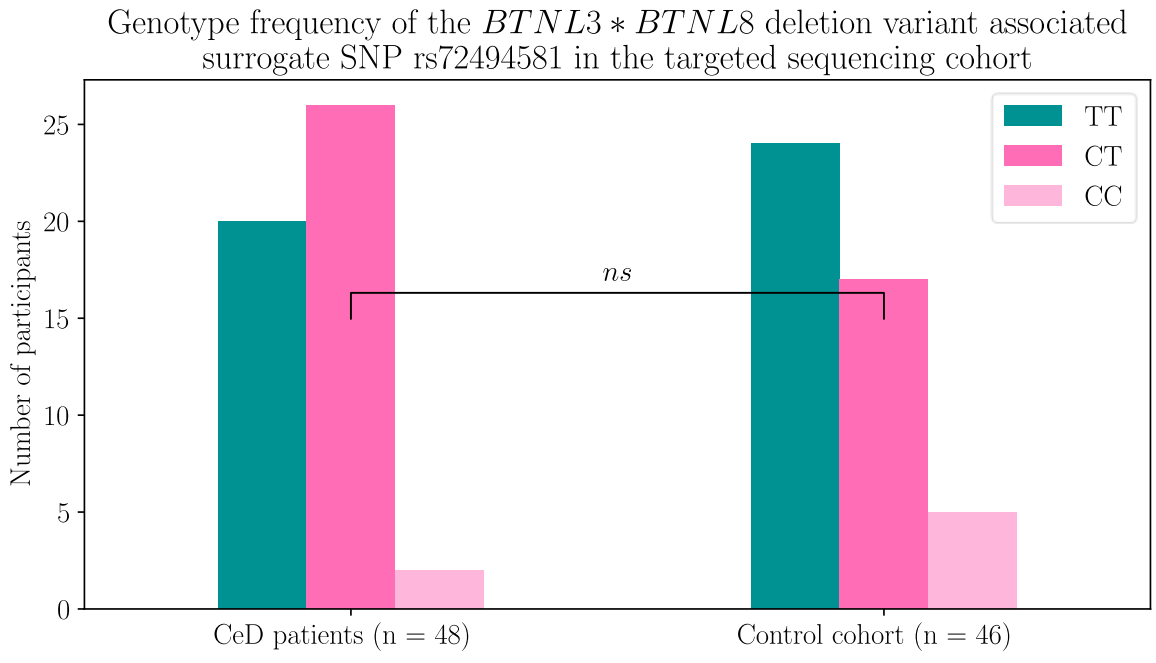

Figure A2. There were no significant differences in the frequency of the *BTNL8*BTNL3* copy number variants between CeD patients (n = 48) and controls (n = 46). The *BTNL8*BTNL3* deletion variant encodes a truncated

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BTNL8-BTNL3 fusion protein [41]. Dart, *et al.* [53] identified rs72494581 in the intronic region of *BTNL3*, which serves as a surrogate SNP and the alleles are associated with the *BTNL8*BTNL3* copy number variants. The major allele, the *T* allele, is associated with the full length *BTNL3* and *BTNL8* genes, while the minor allele, the *C* allele, is associated with the *BTNL8*BTNL3* deletion. Fisher's exact testing showed that there were no significant differences ($p = 0.2144$) in the frequency of the *BTNL8*BTNL3* deletion variant associated rs72494581 genotypes in the CeD and control groups.

Table A1. Less than 40% of sites found in CeD samples were shared with controls. Shared polymorphic sites between CeD and control samples in a) the whole hybridization capture dataset, b) in non-synonymous coding sites, c) in read depth filtered non-synonymous coding sites. Read depth was defined as the number of sequence reads per site. Read depth filtering was applied as a quality control step. Sites, where the read depth (dp) was more than 10, in more than 90% of the samples in each group passed the read depth filter. Sites described above can be multi-allelic, meaning they may have more than one alternative allele. Non-matching overlapping sites were defined as polymorphic sites, where the reference and/or alternate alleles identified in each group were different alleles. For example, a non-matching overlapping SNP would have the same base pair position on the chromosome with the same reference allele, but the alternate allele in one group is different from the other group's. Comparisons were carried out using vcftools [89].

a)

	Number of butyrophilin family sites	Sites unique to group	Shared sites	Non-matching overlapping sites
Coeliac	1168	701	435	32
Control	769	302		

b)

	Number of butyrophilin family non-synonymous coding sites	Sites unique to group	Shared sites
Coeliac	108	79	21
Control	58	29	

c)

	Number of butyrophilin family non-synonymous coding sites (>90% samples in group dp >10)	Sites unique to group	Shared sites
Coeliac	60	54	6
Control	21	15	

Table A2. Percentage data of burden testing butyrophilin variants in CeD samples against controls from the hybridization capture dataset.

Gene	Qual. SNPs	CeD %(≥1 HET)	CeD %(≥2 HET)	CeD %(HOM ALT)	CeD total qual. allele freq	Control %(≥1 HET)	Control %(≥2 HET)	Control %(HOM ALT)	Control total qual. allele freq	Dominant model p-value	Recessive model p-value
BTN2A1	**3**	**45.8**	**43.8**	**6.3**	**0.281**	**10.9**	**8.7**	**0.0**	**0.047**	**1.46E-05**	**3.70E-08**

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BTN3A2	1	10.4	0.0	2.1	0.073	19.6	0.0	2.2	0.120	0.929	0.946
ERMAP	1	43.8	0.0	16.7	0.385	43.5	0.0	15.2	0.370	0.516	0.988

Burden testing was carried out on butyrophilin variants in CeD samples against controls from the dataset with read depth filtering. During read depth filtering in only sites, where more than 90% of samples had a read depth coverage of >10, were selected. Percentage values in columns 3-5 and 7-9 show the percentage of individuals with each genotype within the CeD and the control groups of the dataset respectively. Significant results are highlighted in bold. Abbreviations: CeD: coeliac disease; freq: frequency; HET: heterozygous; HOM ALT: homozygous for the alternative allele; N: number; qual.: qualifying; SNP: single nucleotide polymorphism

Table A3. Per sample genotype of participants at significant *BTN2A1* qualifying SNPs from Table 5b Abbreviations: het: heterozygous; hom alt: homozygous for the alternative allele; hom ref: homozygous for the reference allele.

a) CeD participants

Position	**6:26463432**	**6:26468098**	**6:26468317**
rsID	**rs13195509**	**rs3734542**	**rs3734543**
CB22	hom alt	hom alt	hom alt
CB24	hom alt	hom alt	hom alt
CB26	het	het	het
CB27	het	het	het
CB29	het	het	het
CB31	het	het	het
CB32	hom ref	hom ref	hom ref
CB33	hom ref	hom ref	hom ref
CB34	het	het	het
CB35	het	het	het
CB36	het	het	het
CB37	het	het	het
CB38	het	het	het
CB39	hom ref	hom ref	hom ref
CB40	hom ref	hom ref	hom ref
CB41	hom ref	hom ref	hom ref
CB43	hom ref	hom ref	hom ref
CB44	hom ref	hom ref	hom ref
CB45	het	het	het
CB46	hom ref	hom ref	hom ref
CB48	hom ref	hom ref	hom ref
CB49	hom ref	hom ref	hom ref
CB50	hom ref	hom ref	hom ref
CB51	het	het	het
CB52	hom ref	hom ref	hom ref
CB53	het	het	het
CB54	het	het	het
CB55	het	het	het
CB56	hom ref	hom ref	hom ref
CB57	hom ref	hom ref	hom ref
CB58	het	het	het

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CB59	hom ref	hom ref	hom ref
CB60	hom ref	hom ref	hom ref
CB61	het	het	het
CB62	het	het	het
CB63	hom alt	hom alt	hom alt
CB65	hom ref	hom ref	hom ref
CB67	het	het	het
CB69	het	het	het
CB70	hom ref	hom ref	hom ref
CD1	hom ref	het	hom ref
CD2	hom ref	hom ref	hom ref
CD3	hom ref	hom ref	hom ref
CD4	het	het	het
CD5	het	het	het
CD6	hom ref	hom ref	hom ref
CD7	hom ref	hom ref	hom ref
CD8	hom ref	hom ref	hom ref

b) Control participants

Position	**6:26463432**	**6:26468098**	**6:26468317**
rsID	**rs13195509**	**rs3734542**	**rs3734543**
NB11	hom ref	hom ref	hom ref
NB13	hom ref	hom ref	hom ref
NB14	hom ref	hom ref	hom ref
NB16	hom ref	hom ref	hom ref
NB19	hom ref	hom ref	hom ref
NB2	hom ref	hom ref	hom ref
NB20	hom ref	hom ref	hom ref
NB21	het	het	het
NB22	hom ref	hom ref	hom ref
NB25	hom ref	hom ref	hom ref
NB28	het	het	het
NB29	hom ref	hom ref	hom ref
NB3	hom ref	hom ref	hom ref
NB30	hom ref	hom ref	hom ref
NB31	hom ref	hom ref	hom ref
NB32	hom ref	hom ref	hom ref
NB34	hom ref	hom ref	hom ref
NB35	hom ref	hom ref	hom ref
NB37	hom ref	hom ref	hom ref
NB38	hom ref	hom ref	hom ref
NB39	hom ref	hom ref	hom ref
NB41	hom ref	hom ref	hom ref
NB42	hom ref	hom ref	hom ref
NB44	hom ref	hom ref	hom ref
NB46	hom ref	hom ref	hom ref
NB47	hom ref	hom ref	hom ref
NB48	hom ref	hom ref	hom ref

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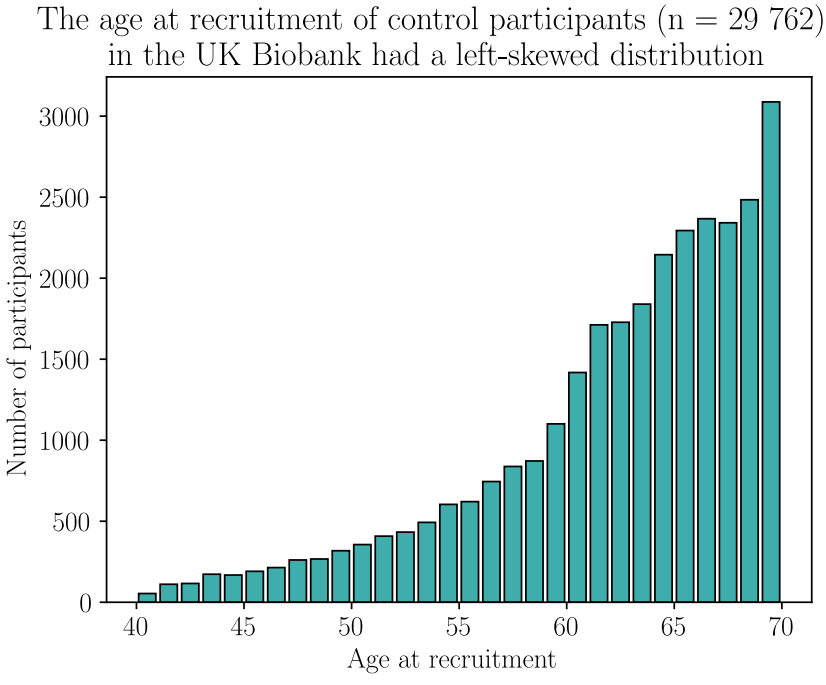
NB49	hom ref	hom ref	hom ref
NB5	hom ref	hom ref	hom ref
NB50	het	het	het
NB52	hom ref	hom ref	hom ref
NB56	hom ref	hom ref	hom ref
NB58	hom ref	hom ref	hom ref
NB68	hom ref	hom ref	hom ref
NB69	hom ref	hom ref	hom ref
NB7	hom ref	hom ref	hom ref
NB70	hom ref	hom ref	hom ref
NB71	hom ref	hom ref	hom ref
ND1	hom ref	hom ref	hom ref
ND10	hom ref	hom ref	hom ref
ND2	hom ref	hom ref	hom ref
ND5	het	het	het
ND6	hom ref	hom ref	hom ref
ND7	hom ref	hom ref	hom ref
ND8	hom ref	hom ref	hom ref
ND9	hom ref	het	hom ref

Appendix B – Demographic Data of the Selected UK Biobank Participants

Firstly, the age at recruitment of the participants were analysed. The distribution of the age at recruitment of neither controls nor of CeD patients followed normal distribution (Figure B1). The mean age at recruitment for controls was 62, which was significantly higher than that of CeD patients (t = 27.297, df = 3539.3, p < 2.2E-16), whose average age at recruitment was 58.

a)



b)

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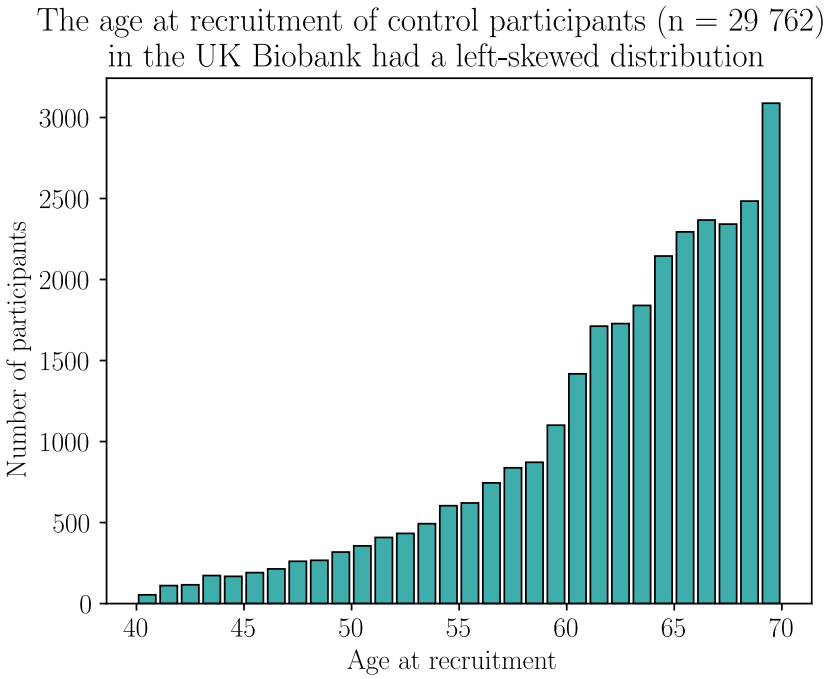

c)

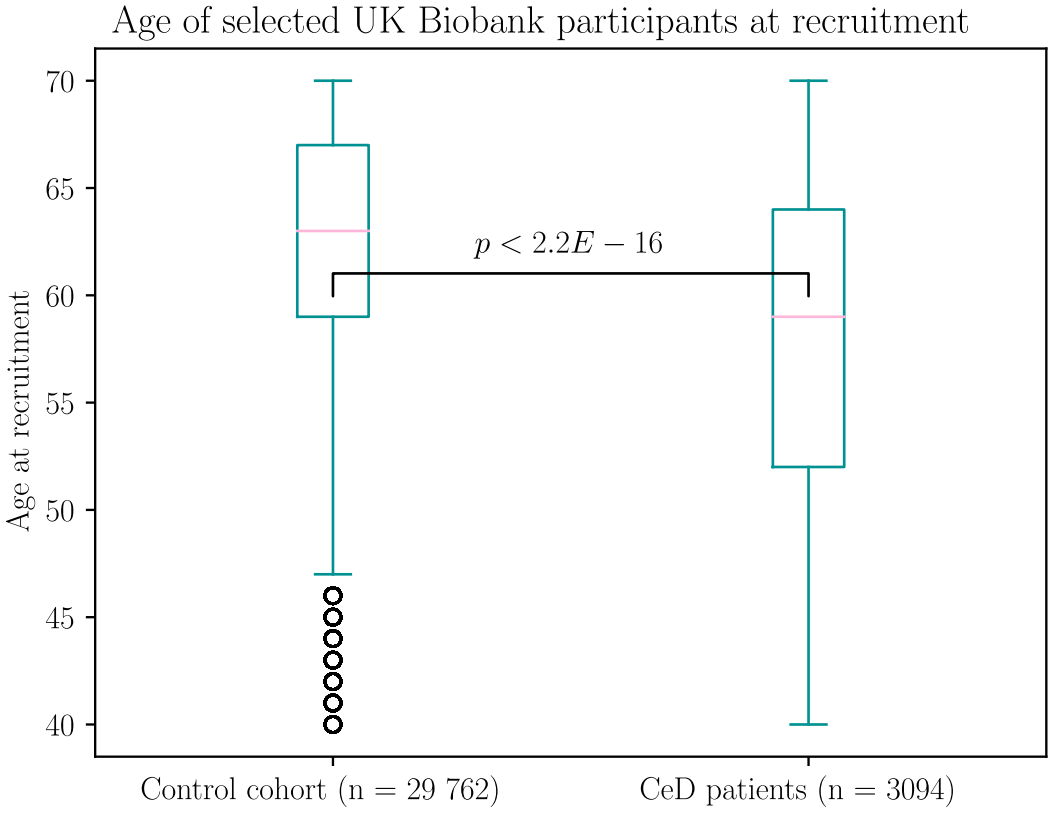

Figure 1. CeD patients were significantly younger than control individuals when they were recruited for the initial UK Biobank study. The distribution of the age at recruitment of a) 29 762 control participants and b) 3094 CeD patients from the UK Biobank dataset did not follow normal distribution. c) The mean age at recruitment was significantly higher in control individuals (t = 27.297, df = 3539.3, p-value < 2.2E-16).

Secondly, the sex of CeD and control participants in the UK Biobank was investigated (Figure B2). Interestingly, the proportion of female participants was significantly higher in the CeD group

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(64.8%, 2005/3094) than in the control group (40.4%, 12 010/29 762) (X-squared = 683.91, df = 1, $p <$ 2.2E-16).

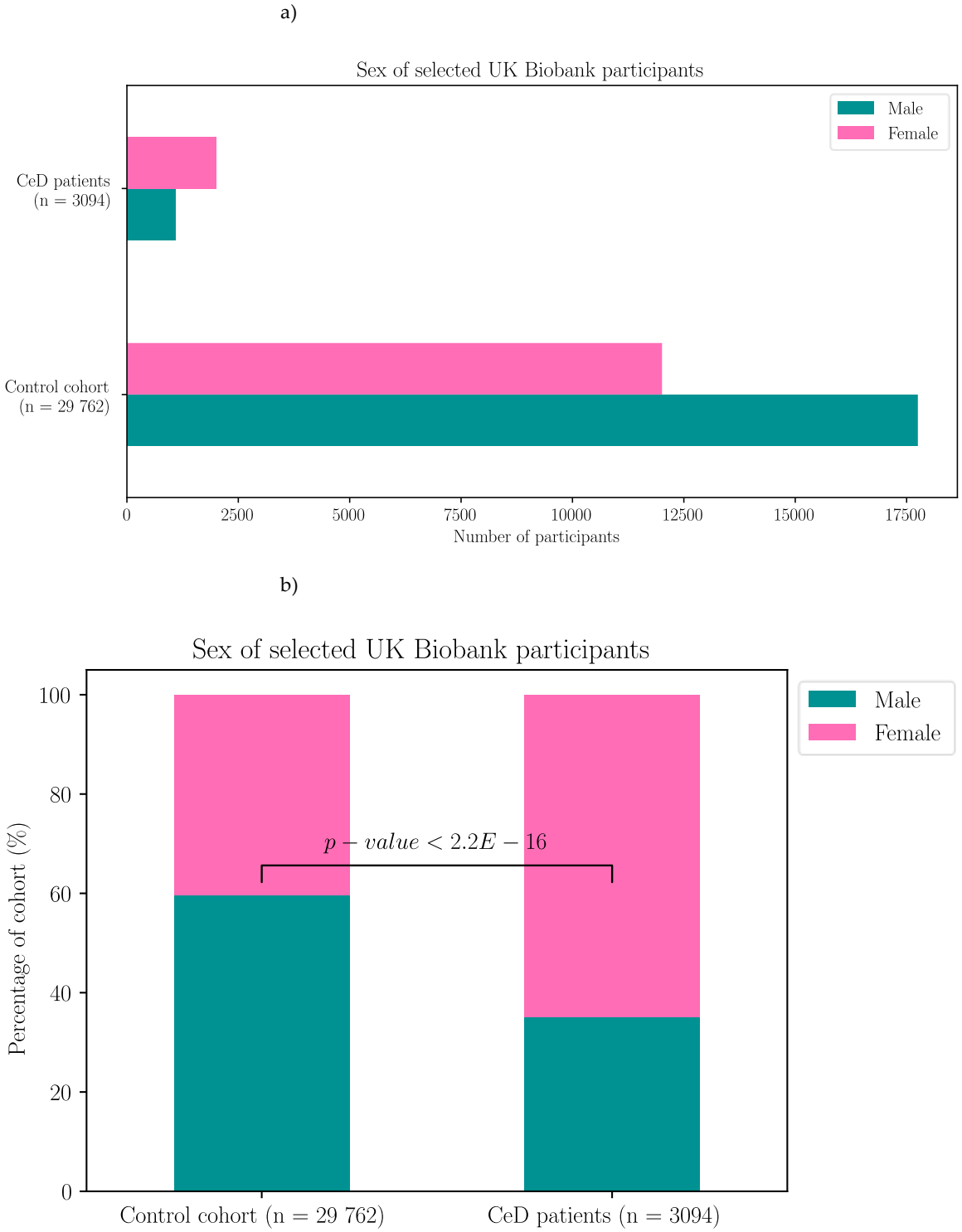

Figure 2. A significantly higher proportion of individuals diagnosed with CeD were female participants, compared with the control cohort in the UK Biobank dataset. The sex of 3094 CeD patients and 29 762 control participants was analysed from the UK Biobank. The figure shows the a) number and b) percentage of each sex for CeD and control participants. The CeD group in the UK Biobank had a significantly higher proportion of female participants (X-squared = 683.91, df = 1, $p <$ 2.2E-16).

Thirdly, the ethnic background of the UK Biobank participants was analysed. In both CeD and control groups, the majority of participants had White British backgrounds, at 91.8% (26 850/29 762)

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and 90.2% (2841/3094) respectively (Figure B3). In both groups, Irish and any other white background were the second and third most frequent ethnic backgrounds respectively. When the ethnic background of CeD and control participants were compared (X-squared = 42.294, df = 21, $p = 0.00384$), no significant difference was present after Bonferroni correction for multiple testing ($p > 0.05$).

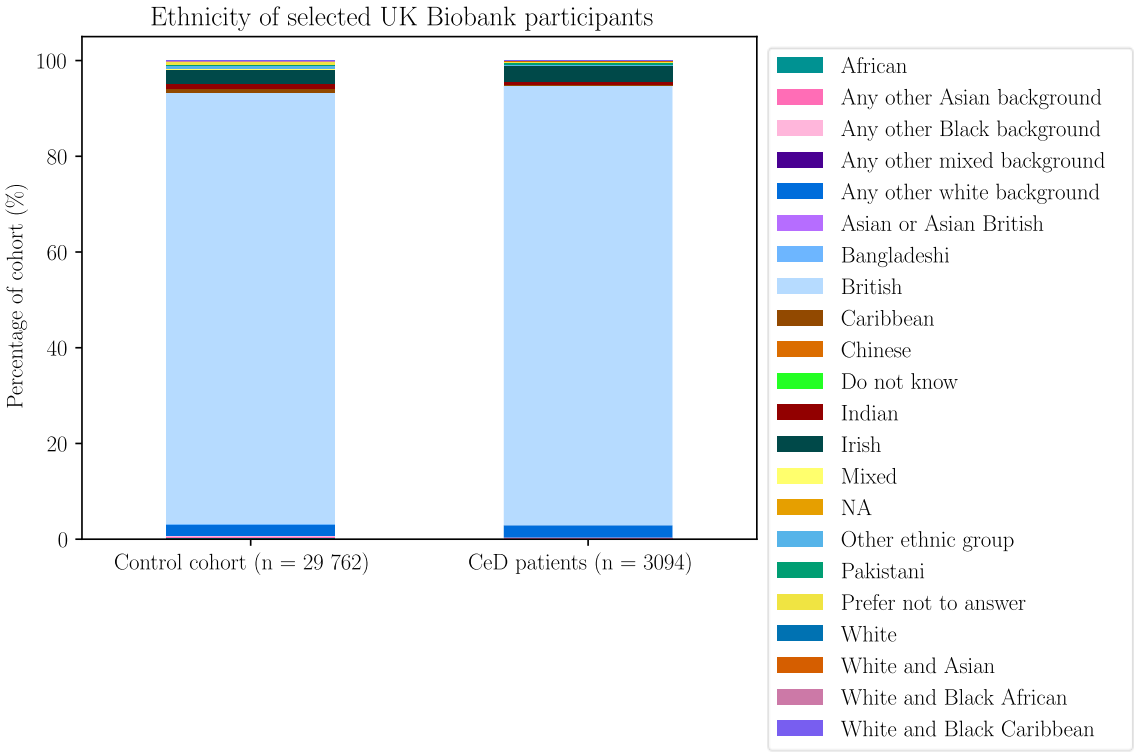

Figure 3. White British was the most common ethnic background in the UK Biobank participants regardless of CeD status. The ethnic backgrounds of 3094 CeD patients and 29 762 control participants were analysed from the UK Biobank. There were no significant differences in the ethnic background of CeD and control participants after Bonferroni correction.

Finally, the dietary web questionnaire answers of CeD and control individuals were examined for any differences between the two groups. GFD was excluded from the statistical analysis of dietary differences between CeD and control individuals in the UK Biobank. Firstly, being on a GFD was one of the exclusion criteria for the control cohort, to exclude potential, undiagnosed CeD cases. Therefore this diet would have zero occurrences in the control group. Secondly, CeD patients generally follow a GFD, as it is currently the only treatment for CeD [4]. This created a higher occurrence of GFD in CeD patients compared to controls selected from the UK Biobank dataset. The majority of UK Biobank participants did not adhere to any special diet, with 94.7% of controls (28 185/29 762) and 71.2% of CeD patients (2204/3094) reporting no special diet (Figure B5). A low calorie diet was the most common special diet in controls (3.1%, 926/29 762).

A GFD was the most common special diet in CeD participants of the UK Biobank (22.2%, 686/3094), with an additional 3.6% (112/3094) of patients following a GFD in addition to other special diets. The second and third most common special diet in the CeD group was a combination of a GFD with a low calorie diet, and a GFD with a lactose-free diet, at 1.5% (45/3094) and 1.3% (40/3094) respectively. When any variation of a GFD was excluded from the CeD group, the most common diet was a low calorie diet (1.3%, 39/3094). Interestingly, 74.2% (2296/3094) of CeD participants did not follow a diet that excluded gluten.

When excluding a GFD from the analyses, the proportions of participants following the special diets within the control group was significantly different from the CeD group (X-squared = 23.303, df = 11, $p = 0.01602$). The lactose-free–low calorie, vegan, lactose-free–low calorie–vegetarian, lactose-free–vegetarian and lactose-free–low calorie–vegan diets were only found in the control group after

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excluding CeD patients adhering to a GFD. This could be due to CeD patients following the aforementioned diets in addition to being on a GFD (Table B1).

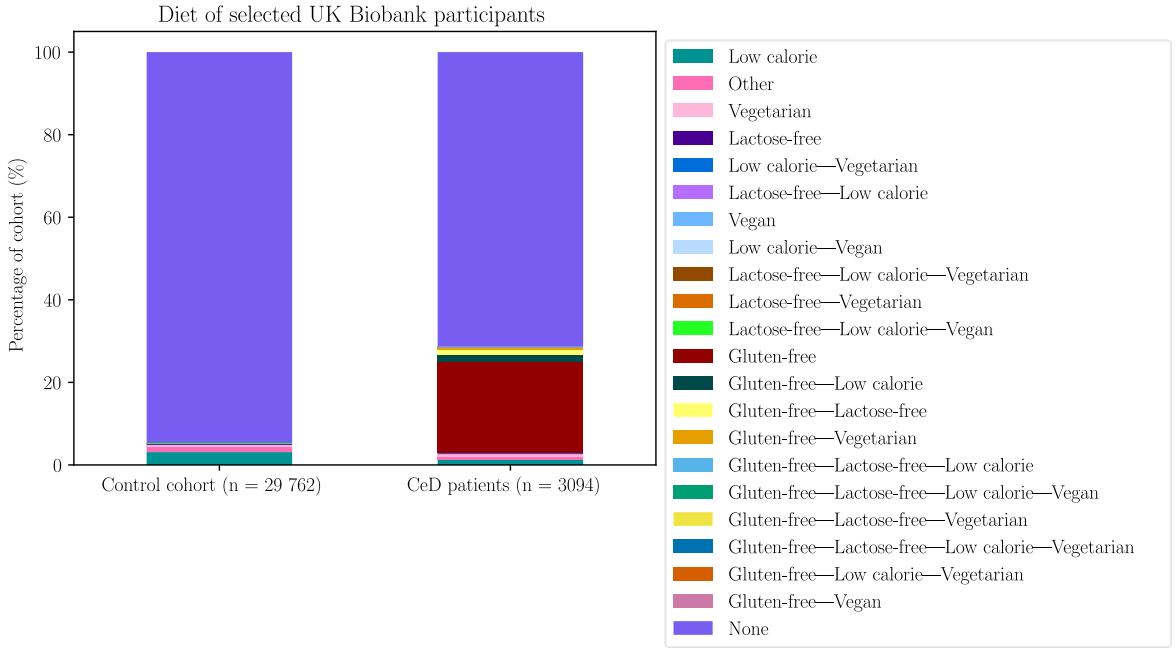

Figure 4. The majority of UK Biobank participants reported no special diet in both control and CeD groups. The diet of 3094 CeD patients and 29 762 control participants was analysed from the UK Biobank. The majority of UK Biobank participants reported no special diet in both control (94.7%, 28 185/29 762) and CeD (71.2%, 2204/3094) groups. When participants following a gluten-free diet were excluded from the analysis, the proportions of participants following each special diet were significantly different between the CeD and control groups (X-squared = 23.303, df = 11, p = 0.01602). This was likely caused by having to exclude patients who followed multiple diets that included a gluten-free diet. Participants on a gluten-free diet were excluded from the control group.

Table 1. The significant difference between the special diets of controls (n = 29 762) and CeD participants not on a gluten-free diet (n = 2296) may stem from CeD patients following multiple diets in addition to following a gluten-free diet.

			Without GFD		With GFD	
Diet	**N controls on diet**	**% Controls on diet**	**N CeD on diet**	**% CeD on diet (out of 2296)**	**N CeD on diet**	**% CeD on diet (out of 3094)**
Lactose-free–low calorie	20	0.067	0	0	5	0.162
Vegan	15	0.050	0	0	1	0.032
Lactose-free–low calorie–vegetarian	2	0.007	0	0	1	0.032
Lactose-free–vegetarian	2	0.007	0	0	1	0.032
Lactose-free–low calorie–vegan	1	0.003	0	0	2	0.065

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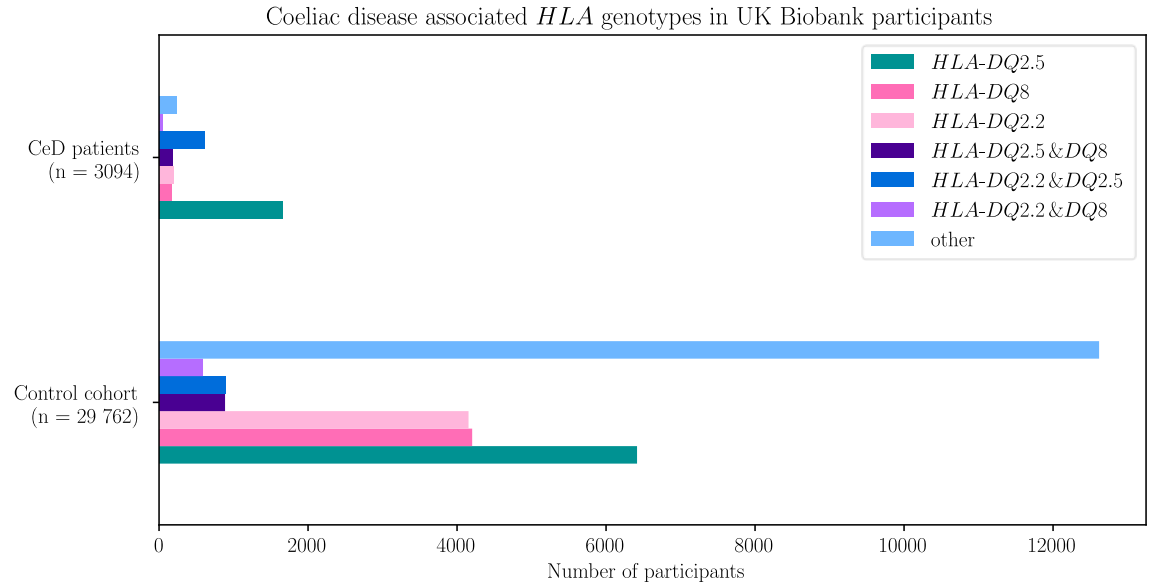
Appendix C – Supplementary Materials for Results Section 2.2

a)



b)

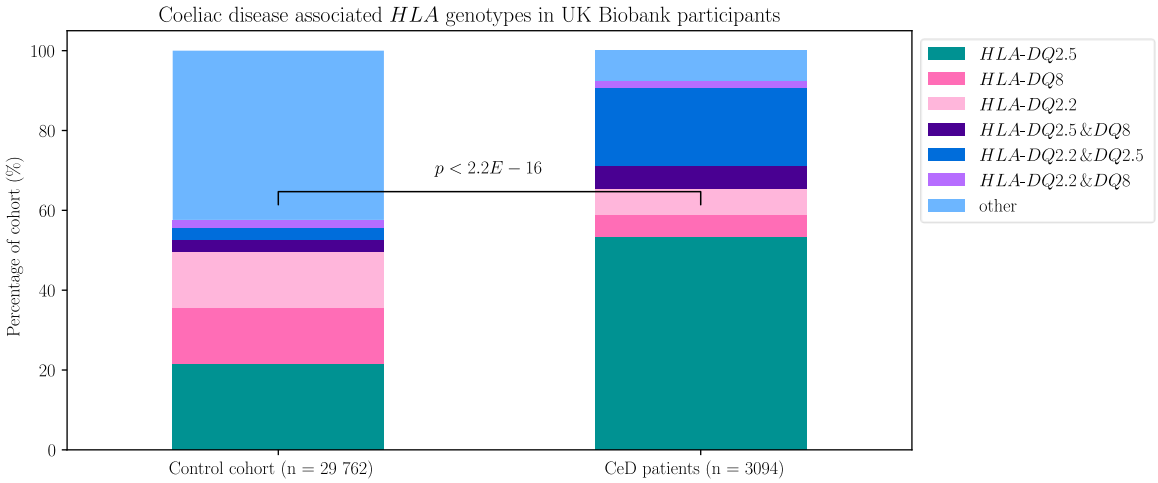


Figure 1. CeD patients (n = 3094) had significantly higher proportions of CeD risk associated *HLA* genotypes compared to controls (n = 29 762) in the UK Biobank's 500,000 genome-wide genotyping dataset. The a) number and b) percentage of participants with CeD risk HLA genotypes were significantly more frequent in CeD patients compared to control participants (X-squared = 4062.5, df = 6, $p < 2.2E\text{-}16$). All participants' *HLA* genotypes were called using the HLA imputation values provided by the UK Biobank.

Table 1. Coefficients of the binomial regression model investigating CeD risk *HLA* genotypes as a predictor variable for CeD status in the UK Biobank dataset. Results of the binomial generalised linear model testing the association between CeD status and CeD risk associated HLA genotypes in the UK Biobank dataset. Abbreviations: NA: not applicable; ns: not significant.

HLA genotype	Coefficient estimate	Standard error	z value	p-value	CeD risk
HLA-DQ2.2, HLA-DQ2.5	2.649	0.090	29.550	< 2E-16	Increase
HLA-DQ2.2, HLA-DQ8	0.570	0.164	3.474	5.13E-04	Increase
HLA-DQ2.5	1.682	0.078	21.662	< 2E-16	Increase

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HLA-DQ2.5, HLA-DQ8	1.456	0.109	13.352	< 2E-16	Increase
HLA-DQ8	-0.163	0.107	-1.533	0.125	ns
Other *HLA* genotype	-0.949	0.098	-9.677	< 2E-16	Decrease
Constant	-3.039	0.073	-41.872	< 2E-16	NA

Table 2. Single variant analysis of butyrophilin SNPs and CeD status without taking the *HLA* loci into account using the UK Biobank dataset. SNPs significantly associated with CeD status are in bold. Bonferroni correction was applied due to multiple testing.

SNP name	Gene	OR	upper	lower	adjusted p-value	ln(OR)
rs10484441_G	*BTN2A1*	1.138386	1.249955	1.038835	0.60789	0.129612
rs12660069_C	*BTN2A1*	1.106915	1.299592	0.948994	1	0.101577
rs13195402_G	***BTN2A1***	**0.397002**	**0.424644**	**0.371261**	**4.67E-158**	**-0.92381**
rs13195509_G	***BTN2A1***	**0.424358**	**0.45231**	**0.398239**	**1.61E-151**	**-0.85718**
rs13437351_G	*BTN2A1*	1.485538	1.91326	1.176198	0.14151	0.395777
rs1407045_A	***BTN2A1***	**1.314112**	**1.385827**	**1.246309**	**6.07E-22**	**0.273161**
rs142951857_A	*BTN2A1*	1.391065	3.107641	0.723169	1	0.33007
rs143104579_G	*BTN2A1*	1.14068	1.405753	0.935552	1	0.131625
rs146399224_T	*BTN2A1*	10963.24	NA	0.003938	1	9.302303
rs148111655_G	*BTN2A1*	1.130613	2.537072	0.583563	1	0.12276
rs2273558_A	***BTN2A1***	**0.673125**	**0.711994**	**0.636428**	**1.69E-41**	**-0.39582**
rs2893856_T	***BTN2A1***	**0.839708**	**0.911101**	**0.772766**	**0.0032259**	**-0.1747**
rs2893857_C	*BTN2A1*	1.142895	1.255236	1.042695	0.48083	0.133565
rs3734539_C	*BTN2A1*	4032.285	NA	0.007	1	8.302088
rs3734542_G	***BTN2A1***	**0.425283**	**0.453292**	**0.39911**	**8.59E-151**	**-0.855**
rs3734543_G	***BTN2A1***	**0.430012**	**0.458993**	**0.402974**	**1.59E-140**	**-0.84394**
rs3799380_T	***BTN2A1***	**0.577632**	**0.611726**	**0.545555**	**8.59E-77**	**-0.54882**
rs56296968_C	***BTN2A1***	**0.54644**	**0.579504**	**0.515375**	**9.70E-89**	**-0.60433**
rs6456724_T	***BTN2A1***	**0.838697**	**0.91004**	**0.771803**	**0.00287**	**-0.17591**
rs6907857_T	*BTN2A1*	1.433106	1.834039	1.140956	0.29414	0.359844
rs6911470_C	*BTN2A1*	1.472809	1.963726	1.132407	0.57829	0.387171
rs6929846_T	***BTN2A1***	**0.813898**	**0.875261**	**0.756001**	**3.60E-06**	**-0.20592**
rs7773913_C	*BTN2A1*	1.433061	1.833981	1.14092	0.29439	0.359813
rs7773938_C	***BTN2A1***	**0.548927**	**0.582092**	**0.517765**	**1.15E-87**	**-0.59979**
rs77870445_T	*BTN2A1*	1.219723	1.609943	0.942473	1	0.198624
rs9348718_A	*BTN2A1*	1.267016	1.454243	1.109411	0.06114	0.236664
rs9358943_C	*BTN2A1*	1.768069	31.86429	0.363137	1	0.569888
rs9358944_A	***BTN2A1***	**0.546443**	**0.579355**	**0.515512**	**1.55E-89**	**-0.60432**
rs9358945_A	***BTN2A1***	**0.54552**	**0.578367**	**0.514649**	**4.37E-90**	**-0.60602**
rs9461254_G	*BTN2A1*	1.464419	1.953756	1.125151	0.66799	0.381458
rs10456045_G	***BTN3A1***	**0.638826**	**0.674371**	**0.605202**	**2.97E-57**	**-0.44812**
rs10807008_G	*BTN3A1*	1.091584	1.192289	1.001092	1	0.087629
rs12200782_C	*BTN3A1*	1.138999	1.250121	1.039809	0.56581	0.13015
rs12207930_C	*BTN3A1*	1.147602	1.241418	1.062201	0.05418	0.137674
rs12208447_C	*BTN3A1*	1.284779	1.624554	1.030908	1	0.250586
rs12214924_T	*BTN3A1*	1.147514	1.241076	1.062324	0.05283	0.137597
rs143476765_A	*BTN3A1*	1.108868	4.616795	0.396888	1	0.10334
rs144114619_T	*BTN3A1*	1.212854	2.147941	0.740604	1	0.192976
rs145059723_A	*BTN3A1*	1.412767	4.034311	0.629895	1	0.34555

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rs1741738_A	*BTN3A1*	1.144367	1.242176	1.05573	0.11623	0.134851
rs17610161_G	*BTN3A1*	1.097034	1.199223	1.005306	1	0.09261
rs1796520_C	***BTN3A1***	**0.758646**	**0.800004**	**0.719297**	**2.40E-22**	**-0.27622**
rs3799378_A	***BTN3A1***	**0.585559**	**0.61957**	**0.553499**	**2.92E-75**	**-0.53519**
rs3857549_C	***BTN3A1***	**1.247408**	**1.401099**	**1.114594**	**0.01526**	**0.221068**
rs3902051_A	*BTN3A1*	1.090365	1.187959	1.002366	1	0.086513
rs41266839_G	***BTN3A1***	**0.396746**	**0.423498**	**0.37178**	**2.12E-168**	**-0.92446**
rs4609015_T	***BTN3A1***	**1.151594**	**1.245578**	**1.066033**	**0.03817**	**0.141147**
rs4712990_C	*BTN3A1*	1.101764	1.203269	1.010539	1	0.096912
rs55676749_T	*BTN3A1*	1.13993	1.38368	0.948273	1	0.130967
rs56161420_G	*BTN3A1*	1.138909	1.230815	1.055142	0.09381	0.130071
rs6900725_T	***BTN3A1***	**1.149401**	**1.242804**	**1.064341**	**0.04333**	**0.139241**
rs6912853_C	***BTN3A1***	**1.16488**	**1.257236**	**1.080577**	**0.00785**	**0.152618**
rs6920986_C	***BTN3A1***	**1.148238**	**1.241884**	**1.062975**	**0.04995**	**0.138228**
rs6921148_T	*BTN3A1*	1.165176	1.411589	0.971033	1	0.152872
rs742090_A	***BTN3A1***	**0.759078**	**0.800536**	**0.719639**	**3.58E-22**	**-0.27565**
rs7770214_G	*BTN3A1*	1.145779	1.239155	1.060758	0.06048	0.136085
rs80153343_G	*BTN3A1*	1.136899	1.439622	0.910733	1	0.128305
rs11758089_T	***BTN3A2***	**1.192878**	**1.288514**	**1.105674**	**0.00063**	**0.176369**
rs12176317_A	***BTN3A2***	**0.445307**	**0.474099**	**0.41837**	**6.72E-140**	**-0.80899**
rs12194095_C	*BTN3A2*	1.118798	1.235914	1.015164	1	0.112255
rs12199613_C	***BTN3A2***	**0.67037**	**0.706851**	**0.635751**	**1.76E-47**	**-0.39993**
rs12205731_G	*BTN3A2*	1.114755	1.232063	1.011029	1	0.108634
rs144016445_G	*BTN3A2*	81101.36	NA	217.0251	1	11.30346
rs1977_A	***BTN3A2***	**0.445697**	**0.474831**	**0.418455**	**1.17E-136**	**-0.80811**
rs1979_G	***BTN3A2***	**0.44537**	**0.474171**	**0.418426**	**8.23E-140**	**-0.80885**
rs1985732_A	***BTN3A2***	**0.63289**	**0.668226**	**0.599467**	**2.87E-59**	**-0.45746**
rs2073526_G	***BTN3A2***	**0.74435**	**0.785579**	**0.705115**	**9.15E-25**	**-0.29524**
rs35183513_G	*BTN3A2*	1.102861	1.203659	1.012184	1	0.097908
rs58367598_T	*BTN3A2*	1.234256	1.449383	1.057523	0.89091	0.210469
rs7765566_G	*BTN3A2*	1.269787	1.499163	1.083157	0.39847	0.23885
rs9104_G	*BTN3A2*	1.092904	1.187567	1.007255	1	0.088838
rs9358934_G	***BTN3A2***	**0.447824**	**0.476845**	**0.420678**	**2.34E-137**	**-0.80335**
rs9379855_T	***BTN3A2***	**0.447682**	**0.47666**	**0.420575**	**8.85E-138**	**-0.80367**
rs9379858_T	***BTN3A2***	**0.448449**	**0.477474**	**0.421299**	**3.19E-137**	**-0.80196**
rs9379859_C	***BTN3A2***	**0.447843**	**0.476903**	**0.420662**	**5.37E-137**	**-0.80331**
rs9379861_G	*BTN3A2*	1.225507	1.619363	0.945713	1	0.203355
rs9393713_G	***BTN3A2***	**0.442958**	**0.471602**	**0.416159**	**1.07E-141**	**-0.81428**
rs9393714_G	***BTN3A2***	**0.443419**	**0.47214**	**0.416551**	**6.99E-141**	**-0.81324**
rs186813312_C	*BTNL3*	0.103966	NA	NA	NA	-2.26369
rs199970076_G	*BTNL3*	0.544013	10.42476	0.087697	1	-0.60878
rs201534771_G	*BTNL3*	0.108957	NA	NA	NA	-2.2168
rs201813197_C	*BTNL3*	1.141842	4.74926	0.409599	1	0.132642
rs35157246_C	*BTNL3*	1.069831	1.242264	0.926052	1	0.067501
rs4700774_G	*BTNL3*	0.943446	0.999778	0.89061	1	-0.05822
rs59220426_C	*BTNL3*	1.006054	1.132599	0.896669	1	0.006036
rs73815153_G	*BTNL3*	1.009988	1.138564	0.899028	1	0.009938
rs7713324_A	*BTNL3*	1.004294	1.130736	0.895001	1	0.004284

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rs7726604_C	*BTNL3*	1.004751	1.13121	0.895444	1	0.00474
rs112469887_G	*BTNL8*	1.084234	1.330225	0.893133	1	0.080874
rs113071395_G	*BTNL8*	0.867372	1.006007	0.751681	1	-0.14229
rs113534626_A	*BTNL8*	1.019956	1.206531	0.868252	1	0.019759
rs141492316_T	*BTNL8*	0.891806	1.095588	0.733483	1	-0.11451
rs145199317_A	*BTNL8*	0.907765	1.254932	0.673198	1	-0.09677
rs151174174_C	*BTNL8*	0.770441	0.932722	0.641625	0.63031	-0.26079
rs17704291_C	*BTNL8*	0.940216	0.996283	0.88763	1	-0.06165
rs200633883_C	*BTNL8*	0.311552	1.114968	0.108457	1	-1.16619
rs201214790_T	*BTNL8*	4044.713	NA	1.08E-07	1	8.305166
rs201891387_G	*BTNL8*	0.62355	2.663098	0.210953	1	-0.47233
rs2276995_A	*BTNL8*	0.983681	1.038422	0.931987	1	-0.01645
rs2619739_C	*BTNL8*	1.101246	1.212402	1.002386	1	0.096442
rs7724813_G	*BTNL8*	1.078621	1.169543	0.996169	1	0.075683

Table 3. SNP and allele count data for the significant SNPs from the non-HLA model. These SNPs were significantly associated with CeD status in single variant testing of the UK Biobank dataset. The SNPs in bold remained significantly associated with CeD status in the binomial regression models that took the *HLA* genotype of individuals into account.

SNP, reference allele	Gene	Number of the SNP in control	Number of the SNP in CeD	Total allele count in control	Total allele count in CeD	Total number of the SNP in the UK Biobank	Total allele count in UK Biobank
rs13195402_G	***BTN2A1***	**52060**	**4631**	**58392**	**6028**	**56691**	**64420**
rs13195509_G	***BTN2A1***	**52271**	**4654**	**59474**	**6176**	**56925**	**65650**
rs1407045_A	*BTN2A1*	30596	3603	59306	6172	34199	65478
rs2273558_A	*BTN2A1*	34599	3248	51134	5570	37847	56704
rs2893856_T	*BTN2A1*	7815	697	59452	6182	8512	65634
rs3734542_G	***BTN2A1***	**52209**	**4656**	**59432**	**6180**	**56865**	**65612**
rs3734543_G	***BTN2A1***	**52002**	**4644**	**59146**	**6114**	**56646**	**65260**
rs3799380_T	*BTN2A1*	46887	4213	59354	6164	51100	65518
rs56296968_C	***BTN2A1***	**47941**	**4295**	**59422**	**6170**	**52236**	**65592**
rs6456724_T	*BTN2A1*	7813	696	59418	6178	8509	65596
rs6929846_T	*BTN2A1*	10355	903	59460	6180	11258	65640
rs7773938_C	*BTN2A1*	47953	4289	59472	6162	52242	65634

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rs9358944_A	***BTN2A1***	**47929**	**4294**	**59462**	**6182**	**52223**	**65644**
rs9358945_A	***BTN2A1***	**47944**	**4292**	**59478**	**6182**	**52236**	**65660**
rs10456045_G	*BTN3A1*	41458	3680	59434	6174	45138	65608
rs1796520_C	*BTN3A1*	28075	2504	59240	6176	30579	65416
rs3799378_A	***BTN3A1***	**45113**	**4017**	**59206**	**6152**	**49130**	**65358**
rs3857549_C	*BTN3A1*	55572	5848	59430	6170	61420	65600
rs41266839_G	***BTN3A1***	**52985**	**4720**	**59430**	**6178**	**57705**	**65608**
rs4609015_T	*BTN3A1*	50759	5371	59452	6170	56130	65622
rs6900725_T	*BTN3A1*	50682	5378	59392	6182	56060	65574
rs6912853_C	*BTN3A1*	50145	5329	59434	6176	55474	65610
rs6920986_C	*BTN3A1*	50787	5381	59464	6182	56168	65646
rs742090_A	*BTN3A1*	28172	2506	59438	6174	30678	65612
rs11758089_T	*BTN3A2*	50176	5354	59432	6182	55530	65614
rs12176317_A	***BTN3A2***	**51604**	**4597**	**59488**	**6182**	**56201**	**65670**
rs12199613_C	***BTN3A2***	**36321**	**3178**	**59396**	**6178**	**39499**	**65574**
rs1977_A	***BTN3A2***	**50506**	**4497**	**58436**	**6074**	**55003**	**64510**
rs1979_G	***BTN3A2***	**51551**	**4590**	**59448**	**6176**	**56141**	**65624**
rs1985732_A	***BTN3A2***	**41492**	**3674**	**59442**	**6174**	**45166**	**65616**
rs2073526_G	*BTN3A2*	26272	2288	59434	6176	28560	65610
rs9358934_G	***BTN3A2***	**51477**	**4591**	**59412**	**6174**	**56068**	**65586**
rs9379855_T	***BTN3A2***	**51458**	**4579**	**59392**	**6162**	**56037**	**65554**
rs9379858_T	***BTN3A2***	**51474**	**4586**	**59422**	**6170**	**56060**	**65592**
rs9379859_C	***BTN3A2***	**51530**	**4595**	**59452**	**6174**	**56125**	**65626**
rs9393713_G	***BTN3A2***	**51601**	**4590**	**59472**	**6178**	**56191**	**65650**
rs9393714_G	***BTN3A2***	**51581**	**4594**	**59456**	**6180**	**56175**	**65636**

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Table 4. Single variant analysis of butyrophilin SNPs and CeD status in binomial regression models that took the *HLA* loci into account using the UK Biobank dataset. SNPs significantly associated with CeD status are in bold. Bonferroni correction was applied due to multiple testing.

SNP name	Gene	OR	upper	lower	adjusted p-value	ln(OR)
rs10484441_G	*BTN2A1*	0.979307	1.082186	0.887707	1	-0.02091
rs12660069_C	*BTN2A1*	0.987937	1.171444	0.837975	1	-0.01214
rs13195402_G	***BTN2A1***	**0.812801**	**0.876887**	**0.753657**	**8.15E-06**	**-0.20727**
rs13195509_G	***BTN2A1***	**0.824983**	**0.886694**	**0.767819**	**1.62E-05**	**-0.19239**
rs13437351_G	*BTN2A1*	1.359939	1.781839	1.056236	1	0.30744
rs1407045_A	*BTN2A1*	1.061681	1.125065	1.001972	1	0.059854
rs142951857_A	*BTN2A1*	1.189393	2.744421	0.589877	1	0.173443
rs143104579_G	*BTN2A1*	1.045598	1.306706	0.844534	1	0.044589
rs146399224_T	*BTN2A1*	2678.29	NA	0.001107	1	7.892934
rs148111655_G	*BTN2A1*	1.06888	2.495456	0.522042	1	0.066611
rs2273558_A	*BTN2A1*	0.924046	0.983637	0.868186	1	-0.07899
rs2893856_T	*BTN2A1*	0.978844	1.068109	0.895906	1	-0.02138
rs2893857_C	*BTN2A1*	0.983276	1.0868	0.891136	1	-0.01687
rs3734539_C	*BTN2A1*	12320.17	NA	2.26E-05	1	9.418993
rs3734542_G	***BTN2A1***	**0.828358**	**0.890318**	**0.770964**	**2.94E-05**	**-0.18831**
rs3734543_G	***BTN2A1***	**0.845824**	**0.910556**	**0.785966**	**0.000823**	**-0.16744**
rs3799380_T	*BTN2A1*	0.906299	0.966317	0.850232	0.260718	-0.09839
rs56296968_C	***BTN2A1***	**0.889118**	**0.949206**	**0.833053**	**0.042016**	**-0.11753**
rs6456724_T	*BTN2A1*	0.975504	1.064451	0.892856	1	-0.0248
rs6907857_T	*BTN2A1*	1.296362	1.68837	1.012123	1	0.259562
rs6911470_C	*BTN2A1*	1.225626	1.666065	0.92177	1	0.203452
rs6929846_T	*BTN2A1*	0.956691	1.034347	0.884033	1	-0.04427
rs7773913_C	*BTN2A1*	1.29982	1.692843	1.014844	1	0.262226
rs7773938_C	*BTN2A1*	0.892443	0.95269	0.83623	0.062934	-0.11379
rs77870445_T	*BTN2A1*	0.971098	1.300178	0.738108	1	-0.02933
rs9348718_A	*BTN2A1*	1.100624	1.274443	0.954703	1	0.095877
rs9358943_C	*BTN2A1*	0.455962	8.266602	0.091903	1	-0.78535
rs9358944_A	***BTN2A1***	**0.888825**	**0.948639**	**0.833001**	**0.038293**	**-0.11786**
rs9358945_A	***BTN2A1***	**0.886765**	**0.946407**	**0.831099**	**0.02906**	**-0.12018**
rs9461254_G	*BTN2A1*	1.206876	1.642976	0.90642	1	0.188035
rs10456045_G	*BTN3A1*	0.918688	0.975087	0.865666	0.527112	-0.08481
rs10807008_G	*BTN3A1*	0.94089	1.033981	0.857412	1	-0.06093
rs12200782_C	*BTN3A1*	0.987553	1.090084	0.896183	1	-0.01253
rs12207930_C	*BTN3A1*	0.994811	1.081946	0.91566	1	-0.0052
rs12208447_C	*BTN3A1*	0.971864	1.246094	0.767755	1	-0.02854
rs12214924_T	*BTN3A1*	0.997777	1.08479	0.918712	1	-0.00223
rs143476765_A	*BTN3A1*	0.541997	2.391824	0.175983	1	-0.61249
rs144114619_T	*BTN3A1*	0.937876	1.708275	0.552209	1	-0.06414
rs145059723_A	*BTN3A1*	0.851224	2.534279	0.356178	1	-0.16108
rs1741738_A	*BTN3A1*	1.055187	1.152999	0.966854	1	0.053718
rs17610161_G	*BTN3A1*	0.948685	1.04337	0.863865	1	-0.05268
rs1796520_C	*BTN3A1*	0.925332	0.980491	0.873166	0.877114	-0.0776
rs3799378_A	***BTN3A1***	**0.866517**	**0.922476**	**0.814111**	**0.000704**	**-0.14327**
rs3857549_C	*BTN3A1*	1.20727	1.366146	1.0703	0.249929	0.188361

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rs3902051_A	*BTN3A1*	0.947893	1.038919	0.865991	1	-0.05351
rs41266839_G	***BTN3A1***	**0.806793**	**0.868508**	**0.749711**	**1.06E-06**	**-0.21469**
rs4609015_T	*BTN3A1*	0.999771	1.087084	0.920447	1	-0.00023
rs4712990_C	*BTN3A1*	0.953022	1.047148	0.8686	1	-0.04812
rs55676749_T	*BTN3A1*	1.055136	1.295119	0.867029	1	0.05367
rs56161420_G	*BTN3A1*	1.003319	1.090026	0.92446	1	0.003313
rs6900725_T	*BTN3A1*	0.998519	1.085343	0.919613	1	-0.00148
rs6912853_C	*BTN3A1*	1.060948	1.150125	0.979684	1	0.059162
rs6920986_C	*BTN3A1*	0.997232	1.08425	0.918167	1	-0.00277
rs6921148_T	*BTN3A1*	1.075901	1.317605	0.885986	1	0.073159
rs742090_A	*BTN3A1*	0.927391	0.982792	0.875004	1	-0.07538
rs7770214_G	*BTN3A1*	0.992737	1.079363	0.914026	1	-0.00729
rs80153343_G	*BTN3A1*	1.144379	1.465945	0.904679	1	0.134862
rs11758089_T	*BTN3A2*	1.093163	1.188249	1.00675	1	0.089075
rs12176317_A	***BTN3A2***	**0.820862**	**0.880647**	**0.765377**	**3.50E-06**	**-0.1974**
rs12194095_C	*BTN3A2*	0.971352	1.079478	0.875824	1	-0.02907
rs12199613_C	***BTN3A2***	**0.884297**	**0.93714**	**0.834446**	**0.003312**	**-0.12296**
rs12205731_G	*BTN3A2*	0.970415	1.079025	0.874528	1	-0.03003
rs144016445_G	*BTN3A2*	38750.66	NA	151.7424	1	10.5649
rs1977_A	***BTN3A2***	**0.816781**	**0.87678**	**0.761121**	**2.06E-06**	**-0.20238**
rs1979_G	***BTN3A2***	**0.820729**	**0.880499**	**0.765255**	**3.40E-06**	**-0.19756**
rs1985732_A	***BTN3A2***	**0.896055**	**0.951463**	**0.84398**	**0.033488**	**-0.10975**
rs2073526_G	*BTN3A2*	0.925312	0.980986	0.872648	0.940603	-0.07762
rs35183513_G	*BTN3A2*	0.951537	1.044843	0.867784	1	-0.04968
rs58367598_T	*BTN3A2*	1.089293	1.290835	0.924221	1	0.085529
rs7765566_G	*BTN3A2*	1.145085	1.363507	0.967785	1	0.135479
rs9104_G	*BTN3A2*	0.945089	1.032973	0.86575	1	-0.05648
rs9358934_G	***BTN3A2***	**0.824601**	**0.884767**	**0.76877**	**7.53E-06**	**-0.19286**
rs9379855_T	***BTN3A2***	**0.82361**	**0.883636**	**0.767905**	**6.04E-06**	**-0.19406**
rs9379858_T	***BTN3A2***	**0.825671**	**0.885867**	**0.769811**	**8.99E-06**	**-0.19156**
rs9379859_C	***BTN3A2***	**0.824802**	**0.885058**	**0.768893**	**8.10E-06**	**-0.19261**
rs9379861_G	*BTN3A2*	1.039521	1.399242	0.785634	1	0.03876
rs9393713_G	***BTN3A2***	**0.814157**	**0.873453**	**0.759123**	**9.27E-07**	**-0.2056**
rs9393714_G	***BTN3A2***	**0.818022**	**0.877663**	**0.762671**	**2.08E-06**	**-0.20087**
rs186813312_C	*BTNL3*	0.387143	NA	NA	NA	-0.94896
rs199970076_G	*BTNL3*	0.890096	19.02105	0.105649	1	-0.11643
rs201534771_G	*BTNL3*	0.373628	NA	NA	NA	-0.98449
rs201813197_C	*BTNL3*	0.906025	3.98319	0.295074	1	-0.09869
rs35157246_C	*BTNL3*	1.061269	1.243749	0.909647	1	0.059466
rs4700774_G	*BTNL3*	0.953121	1.014114	0.896074	1	-0.04801
rs59220426_C	*BTNL3*	0.964396	1.094724	0.852069	1	-0.03625
rs73815153_G	*BTNL3*	0.979858	1.113514	0.864825	1	-0.02035
rs7713324_A	*BTNL3*	0.962434	1.092563	0.850278	1	-0.03829
rs7726604_C	*BTNL3*	0.963325	1.093541	0.851096	1	-0.03736
rs112469887_G	*BTNL8*	1.04641	1.299262	0.850631	1	0.045365
rs113071395_G	*BTNL8*	0.908391	1.065436	0.777899	1	-0.09608
rs113534626_A	*BTNL8*	1.008072	1.204841	0.848563	1	0.00804
rs141492316_T	*BTNL8*	0.930335	1.160408	0.752616	1	-0.07221

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rs145199317_A	*BTNL8*	0.884686	1.250181	0.639686	1	-0.12252
rs151174174_C	*BTNL8*	0.828069	1.016412	0.679378	1	-0.18866
rs17704291_C	*BTNL8*	0.952291	1.013025	0.895481	1	-0.04888
rs200633883_C	*BTNL8*	0.419543	1.675223	0.123618	1	-0.86859
rs201214790_T	*BTNL8*	740.6942	NA	1.97E-08	1	6.607588
rs201891387_G	*BTNL8*	0.495896	2.269513	0.147322	1	-0.70139
rs2276995_A	*BTNL8*	0.984754	1.043162	0.929755	1	-0.01536
rs2619739_C	*BTNL8*	1.078254	1.194765	0.974928	1	0.075343
rs7724813_G	*BTNL8*	1.054983	1.150319	0.968751	1	0.053525

Table 5. Single variant analysis of butyrophilin SNPs and CeD status using binomial regression models on the HLA-DQ2.5-matched case-control cohort of the UK Biobank database. SNPs significantly associated with CeD status are in bold. Bonferroni correction was applied due to multiple testing.

SNP name	gene	OR	upper	lower	adjusted p-value	ln(OR)
rs10484441_G	*BTN2A1*	1.026879	1.182655	0.894513	1	0.026524
rs12660069_C	*BTN2A1*	0.954382	1.207579	0.761803	1	-0.04669
rs13195402_G	***BTN2A1***	**0.757206**	**0.831328**	**0.689864**	**5.10E-07**	**-0.27812**
rs13195509_G	***BTN2A1***	**0.77459**	**0.846648**	**0.708837**	**1.75E-06**	**-0.25542**
rs13437351_G	*BTN2A1*	1.400742	2.090478	0.973921	1	0.337002
rs1407045_A	*BTN2A1*	1.080193	1.170756	0.996977	1	0.07714
rs142951857_A	*BTN2A1*	1.287748	4.431929	0.486536	1	0.252895
rs143104579_G	*BTN2A1*	1.331616	1.885897	0.963027	1	0.286393
rs146399224_T	*BTN2A1*	0.25741	NA	NA	NA	-1.35708
rs148111655_G	*BTN2A1*	0.944297	4.178925	0.294481	1	-0.05731
rs2273558_A	*BTN2A1*	0.892885	0.971388	0.820718	0.848901	-0.1133
rs2893856_T	*BTN2A1*	0.927404	1.048809	0.818043	1	-0.07537
rs2893857_C	*BTN2A1*	1.040565	1.199279	0.905854	1	0.039764
rs3734539_C	*BTN2A1*	27166.76	NA	9.99E-14	1	10.20975
rs3734542_G	***BTN2A1***	**0.77697**	**0.849181**	**0.711073**	**2.52E-06**	**-0.25235**
rs3734543_G	***BTN2A1***	**0.792317**	**0.867952**	**0.723463**	**5.43E-05**	**-0.23279**
rs3799380_T	*BTN2A1*	0.869548	0.945573	0.799787	0.107572	-0.13978
rs56296968_C	***BTN2A1***	**0.852755**	**0.928301**	**0.783513**	**0.02331**	**-0.15928**
rs6456724_T	*BTN2A1*	0.924581	1.045479	0.815654	1	-0.07841
rs6907857_T	*BTN2A1*	1.401132	2.091061	0.974192	1	0.33728
rs6911470_C	*BTN2A1*	1.565436	2.679723	0.978088	1	0.448164
rs6929846_T	*BTN2A1*	0.870873	0.973832	0.777255	1	-0.13826
rs7773913_C	*BTN2A1*	1.408409	2.10183	0.979326	1	0.34246
rs7773938_C	***BTN2A1***	**0.854233**	**0.929778**	**0.784985**	**0.026611**	**-0.15755**
rs77870445_T	*BTN2A1*	1.022451	1.530146	0.704151	1	0.022203
rs9348718_A	*BTN2A1*	1.308813	1.636793	1.057849	1	0.26912
rs9358943_C	*BTN2A1*	0.257602	NA	NA	NA	-1.35634
rs9358944_A	***BTN2A1***	**0.850121**	**0.924966**	**0.781482**	**0.016059**	**-0.16238**
rs9358945_A	***BTN2A1***	**0.847905**	**0.922571**	**0.77943**	**0.012607**	**-0.16499**
rs9461254_G	*BTN2A1*	1.565001	2.678973	0.977818	1	0.447886
rs10456045_G	*BTN3A1*	0.872024	0.944198	0.805371	0.074344	-0.13694
rs10807008_G	*BTN3A1*	0.988264	1.128972	0.867514	1	-0.01181
rs12200782_C	*BTN3A1*	0.969241	1.112166	0.84719	1	-0.03124
rs12207930_C	*BTN3A1*	1.059407	1.19361	0.94228	1	0.05771

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rs12208447_C	*BTN3A1*	1.183033	1.71485	0.838562	1	0.168081
rs12214924_T	*BTN3A1*	1.059617	1.192843	0.943249	1	0.057908
rs143476765_A	*BTN3A1*	0.385935	2.931895	0.063895	1	-0.95209
rs144114619_T	*BTN3A1*	0.800306	1.80142	0.391948	1	-0.22276
rs145059723_A	*BTN3A1*	1.546934	29.22579	0.264012	1	0.436275
rs1741738_A	*BTN3A1*	1.126398	1.288465	0.987689	1	0.119025
rs17610161_G	*BTN3A1*	0.999336	1.142965	0.876283	1	-0.00066
rs1796520_C	*BTN3A1*	0.901974	0.977599	0.831877	1	-0.10317
rs3799378_A	***BTN3A1***	**0.829346**	**0.900397**	**0.763968**	**0.00081**	**-0.18712**
rs3857549_C	*BTN3A1*	1.307821	1.557482	1.105147	0.217446	0.268362
rs3902051_A	*BTN3A1*	0.979611	1.113404	0.864043	1	-0.0206
rs41266839_G	***BTN3A1***	**0.753767**	**0.824792**	**0.68903**	**7.25E-08**	**-0.28267**
rs4609015_T	*BTN3A1*	1.055122	1.187787	0.939241	1	0.053657
rs4712990_C	*BTN3A1*	1.009758	1.153906	0.886126	1	0.00971
rs55676749_T	*BTN3A1*	1.356015	1.867802	1.007293	1	0.30455
rs56161420_G	*BTN3A1*	1.060354	1.193295	0.944184	1	0.058603
rs6900725_T	*BTN3A1*	1.061349	1.194313	0.945175	1	0.059541
rs6912853_C	*BTN3A1*	1.087423	1.216203	0.974151	1	0.08381
rs6920986_C	*BTN3A1*	1.062734	1.196651	0.9458	1	0.060845
rs6921148_T	*BTN3A1*	1.340282	1.834522	1.000858	1	0.29288
rs742090_A	*BTN3A1*	0.903554	0.979423	0.833241	1	-0.10142
rs7770214_G	*BTN3A1*	1.053031	1.185369	0.937421	1	0.051673
rs80153343_G	*BTN3A1*	0.979216	1.347528	0.724804	1	-0.021
rs11758089_T	*BTN3A2*	1.191327	1.352007	1.052499	0.618146	0.175068
rs12176317_A	***BTN3A2***	**0.766628**	**0.836949**	**0.702371**	**2.82E-07**	**-0.26575**
rs12194095_C	*BTN3A2*	0.943672	1.092386	0.818034	1	-0.05798
rs12199613_C	***BTN3A2***	**0.842231**	**0.911373**	**0.77819**	**0.002057**	**-0.1717**
rs12205731_G	*BTN3A2*	0.933998	1.081909	0.809117	1	-0.06828
rs144016445_G	*BTN3A2*	73914.07	NA	1.75E-06	1	11.21066
rs1977_A	***BTN3A2***	**0.764904**	**0.835801**	**0.700168**	**2.99E-07**	**-0.268**
rs1979_G	***BTN3A2***	**0.76816**	**0.838582**	**0.70381**	**3.63E-07**	**-0.26376**
rs1985732_A	***BTN3A2***	**0.860169**	**0.932012**	**0.793857**	**0.023502**	**-0.15063**
rs2073526_G	*BTN3A2*	0.90252	0.979048	0.831612	1	-0.10256
rs35183513_G	*BTN3A2*	1.000653	1.140268	0.880486	1	0.000652
rs58367598_T	*BTN3A2*	1.153984	1.470445	0.915197	1	0.143221
rs7765566_G	*BTN3A2*	1.171754	1.497136	0.928192	1	0.158502
rs9104_G	*BTN3A2*	1.010674	1.145196	0.894134	1	0.010617
rs9358934_G	***BTN3A2***	**0.772404**	**0.843549**	**0.707423**	**8.85E-07**	**-0.25825**
rs9379855_T	***BTN3A2***	**0.771306**	**0.842174**	**0.706564**	**6.76E-07**	**-0.25967**
rs9379858_T	***BTN3A2***	**0.774407**	**0.845625**	**0.709352**	**1.18E-06**	**-0.25566**
rs9379859_C	***BTN3A2***	**0.770242**	**0.841254**	**0.705384**	**6.31E-07**	**-0.26105**
rs9379861_G	*BTN3A2*	0.987622	1.586757	0.639198	1	-0.01246
rs9393713_G	***BTN3A2***	**0.760996**	**0.830868**	**0.697149**	**1.06E-07**	**-0.27313**
rs9393714_G	***BTN3A2***	**0.765074**	**0.835356**	**0.700859**	**2.25E-07**	**-0.26778**
rs186813312_C	*BTNL3*	0.257602	NA	NA	NA	-1.35634
rs199970076_G	*BTNL3*	0.272926	6.904492	0.010788	1	-1.29856
rs201534771_G	*BTNL3*	0.273671	NA	NA	NA	-1.29583
rs201813197_C	*BTNL3*	0.514697	3.715301	0.100372	1	-0.66418

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rs35157246_C	*BTNL3*	1.03751	1.287524	0.842551	1	0.036824
rs4700774_G	*BTNL3*	0.978837	1.065525	0.899706	1	-0.02139
rs59220426_C	*BTNL3*	1.017762	1.216405	0.856252	1	0.017606
rs73815153_G	*BTNL3*	1.02474	1.225825	0.861442	1	0.024438
rs7713324_A	*BTNL3*	1.018119	1.216841	0.856546	1	0.017957
rs7726604_C	*BTNL3*	1.020763	1.219953	0.85881	1	0.02055
rs112469887_G	*BTNL8*	1.002918	1.333287	0.765187	1	0.002914
rs113071395_G	*BTNL8*	0.93155	1.162634	0.752338	1	-0.07091
rs113534626_A	*BTNL8*	0.9371	1.185234	0.747845	1	-0.06496
rs141492316_T	*BTNL8*	0.963798	1.314391	0.718601	1	-0.03687
rs145199317_A	*BTNL8*	1.12544	1.912393	0.697448	1	0.118174
rs151174174_C	*BTNL8*	0.729764	0.953457	0.564223	1	-0.31503
rs17704291_C	*BTNL8*	0.969451	1.055155	0.891212	1	-0.03103
rs200633883_C	*BTNL8*	0.171372	1.035119	0.022558	1	-1.76392
rs201214790_T	*BTNL8*	0.256248	NA	NA	NA	-1.36161
rs201891387_G	*BTNL8*	0.171476	1.035747	0.022572	1	-1.76331
rs2276995_A	*BTNL8*	1.002057	1.084332	0.926283	1	0.002055
rs2619739_C	*BTNL8*	0.966968	1.107812	0.846435	1	-0.03359
rs7724813_G	*BTNL8*	1.041603	1.171988	0.927725	1	0.040761

Table 6. SNP and allele count of the SNPs significantly associated with CeD in UK Biobank participants with the *HLA-DQ2.5* genotype. These SNPs were significantly associated with CeD status in the *HLA-DQ2.5*-matched single variant testing of the UK Biobank dataset.

Participants with the *HLA-DQ2.5* genotype							
SNP, reference allele	**Gene**	**Number of the SNP in controls with *HLA-DQ2.5***	**Number of the SNP in CeD with *HLA-DQ2.5***	**Total allele count in control with *HLA-DQ2.5***	**Total allele count in CeD with *HLA-DQ2.5***	**Total number of the SNP in the UK Biobank with *HLA-DQ2.5***	**Total allele count in UK Biobank with *HLA-DQ2.5***
rs13195402_G	*BTN2A1*	9398	2256	12514	3206	11654	15720
rs13195509_G	*BTN2A1*	9408	2265	12820	3296	11673	16116
rs3734542_G	*BTN2A1*	9386	2266	12808	3300	11652	16108
rs3734543_G	*BTN2A1*	9366	2265	12722	3258	11631	15980
rs56296968_C	*BTN2A1*	8609	2107	12798	3290	10716	16088
rs7773938_C	***BTN2A1***	**8606**	**2106**	**12804**	**3290**	**10712**	**16094**
rs9358944_A	*BTN2A1*	8603	2108	12818	3304	10711	16122
rs9358945_A	*BTN2A1*	8607	2106	12818	3302	10713	16120
rs3799378_A	*BTN3A1*	8129	1959	12768	3286	10088	16054

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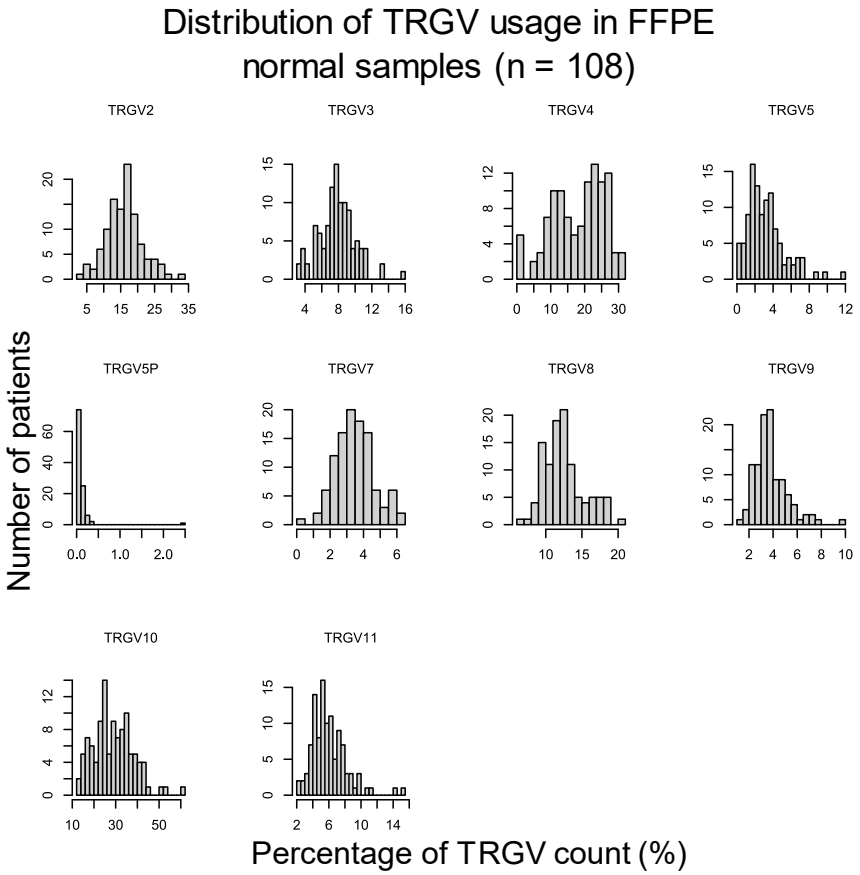
rs41266839_G	*BTN3A1*	9577	2298	12820	3298	11875	16118
rs12176317_A	*BTN3A2*	9347	2242	12826	3302	11589	16128
rs12199613_C	*BTN3A2*	6396	1515	12802	3300	7911	16102
rs1977_A	*BTN3A2*	9135	2197	12574	3248	11332	15822
rs1979_G	*BTN3A2*	9330	2242	12808	3302	11572	16110
rs1985732_A	*BTN3A2*	7353	1777	12816	3294	9130	16110
rs9358934_G	*BTN3A2*	9333	2240	12810	3292	11573	16102
rs9379855_T	*BTN3A2*	9324	2239	12802	3294	11563	16096
rs9379858_T	*BTN3A2*	9321	2242	12804	3296	11563	16100
rs9379859_C	*BTN3A2*	9340	2244	12806	3296	11584	16102
rs9393713_G	*BTN3A2*	9345	2237	12812	3298	11582	16110
rs9393714_G	*BTN3A2*	9346	2241	12818	3300	11587	16118

Appendix D – Supplementary Materials for Results Section 2.3

a)



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b)

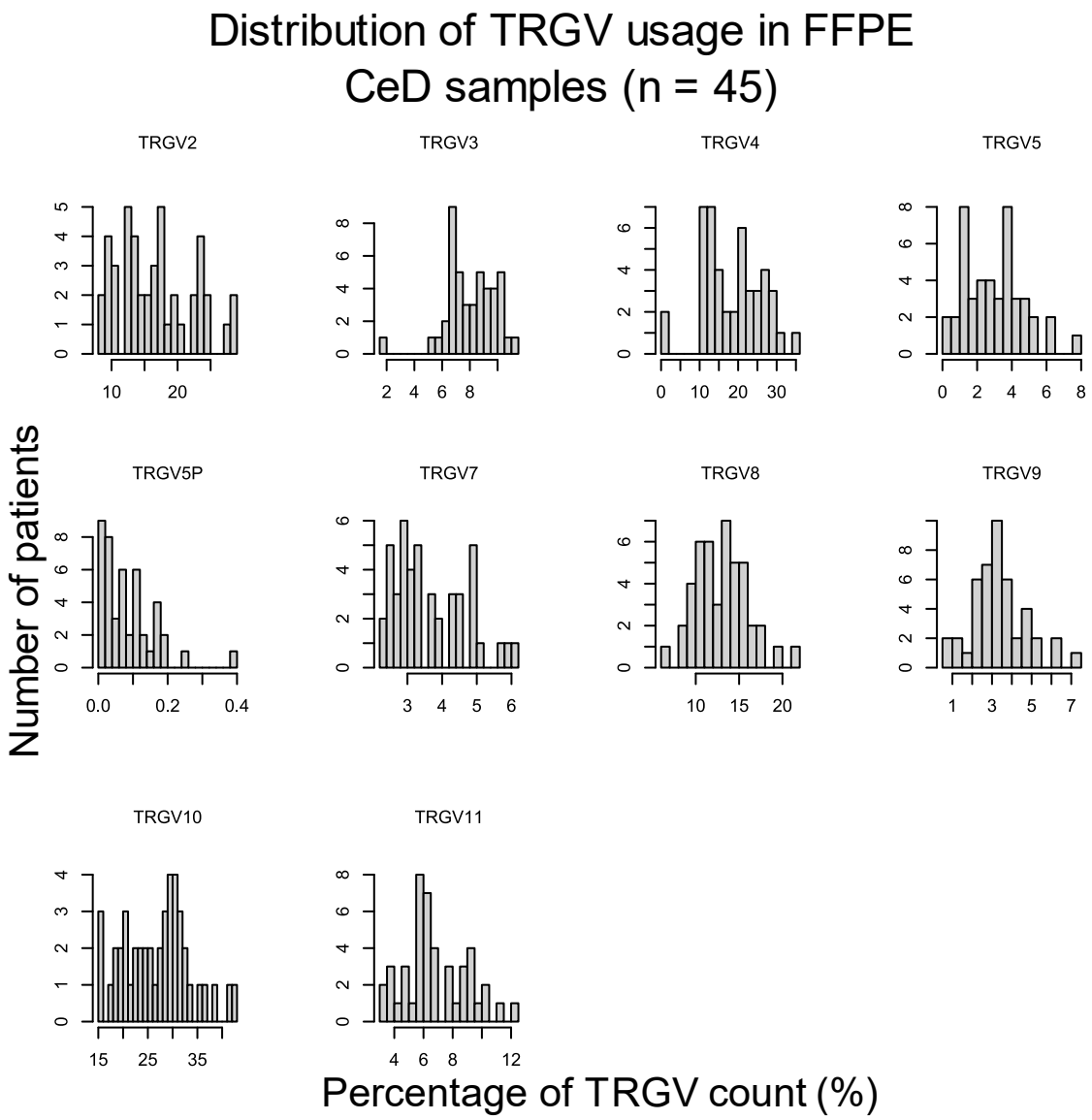

Figure 1. The TRGV usage of a) healthy control (n = 108), and b) CeD FFPE duodenal samples (n = 45) was not normally distributed in the duodenal samples subjected to TRGV usage analysis.

Table 1. There were no significant differences in the TRGV usage of FFPE CeD (n = 45) and healthy control (n = 108) samples after Bonferroni correction was applied. Raw p-values from Mann-Whitney U (MWU) tests were adjusted using Bonferroni correction to account for false positives due to multiple testing.

	FFPE CeD (n = 45) vs FFPE healthy control (n = 108)	
	Raw p-value (MWU)	**Adjusted p-value**
TRGV2	0.775	1
TRGV3	0.411	1
TRGV4	0.812	1
TRGV5	0.906	1
TRGV5P	0.684	1
TRGV7	0.566	1
TRGV8	0.382	1

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TRGV9	0.070	0.70
TRGV10	0.248	1
TRGV11	0.025	0.25

Table 2. There were no significant differences in the HV4 distribution between healthy controls (n = 238) and CeD patients (n = 141). The reference amino acid sequence KYDTYGSTRKNLRMILR is noted as WT in the table. Sequences with amino acid substitutions are provided in full. Pairwise Fisher's exact test with Bonferroni correction was applied on the different HV4 phenotypes in CeD and healthy control patients.

	141 CeD vs 238 healthy control samples	
	Raw p-values (Fisher)	**Adjusted p-values**
WT vs WT, KYDTYGSTRQNLRMIL	0.3925	1
WT vs KYDTYGSTRQNLRMILR	0.3392	1
WT vs KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA	0.3668	1
WT vs WT, KYNTYGSTRKNLRMILR	0.3668	1
WT vs KYDTYGNTRKNLRMILR, WT	1	1
WT vs WT, KYDTYGSTRKSLRMILR	0.6258	1
WT vs KYDTYGSTRKSLRMILR	1	1
WT vs WT, KYDTYGSIRKNLRMILR	0.3668	1
WT, KYDTYGSTRQNLRMILR vs KYDTYGSTRQNLRMILR	0.1697	1
WT, KYDTYGSTRQNLRMILR vs KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA	0.4524	1
WT, KYDTYGSTRQNLRMILR vs WT, KYNTYGSTRKNLRMILR	0.4524	1
WT, KYDTYGSTRQNLRMILR vs WT, KYDTYGNTRKNLRMILR	1	1
WT, KYDTYGSTRQNLRMILR vs WT, KYDTYGSTRKSLRMILR	1	1
WT, KYDTYGSTRQNLRMILR vs KYDTYGSTRKSLRMILR	1	1
WT, KYDTYGSTRQNLRMILR vs WT, KYDTYGSIRKNLRMILR	0.4524	1
KYDTYGSTRQNLRMILR vs KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA	0.25	1
KYDTYGSTRQNLRMILR vs WT, KYNTYGSTRKNLRMILR	0.25	1
KYDTYGSTRQNLRMILR vs WT, KYDTYGNTRKNLRMILR	1	1
KYDTYGSTRQNLRMILR vs WT, KYDTYGSTRKSLRMILR	0.5165	1
KYDTYGSTRQNLRMILR vs KYDTYGSTRKSLRMILR	1	1
KYDTYGSTRQNLRMILR vs WT, KYDTYGSIRKNLRMILR	0.25	1
KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA vs WT, KYNTYGSTRKNLRMILR	1	1
KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA vs WT, KYDTYGNTRKNLRMILR	1	1

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KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA vs WT, KYDTYGSTRKSLRMILR	1	1
KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA vs KYDTYGSTRKSLRMILR	1	1
KYDTYGSTRQNLRMILR, KYDTYGSTR_ELENDTA vs WT, KYDTYGSIRKNLRMILR	1	1
WT, KYNTYGSTRKNLRMILR vs WT, KYDTYGNTRKNLRMILR	1	1
WT, KYNTYGSTRKNLRMILR vs WT, KYDTYGSTRKSLRMILR	1	1
WT, KYNTYGSTRKNLRMILR vs KYDTYGSTRKSLRMILR	1	1
WT, KYNTYGSTRKNLRMILR vs WT, KYDTYGSIRKNLRMILR	1	1
WT, KYDTYGNTRKNLRMILR vs WT, KYDTYGSTRKSLRMILR	1	1
WT, KYDTYGNTRKNLRMILR vs KYDTYGSTRKSLRMILR	1	1
WT, KYDTYGNTRKNLRMILR vs WT, KYDTYGSIRKNLRMILR	1	1
WT, KYDTYGSTRKSLRMILR vs KYDTYGSTRKSLRMILR	1	1
WT, KYDTYGSTRKSLRMILR vs WT, KYDTYGSIRKNLRMILR	1	1
KYDTYGSTRKSLRMILR vs WT, KYDTYGSIRKNLRMILR	1	1

Appendix E – Selecting and Sequencing the Butyrophilin Genes of Interest

Appendix E.1: HPA Expression Profiles of Butyrophilin Family Genes

Table 1. The expression of the butyrophilin family members in intestinal tissues provided by the HPA. Butyrophilin protein expression in a) the duodenum and in b) the small intestine, colon and rectum was extracted from the Tissue section of the Human Protein Atlas database [90,91]. The reliability score of each entry was provided by the HPA. The score was based on the reliability between the RNA sequencing and antibody staining data. For most genes, the HPA provided immunohistochemical evidence for the protein expression of the genes. Only RNA sequencing data were available for *BTNL2*, *BTNL3*, and *BTNL9* expression, denoted with *. Evidence linking the butyrophilins to immune cell function and CeD risk were also used to determine inclusion in the custom sequencing panel [19,21,30]. The expression of butyrophilin family members in b) is shown only for the genes that were selected for the custom probe panel. [Data accessed in 2021].

a) Butyrophilin family expression in the duodenum

	Reliability as defined by the HPA	**Protein expression in duodenum (IHC) *if RNA data only***	**Comment**	**Included?**
ERMAP	Uncertain	High	Uncertain Tissue Atlas reliability score High expression in digestive tissues Low-medium expression in immune cells	Yes
MOG	Enhanced	None	Not expressed in immune cells or digestive tissues	Yes

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			Genomic variance control for the significance of butyrophilin variation in CeD risk	
BTN1A1	Supported	None	Not expressed in digestive tissues or immune cells	No
BTN2A1	Approved	Medium	Included due to reliability score and expression Implicated in the stimulation of Vγ9Vδ2+ T cells [37]	Yes
BTN2A2	Uncertain	High	Uncertain Tissue Atlas reliability score Medium-high expression in digestive tissues Expressed in immune cells	Yes
BTN3A1	Approved	Medium	Included due to reliability score and expression Implicated in the stimulation of Vγ9Vδ2+ T cells [30]	Yes
BTN3A2	Uncertain	Medium	Uncertain Tissue Atlas reliability score Implicated in the stimulation of Vγ9Vδ2+ T cells [27]	Yes
BTN3A3	Enhanced	Medium	Included due to reliability score and expression	Yes
BTNL2	Pending	*None*	Pending Tissue Atlas reliability score *HLA* independent significant association with CeD [19]	Yes
BTNL3	Pending	*High*	Pending Tissue Atlas reliability score No protein expression data available for the intestinal tissues Previously documented role in CeD [21]	Yes
BTNL8	Enhanced	Medium	Previously documented role in CeD [21]	Yes
BTNL9	Pending	*Low*	Pending Tissue Atlas reliability score No protein expression data available for the intestinal tissues Not expressed in immune cells	No
BTNL10	NA	NA	No entry	No
SKINT1L	NA	NA	No entry	No
BTN2A3P	NA	NA	No entry	No

b) Expression of selected butyrophilin family members in the small intestine, colon and rectum

Tissue expression (IHC) *if RNA data only*						Included in the panel?
	Reliability as defined by the HPA	Small intestine (glandular)	Duodenum (glandular)	Rectum (glandular)	Colon (glandular)	
ERMAP	Uncertain	High	High	High	High	Yes
MOG	Enhanced	none	none	none	none	Yes
BTN2A1	Approved	Low	Medium	Medium	Medium	Yes

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BTN2A2	Uncertain	High	High	Medium	Medium	Yes
BTN3A1	Approved	High	Medium	High	High	Yes
BTN3A2	Uncertain	High	Medium	Medium	Medium	Yes
BTN3A3	Enhanced	Medium	Medium	Medium	Medium	Yes
BTNL2	Pending	*Very low*	none	none	none	Yes
BTNL3	Pending	*High*	*High*	*High*	*High*	Yes
BTNL8	Enhanced	Medium	Medium	none	none	Yes

Table 2. The expression of butyrophilin family genes of interest in immune cells provided by the HPA. The butyrophilin family mRNA expression data in immune cells were accessed from the Human Protein Atlas (HPA) [90,91]. The reliability score of each entry was provided by the HPA. The score was based on the reliability between the RNA sequencing and antibody staining data. The RNA expression data from T cells, DCs, NK cells, and macrophages were selected from the Single cell type section of the gene entries. The RNA expression data from T-regs and γδ T cells were accessed from the Immune cell type section of the gene entries. [Data accessed in 2021]

Immune cell expression (RNA sequencing)								Included in the panel?
	Reliability as defined by the HPA	γδ T cells	T cells	T-reg	DCs	Macrophages	NK cells	
ERMAP	Uncertain	Low	Medium	Medium	Medium	Medium	Low	Yes
MOG	Enhanced	Very low	None	Very low	None	None	None	Yes
BTN2A1	Approved	Medium	Medium	Medium	Medium	High	Low	Yes
BTN2A2	Uncertain	Low	Medium	Medium	High	High	Medium	Yes
BTN3A1	Approved	High	High	High	Low	Medium	High	Yes
BTN3A2	Uncertain	High	High	High	Medium	High	High	Yes
BTN3A3	Enhanced	High	High	High	Medium	Medium	High	Yes
BTNL2	Pending	None	None	None	None	None	None	Yes
BTNL3	Pending	None	None	None	None	None	None	Yes
BTNL8	Enhanced	None	None	None	None	None	None	Yes

Appendix E.2: Modified Nonacus Cell3 Hybridization Capture and Illumina Sequencing

To summarise the modified protocol, the DNA quality of all samples were measured initially, to acquire 200ng input DNA for the fragmentation step (1.B) of the hybridization capture protocol. Fragmentation time in step 1.B was modified to 30 minutes to achieve 200bp DNA fragments. Afterwards, the Genomic Tapestation kit (Agilent Technologies) was used to check the correct fragment size.

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Next in step 1.C, unique molecular identifier (UMI) adapters were ligated to the DNA fragments. During the magnetic bead clean up using NGS Target Pure Clean-Up Beads (Nonacus) the adapter ligated DNA fragments were incubated for 6 minutes on the magnetic strip. Nuclease free water was used in the final step of the clean-up.

The pre-hybridization PCR in step 1.D was carried out for 4 cycles. Afterwards, the DNA concentration of each reaction was measured in step 1.E. Samples with DNA concentrations lower than 10 ng/μL were subjected to additional rounds of amplification, and steps 1.D and 1.E were repeated. CB22, CB24, CB26, CB27 and CB30 had low DNA concentration after the amplification step, therefore they were subjected to 5 additional cycles of PCR. Sample NB7 was also subjected to 6 more cycles of PCR.

In step 2.A, the samples were pooled together, each library containing DNA fragments from 8 patients. For each sample, 125ng of DNA was used, and the pooled libraries were dried using a vacuum concentrator for 30 minutes. Each pooled library was hybridized overnight at 65 °C with the designed butyrophilin probes and a 1:50 dilution of *HLA* probes provided by Nonacus.

In step 2.B, the hybridized library was captured on Dynabeads M-270 Streptavidin beads (Invitrogen/Thermo Fisher Scientific). Post-hybridization PCR was carried out for 20 cycles in step 2.C. This was followed by bead clean up using NGS Target Pure Clean-Up Beads. Similarly to step 1.C, the beads and the amplified captured library were incubated for 6 minutes on the magnetic strip. Nuclease free water was used in the final step of the clean-up.

In step 2D, the concentration of the captured library was measured using Qubit, and the size of the DNA fragments in each hybridisation library was quantified using Tapestation.

Illumina MiSeq sequencing required each captured hybridisation library to be diluted to 10nM concentration. The concentration of each sample in nM was calculated using the following equation:

$$concentration\ (nM) = \frac{concentration\ (ng/\mu L)}{660 * DNA\ fragment\ size\ (bp)} * 10^6$$

where the concentration (ng/μL) was the DNA concentration of the captured library as quantified by Qubit, and the DNA fragment size (bp) was the average DNA fragment size as measured by Tapestation.

After each of the 12 hybridized libraries were diluted to 10nm, 2μL of each diluted library were mixed together. Afterwards, 10μL was sent to the Department of Biochemistry for sequencing using the Illumina MiSeq system.

Appendix E.3: Measuring DNA Quantity and Fragment Size

Qubit 2.0 fluorometer (Invitrogen) was used to measure nucleic acid quantity, using the Qubit dsDNA High Sensitivity Quantification Assay kit (Invitrogen) according to manufacturers' instructions.

The 4200 Tapestation System (Agilent Technologies) was used to measure the fragment size of DNA samples. The Genomic DNA ScreenTape Analysis kit (Agilent Technologies) was used to measure the fragment sizes of DNA samples after step 1.B of the Nonacus hybridization capture protocol (Nonacus). The D1000 ScreenTape Assay kit (Agilent Technologies) was used to measure the DNA sizes of the pooled hybridization libraries in step 2.D of the Nonacus hybridization capture protocol.

Appendix F – Detailed Germline Short Variant Discovery Protocol

Appendix F.1: Per Sample Preprocesses and Variant call Using GATK

The variant discovery process for the targeted sequencing cohort was split into two parts. In the first part, each patient sample was processed separately. The variants were called per sample as recommended by the GATK v4.2.6.0 documentation. In the second part of the variant discovery process, the variant-called samples were consolidated, and genotyping was performed jointly for the whole cohort (Appendix F.2).

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The workflow management software Snakemake was used to orchestrate the per-sample preprocessing and variant calling part of the pipeline (Figure F1) [92].

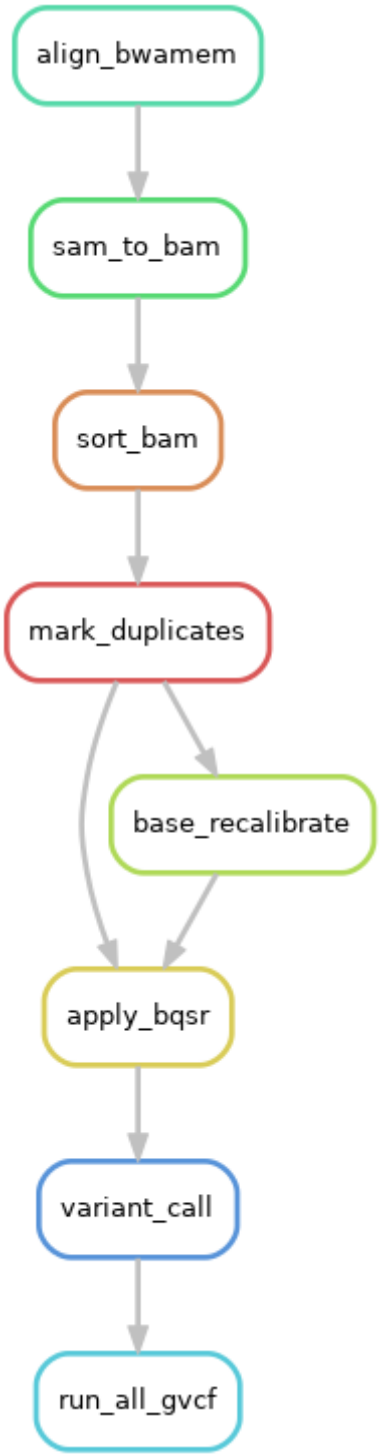

Figure 1. The genetic variants were called per sample for the hybridization capture samples by adapting GATK best practices. Each box symbolises a step or rule in the Snakemake workflow [92]. The directed acyclic graph was created using the Snakemake software's built in commands.

Based on the preprocessing methods of Cucco, *et al.* [74,75], the adapter trimmed raw sequencing files were mapped to the Genome Reference Consortium Human Build 38 (GRCh38) human reference genome using the Burrow-Wheeler Aligner (bwa) v0.7.17 program in the `align_bwamem` rule [93,94]. The resulting sequence alignment map (SAM) files were converted to binary alignment maps (BAM) (`sam_to_bam`), then sorted (`sort_bam`) and indexed using the SAMtools version 1.16.1

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program [78]. The mapping efficiency of the sorted BAM files were assessed using the command `samtools stats`.

Next, we used GATK v4.2.6.0 to carry out germline short-variant discovery in accordance with GATK best practices [72]. First, any duplicate reads that were derived from the same original DNA sample were marked using the MarkDuplicates tool (`mark_duplicates`). This was followed by calculating (`base_recalibrate`) and correcting any errors detected in the base quality scores (`apply_bqsr`) using the BaseRecalibrator and ApplyBQSR tools respectively. Following these preprocessing steps, the SNP and indel variants were called for each sample using the HaplotypeCaller tool in GVCF mode (`variant_call`).

Appendix F.2: Joint Genotyping Using GATK and Variant Annotation Using Vcftools and ANNOVAR

In the second part of the variant discovery process, the samples that had undergone variant calling were subjected to consolidation, followed by joint genotyping. Here the samples were separated into CeD and control groups before consolidating the samples into a joint dataset.

The joint genotyping was carried out using GATK programs with default settings (Figure F2). First, the germline cohort data was created by consolidating the per-sample genomic variant call format (GVCF) files created by the HaplotypeCaller tool as described above. The sample consolidation step was carried out by the GenomicsDBImport tool (`consolidate_gvcfs`). The resulting cohort database was passed to the GenotypeGVCFs joint genotyping tool (`jointcall_cohort`). Next, the raw variants were filtered in a two-step process. First the variant quality scores on the log-odds scale (VQSLOD) were calculated using the VariantRecalibrator tool (`variant_recalibration`). A filtering threshold was applied to these variant quality scores to produce a set of high quality variant calls using the ApplyVQSR tool (`applyvqsr`). The output of the above joint cohort processes was a recalibrated VCF file, that contained all genotyping data of the cohort. The recalibrated VCF files were annotated by applying VCFtools v0.1.17 in frequency (`run_vcffreq`), count (`run_vcfcounts`) and comparison (`compare_vcf`) mode [89]. Variant annotation was also carried out using the table_annovar program from ANNOVAR version 2020Jun08 with default settings (`table_annovar`) [95].

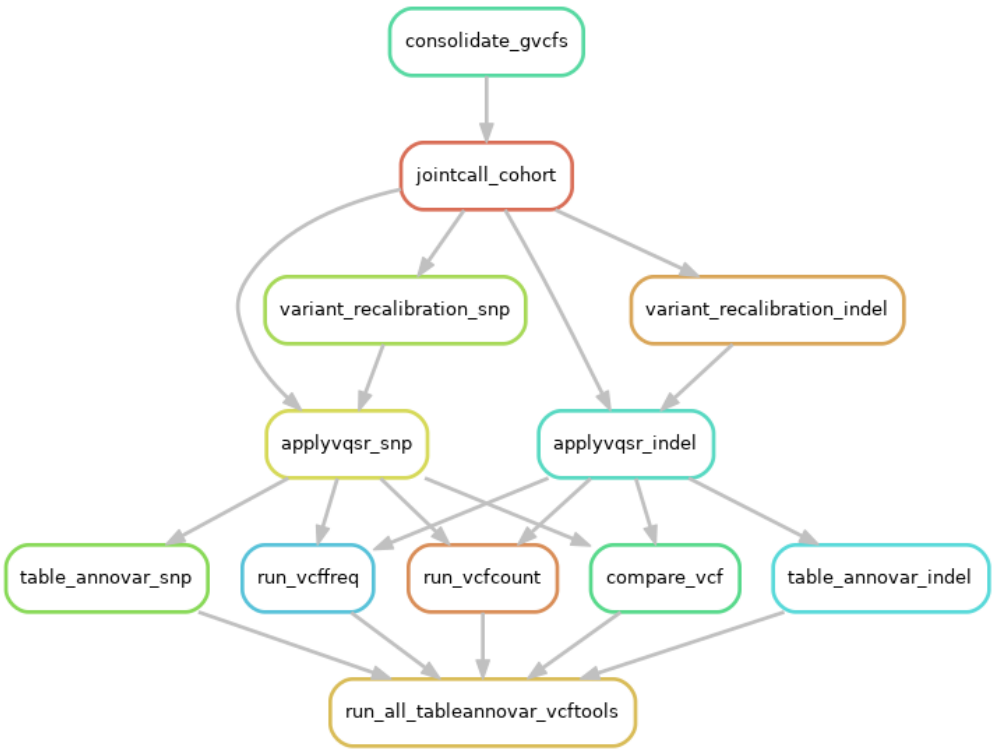

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Figure 2. The samples of the targeted sequencing cohort (n = 94) were jointly genotyped and the variants were annotated using an adapted GATK workflow. Each box symbolises a step or rule in the Snakemake workflow [92]. The directed acyclic graph was created using the Snakemake software's built in commands.

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