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ARTICLE

Butyrophilin Downregulation in Chronic Lymphocytic Leukaemia: An Important Barrier to γδ T Cell-Mediated Cytotoxicity

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ABSTRACT: Introduction: Butyrophilins (BTNs) belong to the immunoglobulin superfamily; they play crucial roles in immune regulation, especially in γδ T cell activation. While their expression has been studied in solid tumours, their involvement in hematologic malignancies remains poorly understood. **Objectives:** We hypothesised that BTNs are dysregulated in chronic lymphocytic leukaemia (CLL), contributing to γδ T cell dysfunction and potentially influencing disease progression. **Methods:** In this study, we analyzed publicly available microarray and RNA-seq datasets to investigate the expression patterns of *BTN* genes in CLL. **Results:** Our findings reveal significant dysregulation of *BTN* gene expression in CLL, with *BTN2A1*, *BTN3A1*, *BTN3A2*, and *BTN3A3* being markedly downregulated in peripheral blood mononuclear cells (PBMCs) and bone marrow samples from CLL patients compared to healthy volunteers, while *BTN1A1* was upregulated. Furthermore, *BTN2A2* was selectively downregulated in neoplastic B cells, whereas *BTN3A1* was upregulated in T cells from CLL patients compared to healthy volunteers. Notably, lower *BTN* expression was associated with an unmutated *IGVH* status and male sex. Kaplan-Meier survival analysis demonstrated that higher expression of *BTN2A1*, *BTN3A1*, *BTN3A2*, and *BTN3A3* correlated with a significantly longer overall survival. **Conclusions:** Given the established role of BTN2A1 and BTN3A1 in the phosphoantigen-mediated activation of Vδ2 γδ T cells, their downregulation may contribute to γδ T cell dysfunction in CLL. These results highlight the potential prognostic value of *BTN* gene expression in CLL and underscore the need for further studies exploring its mechanistic role in disease progression and immune evasion.

KEYWORDS: Butyrophilins; chronic lymphocytic leukemia (CLL); γδ T cells; BTN2A1; BTN3A1; RNA-seq

1 Introduction

Butyrophilins (BTN) are a family of proteins encoded by 7 genes: *BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A1, BTN3A2, BTN3A3*. Since *BTN2A3* is a pseudogene, there are only six functional BTNs [1]. They belong to the superfamily of immunoglobulins and structurally resemble the regulatory B7 family of molecules [2,3]. BTNs are strongly expressed in lymphoid tissues, including B cells and T cells [2]. Among them, *BTN1A1* is rarely expressed in leukocytes, whereas *BTN2A2* is exclusively expressed in these cells. All other BTNs are widely expressed in different tissues, both healthy and cancerous [1]. So far, BTN expression has been primarily studied in solid tumours [4]. For instance, low expression of *BTN3A3* is a negative prognostic factor in non-small cell lung cancer [5]. Similarly, higher expression of *BTN3A1* in bladder cancer indicates better overall survival [6].

BTN2A1, BTN3A1, BTN3A2, and BTN3A3 are involved in γδ T cell stimulation, while BTN1A1 and BTN2A2 exert inhibitory effects [3]. BTN2A1 and BTN3A1 are crucial for the phosphoantigen-dependent

activation of human Vδ2 cells [3,7]. Even though the function of BTN3A1 had been recognised for some time, only the discovery of BTN2A1's importance was a breakthrough [8]. Under physiological conditions, the phosphoantigen level in normal human cells remains low. However, in numerous infections or neoplastic processes, the concentration of phosphoantigens increases. Phosphoantigens bind the intracellular B30.2 domain of BTN3A1, which leads to its heterodimerisation or heteropolymerisation with BTN2A1 and possibly also BTN3A2. This complex is then recognized by the Vγ9Vδ2 TCR unit, resulting in Vδ2 activation [9–11].

γδ T cells constitute a small fraction of total T cells (approx. 2%–5%), yet they are a crucial part of the immune system due to their ability to rapidly respond to infections, e.g., tuberculosis, and cancer. While they are essential in normal immunosurveillance, γδ T cells may also be implicated in the pathogenesis of various diseases, including multiple sclerosis or asthma [12–14]. They are typically divided into Vδ1, Vδ2, and Vδ3 based on the variable fragment of the T cell receptor (TCR) δ they use [15]. Each subset recognises distinct, partially conserved antigens: Vδ1 primarily binds to self-antigens such as MIC-A (MHC class I polypeptide–related sequence A) or MIC-B (MHC class I polypeptide–related sequence B), Vδ2 responds to phosphoantigens, whereas the antigen targets of Vδ3 remain poorly characterised [16–19].

γδ T cells, especially the Vδ2 ones, are exhausted and dysfunctional in the course of chronic lymphocytic leukaemia (CLL) [20]. While human γδ T cells, both Vδ1 and Vδ2 ones, from healthy donors recognise and potently kill CLL cell lines like MEC-1 *in vitro*, those derived from CLL patients show severely limited cytotoxic activity [21,22]. CLL patients have previously been divided based on the proliferative capacity of Vδ2 cells into responders and non-responders; the non-responders are more likely to have unmutated *IGVH* (immunoglobulin heavy chain variable region), an important negative prognostic marker in CLL [23–25]. Notably, *IGVH* mutation status is of clinical significance. While the classical front-line therapy consists of a fludarabine, cyclophosphamide, and rituximab regimen, patients with unmutated *IGVH* are less likely to respond well to treatment and fail to achieve long-term remissions. Additionally, they are more frequently affected by severe toxicities, including treatment-related myeloid neoplasms [26]. Various mechanisms underlying impaired proliferation were proposed and tested, but the expression pattern of BTNs in CLL has never been investigated.

We hypothesised that BTNs are significantly dysregulated in CLL patients and that their altered expression contributes to γδ T cell dysfunction, which in turn may affect disease progression and patient survival. To test our assumptions, we extracted the expression data of *BTN* genes from publicly available datasets and, after normalisation, conducted comparative analyses.

2 Methods

2.1 Datasets

Microarray datasets were retrieved from the Gene Expression Omnibus (GEO) Dataset website, a repository maintained by the National Library of Medicine (https://www.ncbi.nlm.nih.gov/gds) [27]. Datasets that included CLL patients were selected, but only untreated samples were considered for the analysis. Additionally, datasets containing healthy controls were included for comparison. The following datasets were analyzed: GSE20211 [28], GSE21029 [29], GSE21942 [30], GSE9476 [31], GSE22255 [32], GSE22356 [33], GSE27567 [34], GSE6691 [35], GSE47552 [36], GSE22529 [37], GSE54017 [38]. Additionally, RNAseq data from a large cohort of CLL patients from the Broad Institute, along with limited clinical data, were obtained from cBioPortal [39,40].

2.2 Bioinformatic Analysis

Series matrix files were downloaded, and gene expression data were normalised using Min-Max scaling (0–10). Normalisation enabled direct comparison of data from different datasets. Probes corresponding to *BTN* family genes were extracted, with probe names selected based on annotations specific to each microarray platform used in the study. The normalisation, probe selection, and metadata extraction were handled by a Python script. If more than one probe corresponded to the same gene, all of them were extracted, and an average expression was calculated. Data for T cells were extracted from a single dataset, thus, they did not require any normalisation and are presented as transcripts per million (TPM). Similarly, the Broad Institute dataset did not require normalisation as it was independently analyzed.

2.3 Statistics

Statistical analysis was performed in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk test was used to assess data distribution. Due to a non-normal distribution, the Mann-Whitney U test was used to calculate *p*-values. Data are presented as individual points with marked median and IQR (interquartile range). Survival analysis was assessed in JASP 0.19.3 (Department of Psychological Methods, University of Amsterdam) with Kaplan-Meier curves, and the statistical significance was then calculated with the Mantel-Haenszel (log-rank) test.

3 Results

3.1 Butyrophilins Are Downregulated in CLL-Derived PBMCs

Initially, we analyzed the total expression of *BTN* genes in peripheral blood mononuclear cells (PBMCs) of CLL patients and healthy volunteers. *BTN2A1*, *BTN3A1*, *BTN3A2*, and *BTN3A3* were all significantly downregulated in CLL patients (Fig. 1B,D–F). This suggests a broad dysregulation of BTNs in CLL pathophysiology. In contrast, *BTN1A1* was upregulated in CLL patients (Fig. 1A), while BTN2A2 did not exhibit any statistically significant differences (Fig. 1C).

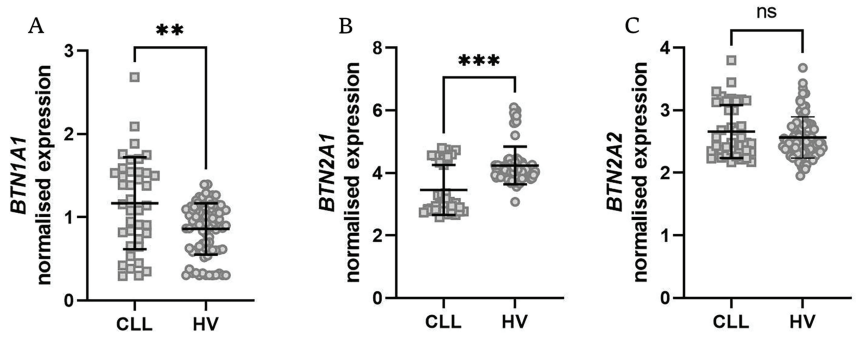


Figure 1: (Continued)

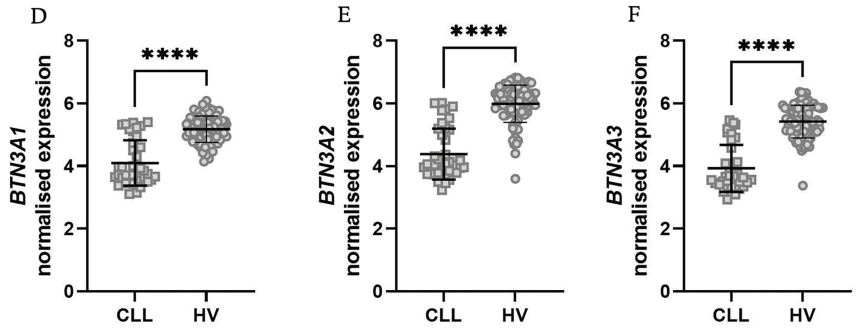


Figure 1: Expression levels of various butyrophilin (BTN) genes in PBMCs from healthy volunteers (HV) and patients with chronic lymphocytic leukaemia (CLL). The normalised expression data for *BTN1A1* (**A**), *BTN2A1* (**B**), *BTN2A2* (**C**), *BTN3A1* (**D**), *BTN3A2* (**E**), and *BTN3A3* (**F**) are displayed as scatter dot plots with a median and interquartile range. Statistical analysis indicates significant upregulation of *BNT1A1* (**A**), while downregulation of *BTN2A1*, *BTN3A1*, *BTN3A2*, and *BTN3A3* (**B**–**E**) in CLL samples compared to HV was noted. *BTN2A2* expression (**C**) shows no significant difference. Statistical significance was assessed using the U Mann-Whitney test. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant

3.2 All Six Butyrophilins are Significantly Dysregulated in the Bone Marrow of CLL Patients

Following our PBMC analysis, we extended our investigation to bone marrow samples from both CLL patients and healthy volunteers. Consistent with our PBMC findings, *BTN1A1* was again significantly upregulated in CLL patients (Fig. 2A), while the remaining five *BTN* genes were significantly downregulated (Fig. 2B–F). A notable difference should be noted for *BTN2A2*, which did not differ in PBMCs, while being substantially downregulated in bone marrow.

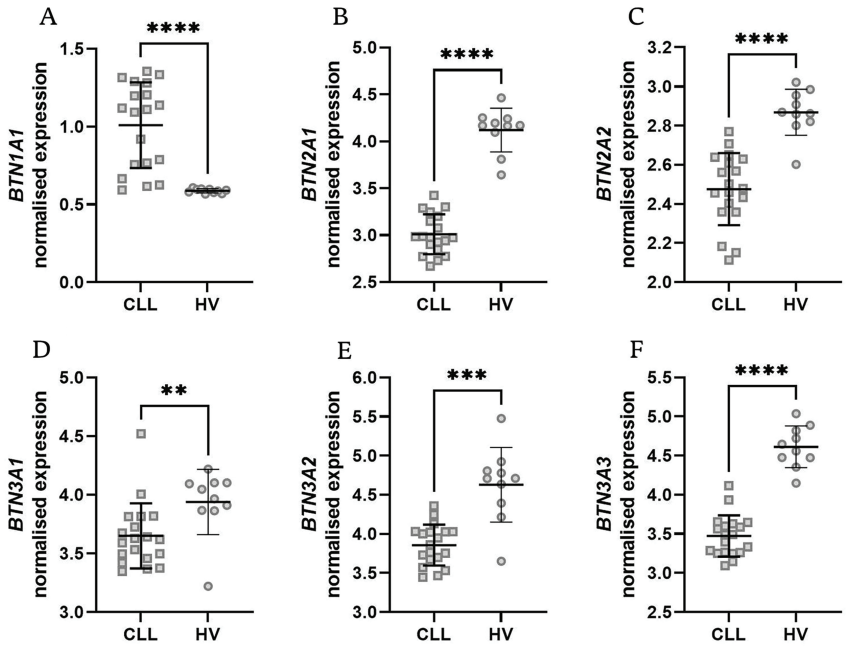


Figure 2: Expression levels of various butyrophilin (*BTN*) genes in bone marrow samples from healthy volunteers (HV) and chronic lymphocytic leukaemia (CLL) patients. *BTN1A1* showed a significant increase in CLL patients (**A**), while *BTN2A1, BTN2A2, BTN3A1, BTN3A2*, and *BTN3A3* showed a significant decrease (**B**–**F**). Normalised expression levels are displayed as scatter dot plots with median and interquartile range. Statistical significance was assessed using the U Mann-Whitney test. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$

3.3 *BTN2A2 Is Downregulated in Isolated Neoplastic B Cells, and BTN3A1 Is Upregulated in T Cells from CLL Patients*

To refine our understanding of *BTN* dysregulation in specific immune compartments, we analyzed BTN expression in isolated neoplastic B cells and T cells obtained from the peripheral blood of CLL patients and healthy volunteers. In contrast to PBMCs, a relatively uniform expression of nearly all butyrophilins was noted in B cells except for *BTN2A2*, which was significantly downregulated in CLL (Fig. 3A–F).

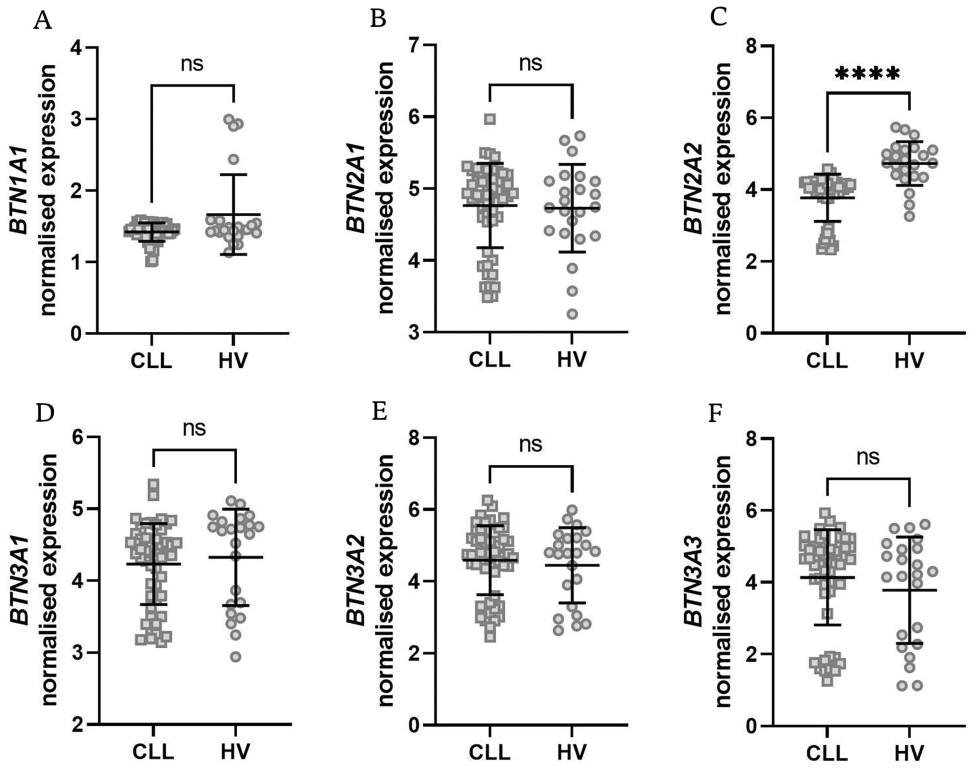


Figure 3: Normalised expression levels of *BTN1A1*, *BTN2A1*, *BTN2A2*, *BTN3A1*, *BTN3A2*, and *BTN3A3* in isolated B cells from healthy donors (HV) and neoplastic B cells from chronic lymphocytic leukaemia (CLL) patients. *BTN2A2* expression was significantly lower in CLL patients (**C**), while no differences were noted for other *BTNs* (**A,B,D–F**). Data are displayed as scatter dot plots with median and interquartile range. Statistical significance was assessed using the U Mann-Whitney test. ****$p < 0.0001$; ns, not significant

BTN1A1 was not expressed in T cells, whereas *BTN3A1* was markedly upregulated in T cells from CLL patients (Fig. 4C). The expression pattern of the remaining *BTN* genes showed no substantial differences (Fig. 4A,B,C,E).

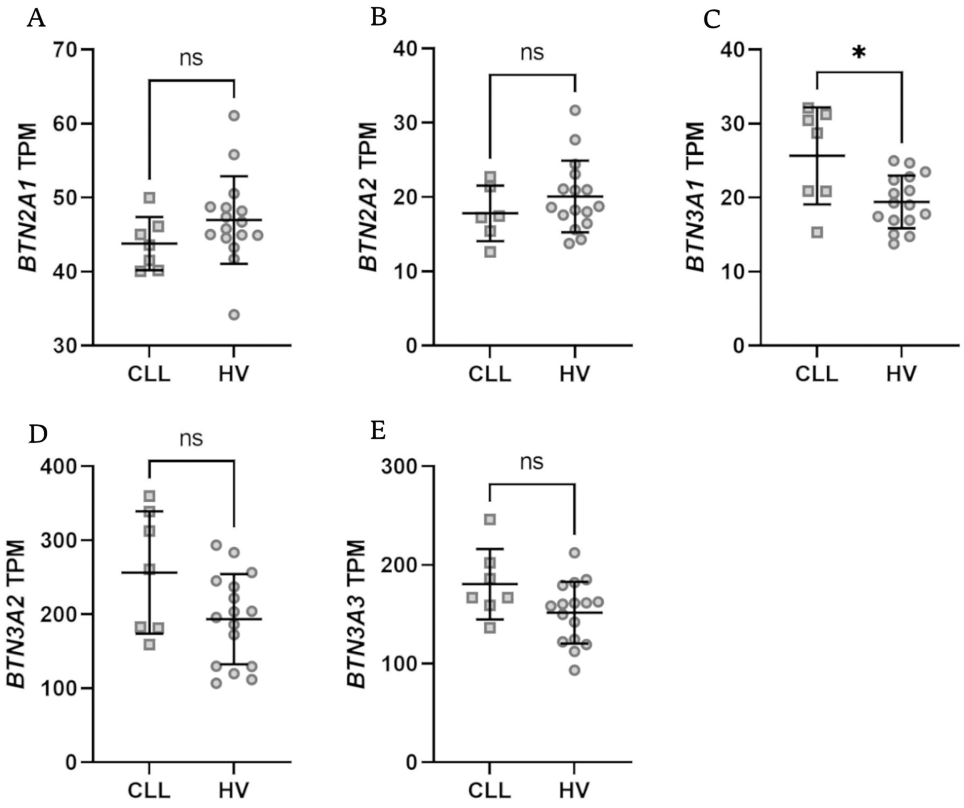


Figure 4: Expression levels of *BTN* genes in isolated T lymphocytes from healthy volunteers (HV) and chronic lymphocytic leukaemia (CLL) patients. There was no expression of *BTN1A1*. Only *BTN3A1* showed a significant difference with notable upregulation in CLL patients (**C**); no differences were noted for *BTN2A1, BTN2A2, BTN3A2, BTN3A3* (**A,B,D,E**). Data are expressed in transcripts per million (TPM), with statistical significance assessed using the Mann-Whitney U test. Data are displayed as scatter dot plots with median and interquartile range. *$p < 0.05$; ns, not significant

3.4 The Expression of BTN Genes Is Downregulated in Patients with Unmutated IGVH as Well as in Male Subjects

Then, we analyzed the Broad Institute dataset that contained gene expression data from PBMCs of 715 CLL patients [40]. We subdivided the patients into those with mutated and unmutated *IGVH* genes. The former group had significantly higher expression of all *BTN* genes (Fig. 5A–F).

Additionally, the dataset included information on patient sex, allowing for further stratification into male and female groups. Interestingly, female subjects had significantly higher expression of *BTN* genes except for *BTN1A1* (Fig. 5G–L).

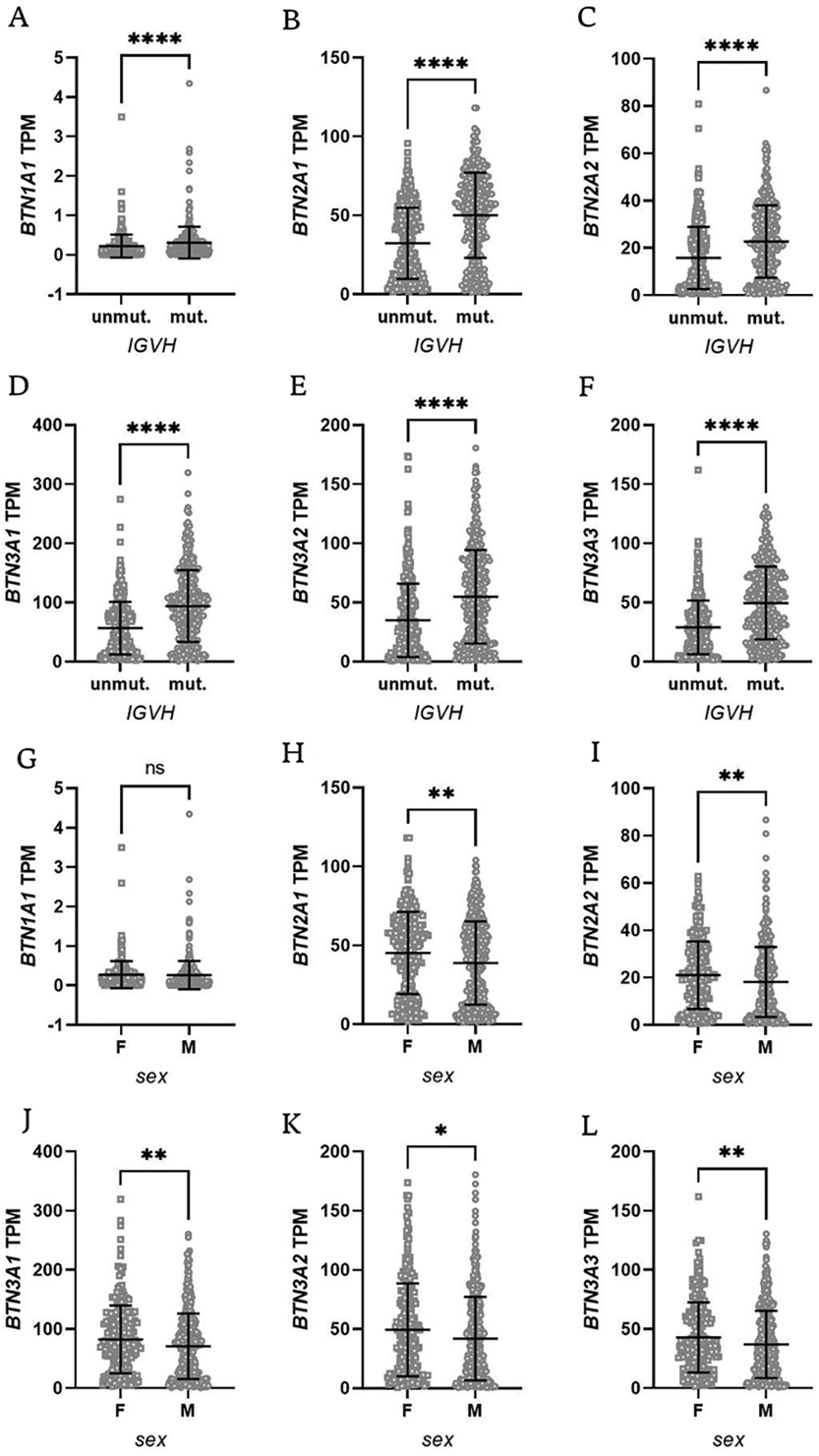
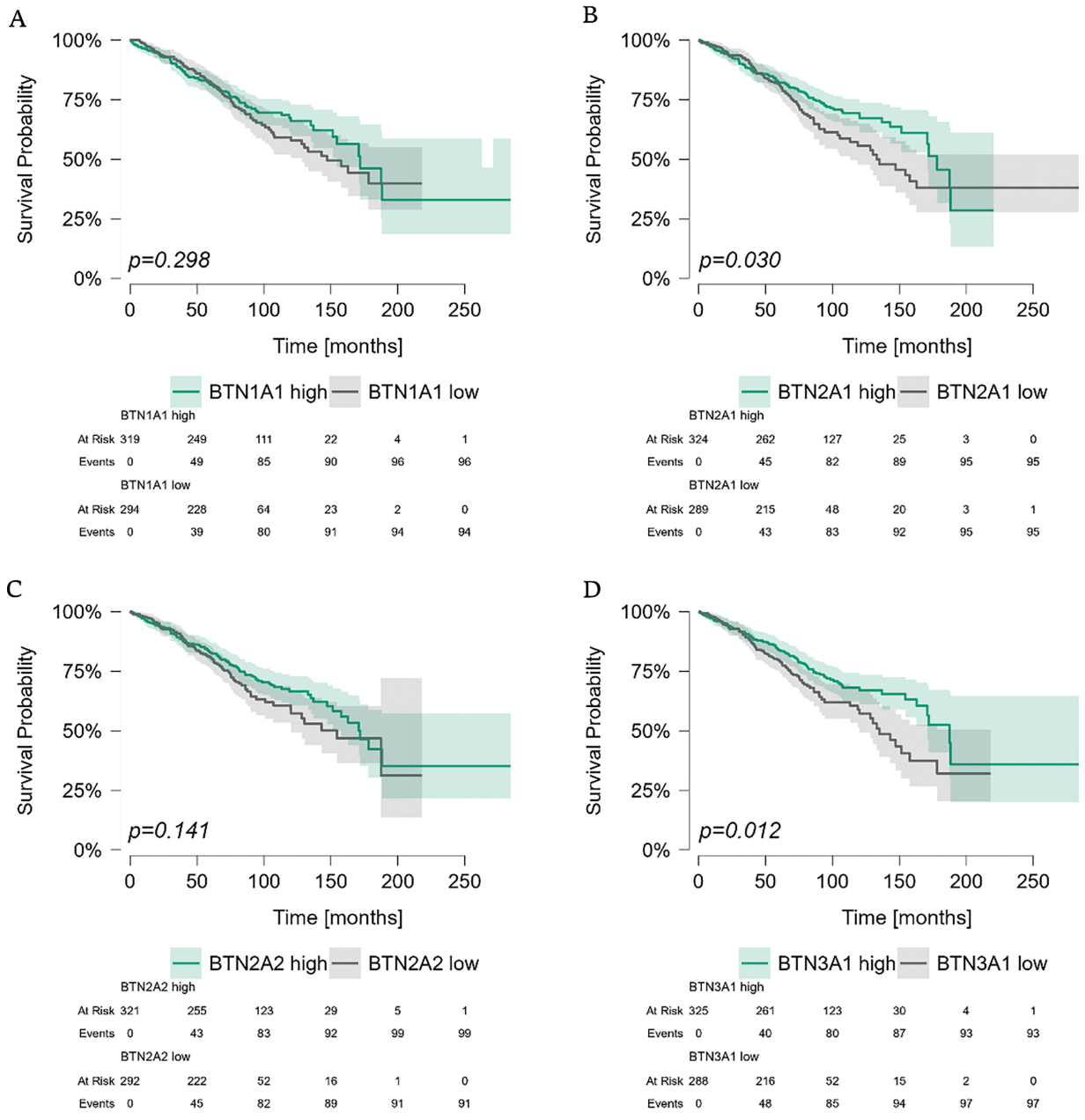


Figure 5: Butyrophilin (BTN) gene expression in CLL patients stratified by *IGVH* mutation status and sex. Patients were grouped based on *IGVH* mutation status (mutated vs. unmutated, panels **A–F**) or sex (female vs. male, panels **G–L**). A significantly lower expression of *BTN* genes was observed in unmutated *IGVH* cases, as well as in male patients. Data are displayed as scatter dot plots with median and interquartile range. Statistical significance was assessed using the Mann-Whitney U test, with significance levels indicated as follows: *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; ns, not significant; F, female; M, male

3.5 *Patients with Higher BTN Expression Had Longer Overall Survival*

Finally, we analyzed the impact of *BTNs* on overall survival (OS) in CLL patients (Fig. 6A–F). The Broad Institute dataset included both survival status and duration, with follow-up data extending up to 20 years for a limited subset of patients. While *BTN1A1* and *BTN2A2* expression did not affect OS, significant differences were observed for the remaining *BTN* genes.



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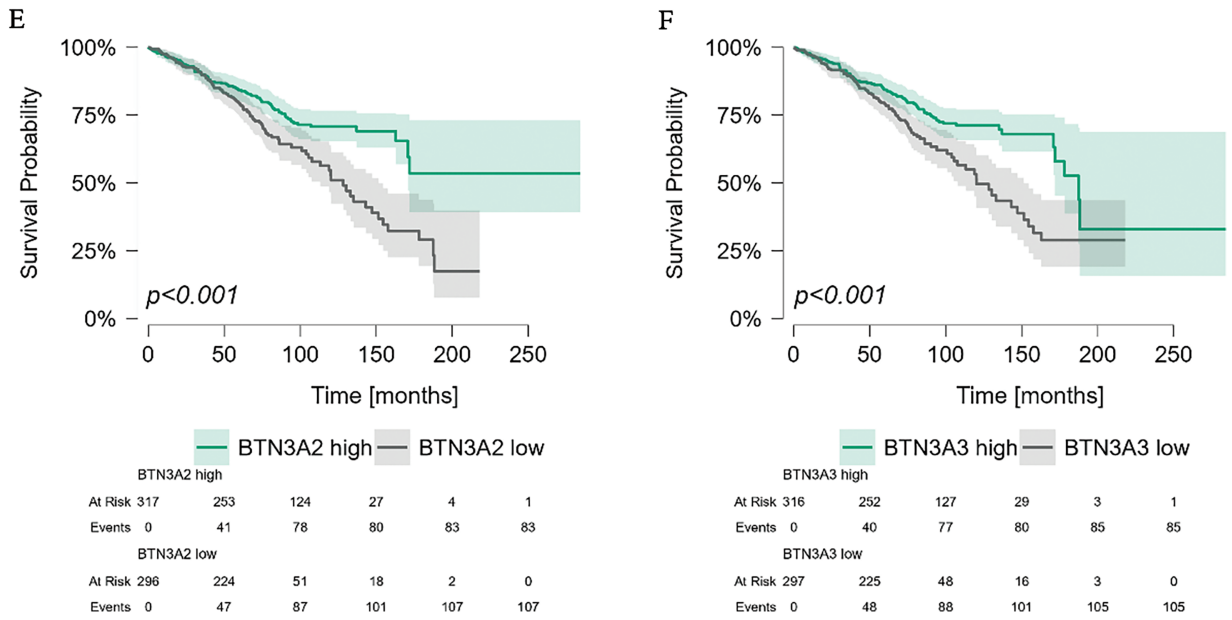
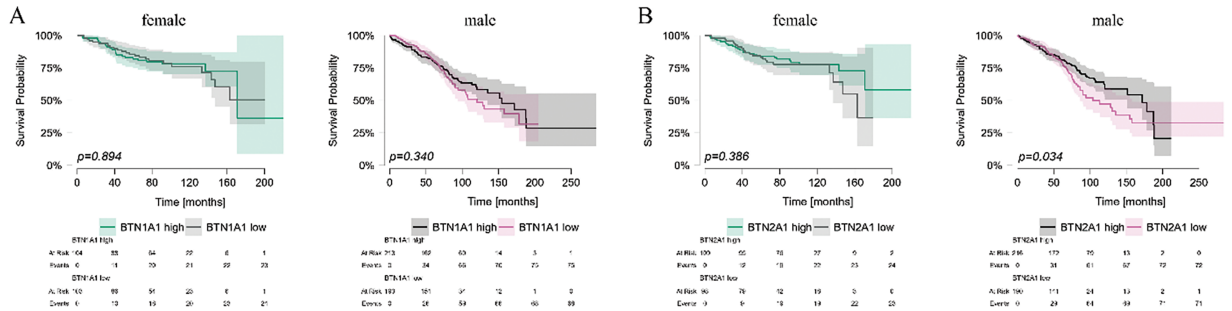


Figure 6: Kaplan-Meier survival analysis of CLL patients stratified by butyrophilin (*BTN*) expression in PBMCs. Patients were divided into high and low-expression groups based on the median expression value for each *BTN* gene. Survival was analyzed using the Kaplan-Meier curves; statistical significance was assessed using the Mantel-Haenszel (log-rank) test. Time is shown in months. Significant differences in survival were observed for *BTN2A1* (p = 0.030), *BTN3A1* (p = 0.012), *BTN3A2* (p < 0.001), and *BTN3A3* (p < 0.001), whereas *BTN1A1* (p = 0.298) and *BTN2A2* (p = 0.141) (**A**–**F**) showed no significant impact on survival

As the expression of *BTN* genes in PBMCs differed between sexes, we stratified patients by sex into male and female groups, and analyzed OS in these subgroups based on the expression levels of individual BTN genes. Generally, results for both sexes were comparable. No differences in OS were noted for *BTN1A1* and *BTN2A2* (Fig. 7A,C), and similar differences for both male and female subgroups were observed in *BTN3A1, BTN3A2*, and *BTN3A3* (Fig. 7D–F). The only visible inter-sex difference was noted for *BTN2A1*, which seems to impact the OS in female patients, but not in males (Fig. 7B).



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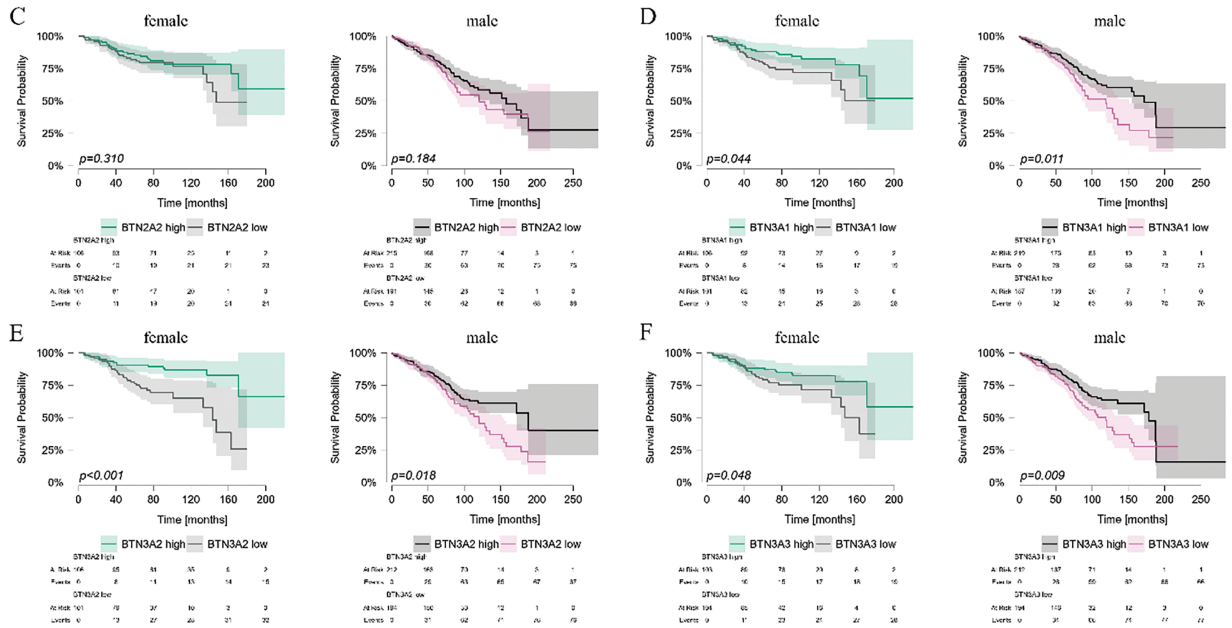

Figure 7: Kaplan-Meier survival analysis of CLL patient survival stratified by butyrophilin (*BTN*) expression in PBMCs, analyzed separately for male and female individuals. Patients were divided into high and low-expression groups based on the median expression value for each *BTN* gene: *BTN1A1* (**A**); *BTN2A1* (**B**); *BTN2A2* (**C**); *BTN3A1* (**D**); *BTN3A2* (**E**); *BTN3A3* (**F**). Survival was analyzed using the Kaplan-Meier curves; statistical significance was assessed using the Mantel-Haenszel (log-rank) test. Time is shown in months. Significant ($p < 0.05$) differences are marked by the indication of *p* values on the graph

4 Discussion

In this study, we identified significant dysregulation of *BTN* gene' expression in CLL. Moreover, this dysregulation had significant clinical implications, as higher *BTN* expression was linked to longer OS.

BTNs are crucial for the phosphoantigen-mediated activation of the Vδ2 subset of γδ T cells. As previously mentioned, γδ T cells in CLL are exhausted and functionally impaired [20]. To date, we lack any mechanistic explanation for this phenomenon. The dysregulation of BTN2A1 and BTN3A1 and their significantly lower expression in CLL are probably at least partially responsible for the γδ T dysfunction. Indeed, as recently proved by Cano et al., the expression of BTN2A1 on cancerous cells is necessary for Vδ2-mediated cytotoxic response [41]. Moreover, their findings indicate that the strength of this response is directly proportional to the surface expression of BTN2A1 on target cells [41].

Interestingly, it is a high expression of BTN3A1 that has negative prognostic value in some solid tumours like glioblastoma, pancreatic ductal adenocarcinoma, or oesophagal cancer [42–44]. On the other hand, according to Liang et al., in pan-cancer analysis of *BTN3A1*, its high expression is mostly a predictor of longer overall survival, e.g., in lung, breast or gastric cancer; at the same time, the negative prognostic value of *BTN3A1* was noted only in testicular germ tumours and low-grade gliomas [45]. This is in line with our observations that *BTN3A1* expression in PBMCs is decreased in CLL patients, even more so in those with negative prognostic markers (*IGVH* unmutated). More importantly, we found that higher *BTN3A1* expression is a predictor of longer overall survival in CLL. Indeed, Malinowska et al. reported a case of acute myeloid leukaemia with translocations that involved the *BTN3A1* gene and caused loss of heterozygosity; they concluded that it may significantly contribute towards leukaemic cell malignancy [46]. Interestingly, *BTN3A2*

and *BTN3A3* demonstrate the most pronounced separation of survival curves; however, the underlying reasons for this remain unclear. Current literature does not provide an explanation, suggesting that further studies are needed to explain this phenomenon.

Similarly to our observations, *BTN2A1*, *BTN3A1*, *BTN3A2*, and *BTN3A3* were significantly downregulated in breast cancer [47]. Moreover, Ren et al. also noted better overall survival in patients with high *BTN2A1*, *BTN3A1*, *BTN3A2*, and *BTN3A3* [47]. This is also in line with our observations and further confirms their potential value and implications. Although each BTN has its own functions, they closely cooperate and synchronised expression seems necessary for proper function, most importantly for γδ T activation [48]. Thus, it is also important to analyse the expression of all *BTN* genes concurrently in the same samples. It may also be valuable to assess the Epstein-Barr virus (EBV) infection in the same samples. EBV is known to regulate the expression of BTN3A1 and BTN2A1 on the one hand and negatively affect the overall survival of CLL patients on the other [49–51]. Both BTN2A2 and BTN1A1 have suppressive potential and inhibit T cell activation and proliferation [52]. Interestingly, we have not observed any impact of *BTN2A2* expression on OS, which is contrary to the observations in other tumours, e.g., glioma [53].

While BTN2A1 is critical for γδ T activation and cytotoxic response against cancer, it is also implicated in the transition of macrophages from pro-inflammatory M1 to suppressive M2 phenotype [41,54]. Moreover, Kreneur et al. noted that with the use of blocking anti-BTN2A1 antibodies, they were able to induce a significant shift back from M2 to M1 [54]. While this may be beneficial in some cases, it would also significantly lower the potential of γδ T cells; thus, caution is needed, especially in cases like CLL, where *BTN2A1* is already lowered. While anti-BTN2A1 antibodies may have limited potential in CLL, they could be useful in tumours with higher *BTN* expression, like glioblastoma [55]. Additionally, γδ T cell infiltration is a positive prognostic marker in various solid tumours, e.g., head and neck cancer, where γδ T infiltration is directly proportional to the expression of *BTNs* in tumour cells [56].

Finally, the pattern of *BTN1A1* expression is worth noting. As mentioned in the introduction, *BTN1A1* expression is negligible in leukocytes. Our data indicate that, indeed, there is no expression of *BTN1A1* in T lymphocytes, but in B cells and total PBMCs, it remains low, yet detectable.

Although our findings contribute to a better understanding of CLL, some limitations must be discussed. The current study was based on a re-analysis of publicly available datasets, which enabled the inclusion of a relatively high number of samples from patients with potentially different sociodemographic backgrounds. At the same time, this approach substantially affected the amount of other data available—the majority of datasets had only very limited clinical information available. The survival analysis is based on a large dataset that includes patients from the USA, Spain, and Germany treated over an extended period. While this diverse dataset allows for the inclusion of a high number of patients with varied sociodemographic backgrounds, it is important to acknowledge that differences in treatment protocols across countries may influence overall survival and introduce potential bias in our results. Another key limitation of our analysis is the use of bulk PBMCs and bone marrow samples, which do not account for variations in CLL cell load among patients. Given that PBMCs in CLL patients are largely composed of malignant B cells, while those from healthy controls contain a diverse mix of immune populations, direct comparisons may be affected by this cellular imbalance. Ideally, the CLL load should be taken into consideration. However, current dataset limitations prevent precise adjustments for CLL cell load. Future validation using isolated cell populations or adjusting for CLL burden would provide a more accurate assessment, and we aim to address this in upcoming studies. Nevertheless, we obtained encouraging results that implicate the need for further studies on fresh CLL samples with concomitant assessment of EBV infection and γδ T cells.

5 Conclusions

A strong dysregulation of *BTNs* in CLL samples indicates their potential clinical importance. This is further corroborated by the survival analysis results. Patients with lower expression of *BTNs* had significantly shorter OS. Moreover, patients with unmutated *IGVH*, thus those with worse prognosis, demonstrated markedly lower expression of *BTNs* as well. Notably, the expression of *BTN* genes in PBMCs had a significant impact on overall survival, highlighting their potential prognostic value. Since *BTN* expression in PBMCs can be easily assessed at diagnosis using a simple qPCR assay, it could potentially be incorporated into clinical practice to enhance risk stratification and improve patient management. Given the established role of BTN2A1 and BTN3A1 in γδ T cell activation, their downregulation may contribute to γδ T cell dysfunction, potentially affecting immune surveillance and disease progression. Further research is needed to validate these results in prospective cohorts and to explore the underlying mechanisms driving *BTN* dysregulation in CLL and their importance for the function of γδ T cells.

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Author Contributions: Conceptualization: Natalia Lehman, Agnieszka Bojarska-Junak, Michał Zarobkiewicz; Data curation: Michał Zarobkiewicz; Formal Analysis: Michał Zarobkiewicz; Funding acquisition: Michał Zarobkiewicz; Investigation: Natalia Lehman, Agnieszka Bojarska-Junak, Michał Zarobkiewicz; Methodology: Michał Zarobkiewicz; Project administration: Michał Zarobkiewicz; Resources: Michał Zarobkiewicz; Software: Michał Zarobkiewicz; Supervision: Michał Zarobkiewicz; Validation: Michał Zarobkiewicz; Visualization: Michał Zarobkiewicz; Writing—original draft: Michał Zarobkiewicz; Writing—review & editing: Natalia Lehman, Agnieszka Bojarska-Junak, Michał Zarobkiewicz. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The datasets used in this study are publicly available and are listed in the Methods section. Further details, including access links, can be found in the referenced sources.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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