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DNA methylation patterns of adult survivors of adolescent/young adult Hodgkin lymphoma compared to their unaffected monozygotic twin.

Jun Wang¹, David Van Den Berg¹, Amie E. Hwang¹, Daniel Weisenberger², Timothy Triche Jr.^{3,4}, Bharat N. Nathwani⁵, David V. Conti¹, Kim Siegmund¹, Thomas M. Mack^{1,6}, Steve Horvath⁷, Wendy Cozen^{1,6}

¹Department of Preventive Medicine, University of Southern California, Los Angeles, California, USA

²Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California, USA

³Department of Translational Genomics, University of Southern California, Los Angeles, California, USA

⁴Van Andel Research Institute, Grand Rapids, Michigan, USA, USA

⁵Department of Pathology, City of Hope National Medical Center, Duarte, California, USA

⁶Department of Pathology, University of Southern California, Los Angeles, California, USA

⁷Department of Biostatistics, University of California at Los Angeles, Los Angeles, California, USA

Abstract

DNA methylation (DNAm) silences gene expression and may play a role in immune dysregulation that is characteristic of adolescent/young adult Hodgkin lymphoma (AYAHL). We used the Infinium HumanMethylation27 BeadChip to quantify DNAm in blood (N=9, mean age 57.4 y) or saliva (N=36, mean age 50.0 y) from long-term AYAHL survivors and their unaffected co-twins. Epigenetic aging (DNAm age) was calculated using previously described methods and compared between survivors and co-twins using paired t-tests and analyses were stratified by sample type, histology, sex, age at sample collection and time since diagnosis. Differences in blood DNAm age were observed between survivors and unaffected co-twins (64.1 vs. 61.3 years, respectively, $P=0.04$), especially in females ($P=0.01$); no differences in saliva DNAm age were observed. Survivors and co-twins had 74 (in blood DNA) and 6 (in saliva DNA) differentially methylated loci. Our results suggest persistent epigenetic aging in AYAHL survivors long after HL cure.

Keywords

Hodgkin Lymphoma; AYA; survivors; twins; blood; saliva; DNA methylation

Corresponding author: Wendy Cozen, D.O., M.P.H., Professor, Dept. of Preventive Medicine, Dept. of Pathology, Keck School of Medicine of USC, University of Southern California, 1441 Eastlake Ave. MC9175, Los Angeles, CA 90089-9175, 323 865 0447, wcozen@usc.edu.

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Introduction

Hodgkin lymphoma (HL) is the most common form of malignant lymphoma affecting people under the age of 30 in developed countries [1, 2] (<https://seer.cancer.gov/statfacts/html/hodg.html>). Monozygotic (MZ) twins of adolescent and young adult (AYA) HL patients (defined as 15–39 years of age at diagnosis) have a ~100-fold risk compared to the general population, while dizygotic (DZ) twins of patients have a ~7-fold risk similar to that for non-twin siblings [3]. Genome-wide association studies (GWAS) have identified genetic risk variants in the *HLA* region and in *IL13*, *REL*, *PVT1*, *GATA 3*, and *TCF3* genes that cumulatively explain less than 5% of genetic risk [4, 5]. There are also strong environmental determinants of HL that include high socioeconomic status, fewer opportunities for microbial exposures in early childhood, and infectious mononucleosis (limited to the Epstein-Barr virus [EBV]+ subtype) [6, 7, 8]. These environmental and genetic factors contribute to the development of a T-helper-Type 2 (Th2) immune phenotype associated with susceptibility among AYAHL patients [7, 9, 10], that may persist after treatment and cure.

The cure rate is generally high, especially among AYAHL, but 5–15% still die of their disease or late effects of therapy within 10 years [11, 12]. A European study reported that premature mortality in young people diagnosed with HL had an economic cost to the European Union of €306,628 per HL death, the second highest of any cancer after melanoma [13]. In addition, late effects cause considerable morbidity, including second primary malignancy, cardiovascular disease and pulmonary fibrosis [11]. For example, AYAHL patients treated with chemotherapy only have a 33-fold increased risk of a late onset leukemia and those treated with both chemotherapy and radiation have a 17-fold increased risk of late onset non-Hodgkin lymphoma (NHL) [12]. Chronic fatigue, depression and persistent immune dysfunction are also long-term consequences of the disease and its treatment [14, 15, 16]. The biological basis for these persistent abnormalities is largely unknown but may involve chronic inflammation [17, 18, 19].

DNA methylation (DNAm) is an epigenetic change characterized by the covalent addition of a methyl group to cytosines within the CpG sequence context that can regulate gene expression [20]. DNAm patterns in genes can reflect the pathways involved in disease. Heritability analyses suggest that 18–19% of the variance in DNAm across the genome in MZ and DZ twin pairs is due to heritable factors [21, 22]. These estimates significantly decrease as twins age, suggesting that cumulative environmental exposures may make impact on DNAm.

Horvath et al. developed a measure of cellular aging by examining a pattern of hyper- or hypo-methylation in specific genes that reflects cellular aging and functions as an epigenetic clock [23], consistent across most tissue types. DNAm age measured in leukocytes has been shown to predict all-cause mortality in later life, even after adjusting for known risk factors [24, 25] implying a relationship to biological aging. Markers of physical and mental fitness are associated with the epigenetic clock (lower abilities associated with accelerated aging) [26]. Environmental exposures such as tobacco use and occupational chemical exposures may influence global (genome-wide) and gene-specific DNAm in blood or epithelial cells

from healthy subjects [27, 28]. Alkylating agents, used in the multi-drug treatment regimen for HL and other cancers, can also affect DNAm [29].

Several specific DNAm changes have been demonstrated in HL tumors [30, 31] and cell lines [32], but DNAm has not been examined in circulating leukocytes of HL patients. Because twins are matched on genome and to a large extent, early life exposures, a comparison between DNAm between MZ twins may be more meaningful than one between unrelated individuals. We previously reported that AY AHL is associated with increased interleukin-6 (IL6) [9] and decreased interleukin-12 (IL12) production [10] using MZ twin pairs and controls. In addition, we showed that a deficit of early life-fecal-oral exposures [7] and lower fecal microbiota diversity [33] were more common in AY AHL survivors compared to their unaffected twins. Here, we compare DNAm patterns in 23,477 CpG loci in long-term AY AHL survivors to those in their unaffected MZ co-twins to determine whether differences in DNAm persist after treatment and cure.

Methods

Study Population

The study was approved by the Institutional Review Board of the Keck School of Medicine, University of Southern California and all participants provided signed written informed consent. Twin pairs discordant for AY AHL were recruited from the International Twin Registry, a volunteer-based registry of twins with chronic diseases throughout the U.S. and Canada developed and maintained at USC [34, 35]. Cases were diagnosed from 1961 to 2004, with a mean year of 1982 and a median year of 1981. Medical records pathology reports, and histopathologic slides were requested and reviewed by a single hematopathologist for diagnostic confirmation. Histological subtype, but not Epstein-Barr virus tumor status, was available for the majority of patients.

We collected blood and/or saliva specimens from 138 HL survivors and their unaffected twins. Blood specimens were shipped by overnight courier to USC, and buffy coats were separated and frozen within 24 hours. Saliva specimens were collected in Oragene (DNA Genotek, Ottawa, Ontario, Canada) kits and returned to USC by mail and were stored in a -20 refrigerator until DNA extraction. Both members of 45 monozygotic (MZ) HL-discordant twin pairs had sufficiently large samples for DNAm determination; nine provided blood and 36 provided saliva samples.

DNAm Profiling

Genomic DNA was extracted from blood and saliva samples and was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. DNA profiling was performed at the USC Epigenome Center Data Production Facility using the Illumina Infinium HumanMethylation27 (HM27) BeadChip that concurrently interrogates 27,578 CpG loci covering 14,475 genes. Detailed information for the assay is available at www.illumina.com. The DNA samples for each AY AHL survivor and their unaffected MZ twin were placed in adjacent wells on the same beadchip to mitigate beadchip-to-beadchip and well variation.

Statistical Analysis

The DNAm level of each CpG locus was calculated as a beta value, the ratio of signal from the methylated probe relative to the sum of both methylated and unmethylated probes. Beta values range from 0 (unmethylated) to 1 (fully methylated). CpG loci on the X and Y chromosomes, those containing a single-nucleotide polymorphism (SNP) within 5 base pairs of the targeted CpG locus and those containing repetitive elements (repeat length ≥ 10) were eliminated from data, leaving 23,477 loci for analysis. We applied non-specific filtering by variance to remove the bottom 67% CpG loci to gain power; because the results remained essentially the same, we present results without the filtering.

To assess the effect of HL or its treatment on DNAm, DNAm beta value differences between HL survivors and their unaffected twins were assessed in three ways using paired t-tests: 1) mean DNAm across all loci contained in the array ($N = 23,477$ loci) (i.e. array-wide mean DNAm); 2) DNAm age using the 353 CpG loci previously developed to measure epigenetic aging [23]; and 3) DNAm differences at each individual CpG locus for 23,477 loci. Briefly, DNAm age was calculated using a pipeline based on a discovery and replication set in multiple tissues using the two Illumina Infinium arrays (27K and 450K), identifying 353 hyper- or hypo-methylated CpG island loci (<http://labs.genetics.ucla.edu/horvath/dnamage>) that strongly predict age ($r^2 = 0.96$). The beta values of the selected loci were weighted and averaged, producing an epigenetic, or DNAm age. The 353 CpG loci comprising the DNAm measure is presented in Supplementary Table 3. All analyses were performed separately for twins providing blood and saliva samples because of the potential difference in leukocyte subset distribution. Array-wide mean DNAm and DNAm age were evaluated among all participants and by age at and time since diagnosis, sex and histology.

It is known that DNA methylation shows substantial variation in different types of leukocytes [36], thus DNAm measured in blood can be confounded by white blood cell composition. Therefore, we applied Houseman's algorithm [37] which uses DNAm patterns to assess the mean frequency of granulocytes, monocytes and lymphocytes, to determine whether there were differences in the distribution of these leukocyte cell types between saliva and blood samples. However, we did not adjust for the distribution in the main case-control analysis because of the small number of pairs with blood samples.

All analyses were conducted using R software (version 3.3.3). We accounted for multiple comparisons when comparing DNAm differences at each of the 23,477 CpG loci between the survivors and unaffected co-twins using a false discovery rate adjusted p-value [38], with statistical significance considered to be $FDR < 0.05$.

Results

Demographics are shown in Table 1. The mean and median age at sample collection of the nine pairs with blood samples were 57.4 (SD: 9.8) and 54 (range: 46–79) years, respectively, and 50.0 (SD: 10.3) and 47.5 (range: 31–68) years for the pairs with saliva samples. Fifty-six percent of the pairs who provided samples were male. All AYAHL survivors were in complete remission and essentially cured. The distribution of follow-up time was skewed in the nine pairs providing blood samples, with a mean and median of 27.6 (SD: 12.0) and 34

(range: 8–42) years, but not for the 36 pairs providing a saliva sample (both mean and median = 24.5 years). Among those with classifiable histology, 85% (6/7) of the AYAHL survivors who provided a blood sample and 62% (18/29) of those who provided a saliva sample had tumors classified as the nodular sclerosis (NS) subtype.

Differences in array-wide and loci-specific DNAm

HL survivors and their unaffected twins did not differ with respect to array-wide mean DNAm when considered together or stratified by sex, age at diagnosis, age at sample collection, or follow-up time (Supplementary Table 1). Overall, beta DNAm levels were about 4% higher in the DNA from blood compared to saliva specimens.

No CpG loci showed significant differences in DNAm between AYAHL survivors and their unaffected co-twins at a FDR of 5%. Thus, we reported CpG loci with a mean DNAm beta value differences -0.05 and $P < 0.05$ ($N = 74$ and 6 loci among twin pairs providing blood and saliva samples, respectively) (Table 2). Among twin pairs with blood samples, AYAHL survivors had higher mean beta values than their unaffected co-twins for 41/74 loci while unaffected co-twins demonstrated higher average beta values than case twins for 33 loci. Several differentially methylated loci in DNA occurred in genes directly or indirectly related to HL, including *TAP1*, *TLR9*, *IFNG*, *RUNX3* and all but the *TLR9* locus were hypomethylated in survivors compared to unaffected twin controls.

Among twin pairs with saliva samples, survivors had higher mean beta values than their unaffected co-twins for 3/6 the loci.

DNAm age

HL survivors had older mean blood DNAm age than their unaffected co-twins (+2.8 years, $P = 0.04$) (Table 3). The age differential persisted when stratified by sex, however, it was only statistically significant among female pairs (females: +2.7 years, $P = 0.01$; males +2.8 years, $P = 0.26$). There was a borderline significant difference between HL survivors and their unaffected co-twins among pairs who were older than the median age at sample collection (+2.4 years, $P = 0.09$). There were no significant differences among the pairs who provided saliva samples, although in each stratum, the AYHL survivor had slightly higher DNAm age compared to their unaffected twin. When mean differences in DNAm age were compared to actual age, the difference was larger in pairs who provided a blood sample (HL survivor + 6.6 years; unaffected twins + 3.9 years), compared to pairs who provided a saliva sample (HL survivor + 3.3 years; unaffected twins + 2.5 years) (Supplementary Figure 1 a and b).

We then assessed the proportion of lymphocytes, monocytes and granulocytes in HL survivors and unaffected co-twins in blood and saliva samples using Houseman's algorithm as described above (Supplementary Table 2). Among AYAHL survivors, blood samples had a higher average proportion of lymphocytes and a lower proportion of granulocytes (32.5% lymphocytes and 60.5% granulocytes) compared to saliva samples (17.8% lymphocytes and 73.7% granulocytes), with borderline significant differences.

Discussion

We examined DNAm in long-term AYAHl survivors and used unaffected twins of cancer survivors as genetically and early life-matched controls. We observed an epigenetic age acceleration in AYAHl survivors compared to their unaffected co-twins in every stratum, (sex, age at and time since diagnosis, histological subtype), limited to DNA from blood specimens. Other studies have reported associations between DNAm age and risk of overall cancer risk [39], B-cell lymphoma [40] and lung cancer among women and cancer mortality in later life [41], even after adjusting for known risk factors. Older tumor tissue DNAm age (older than patient age) was observed in various tumor types (e.g. bladder, bone marrow and ovary) [23]. A recent study found that chemotherapy can induce blood DNAm alterations among ovarian cancer patients associated with overall survival [42].

It is clear that survivors of AYA cancer experience an increased risk of serious health conditions associated with aging, including cardiovascular disease, pulmonary fibrosis, gastrointestinal reflux, and second malignancy, with a prevalence of 66%–88% [43], and that they have a life expectancy significantly lower than that of the general population [44]. It is possible that altered DNAm as a result of therapy may contribute to the late health outcomes. In our study, it is notable that the difference in DNAm age in the AYAHl-discordant twin pairs persisted for decades after cure. There are reports that exposure to Vinca alkaloids (i.e. vincristine), alkylation agents (i.e. dacarbazine), bleomycin and radiation, all part of standard AYAHl therapy, may be associated with epigenetic aging [45]. Thus, the most likely explanation for the difference in DNAm age between survivors and their twins is that it is a result of treatment, given that at least 3 of the 4 agents in standard chemotherapy and radiation therapy result in epigenetic modification[46]. Although we did not have detailed treatment information for each patient, the patients were mainly stage I or IIA, with a handful with IIIA, and during the diagnostic period (1961–2004), treatment with radiation therapy and combination chemotherapy was standard for early stage disease [47]. Alternative explanations are that the accelerated epigenetic age is a result of the disease, or an acquired risk factor prior to diagnosis.

In studies of cancer-free individuals, males showed a higher acceleration in epigenetic age in blood compared to females [48]. Our study was too small to assess whether there were gender-specific differences in accelerated aging in AYAHl survivors, although in the pairs that provided blood, there was a suggestion of a stronger effect among females.

There was no difference in array-wide mean DNAm between survivors and matched unaffected co-twins. However, we did find a large number of specific loci that were differentially methylated, largely limited to the nine pairs who provided DNA from blood specimens, (although statistical significance did not survive adjustment for multiple comparisons). We identified 74 and 6 loci with a mean beta value difference of at least 0.05 and $P < 0.05$ among twin pairs with blood and saliva samples, respectively. Three loci (*TLR9*, *TAP1* and *IFNG*) have been implicated in HL risk in candidate genetic association studies. A *TAP1* genetic variant (allele encoding amino acid Ile at residue 333) was associated with familial NS AYAHl in a case-parent trio study [49]. A SNP in *TLR9* [50], a gene encoding the toll-like receptor 9 molecule that recognizes unmethylated CpG

with larger sample sizes and with blood cell-type specific DNAm profiling are important in understanding the role of DNAm in AYAHF survivorship.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The data that support the findings of this study are available from the corresponding author, W.C., upon reasonable request.

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Table 1.

Characteristics of Hodgkin lymphoma survivor twins and their unaffected co-twins.

	Blood samples N=9 pairs		Saliva samples N=36 pairs	
	Mean	SD	Mean	SD
Age at diagnosis, y	29.9	11.7	25.5	8.1
Age at sample collection, y	57.4	9.8	50	10.3
Male	58.6	11	50.9	9.6
Female	56	7.7	49.3	10.8
Years follow-up since diagnosis, y	27.6	12	24.5	8.8
	Median	Range	Median	Range
Age at diagnosis, y	26	17–52	23.5	14–44
Age at sample collection, y	54	46–79	47.5	31–68
Male	54	51–79	47.5	33–68
Female	56.5	46–65	47	31–67
Years follow-up since diagnosis, y	34	8–42	24.5	3–46
	N	%	N	%
Sex				
Male	5	55.6	16	44.4
Female	4	44.4	20	55.6
Histology				
Nodular sclerosis	6	66.7	18	50
Other	1	11.1	11	30.6
Unknown	2	22.2	7	19.4

Table 2.Individual loci¹ with significant within pair blood or saliva DNAm difference.

Loci	Mean beta difference (Case – Co-twin)	P-value	Gene ID ²	Gene Symbol	Located in CpG Island
<i>Twin pairs with blood sample</i>					
cg25298754	−0.058	0.001	79413	ZBED2	no
cg13500819	−0.078	0.001	51237	MGC29506	no
cg23667432	−0.063	0.001	250	ALPP	yes
cg00168942	−0.055	0.002	219770	GJD4	no
cg02196805	−0.056	0.002	1437	CSF2	no
cg03112433	−0.089	0.003	5218	CDK14	no
cg16529592	−0.070	0.003	864	RUNX3	no
cg18087514	−0.113	0.004	8564	KMO	no
cg20289911	−0.054	0.004	83541	FAM110A	no
cg19421752	−0.055	0.004	348932	SLC6A18	yes
cg03954858	−0.053	0.005	113828	FAM83F	no
cg05824215	−0.070	0.005	1235	CCR6	no
cg12387247	−0.061	0.005	2208	FCER2	no
cg20099806	−0.062	0.005	57003	CCDC47	no
cg07618900	−0.062	0.008	144453	BEST3	no
cg26227465	−0.056	0.011	3458	IFNG	no
cg09588653	−0.066	0.012	6534	SLC6A7	no
cg07826255	−0.054	0.012	6442	SGCA	no
cg22062068	−0.062	0.017	27288	RBMXL2	yes
cg20587168	−0.051	0.017	284382	ACTL9	yes
cg07330329	−0.064	0.023	79097	TRIM48	no
cg01281904	−0.067	0.023	4986	OPRK1	yes
cg24202119	−0.059	0.024	133690	CAPSL	no
cg22586527	−0.073	0.024	55553	SOX6	no
cg02735486	−0.083	0.026	287	ANK2	yes
cg18829411	−0.058	0.029	3150	HMG1	yes
cg04180953	−0.056	0.029	1823	DSC1	no
cg16853860	−0.067	0.032	6890	TAP1	no
cg23228178	−0.060	0.033	23569	PADI4	no
cg07637239	−0.053	0.034	338567	KCNK18	no
cg26251865	−0.057	0.037	56269	IRGC	no
cg19850370	−0.051	0.038	8671	SLC4A4	no
cg02334775	−0.052	0.042	3669	ISG20	yes
cg06244417	0.054	0.003	2219	FCN1	no
cg03883348	0.053	0.004	147700	KLC3	yes
cg10978346	0.068	0.005	8821	INPP4B	no
cg16404106	0.054	0.006	60494	CCDC81	yes
cg21578541	0.051	0.006	54106	TLR9	no

Loci	Mean beta difference (Case – Co-twin)	P-value	Gene ID ²	Gene Symbol	Located in CpG Island
cg09573795	0.069	0.006	4487	MSX1	yes
cg18267381	0.050	0.011	79750	ZNF385D	yes
cg22752533	0.061	0.012	57468	SLC12A5	yes
cg21750887	0.061	0.013	57094	CPA6	no
cg22285621	0.074	0.014	54961	SSH3	yes
cg27553955	0.053	0.014	170850	KCNG3	yes
cg08349806	0.056	0.014	255027	MPV17L	yes
cg12799895	0.061	0.015	4885	NPTX2	yes
cg10019507	0.050	0.016	6754	SSTR4	yes
cg18934187	0.105	0.016	147323	STARD6	no
cg15309006	0.053	0.017	63928	CHP2	yes
cg06958829	0.051	0.018	1101	CHAD	yes
cg20483374	0.053	0.020	114902	C1QTNF5	yes
cg12324629	0.072	0.020	9706	ULK2	yes
cg15379633	0.088	0.021	9609	RAB36	yes
cg24777950	0.066	0.022	1511	CTSG	no
cg18640030	0.060	0.024	1392	CRH	no
cg08015496	0.062	0.024	1314	COPA	no
cg12594641	0.072	0.025	130574	LYPD6	yes
cg26556719	0.062	0.028	56136	PCDHA13	yes
cg24120841	0.088	0.028	7068	THRB	yes
cg14914852	0.057	0.030	124626	ZPBP2	yes
cg01615704	0.060	0.030	7851	MALL	yes
cg24474182	0.078	0.030	53829	P2RY13	no
cg15250507	0.068	0.032	55765	C1orf106	yes
cg12164282	0.088	0.034	7837	PXDN	yes
cg12163490	0.053	0.036	1009	CDH11	yes
cg12006284	0.052	0.041	7490	WT1	yes
cg12788313	0.060	0.046	4485	MST1	no
cg02240622	0.056	0.046	5330	PLCB2	no
cg25044651	0.061	0.047	206338	LVRN	yes
cg03958979	0.053	0.047	7101	NR2E1	yes
cg14576824	0.059	0.047	26750	RPS6KC1	yes
cg23606023	0.069	0.047	863	CBFA2T3	no
cg14603345	0.099	0.049	22903	BTBD3	yes
cg03665457	0.101	0.049	158160	HSD17B7P2	yes
<i>Twin pairs with saliva sample</i>					
cg19096475	0.078	0.0003	79827	ASAM	yes
cg06398181	0.053	0.0003	7776	ZNF236	no
cg15811427	0.058	0.001	2357	FPR1	no
cg12461141	–0.058	0.005	10346	TRIM22	no
cg19319069	–0.060	0.025	7328	UBE2H	yes

Loci	Mean beta difference (Case – Co-twin)	P-value	Gene ID ²	Gene Symbol	Located in CpG Island
cg20169062	–0.070	0.046	8988	HSPB3	no

¹ Only individual loci with mean intra-pair beta value differences ≥ 0.05 and paired t-test $P < 0.05$ were presented.

² from Entrez Gene, <https://www.ncbi.nlm.nih.gov/gene>

Table 3.

DNAm age in Hodgkin lymphoma survivors and their unaffected co-twins.

		Mean DNAm Age (SD)			
		Blood (N=9 pairs)		Saliva (N=36 pairs)	
(N = no of pairs)		HL Survivors	Unaffected co-twin	HL Survivors	Unaffected co-twin
	P				P
All pairs	0.04	64.1 (9.2)	61.3 (8.1)	53.3 (9.5)	52.6 (9.9)
Sex					
Females (N = 4)	0.01	63.7 (11.9)	61.0 (11.2)	52.7 (11.1)	52.0 (10.7)
Males (N = 5)	0.26	64.4 (8.0)	61.6 (6.0)	54.0 (7.3)	53.3 (9.1)
Histology					
Nodular Sclerosis (N = 6)	0.10	60.6 (9.6)	58.6 (8.5)	54.2 (8.8)	53.5 (10.0)
Other (N = 3)	0.30	71.0 (1.3)	66.8 (4.1)	52.3 (10.3)	51.7 (10.0)
Age at sample collection ¹					
< Median (N = 4)	0.27	59.6 (9.3)	56.4 (6.0)	46.6 (7.5)	45.4 (6.4)
Median (N = 5)	0.09	67.7 (8.3)	65.3 (7.7)	59.9 (6.0)	59.7 (7.2)
By time since diagnosis ³					
< median (N = 4)	0.11	62.6 (10.5)	58.3 (8.2)	51.0 (10.8)	49.7 (10.8)
median (N = 5)	0.31	65.2 (9.2)	63.7 (8.0)	55.5 (7.7)	55.5 (8.1)

¹Median age at sample collection among twin pairs with blood sample: 54 y

²Median age at sample collection among twin pairs with saliva sample: 47.5 y

³Median years since diagnosis among twin pairs with blood sample: 34 y

⁴Median years since diagnosis among twin pairs with saliva sample: 24.5 y