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Article

Epigenome-Wide Association Studies of Proteasome Inhibitor-Related Cardiotoxicity in Patients with Multiple Myeloma

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Simple Summary

Proteasome inhibitors (PI) help patients with multiple myeloma live longer, but some develop heart problems. We asked whether epigenetic changes in blood can flag this risk. Our study analyzed germline DNA samples from 79 participants of the Prospective Study of Cardiac Events During Proteasome Inhibitor Therapy for Relapsed Multiple Myeloma (PROTECT), including 49 treated with carfilzomib and 30 with bortezomib. We performed epigenome-wide association studies (EWAS) to identify differentially methylated sites, regions and pathways associated with carfilzomib- or bortezomib-related cardiotoxicity. The main outcome was cardiovascular adverse events (CVAEs), including heart failure, and arrhythmia. We then performed a meta-analysis to highlight signals common to CVAE associated with both medicines. Our study uncovered epigenetic signals linked to the heart effects of proteasome inhibitors. If confirmed and validated in larger groups, these signals could serve as blood-based biomarkers for PI related CVAE.

Abstract

Background/Objectives: Carfilzomib (CFZ) and bortezomib (BTZ) are proteasome inhibitors used as the first-line therapy for relapsed or refractory multiple myeloma (MM) but are associated with cardiovascular adverse events (CVAEs). This study aims to identify differentially methylated positions (DMPs) and regions (DMRs), and enriched pathways in patients who developed CFZ- and BTZ- related CVAEs. **Methods:** Baseline germline DNA methylation profiles from 79 MM patients (49 on CFZ and 30 on BTZ) in the Prospective Study of Cardiac Events During Proteasome Inhibitor Therapy (PROTECT) were analyzed. Epigenome-wide analyses within each group identified DMPs, DMRs, and enriched pathways associated with CVAEs compared with individuals without CVAEs. **Results:** Four DMPs were significantly associated with CFZ-CVAE: cg15144237 within *ENSG00000224400* ($p = 9.45 \times 10^{-10}$), cg00927646 within *TBX3* ($p = 9.78 \times 10^{-8}$), and cg10965131 within *WDR86* ($p = 1.00 \times 10^{-7}$). One DMR was identified in the *FAM166B* region ($p = 5.46 \times 10^{-7}$). There was no

evidence of any DMPs in BTZ-CVAE patients, however two DMPs and one DMR reached a suggestive level of significance ($p < 1.00 \times 10^{-5}$): cg09666417 in *DNAJC18* ($p = 3.41 \times 10^{-7}$) and cg12987761 in *USP18* ($p = 5.00 \times 10^{-7}$), and a DMR mapped to the *WDR86/WDR86-AS1* region ($p = 8.11 \times 10^{-8}$). Meta-analysis did not find any significant DMPs, with the top CpG being cg17933807 in *GNL2* ($p = 7.38 \times 10^{-5}$). Pathway enrichment analyses identified peroxisome, MAPK, Rap1, adherens junction, phospholipase D, autophagy, and aldosterone-related pathways to be implicated in CVAEs. **Conclusions:** Our study identified distinct DMP, DMR, and pathway enrichment associated with CVAE, suggesting epigenetic contributors to CVAEs and supporting the need for larger validation studies.

Keywords: cardio-oncology; carfilzomib; bortezomib; epigenetic; DNA methylation; EWAS

1. Introduction

Multiple myeloma (MM) is a hematological lymphoid malignancy of plasma cells and is the second most common hematological malignancy in the United States [1,2]. Proteasome inhibitors (PIs) are one of the most important drug classes for the treatment of MM that emerged in the past two decades and are a component of widely used regimens across the MM landscape [3]. So far, three PIs, bortezomib (BTZ), carfilzomib (CFZ), and ixazomib (IXA) have been approved by the United States Food and Drug Administration [4]. The first PI, BTZ, is a reversible boronic acid dipeptide inhibitor of the proteasome, and was approved in 2003 for the treatment of refractory MM [4] and in 2006 for newly diagnosed MM. CFZ, an irreversible epoxyketone, is a second-generation PI approved in 2012 for the treatment of relapsed and refractory MM patients with at least two prior therapies [5]. IXA, an oral PI, was approved in 2015 for use in combination regimens for patients with relapsed MM [6].

Although PIs have improved outcomes in those with MM, they are associated with an increased risk of cardiovascular adverse events (CVAEs) such as hypertension, congestive heart failure, and arrhythmias [7–10]. In a systematic review and meta-analysis of 24 prospective CFZ clinical trials including 2,594 patients with MM, the incidence of all-grade and high-grade CFZ-CVAEs was 18.1% and 8.2%, respectively [11]. The event rate is even higher in prospective/observational settings where patients with a prior history of cardiovascular disease are not excluded. For example, in the prospective study of cardiac events during proteasome inhibitor therapy (PROTECT) study, 51% of MM patients receiving CFZ experienced a CVAE versus 17% treated with BTZ; the median time to first CVAE was 31 days, and 86% occurred within the first 3 months. Patients who developed a CVAE had significantly worse progression-free and overall survival than those without CVAE, underscoring the clinical impact of PI cardiotoxicity [12].

Emerging evidence suggests that epigenetic modifications, such as DNA methylation, may contribute to anticancer drug-induced cardiotoxicity [13]. This study aims to investigate the baseline germline methylation profiling associated with CVAE in MM patients treated with CFZ or BTZ-based therapy, focusing on differentially methylated positions (DMPs)-and regions (DMRs) and enriched pathways.

2. Materials and Methods

2.1. Study Population

The PROTECT study is a prospective, observational, multi-institutional study conducted between September 2015 and March 2018 to evaluate the risk factors and outcomes in 95 patients with MM treated with BTZ-based or CFZ-based therapy [12]. The PROTECT study was conducted at Vanderbilt University Medical Center and the University of Pennsylvania Abramson Cancer Center and was approved by the Institutional Review Board at these institutions [12]. Briefly, study participants were included if they had refractory or relapsed MM, defined according to the International Myeloma Working Group Criteria [14], and were initiating treatment with the

physician choice of CFZ- or BTZ-based therapy. Cardiovascular assessments were performed by a cardiologist at every treatment cycle and at the time of any suspected CVAE.

2.2. Methylation Profiling and Quality Control

This study included 79 patients with sufficient germline DNA quantity for methylation analysis, including 49 treated with CFZ and 30 treated with BTZ. DNA methylation was assessed using the Illumina Infinium® HumanMethylationEPIC v2.0 BeadChip. After processing the raw data, a mean detection p-value threshold of > 0.01 was used to exclude poor-quality samples; and no samples were excluded. Methylation data were normalized using the quantile normalization method with the minfi package [15]. The internal function of the minfi package was used to confirm sex by checking the concordance between genetically estimated sex and the self-reported sex. Next, we applied a strict p-value cutoff to exclude low-quality probes, removing approximately 5,419 probes that were considered unreliable due to background noise. A total of 23,718 probes located on the sex chromosomes were also removed. In addition, 12,979 probes that overlap with known SNPs were excluded to avoid potential confounding. A total of 42,045 probes identified by Illumina as flagged or inaccurate were excluded. After these quality control steps, 845,888 CpG sites were included in the following analyses. The final DNA methylation beta values, which represent the percentage of methylation at each site, were calculated. Beta values were also transformed to M values, which are the log ratios of the methylated to the unmethylated intensities [16]. M values were used in all statistical analyses due to their better statistical properties. Beta values were also reported to better interpret the results. Batch effects were corrected on both M and beta values using the Harman package [17].

2.3. Statistical Analysis

2.3.1. Descriptive Statistics

All analyses were performed separately for patients treated with CFZ or BTZ. Patient characteristics for continuous variables were summarized using the mean and standard deviation (mean $\pm$ SD). For categorical variables, frequency and percentage were presented. To assess group differences between the CVAE and No-CVAE groups, t- test was used for continuous variables and chi-squared test or Fisher's exact test were used for categorical variables as appropriate.

2.3.2. Methylation Profiling Analysis

Three analyses were conducted for CFZ- and BTZ-treated patients separately: DMPs, DMRs, and pathway enrichment analysis. The limma package was used to identify DMPs through multiple linear regression,[18] with M values used for each CpG site as the outcome variable and subject status (CVAE or No-CVAE) as the independent variable, adjusting for age, sex, and brain natriuretic peptide level higher than normal cut-off (BNPCUT). BNPCUT was categorized as high or normal based on clinical thresholds, defined as high when BNP > 100 ng/L or NT-proBNP > 125 ng/L and normal otherwise. After that, we applied the BACON package to correct for inflation and bias, ensuring more accurate and reliable differential methylation results [19].

The DMRcate package applied the same model to identify DMRs for putative CpGs with $P > 0.01$ [20]. To investigate pathways associated with differentially methylated probes, we performed KEGG-based over-representation analysis on DMPs with $P < 0.001$ using pathfinder on genes mapped from EWAS signals in each cohort. Gene duplicates were addressed in pathfinderR by selecting the lowest p-value per gene for pathway enrichment analysis.

To identify CpG sites that are common between CFZ-CVAE and BTZ-CVAE, we conducted meta-analyses using the meta package in R [21]. The analysis was restricted to CpG sites common to both cohorts, combining summary estimates from patients treated with CFZ and BTZ. Between-study heterogeneity was summarized by the between-study variance (τ^2), the inconsistency statistic (I^2), and Cochran's Q with its P value for heterogeneity. Due to the heterogeneity of the effects between

the two groups, an inverse-variance weighted random-effects model was used in the meta-analysis, with a fixed-effect model used in the sensitivity analysis.

3. Results

3.1. Bassline Characteristics

Of the 79 MM patients included, 49 received CFZ and 30 received BTZ. Among the 49 CFZ-treated patients, 23 (47%) developed a CVAE. Among the 30 BTZ-treated patients, 5 (17%) developed a CVAE.

Patient characteristics did not show a significant difference in age, sex, race, smoking status or history of hypertension. However, higher percentages of patients who developed CVAE had levels of baseline Brain natriuretic peptides (BNP) above normal in both cohorts (CFZ: $p = 0.006$; BTZ: $p = 0.03$), as shown in Table 1.

Table 1. Baseline characteristics of study participants.

Baseline patients' characteristics (n = 79)						
	CFZ (n=49)			BTZ (n=30)		
	CVAE (n=23)	No-CVAE (n=26)	p	CVAE (n=5)	No-CVAE (n=25)	p
Continuous						
Age (years)	66.40 ± 9.30	63.85 ± 9.93	0.36	71.20 ± 13.60	61.88 ± 9.91	0.08
Categorical						
Sex			0.48			>0.99
Female	5 (21.7%)	7 (26.9%)		2 (40.0%)	12 (48.0%)	
Male	18 (78.3%)	19 (73.1%)		3 (60.0%)	13 (52.0%)	
Race			0.67			0.63
White	21 (91.3%)	22 (84.6%)		5 (100.0%)	19 (76.0%)	
African American	2 (8.7%)	4 (15.4%)		0 (0.0%)	5 (20.0%)	
Other	0 (0.0%)	0 (0.0%)		0 (0.0%)	1 (4.0%)	
Smoking status			0.07			>0.99
Yes	14 (60.9%)	8 (30.8%)		1 (20.0%)	4 (16.0%)	
No	9 (39.1%)	18 (69.2%)		4 (80.0%)	21 (84.0%)	
History of HTN			0.35			0.13
Yes	11 (47.8%)	8 (30.8%)		4 (80.0%)	8 (32.0%)	
No	12 (52.2%)	18 (69.2%)		1 (20.0%)	17 (68.0%)	
Brain natriuretic peptide*			0.006			0.03
High	12 (52.2%)	3 (11.5%)		4 (80.0%)	8 (32.0%)	
Normal	11 (47.8%)	23 (88.4%)		1 (20.0%)	17 (68.0%)	

*Brain natriuretic peptide (BNP) was categorized as high or normal based on clinical thresholds, defined as high when BNP > 100 ng/L or NT-proBNP > 125 ng/L and normal otherwise.

3.2. Differentially Methylated Probes and Regions in CFZ-CVAE

3.2.1. DMPs

Of the 840,462 CpG sites tested, the genome-wide methylation analysis revealed 38 DMPs associated with CFZ-related CVAEs at 1×10^{-5} (Figure 1A, Table S1). BACON correction improved model calibration, reducing genomic de-inflation (λ from 0.82 to 0.98, BACON inflation estimate = 0.92) (Figure S1). Among these, four were significant after Benjamin-Hochberg false discovery rate

(FDR) ≤ 0.05 (Table 2), with three DMPs hypermethylated and one hypomethylated in the CVAE patients compared to no-CVAE patients. The beta values of these CpG sites in CVAE vs. no-CVAE patients are illustrated in Figure 2A.

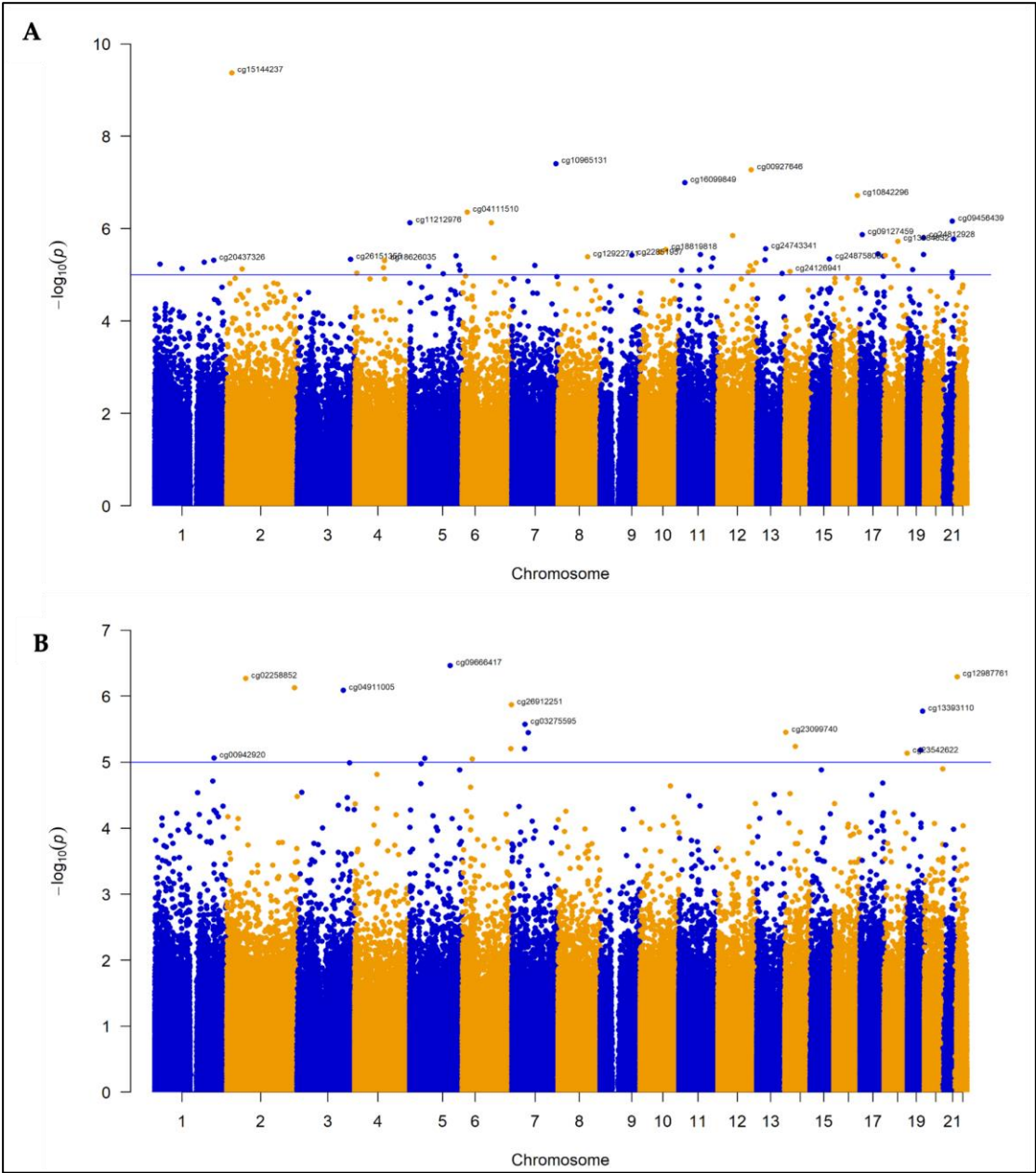


Figure 1. Manhattan plots for the two EWAS of CVAEs: (A). CFZ-CVAE; (B). BTZ-CVAE. The x-axis represents the chromosome position, and the y-axis represents the $-\log_{10}(p)$ value. Each blue or yellow dot represents a CpG site across chromosomes.

The top DMP, cg15144237, is located within an intronic region of the lncRNA ENSG00000224400 and is hypermethylated in CVAE patients compared with no-CVAE patients ($p = 9.45 \times 10^{-10}$, $\log_{2}FC = 0.39$, FDR = 0.001) (Table 2).

The second DMP, cg00927646, lies in an intergenic region ~13.6 kb downstream of the nearest gene, T-box transcription factor 3 (*TBX3*), and is also hypermethylated ($p = 9.78 \times 10^{-8}$, $\log_{2}FC = 0.51$, FDR = 0.028). The third DMP, cg10965131, is located within an exonic CpG island of WD Repeat Domain 86 (*WDR86*) and was hypomethylated in the CVAE patients ($p = 1.79 \times 10^{-7}$, $\log_{2}FC = 0.47$, FDR = 0.038). Two additional CpGs has suggestive level of significance, even though they were not

significant by FDR: cg10842296 in the TSS1500 shore region of dynein light chain roadblock-type 2 (*DYNLRB2*) ($p = 3.18 \times 10^{-7}$, $\log_{2}FC = 0.54$, FDR = 0.054) was hypermethylated, and cg09456439 in an intergenic region between *DYRK1A* and *KCNJ6* ($p = 1.52 \times 10^{-6}$, $\log_{2}FC = -0.34$, FDR = 0.10) was hypomethylated in the CVAE patients.

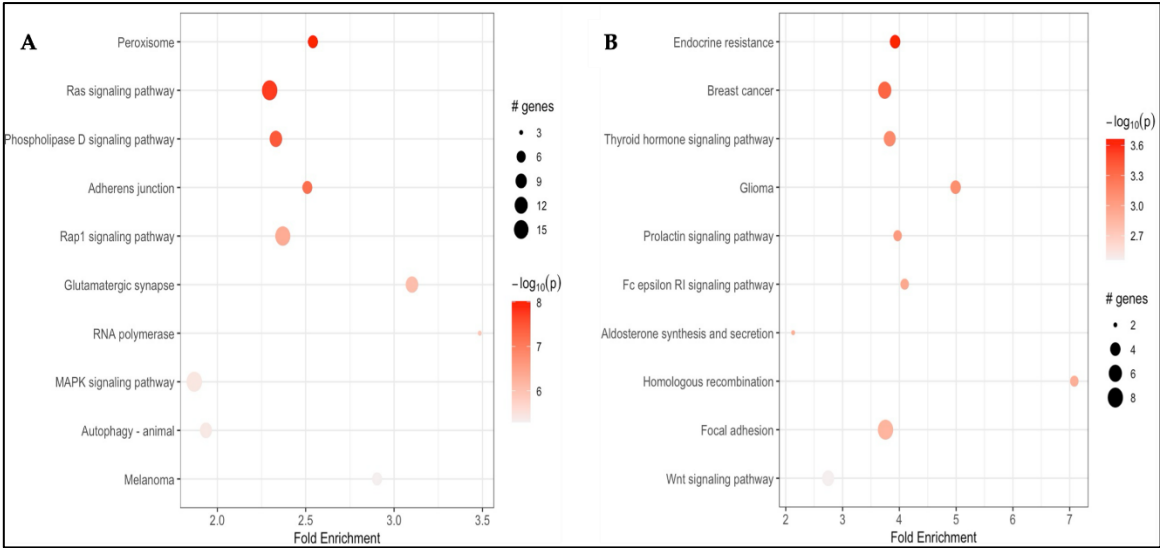


Figure 2. Boxplots showing the methylation differences in the beta values of the top DMPs: (A) in the CFZ analysis; (B) in BTZ analysis.

3. 2.2. DMR

We identified 96 DMRs associated with CFZ-CVAEs, of which 67 were hypermethylated and 29 were hypomethylated. Seven DMRs met a suggestive significance level (Table S2). One DMR consisting of six CpGs showed a plausible association, overlapping *FAM166B* ($p = 5.46 \times 10^{-7}$, mean diff = 0.05) (Table S2).

3.2.3. Pathway Enrichment Analysis

Pathway analysis using pathfindR identified 10 KEGG pathways enriched among genes annotated to CFZ-CVAE-associated DMPs, including Peroxisome (hsa04146; fold enrichment = 2.54, $p = 9.63 \times 10^{-9}$), Ras signaling (hsa04014; fold enrichment = 2.30, $p = 1.83 \times 10^{-8}$), Phospholipase D signaling (hsa04072; fold enrichment = 2.33, $p = 3.73 \times 10^{-8}$), adherens junction (hsa04520; fold enrichment = 2.51, $p = 6.62 \times 10^{-8}$), Rap1 signaling (hsa04015; fold enrichment = 2.37, $p = 4.60 \times 10^{-7}$), glutamatergic synapse (hsa04724; fold enrichment = 3.10, $p = 8.87 \times 10^{-7}$), RNA polymerase (hsa03020; fold enrichment = 3.48, $p = 1.17 \times 10^{-6}$), MAPK signaling (hsa04010; fold enrichment = 1.87, $p = 3.63 \times 10^{-6}$), autophagy (hsa04140; fold enrichment = 1.94, $p = 4.07 \times 10^{-6}$) and melanoma (hsa05218; fold enrichment = 2.90, $p = 5.09 \times 10^{-6}$) (Figure 3A).

Table 2. Summary of DMPs associated with CVAE in CFZ and BTZ analyses and meta-analysis.

No	CpG ID	CH	Positi on	Gene Name	CpG relatio n	Feature	CFZ analysis		BTZ analysis		Meta-analysis				
							log FC	<i>p</i>	FD R	log FC	<i>p</i>	FD R	log ^F C	<i>p</i>	FD R
1	cg151442 37	2	164001 25	ENSG00000224400	Opens ea	Intron	0.3 9	9.45 x 10 ⁻¹⁰	0.00 1	- 0.10	0.58	0.97	0.18	0.47	0.9 8
2	cg009276 46	12	114656 631	TBX3	Opens ea	Intergeni c	0.5 1	9.78 x 10 ⁻⁸	0.02 8	- 0.36	0.047	0.93	0.09	0.84	0.9 9

3	cg10965131	7	151381909	WDR86	Island	Exon	0.53	1.00 x 10 ⁻⁷	0.028	-0.53	0.93	>0.99	-0.32	0.20	0.98
4	cg16099849	11	20609207	SLC6A5	Open sea	Intron	0.47	1.79 x 10 ⁻⁷	0.038	0.03	0.89	0.99	0.28	0.20	0.98
5	cg10842296	16	80540122	DYNLRB2	Shore	TSS1500	0.54	3.18 x 10 ⁻⁷	0.054	0.34	0.47	0.96	0.21	0.58	0.98
6	cg09456439	21	37565327	DYRK1A, KCNJ6	Shore	Intergenic	0.34	1.52 x 10 ⁻⁶	0.1	0.03	0.86	0.99	-0.20	0.28	0.98
7	cg09666417	5	139439593	DNAJC18	Open sea	TSS200	0.05	0.66	>0.99	-0.96	3.41 x 10 ⁻⁷	0.14	-0.44	0.38	0.98
8	cg12987761	22	18148690	USP18	Shore	Intron	0.03	0.98	>0.99	-0.84	5.00 x 10 ⁻⁷	0.14	-0.41	0.33	0.98
9	cg05020252	2	232634573	EFHD1	Island	Intron	0.04	0.69	>0.99	-0.91	7.40 x 10 ⁻⁷	0.14	0.32	0.38	0.98
10	cg17933807	1	37596074	GNL2	Island	TSS200	0.49	7.38 x 10 ⁻⁵	0.314	-0.68	0.002	0.93	0.11	5.79 x 10 ⁻⁷	0.32
11	cg06683313	17	18316066	SMCR8, TOP3A	Shore	Exon, TSS200	0.24	4.43 x 10 ⁻⁵	0.28	-0.30	0.01	0.93	0.05	1.70 x 10 ⁻⁶	0.32

CpG ID: CpG name in Illumina database; CHR: Chromosome; MAPINFO: Genomic coordinates; FC: fold change; *p*: P-value.

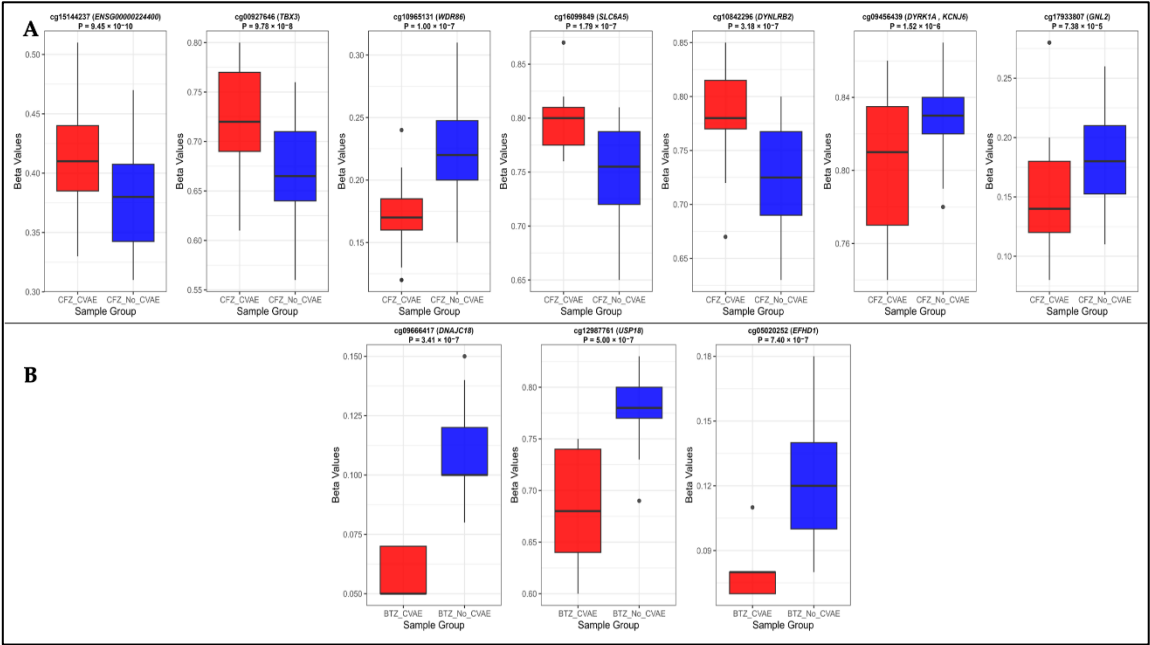


Figure 3. Pathway enrichment analysis for (A) the CFZ analysis; (B) the BTZ analysis.

3.3. Differentially Methylated Probes and Regions in BTZ-CVAE

3.3.1. DMPs

None of DMPs were significant after FDR-adjustment. BACON correction improved model calibration, reducing genomic inflation (λ from 1.51 to 1.09, BACON inflation estimate = 1.19) (Figure S2). However, eighteen DMPs were identified at the suggestive level of 1×10^{-5} , (Table S3). Eleven

were hypomethylated and seven were hypermethylated. Three CpGs at this suggestive level were hypomethylated, namely cg09666417 in the TSS200 region of DnaJ Heat Shock Protein Family Member C18 (*DNAJC18*) ($p = 3.41 \times 10^{-7}$, $\log_{2}FC = -0.96$), cg12987761 in an intronic shore region of ubiquitin-specific peptidase 18 (*USP18*) ($p = 5.00 \times 10^{-7}$, $\log_{2}FC = -0.84$), and cg05020252 in an intronic CpG island within EF-hand domain family member D1 (*EFHD1*) ($p = 7.40 \times 10^{-7}$, $\log_{2}FC = -0.91$), as shown in Figure 2B.

3.3.2. DMRs

We identified 33 DMRs associated with BTZ-CVAEs. Of these, 15 were hypermethylated, 18 were hypomethylated, and 17 met the suggestive significance level (Table S4). One DMR comprising 12 CpGs overlapped *WDR86-AS1/WDR86* ($p = 8.11 \times 10^{-8}$, mean diff = -0.07).

3.3.3. Pathway Enrichment Analysis

Using pathfindR, we found enrichment of 10 KEGG pathways among genes annotated to BTZ-CVAE-associated DMPs. These were endocrine resistance (*hsa01522*; fold enrichment = 3.93, $p = 2.18 \times 10^{-4}$), breast cancer (*hsa05224*; 3.74, $p = 4.45 \times 10^{-4}$), thyroid hormone signaling (*hsa04919*; 3.83, $p = 7.42 \times 10^{-4}$), glioma (*hsa05214*; 4.99, $p = 7.81 \times 10^{-4}$), prolactin signaling (*hsa04917*; 3.97, $p = 9.31 \times 10^{-4}$), Fc epsilon RI signaling (*hsa04664*; 4.09, $p = 1.16 \times 10^{-3}$), aldosterone signaling (*hsa04925*; 2.13, $p = 1.26 \times 10^{-3}$), homologous recombination (*hsa03440*; 7.08, $p = 1.27 \times 10^{-3}$), focal adhesion (*hsa04510*; 3.76, $p = 1.38 \times 10^{-3}$) and Wnt signaling (*hsa04310*; 2.75, $p = 3.46 \times 10^{-3}$) (Figure 3B).

3.4. Meta-Analysis

We conducted a meta-analysis across the 840,462 CpG sites profiled in the CFZ and BTZ cohorts using the meta R package [21]. Given that these cohorts include patients treated with different PIs, we evaluated both fixed-effect and random-effects models to account for potential heterogeneity in methylation responses related to drug-specific CVAE phenotypes (Supplementary materials-II). Due to the expected variation between CFZ- and BTZ, we report the results based on the random-effects model, which provides a more conservative estimate in the presence of between-group heterogeneity. None of the CpG sites reached statistical significance after FDR correction ($FDR < 0.05$). However, 23 DMPs showed suggestive associations at a p-value threshold of 1×10^{-5} (Table S5). The top DMP, cg17933807, lies in a CpG island at the TSS200 region of *GNL2* ($p = 5.79 \times 10^{-7}$, $\log_{2}FC = 0.11$). Cg06683313 lies in an exon of *SMCR8* and in the *TOP3A* TSS200 region ($p = 1.70 \times 10^{-6}$, $\log_{2}FC = 0.05$).

4. Discussion

To our knowledge, this is the first study to examine germline epigenetic changes associated with CFZ- or BTZ-related CVAEs. We leveraged data from MM patients enrolled in the prospective PROTECT cohort. We analyzed the germline DNA methylation profile of MM patients in the PROTECT cohort to identify differentially methylated probes and regions associated with CFZ- and BTZ-related CVAEs. Additionally, we conducted a meta-analysis of EWAS summary statistics from both cohorts to evaluate if there is any CpG site that is associated with CFZ-CVAE and BTZ-CVAE.

Several DMPs and DMRs identified by our study map to genes reported to be associated with cardiac traits: conduction (*TBX3*) [22,23]; cardiomyocyte cell-cycle control (*DYRK1A*, with *DYRK1A* previously linked to cardiomyopathy [24]); the ubiquitin-proteasome/ER axis (*DNAJC18* [25], *USP18* [26], *NUB1* [27,28]); mitochondrial function (*EFHD1* [29], *TOP3A* [30]); ribosome biogenesis (*GNL2*) [31]; lipid handling (*DYNLRB2-2*) [32]; and necroptosis and cardiac signaling (*FAM166B*) [33]. Additional loci include regions near *WDR86/WDR86-AS1*, with *WDR86-AS1* reported as a downregulated biomarker in myocardial infarction [34], and a site within the intronic lncRNA *ENSG00000224400*, which shows rich regulatory markers.

The top DMP identified in the CFZ analysis, cg15144237, is located within an intronic region of the lncRNA *ENSG00000224400*. According to GTEx data, *ENSG00000224400* is not expressed in

whole blood [35]. It is positioned near a candidate cis-regulatory element (cCRE) with enhancer-like chromatin signatures, including H3K4me1, H3K27ac, and DNase I hypersensitivity, suggesting potential regulatory function. LncRNAs are recognized as important regulatory molecules that can recruit chromatin-modifying complexes, and influences gene expression [36]. Reference methylation data from the EWAS Data Hub show that cg15144237 is typically hypomethylated across multiple tissues, including whole blood [37]. Additionally, EWAS Atlas reports hypomethylation of this site in association with traits such as aging and smoking [37]. Therefore, the hypermethylation detected in CFZ-treated patients who developed CVAEs may reflect a treatment-associated epigenetic response, potentially involving altered regulatory activity at this locus. However, how this DMP is involved in CFZ-related CVAEs remains unclear and requires further investigation.

The second CpG, cg00927646, was hypermethylated in patients who developed CVAE and is located in an intergenic region. The nearest gene *TBX3* is expressed in the heart, where it controls the formation of the sinoatrial node and atrioventricular conduction system, and its disruption can lead to arrhythmias [23,38]. As a transcriptional repressor, *TBX3* prevents the atrioventricular bundle from losing its conduction phenotype and becoming contractile myocardium by repressing “working myocardium” genes in *TBX3*-deficient embryos [23]. In addition, in a murine model of doxorubicin-induced cardiomyopathy, cardiac progenitor cells underwent transcriptional reprogramming characterized by upregulation of *TBX3*, among other cardiac transcription factors, indicating its involvement in the adaptive response to anthracycline-induced myocardial injury [39]. These findings support *TBX3* as a biologically plausible mediator of cardiotoxicity in patients treated with CFZ.

The third DMP is cg10965131, is located within exon of *WDR86* at CpG island and exhibited hypomethylation direction. This site overlaps with distal enhancer-associated chromatin features, including H3K4me1, H3K27ac, and DNase I hypersensitivity, suggesting potential regulatory relevance. Notably, this site is ~ 27 kb upstream of *WDR86-AS1*. *WDR86-AS1* is lncRNA that has identified as a downregulated biomarker in myocardial infarction (MI) patients [34,40]. Furthermore, cg10965131 is located downstream of Negative Regulator of Ubiquitin Like Proteins 1 (*NUB1*) ~5 kb. *NUB1* functions as adaptors that transport NEDD8- and FAT10-conjugated proteins to the proteasome [27,28]. As the proteasome is irreversibly blocked by CFZ this may lead to accumulation of undegraded proteins thereby increasing stress in cardiomyocytes and contributing cardiotoxicity.

Two suggestive DMPs with plausible biological relevance were identified: cg10842296 and cg09456439. cg10842296 is located in the TSS1500 region, specifically in the promoter of *DYNLRB2*, and was hypermethylated. *DYNLRB2-2* is a lncRNA that regulates *ABCA1*, a key gene involved in cholesterol efflux and protection against atherosclerosis [32]. According to GTEx, *DYNLRB2* is not expressed in blood, though its potential activation in response to cardiac stress or injury. The second site, cg09456439, is an intergenic CpG located downstream of both *DYRK1A* and *KCNJ6*. It was hypomethylated in CVAE patients and overlaps with a GeneHancer-annotated enhancer predicted to regulate *DYRK1A*. In the literature, *DYRK1A* overexpression has been implicated in cardiac remodeling through two distinct mechanisms. *DYRK1A* overexpression has been reported to prevent cardiomyocyte hypertrophy by suppressing NFAT nuclear activity [41]. Another study using transgenic mice found that *DYRK1A* overexpression led to Rb/E2f signaling suppression, reducing cardiomyocyte proliferation and causing cardiomyopathy [24]. These sites represent novel methylation findings, as neither has been previously associated with any phenotype.

The genes associated with CFZ-CVAE were found to be enriched in peroxisome, Ras/MAPK, Rap1, adherens junction, Phospholipase D, and Autophagy. This finding suggests the involvement of proteotoxic and ER stress, mitochondrial and oxidative stress in the development of CFZ-CVAEs. Peroxisomes manage very-long-chain lipids and detoxify ROS [42]. MAPK signaling is a core stress and hypertrophic pathway in cardiovascular tissues [43]. Enrichment of Rap1 signaling and adherens-junction pathways may reflect carfilzomib-induced endothelial dysfunction, supported by prospective human evidence that carfilzomib impairs endothelial function [44]. Autophagy interacts with the ubiquitin-proteasome system and may buffer proteasome stress [45]. Similar enrichment of

Ras, Rap1, and MAPK signaling pathways has been reported in CFZ-treated human cardiomyocyte-like cells in vitro, suggesting potential mechanistic link to CFZ-associated CVAE [46].

The CpG signals and enriched pathways are consistent with known effects of proteasome inhibition. Proteasome blockade can raise proteotoxic load and triggers ER stress, and methylation at ubiquitin–proteasome or ER-axis genes such as *DNAJC18*, *USP18*, and *NUB1* may reflect stress responses [25–28]. CFZ shows enrichment of autophagy and MAPK pathways that fit compensatory proteostasis and stress or hypertrophic signaling [43,45]. Signals at *GNL2* might indicate nucleolar and ribosome-biogenesis pressure [31]. *TOP3A* and *EFHD1* map to mitochondrial and Ca²⁺ or ROS stress and align with peroxisome enrichment that supports redox handling [29,30,42]. Rap1 and adherens-junction pathways fit with reported CFZ-associated endothelial dysfunction, and TBX3-proximal methylation suggests conduction vulnerability [23,38,44].

The epigenetic changes in BTZ were less significant compared to CFZ that could be due to smaller sample size of patients who developed the CVAE. None of DMPs reached significance after FDR adjustment, so we focused on the CpG sites that met the suggestive threshold of $p < 1 \times 10^{-5}$. The top hit DMP in the BTZ analysis is cg09666417, which is located at the TSS200 region of *DNAJC18*. According to GeneHancer and ENCODE, this CpG site overlaps the promoter region of the gene. *DNAJC18* is predicted to encode an endoplasmic reticulum (ER)-membrane J-domain co-chaperone that may bind heat shock protein 70 (Hsp70) and assist in proteostasis [25]. The ER plays a central role in handling misfolded proteins. A large genome-wide association meta-analysis conducted by the Heart Failure Molecular Epidemiology for Therapeutic Targets (HERMES) consortium identified a common variant at the *DNAJC18* locus (rs10900864) that reached genome-wide significance for association with dilated cardiomyopathy [47]. Another large-scale genetic study using single-cell RNA sequencing showed cardiomyocyte expression of *DNAJC18* and pathway-level involvement in proteostasis-related processes in dilated cardiomyopathy [48]. Both studies detected *DNAJC18* expression in cardiomyocytes.

Moreover, carriers of loss-of-function alleles of *DNAJC18* exhibited altered left ventricular (LV) function, characterized by increased LV end-systolic volume and decreased systolic volume index [49]. Together, these findings suggest that *DNAJC18* is a cardiomyocyte-enriched stress-response gene, and its altered methylation or function may contribute to the pathogenesis of dilated cardiomyopathy by impairing proteostasis and myocardial resilience.

The second hit DMP in BTZ analysis was cg12987761 which is located in the intron of *USP18* and was hypomethylated in BTZ-CVAE patients. *USP18* is a deubiquitinating enzyme known for its role in removing ISG15 modifications and for regulating the ubiquitin–proteasome system [26]. *USP18* modulates the cellular response to BTZ, where its knockdown increases sensitivity to BTZ-induced apoptosis and enhances extrinsic apoptotic signaling, while overexpression confers resistance [50,51]. *USP18* is expressed in the heart and also plays a protective role in the heart, as cardiomyocyte-specific overexpression of *USP18* in mice mitigates myocardial hypertrophy, fibrosis, and ventricular dilation—whereas its deficiency exacerbated these changes [52]. Thus, hypomethylation of cg12987761 in BTZ-CVAE patients suggests altered epigenetic regulation of *USP18* that may be associated with BTZ-associated cardiotoxicity.

cg05020252 is located in intron of *EFHD1* and exhibited hypomethylation status. This gene encodes a member of the EF-hand super family of calcium binding proteins, which regulates mitoflash activity, transient events linked to mitochondrial ROS production [29]. A study using *EFHD1* knockout mice found that did not impair heart structure or function. *EFHD1* deficiency resulted in reduced mitochondrial calcium levels, decreased reactive oxygen species (ROS) production, Lower mitoflash frequency and greater resistance to ischemic injury [53]. This makes *EFHD1* a possible target for cardioprotective therapies.

Our meta-analysis did not identify any significant DMPs. However, two CpGs reached suggestive level of significance ($p < 1 \times 10^{-5}$). The top DMP was cg17933807 within *GNL2* TSS200, located in a CpG island and it was hypomethylated in the CVAE patients across the two cohorts. According to ENCODE, this DMP lies in a promoter region with strong DNase signal that indicates

regulatory activity [54]. *GNL2* encodes a nucleolar GTPase that mediates pre-60S ribosome maturation and contributes to chromatin organization, and its function is most closely linked to cell proliferation [31]. Another DMP, cg06683313, located in an exon of *SMCR8* and within *TOP3A* TSS200 was also hypomethylated consistently in CVAE patients across cohorts. *TOP3A* encodes topoisomerase III α and it supports mitochondrial DNA replication fork progression and decatenation [30]. PIs provoke ER and cellular stress while lowering mitochondrial membrane potential and ATP in human cardiomyocytes. Taken together, these signals at *GNL2* and *TOP3A* map to nucleolar and mitochondrial stress axes that align with the biology of PI-related CVAE.

The fact that we did not identify any significant DMPs in the meta-analysis of CFZ-CVAE and BTZ-CVAE was not surprising. While CFZ and BTZ both target the proteasome, they differ in how they bind the proteasome which results in a distinct safety and efficacy profile. CFZ is more efficacious than BTZ but is associated with higher incidence and severity of cardiotoxicity than BTZ. BTZ reversibly inhibits the $\beta 5$ (chymotrypsin-like) subunit, while CFZ irreversibly blocks the $\beta 5$, $\beta 2$ (trypsin-like), and $\beta 1$ (caspase-like) subunits, leading to broader and more prolonged inhibition. CFZ-mediated co-inhibition of $\beta 5$ and $\beta 2$ subunits of proteasome was found to impair cardiomyocyte contractility in human and murine *in vitro* and *in vivo* models[55].

This study comes with some limitations. First, the sample size is relatively modest, which may increase the likelihood of type II error. Second, the samples were collected from blood, so DNA methylation may not fully reflect processes in cardiac tissue; residual confounding by blood-cell composition or tissue specificity is possible.

5. Conclusions

Our study identified differentially methylated genes associated with CFZ- or BTZ-related CVAEs. The findings suggest that the development of CFZ- or BTZ-CVAE may be epigenetically regulated and that CVAE associated with CFZ and BTZ had distinct methylated profiles. These results are preliminary, and further investigation in larger cohorts and functional validation are needed to validate these associations.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Conflicts of Interest: R.F.C. is currently employed at AbbVie and is a stock shareholder of AbbVie. R.F.C. was employed at Vanderbilt University Medical Center at the time this research was conducted. S.M.R. is an advisory board member for Janssen, Sanofi, Roche Diagnostics, EUSA pharma. All other authors declared no competing interests for this work.

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