Facial Clefting and the Vietnam War: A Study of DNA Methylation Patterns and Intergenerational Stress

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Erin Rachel Kello, PhD

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Changes in DNA methylation patterns have been linked to extreme stress. These patterns are heritable by the next generation. Facial clefting has been linked to changes in methylation patterns affecting craniofacial genes. In this study we explored: 1. If methylation patterns in offspring are associated with maternal exposure to extreme stress 2. If altered methylation patterns are associated with clefting in offspring 3. If the changes preferentially altered craniofacial genes. The present study used peripheral blood samples from 4 cohorts of children. Samples were randomly chosen from a larger group of 505 samples. Group 1 (N=7) have mothers born during the Vietnam war and have CL/P. Group 2 (N=8) have mothers born after the Vietnam war and have CL/P. Group 3 (N=8) have mothers born during the Vietnam war and do not have CL/P. Group 4 (N=8) have mothers born after the Vietnam war and do not have CL/P. We carried out an epigenome wide association study (EWAS) to test the association between DNA methylation pattern, exposure and cleft status, utilizing comparisons between the larger exposed and not exposed cohorts, and cross-wise comparisons between each of the smaller cohorts. Significant results were obtained at the FDR .05 level confirming that overall methylation patterns in children born to mothers who were exposed to war stress and children born to mothers who were not exposed to war stress are different. The affected genes represent an array of core biological functions from cell growth and proliferation to neurological and craniofacial development. The results for the cross-wise comparisons of groups are less conclusive, likely because of the small

sample size. However, several probes were significant at the FDR .05 level. The results for the clefts vs. non-cleft groups may have identified novel loci that are associated clefting in this population. The next step in this study is to evaluate the larger group of samples to ascertain if these associations hold.

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Preface

I would like to thank my committee members: Dr. Michael Siegel, Dr. Alexander Vieira, Dr. Mark Mooney and Dr. Joseph Alter for their continued support for this project. I would especially like to thank Mike Siegel for his support and encouragement as explored potential dissertation research concentrations. I would like to thank Dr. Suzuki Satoshi for access to the samples used in this project. I would like to thank Kathleen Deeley for assistance with the lab work for this project. I would like to thank Maria Chickina for her assistance in data analysis.

1.0 Background

In 1942, Conrad Waddington coined the term epigenetics. He defined this term as "the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington 1942: 11). Waddington's experiments on fruit flies set the stage for the development of the field of epigenetics.

In 1975, two papers independently proposed a system of epigenetic regulation based on DNA methylation (Holliday and Pugh 1975, Riggs 1975). Holiday and Pugh proposed a system of DNA methylation that operates as a developmental clock, turning genes on and off after specified time periods. They suggested this control system operates solely though methylation without DNA sequence changes. Riggs (1975) also proposed a system of epigenetic control based on methylation. He proposed the existence of two methylases in eukaryotes (these were only known from bacteria at the time). One enzyme would perform maintenance methylation on hemi-methylated sites and another would perform de novo methylation, which Riggs postulated would be a more difficult process than maintenance methylation. Riggs used the X inactivation process in mammals to argue that DNA methylation proscribes the binding of regulatory proteins to transcription sites, silencing associated genes.

DNA methylation is defined as the addition of a methyl group to a nucleotide (Brero, Leonhardt, and Cardoso 2006). In mammals, DNA methylation takes places mainly on cytosine bases. Methyl groups are added to the cytosine base at the C_5 position. This forms the compound 5-methyl cytosine (5mc). Hotchkiss (1948) was the first to report the presence of this chemical compound in mammalian DNA. Methylated cytosine bases are mostly found in CpG dinucleotides and methylated in both strands of DNA (Brero, Leonhardt, and Cardoso 2006). CpG dinucleotides

are areas of DNA where a cytosine nucleotide is located adjacent to a guanine nucleotide in the 5’ to 3’ direction. CpG islands are regions of DNA, about 1 kilo base in length, with a high frequency of CpG sites. CpG islands are often located in promoter region of genes. In mammals, approximately 2-5 percent of cytosine bases are methylated (Holliday 2002)

The default state of CpG islands is unmethylated in all cell types (Deaton and Bird 2011). Methylation status variation at a given CpG island is known as methylation variable position (MVP). In most cases, CpG islands are methylated on both DNA strands; however, in the rare case they are not, this is referred to as hemimethylation. When multiple adjacent CpG islands are methylated, this is known as a differentially methylated region (DMR). DMRs vary greatly in length. CpG shores are regions adjacent to CpG islands; they display higher variation in methylation status than CpG islands. DMRs commonly occur in CGI shores.

Jones (1999) describes a complex pattern of CpG position, methylation status and type of control. He states methylation of a CpG island located downstream of the transcriptional initiator of a gene does not inhibit transcription. Methylation in the transcribed region can inhibit expression. Methylation of as few as 7 percent of CpG sites associated with a promoter region is linked to transcriptional repression.

Orofacial clefting (CL/P), which includes cleft lip only, cleft palate only and cleft lip and palate, is a set of developmental abnormalities that affects the primary and secondary palate (Mossey et al., 2009). The overall prevalence in the population for all clefts is 9.92 per 10,000 (Mastroiacovo et al., 2011). The prevalence of cleft lip only is 3.28 per 10,000 while cleft lip and palate is 6.64 per 10,000. Northern Europeans, Asians, Native Americans, and Aboriginal Australians are more commonly affected by cleft lip and palate while those of African descent have more instances of cleft lip only.

Clefting has a multifactorial etiology. Several missense mutations have been linked with an increased risk of the condition (Vieira 2008). Clefting also has an environmental component. Environmental factors known to increase the risk of clefting include: tobacco smoke, alcohol, poor nutrition, viral infection and some medicinal drugs (Mossey et al., 2009).

Some of these environmental factors work through the mechanism of DNA methylation. A mouse model (Plamondon et al., 2011) found a link between genotype, methylation status at a transposable element site and the penetrance rate of cleft lip and palate. Another mouse model found that maternal exposure to retinoic acid led to perturbations in DNA methylation levels during development. In the exposed mice methylation remained mostly static during palatogenesis. In the control mice, DNA methylation was much more dynamic over time

In humans, a study investigating the link between CL/P penetrance and methylation levels identified 578 methylation variable positions (MVPs, also the same as DMPs here) associated with non-syndromic cleft lip and palate (Alvizi et al., 2017). The MVPs were associated with genome regions implicated in craniofacial development. Also, in humans, different cleft subtypes (cleft lip only, cleft lip and palate, and cleft palate only) have different methylation profiles (Sharp et al., 2017).

This study investigates the link between the environmental input of extreme stress, it's effect on DNA methylation patterns and the outcome of CL/P using five null hypotheses:

1. DNA methylation at CpG sites in children whose mothers were born during the Vietnam war (Groups 1 and 3) will not significantly differ from the children whose mothers were born after the Vietnam war (Groups 2 and 4).
2. DNA methylation at CpG sites in children whose mothers were born during the Vietnam war and have CL/P (Group 1) will not differ significantly from children whose mothers were born after the war and have CL/P (Group 2).
3. DNA methylation at CpG sites in children whose mothers who were born during the Vietnam war and have CL/P (Group 1) will not significantly differ from children whose mothers who were born during the Vietnam war and do not have CL/P (Group 3).

4. DNA methylation at CpG sites in children whose mothers were born after the war and have CL/P (Group 2) and children whose mothers were born after the war and do not have CL/P (Group 4).
5. DNA methylation in the promoter region of genes associated with craniofacial development will be the same in all groups.

For each test, the level of significance is a False Discovery Rate (FDR) of .05. In identifying clusters of probes that form differentially methylated regions an FDR of .025 with an additional criterion of a minimum of 10 differentially methylated probes with a FDR of .025 was used.

1.1 Materials and methods

1.1.1 Cohorts

The present study used four cohorts with eight samples in each cohort. Dr. Satoshi Suzuki randomly chose the samples from a larger group of 505 samples. Group one consists of eight subjects who have mothers born during the Vietnam war and have CL/P, ranging in age from .5 years to 15 years. Group two consists of eight subjects who have mothers born after the Vietnam war and have CL/P, ranging in age from .4 years to 3 years. Group three consists of eight subjects who have mothers born during the Vietnam war and do not have CL/P, ranging in age from 22 years to 32 years. Group four consists of 8 subjects who have mothers born after the Vietnam war and do not have CL/P ranging in age from .5 years to 2 years. All cohorts contain a mix of males and females and a mix of cleft types.

Table 1: Study cohorts.

Group	Notation	# of samples	Description
1	ECLP	7* *includes only samples that passed QC	Children born to mothers born during the war with clefts
2	NECLP	8	Children born to mothers born after the war with clefts
3	ENC	8	Children born to mothers born during the war without clefts
4	NENCLP	8	Children born to mothers born after the war without clefts

1.2 Exposure

The American War in Vietnam took place between 1955 and 1975 (Bradley 2009). During this time, the Vietnamese people were subject to a multitude of horrific experiences. During the war, approximately 4 million Vietnamese citizens were displaced from rural villages and moved to refugee camps in cities. The Americans declared large areas of the countryside "free-fire" zones and indiscriminately used the defoliant Agent Orange in these areas. Although many Vietnamese had been moved to camps, as much as 75 percent of the population was still living in these areas. They were exposed to Agent Orange and subject to American bombing campaigns. American ground forces further brutalized local populations with rape, torture, mutilations and indiscriminate killing. Surviving Vietnamese citizens in the American occupied areas were later put into crowded camps, which lacked basic resources such as clean water and toilets. Rural peasants were a source of recruitment for the Army of the Republic of Vietnam (Bradley 2009). This was a force

comprised of mostly conscripted Vietnamese that aided the American armies. This mass conscription greatly reduced the available labor force in villages, in turn reducing agricultural output. This led to mass starvation in the countryside.evels of stress and anxiety and must had feared for their lives. It is reasonable to conclude that the mothers born during the war were exposed to the above traumas through their pregnant mothers.

The sample groups span north and south Vietnam including: Binh (#14 in figure) province in the North and Ho Chi Minh City (#16 in figure) and Bentre (#20 in figure) province in the south. The provinces were affected by the use of the chemical Agent Orange during the war (Grotto et al., 2009). The use of Agent Orange ceased in 1971. All mothers included in the study were born after 1971. The subjects may have secondary exposure to latent Agent Orange in the soil and water supply, but there is not a way to quantify this.

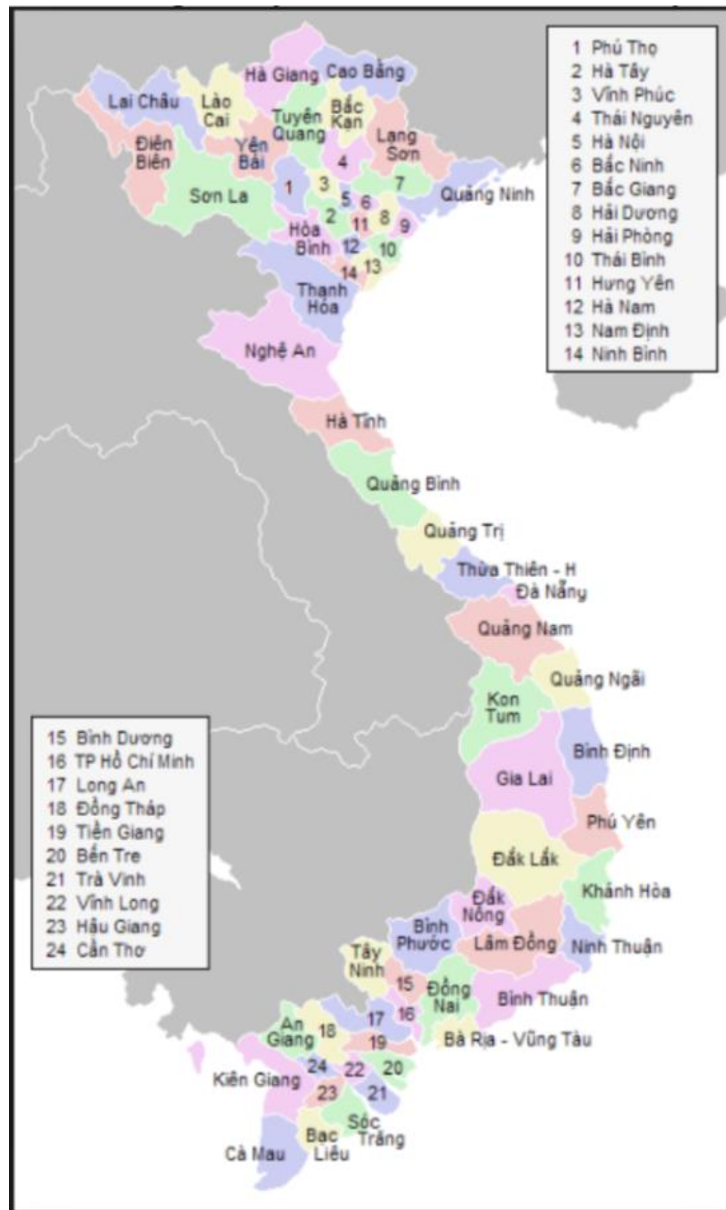

Figure 1: Present day map of provinces in Vietnam.

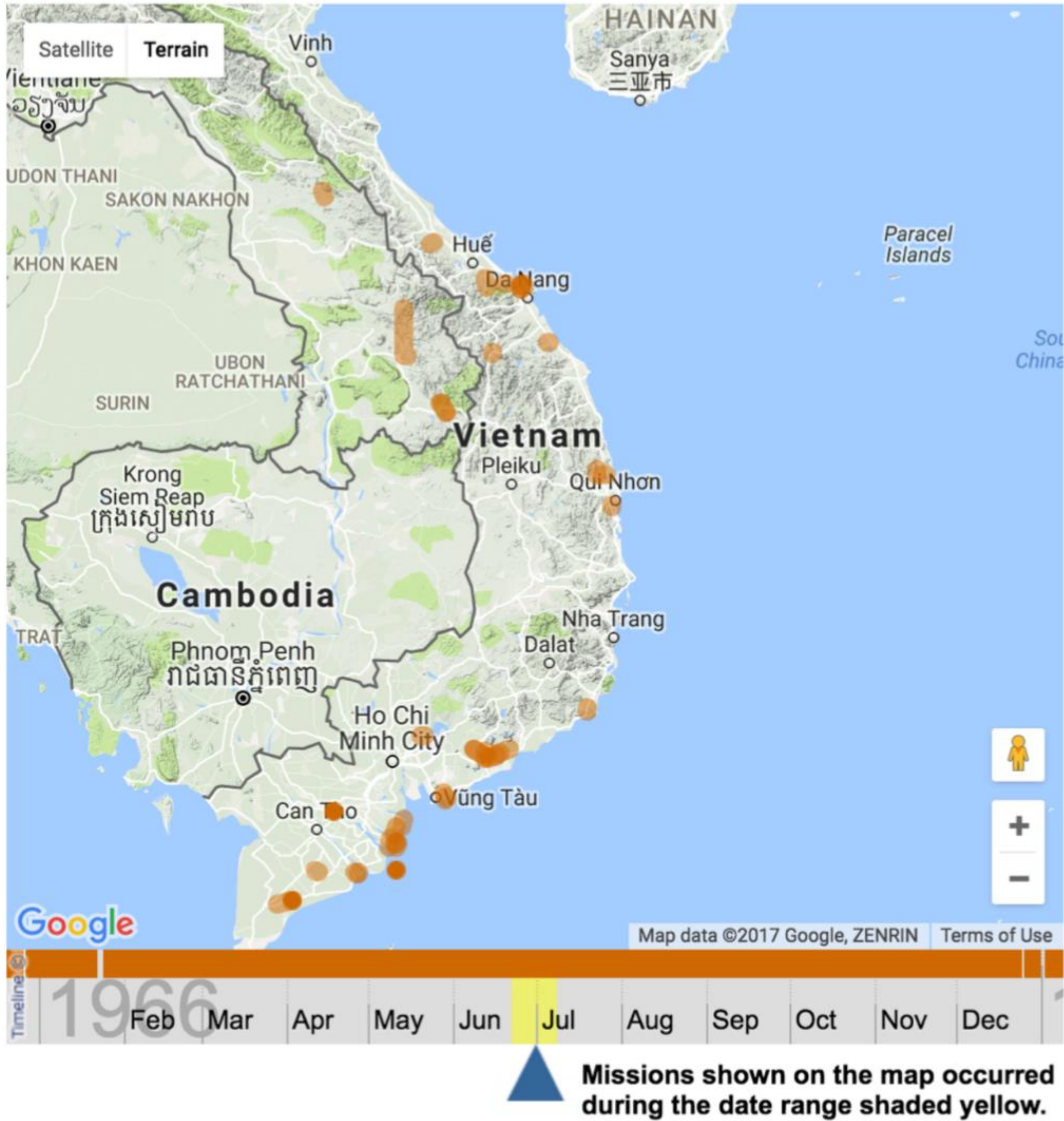

Figure 2: Map of defoliant spraying mission in July 1965. All samples were collected in affected provinces.

1.3 Stress

Extreme stress is known to affect several systems in the body. Hans Selye (1976) outlines the body's general response to stress, stating that regardless of the stimulus that causes the stress,

the initial response by the body is uniform. The hypothalamus is the first mediator in the stress response chain, transforming the initial nervous signals into hormonal messages. These messages then contact the adrenal cortex, which produces corticoids (steroids that act during times of structural stress). Corticoids have been found to persist well beyond the acute period in chronic diseases such as diabetes (Keating and El-Ostra 2013).

Corticoids then mediate other biochemical reactions leading to the process of inflammation, which allows the body to address the stimulus and eventually return to homeostasis. These stress reactions are usually short lived in the body. However, they may become chronic if the stress stimulus is not removed, as is seen in wartime situations. A 2017 study (Glad et al.) found that patients with Cushing's syndrome, caused by an overproduction of cortisol, have altered DNA methylation. In the study those with Cushing's syndrome had lower average methylation levels than controls.

Selye (1976) directly addresses the stress consequences of combat situations. This can be extrapolated to civilian populations that are exposed to war. He reports evidence of increased corticoid production in U.S. soldiers during combat conditions. This response is mediated by the intensity and duration of combat conditions.

A study that evaluated the effects of a prolonged conflict using civilians from Kosovo, Serbia, reported that 26.1 percent met the criteria from PTSD (Morina et al., 2018). Women were more likely than men to experience PTSD. In the study civilians reported exposure to up to 22 different kinds of trauma. The three most reported kinds of trauma were: lack of shelter, lack of food or water, and being close to death.

For the purpose of this study we can say that the mothers of the children who were exposed to the Vietnam War can be reasonably assumed to have been subject to increased stress-induced hormone production for the period of their exposure.

1.4 Intergenerational transmission

This study utilizes an intergenerational exposure. Maternal stress during gestation has been found to affect cortisol levels in offspring (Bowers and Yehuda 2016). Yehuda et al. (2015) report a link between the DNA methylation patterns found in Holocaust survivors with PTSD and their offspring.. The authors report that there is a significant correlation between generational lineage and the methylation levels at specific sites with the parents having higher average methylation levels and the offspring having lower average methylation levels. Another study (Heijmans et al., 2008) using a cohort of Dutch individuals prenatally exposed to nutritional stress during WWII, found low levels of methylation in the offspring of the original cohort at the imprinted *IGF2* locus six decades after the exposure.

1.5 Developmental considerations

1.5.1 The primary palate

Development of the primary palate begins at the fifth week in utero (Mossey et. al. 2009). It involves the frontonasal prominence, the paired maxillary processes and the paired mandibular

processes, which are derived from neural crest mesenchyme. The migration and proliferation of these cells is essential to palatogenesis (Sperber 2002). An essential component of the migration of neural crest cells is their interaction with the surrounding extracellular matrix (ECM) and epithelial tissue. The fusion of these prominences forms the transitory nasal fin, which disintegrates through apoptosis or mesenchymal transition of cells. Disruption of the formation or disintegration of the nasal fin and/or the mesenchymal migration process will result in a cleft.

1.5.2 The secondary palate

Development of the secondary palate begins in the sixth week in utero (Mossey et al., 2009). First, the paired palatal shelves, vertically oriented outgrowths of the maxillary processes, appear. Initially these grow vertically along the sides of the developing tongue. In the eighth week of gestation the palatal shelves elevate to a horizontal position (Sperber 2002). This is a short process, taking place in matter of hours. For the palatal shelves to elevate and fuse, several chemical and mechanical events must take place in a chronologically restricted manner. The elevation of the shelves requires the accumulation of both hyaluronic acid and glycosaminogens in the extracellular matrix (ECM) of the shelves. The tongue must be depressed by mechanical factors during the elevation process.

Epidermal growth factors (EGFs), epidermal growth factors receptors (EGFRs) and transforming growth factors (TGFs) are expressed during all stages of palatal formation (Citterio and Gaillard 1994). The interaction of TGFs with EGFRs produces the matrix metalloproteinases that facilitate palatal closure and degradation of the epithelial seam (Miettinen et al., 1999).

Epithelium along the edges of the palatal shelves must be thickened for proper closure. The epithelial seam must then be removed. The fusion process is marked by an upregulation of keratin

(K5/6) (Gibbins et al., 1999) Fusion proceeds anterioposteriorly. During fusion N-cadherin is upregulated (Montenegro et al., 2000). The formation of the secondary palate is complete when the palatal mesenchyme differentiates into bony and muscular elements of the hard and soft palate. The secondary palate then fuses with the primary palate and the nasal septum. These processes are complete by 10th week in utero.

1.6 Genetics of facial development

Many genes are involved in the development of the primary and secondary palate. In the primary palate, gene families that regulate basic cellular processes of growth, differentiation, and apoptosis control the fate of neural crest cells (Lan et al., 2015). The migration of the neural crest cells is regulated by Homeobox genes (*HOX*), which control patterning.

Genes families expressed in ectomesenchyme of the facial prominences include the following: members of the homeobox family (*HOXA1, HOXA2, HOXB1, HOXB3,* and *HOXB4*), sonic hedgehog (*SHH*), orthodontical (*OXT*), goosecoid (*GSC*), distaless (*DLX*) and muscle segment (MSX) (Schutte and Murray 1999). The list of proteins acting at the cellular level in facial development is as follows: epidermal growth factor (EGF), transforming growth factor alpha and beta families (TGFA and TGFB), fibroblast growth factors (FGFs) and bone morphogenic proteins (BMPs). Additional genes associated with facial development generally include *DLX5* and 6, *FGFR1* and 2, *JAGGED1*, *GLI3*, *PAX3,* and calcium dependent serine protein kinase (*CASK*). Many of these genes have been suggested as playing a role in orofacial clefting. These genes will be prioritized for investigation of differential methylation and craniofacial development. These genes are presented in table x in the appendix.

1.7 Statistical analysis

Biological samples of peripheral blood were collected on Guthrie cards. Dried DNA was sent to the Vieira lab for re-hydration. Bisulfite conversion and the Infinium Methylation EPIC assay, which covers ~850,000 CpG sites, were performed according to Illumina protocols by the University of Pittsburgh Genomics Core. Methylation levels of CpG sites were reported as β-values (β = intensity of the methylated allele (M)/[intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100] (Bibikova et al., 2011).

The data was preprocessed using ChAMP (2.12.2) for R (3.5). ChAMP's default preprocessing utilizes the following methods. Probes that failed to meet a detection p-value of .05 were removed. Cleft lip and palate affects males and females at different rates, so 11,648 probes were removed from the X and Y chromosomes. All probes that did not map to a CpG site were removed. Probes that mapped to known confounding SNPs were removed (Zhou et al., 2017). All multi hit probes were removed (Nordlund et al., 2013). This left (717,600) probes for analysis. One sample from cohort one, AV4 failed quality control and was removed from the dataset leaving 31 samples for analysis.

Data were normalized using the BMIQ method (Teschendorff et al., 2013), which corrects bias between type I and type II probes. A cellular heterogeneity correction was implemented using the RefbaseEWAS method (Houseman et al., 2016).

DNA methylation is significantly associated with age (Horvath 2013). The subjects in this study ranged in age from .5 years to 32 years. Therefore, before implementing the hypothesis test for exposure, a correction for age was implemented using a methodology adapted from Sharp et al. (2017). A hypothesis test was run using clefts as the variable of interest. All DMPs significantly associated with age were removed from the beta matrix.

Differentially methylated positions (DMPs) were identified from beta values using the limma package implemented in ChAMP. This package calculates the p-value for each probe using a linear model. The p-value for this model is the false discovery rate (FDR) detailed in Benjamini and Hochberg, 1995. Differentially methylated regions (DMRs), or probe clusters were identified using the Bumphunter method (Jaffe et al., 2012). This method creates a design matrix and fits a linear model, which groups probes into clusters and then applies a random permutation method to identify significant DMRs. Gene set enrichment analysis (GSEA) was performed in ChAMP using the empirical Bayes method. This method applies a global test to the normalized beta matrix, taking into account the significance level of single probes and correcting for the inequality bias of probe number per gene. This method also considers degree of significance of each CpG site. Differentially methylated interaction hotspots were identified using the EpiMod module in ChAMP. This module utilizes the FEM package (Jiao et al., 2014). The algorithm for the FEM package identifies gene sets with differential methylation and expression patterns based on a protein interaction network. The FEM module was run at the probe level.

1.8 Biological relevance

Significant DMPs were prioritized by functional relevance, using the following methodology adapted from Martino and Saffery (2015). DMPs located within 200 bp of the transcription start site (TSS200) or in the 1st exon were considered the most relevant to associated gene function. These locations have an inverse relationship between methylation and gene function. DMPs located further upstream of the transcription start site (TSS1500) and those in the 5'UTR region were considered of secondary relevance because the relationship between

methylation and gene function at these regions is less clear. DMPs located in the gene body and in the 3' UTR region were given the least significance as methylation here is not known to affect gene function.

Secondarily a measure of CpG density was considered. Regions with high CpG site density that are associated with promoter regions (islands) were prioritized first. Adjacent regions to islands that are located further from the promoter region but still maintain high CpG density (shores) were given second priority. Regions with lower CpG density that are located further from promoter regions (shelves and opensea) were considered the least relevant.

The utility of this approach was confirmed by the identification of the DMP associated with the ELANE gene as the most biologically relevant in the clefting phenotypes and also the identification of this gene as the center of a significant protein interaction network by the FEM algorithm.

2.0 Results

2.1 Exposure groups (Groups 1 and 3 vs. 2 and 4)

2.1.1 Differentially methylated positions (DMPs)

Data analysis confirms there are differences in methylation patterns between the groups of children whose mothers were exposed to the war (one & three) and the groups of children whose mothers were not exposed to the war (two & four). To ascertain the effect of war related stress, without clefting, a global test was run using CLP status as a variable. Probes that were significantly associated with CLP status were removed from the beta matrix before the test for exposure was run. A total of 12,813 significant probes associated with exposure were found at the FDR of .05. Probes were prioritized for further investigation based on location as outlined in the methods section. The 10 most functionally significant probes are presented below in Table 2.

The genes associated with the significant probes are associated with several biological functions. The most notable gene is *LOXL3*, which is part of a family of extracellular amine oxidases, involved in the cross linking of fibrillar collagen types (Lee and Kim 2006). This gene is essential for palatogenesis and spinal column formation in mice (Zheng et al., 2015). Mice with heterozygous deletions of this gene have impaired palatal development and abnormalities in the vertebral column.

Another notable gene is *SIN3A*. This gene plays a role is a transcriptional regulatory protein that forms the scaffold for histone deacetylase repressor complexes involved in epigenetic

regulation (Fleischer et al., 2003). Histone modifications are part of the epigenetic regulatory complex. The relationship between chemical modifications to histones, resulting in higher or lower levels of transcription, and DNA methylation are not currently well understood.

Clefts have been associated with structural brain and cognitive abnormities (Nopoulos, et al., 2007, Roberts et al., 2012). Five genes associated with the top ten most significant probes are involved in neurological processes. *HS3ST2*, *GRM2* and *PALM2* are both highly expressed in the brain (Fagerberg et al., 2014). *FTL* is the ferritin protein, which is involved in intracellular iron storage. Defects in this gene are associated neurodegenerative diseases. *FEZF1* plays a role in hormonal regulation in the brain.

Taken together these results demonstrate that there are significant (at the FDR .05 level) methylation differences between children whose mothers were exposed to the war and those whose mothers were not exposed to war stress.

Table 2: Top 10 most functionally significant probes associated with exposure.

Probe ID	Average exp.	Adj.P.Val	Exposed Avg.	Not Exposed Avg.	Chromosome	Gene	Feature	Cgi
cg26532042	0.106949576	0.001254116	0.123019097	0.0918844	19	*FTL*	TSS200	island
cg21954549	0.171145712	0.001349742	0.209961317	0.134756082	12	*TMTC1*	TSS200	island
cg11849422	0.155472901	0.001742938	0.181273337	0.131284993	9	*PALM2*	TSS200	island
cg26079664	0.199959444	0.002240515	0.28918243	0.116312895	3	*GRM2*	TSS200	island
cg13867963	0.131549169	0.002624394	0.221385824	0.047327304	8	*UNC5D*	TSS200	island
cg02143559	0.242575245	0.002845899	0.301004914	0.187797431	3	*LOC442*	TSS200	island
cg06584891	0.131533798	0.002894416	0.17815881	0.08782285	2	*LOXL3*	TSS200	island
cg23063716	0.093258202	0.002954447	0.106517297	0.0808278	15	*SIN3A*	TSS200	island
cg18422586	0.056828995	0.003204986	0.079875229	0.035223151	16	*HS3ST2*	TSS200	island
cg24934063	0.088838168	0.003208016	0.10509576	0.073596676	7	*FEZF1*	1stExon	island

2.1.2 Differentially methylated regions (DMRs)

A total of 64 significant differentially methylated regions (DMRs) were found at an area p –value cutoff of .025 with at least 10 associated significant probes (Table 2). Three of the DMRs were located on chromosome 6. This chromosome has been linked to clefting in several studies (Marazita et al., 2002, 2004, Letra et al., 2010).

DMR 1 (Figure 3) is associated with the *RNF39* gene. Similar genes in the RING finger family have been found to play a role in synaptic plasticity in rats (Matsuo et al., 2001). DMR 2 (Figure 4) is associated with the *HOXA4* gene, which has a role in hematopoietic stem cell renewal and as an inhibitor of cell differentiation (Iacovino et al., 2009, Fournier et al., 2012). The HOX family of genes is involved in the development of the primary and secondary palate (Sperber 2002). Hypermethylation of the *HOXA4* promoter has also been implicated in many types of leukemia (Strathdee et al., 2007). DMR 3 mapped to an intragenic region. DMR 4 (Figure 5) mapped to the *HLA-DPB1* gene. Polymorphisms of this gene have been linked with chronic beryllium disease (Richeldi et al., 1993). DMR 5 (Figure 6) mapped to the *MIR886* gene (alias *VTRNA2-1*). This gene produces a non-coding RNA that regulates cell growth (Zhang et al., 2014, Yu et al., 2014). This gene is normally imprinted in an allele specific manner with the paternal allele being expressed. In various cancers this gene is often hypermethylated and repressed. Differential methylation of *VTRNA2-1* has been linked to occupation exposure to pesticides (Van der Plaat et al., 2018).

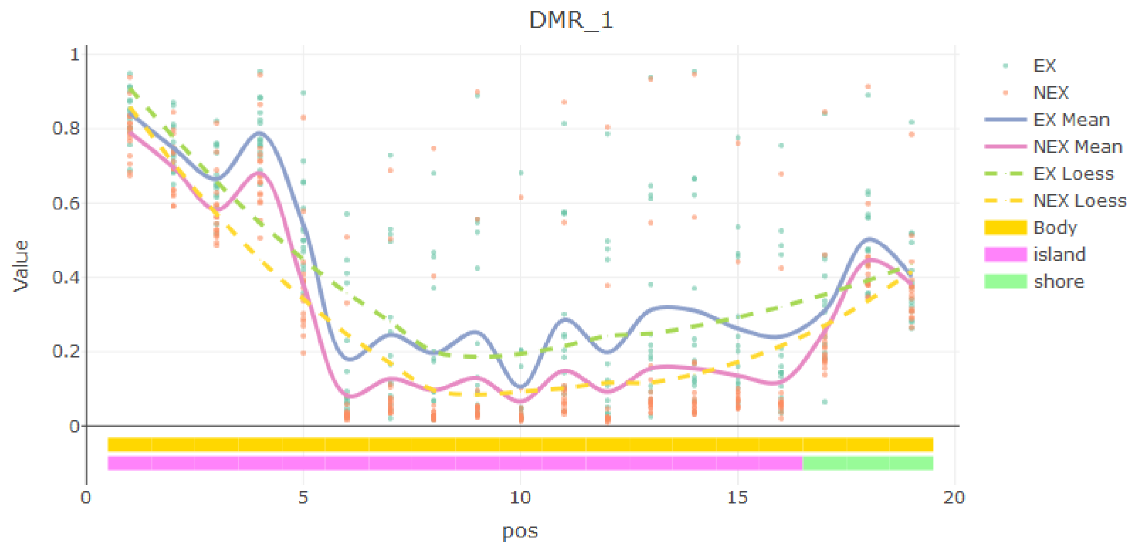

Figure 3: ChAMP Map of DMR 1 with genome features. EX= exposed. NEX= not exposed. Loess = local regression curves based on methylation means.

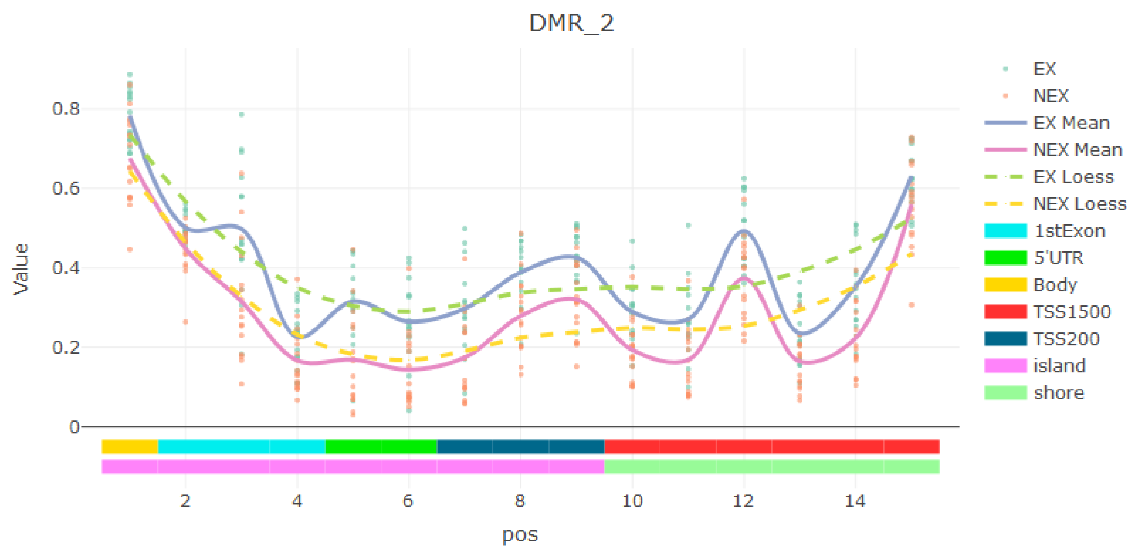

Figure 4: ChAMP Map of DMR 2 with genome features.EX= exposed. NEX= not exposed. Loess = local regression curves based on methylation means.

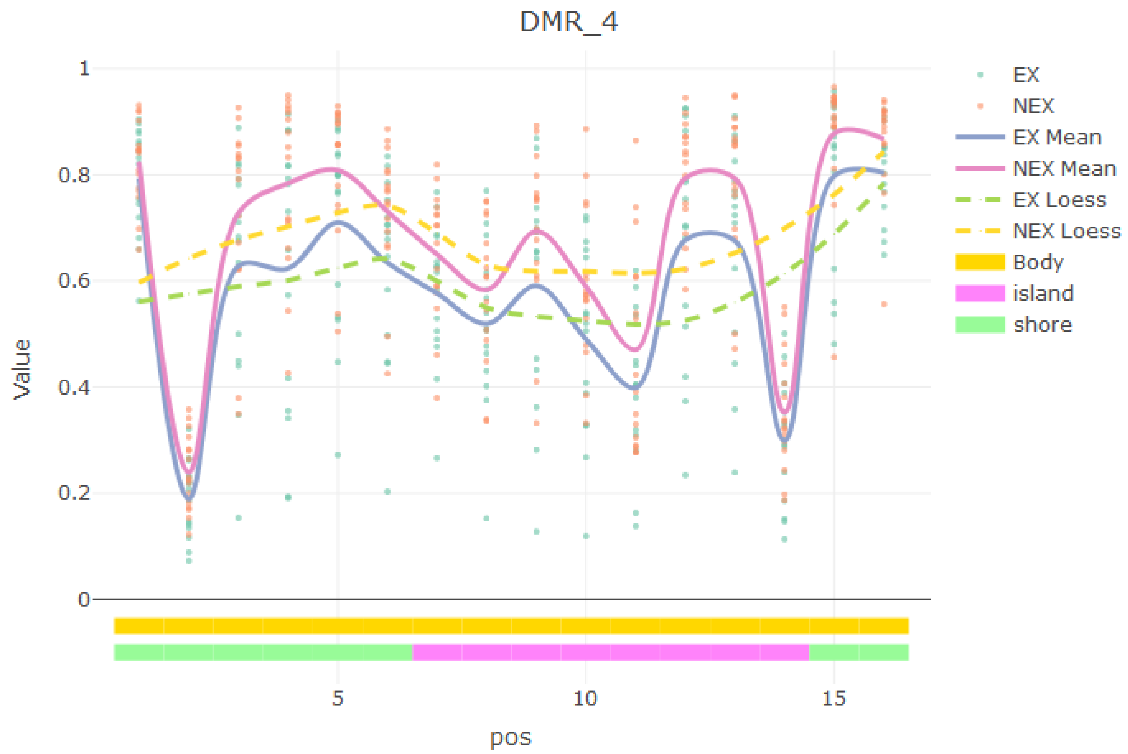

Figure 5: ChAMP Map of DMR 4 with genome features. EX= exposed. NEX= not exposed. Loess = local regression curves based on methylation means.

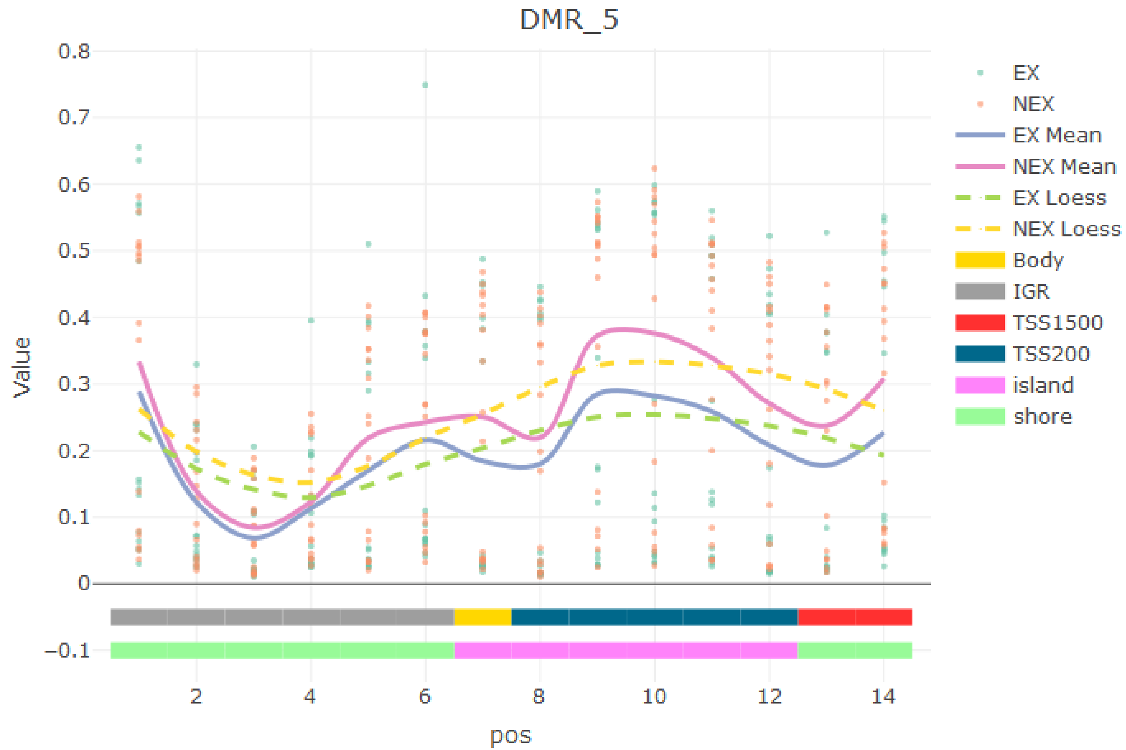

Figure 6: ChAMP Map of DMR 5 with genome features.EX= exposed. NEX= not exposed. Loess = local regression curves based on methylation means.

Table 3: Top 5 significant DMRs associated with exposure. *Area p-value is defined as the percent of candidate regions obtained from the permutations/boostraps that are as extreme as the observed region. **FWER area is defined as the proportion of permutations/bootstraps that had at least one

	Chromosome	# of probes	P-value Area	FWER area	Associated gene(s)
DMR_1	**6**	**19**	**0.000641869**	**0.216**	***RNF39***
DMR_2	**7**	**15**	**0.00190385**	**0.512**	***HOXA4***
DMR_3	**6**	**19**	**0.002339016**	**0.576**	***IGR***
DMR_4	**6**	**16**	**0.002698028**	**0.616**	***HLA-DPB1***
DMR_5	**5**	**14**	**0.002817698**	**0.632**	***MIR886***

2.2 Gene set enrichment analysis (GSEA)

Using the Bayesian iteration of the GSEA (gene set enrichment) module in ChAMP, a list of biologically relevant pathways was generated. The GSEA analysis on exposure groups generated a list of 1423 significant gene sets. Sorting by the Benjamini - Hotchberg adjusted p-value (also known as the FDR) the top ten most significant pathways are described in table 3.

Table 4: Top 10 GSEA results for exposure groups sorted by Benjamini – Hotchberg adjusted p-value.

Pathway	# of genes	BH adj. P-value
BENPORATH_ES_WITH_H3K27ME3	**1009**	**4.13E-58**
BENPORATH_SUZ12_TARGETS	**893**	**8.06E-48**
MIKKELSEN_MEF_HCP_WITH_H3K27ME3	**555**	**8.08E-48**
MEISSNER_BRAIN_HCP_WITH_H3K4ME3_AND_H3K27ME3	**1001**	**5.52E-46**
MARTENS_TRETINOIN_RESPONSE_UP	**762**	**4.78E-41**
BENPORATH_EED_TARGETS	**955**	**5.66E-41**
BENPORATH_PRC2_TARGETS	**588**	**1.82E-40**
MIKKELSEN_MCV6_HCP_WITH_H3K27ME3	**405**	**4.76E-36**
MIKKELSEN_NPC_HCP_WITH_H3K27ME3	**327**	**2.07E-33**
MEISSNER_NPC_HCP_WITH_H3K4ME2_AND_H3K27ME3	**329**	**6.04E-32**

The BENPORATH_ES_WITH_H3K27ME3 gene set is highly expressed in embryonic stem cells and associated with developmental processes (Benporath et al., 2008). The BENPORATH_EED_TARGETS gene set are targets of the polycomb protein EED in embryonic stem cells. The BENPORATH_PRC2_TARGETS gene set are targets

of the Polycomb repression complex and also have a trimethyalted H3K27 mark in their promoters. The BENPORATH_SUZ12_TARGETS are targets of the polycomb protein SUZ12 in embryonic stem cells.

The MIKKELSEN_MEF_HCP_WITH_H3K27ME3 gene set is an embryonic fibroblast cell line with high density CpG promoters (HCP) with a trimethyation mark at position K27 on histone 3 (Mikkelsen et al., 2008). The MIKKELSEN_NPC_HCP_WITH_H3K27ME3 cells are neural progenitor cells with the same histone characteristics. The MIKKELSEN_MCV6_HCP_WITH_H3K27ME3 are embryonic fibroblasts trapped in a differentiated state with the same histone characteristics as above. These are cell lines that are have been investigated in epigenetic chromatin state profiling.

The MARTENS_TRETINOIN_RESPONSE_UP gene set are genes upregulated in NB4 cells (an acute promyelocytic leukemia cell line) in response to retinoic acid (Martens et al., 2010). The dysregualtion of this gene set is implicated in the development of acute promyelocytic leukemia, a subtype of acute myeloid leukemia. The mechanism of action is binding of PML and RARa, a translocation fusion protein, causing changes to H3 acetylation. The compound PML-RARa protein is a repressor various cell processes including differentiation, apoptosis, and self-renewal.

The MEISSNER_NPC_HCP_WITH_H3K4ME2_AND_H3K27ME3 gene set is a neural precursor cell line. Genes in this line have high density CpG promoters with a histone H3 dimethylation mark at K4 and a trimethyation mark at position K27 (Meissner et al., 2008).

2.2.1 EpiMod results

The EpiMod module in ChAMP identified one interactome hotspot associated with exposure centered on the seed gene *MEGF10*. Other genes in the pathway include: *TAOK2*, *BAHD1*, *TMEM132A*, *CUL7*, *ALMS1*, and *FBXW8*. Of the genes in this pathway *MEGF10* and *TMEM132A* are hypomethylated, and *CUL7* is hyper methylated.

MEGF10 encodes a protein that is part of the multiple epidermal growth factor-like domains family (Suzuki and Nakayama 2007). The Megf10 protein is involved in cell adhesion, motility and proliferation. It also is a mediator of apoptotic cell phagocytosis. A knockdown of Megf10 in mouse myoblasts in culture yielded reduced proliferation and migration of these cells (Saha et al., 2017). *TMEM132A* is highly expressed in the adult brain particularly in the cerebellum (Nagase et al., 2000). Several mutations in *CUL7* have been associated with 3M syndrome. This syndrome is characterized by growth retardation and facial dysmorphia (Huber et al., 2005).

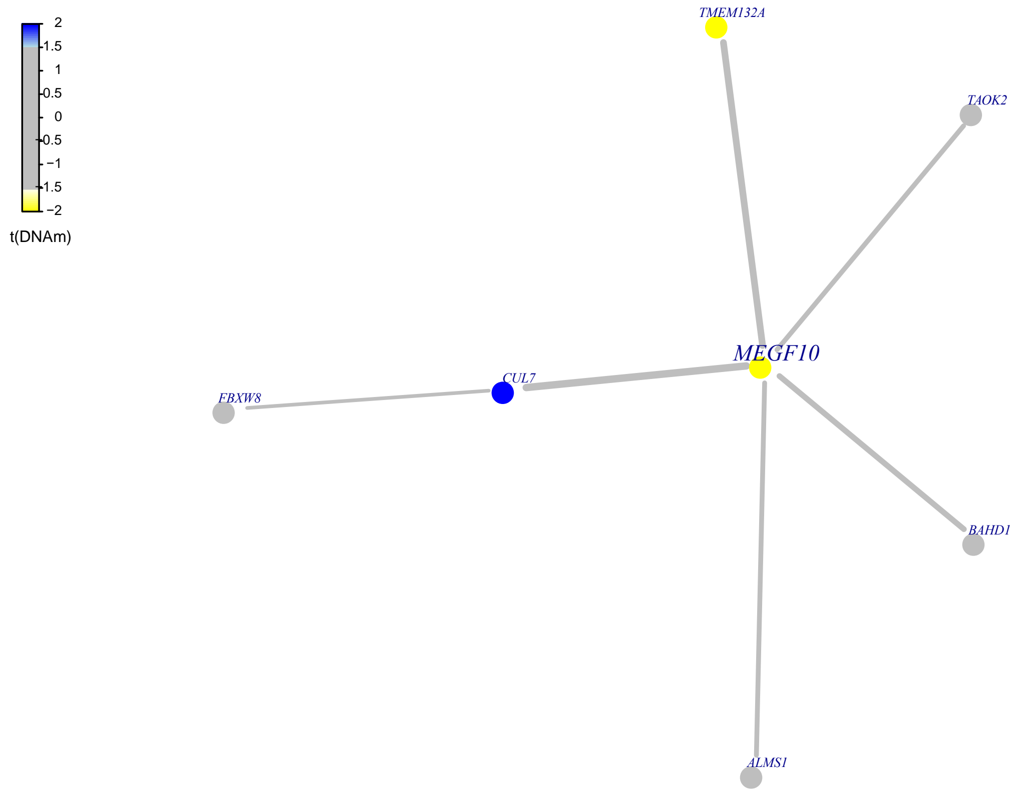

Figure 7: EpiMod for exposure groups.

2.3 Exposed vs non-exposed cleft groups (Gps 1 and 3)

2.3.1 DMPs

There were no significant (at the .05 FDR level) differentially methylated positions between the exposed groups with clefts (1) and the exposed groups without clefts (3). This may be a function of the limited sample size.

2.3.2 DMRs

Two significant DMRs were found at an area p –value cutoff of .025 with at least 10 associated significant probes. The most significant DMR (#1) was associated with *VTRNA1-2* gene, which was also significant in the broader exposure groups. The function is described above. While not directly involved in craniofacial development, this gene is involved in cell growth, which is core process in the formation of the primary and secondary palate. The other DMR (#5) is associated with the *RNF39* gene, also significant in the exposure groups with the function described above.

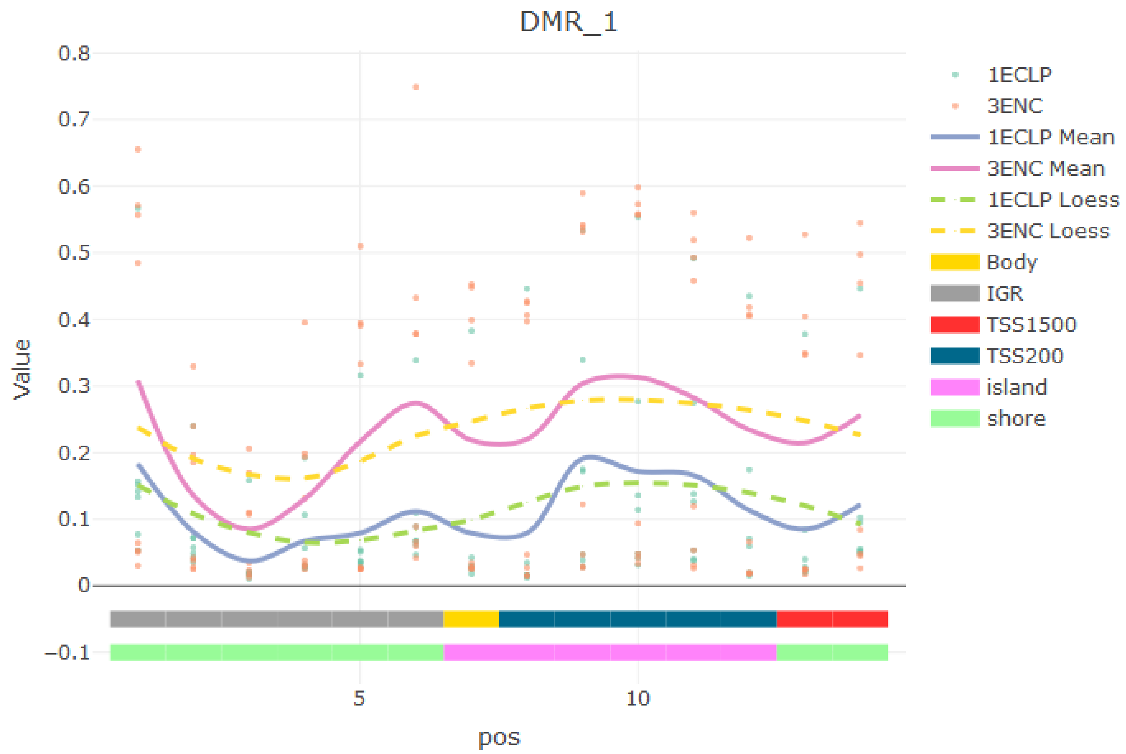

Figure 8: ChAMP Map of DMR 1 for exposed cleft groups with genome features. 1ECLP=Exposed with CLP, 3ENC=Exposed No CLP. Loess = local regression curves based on methylation means.

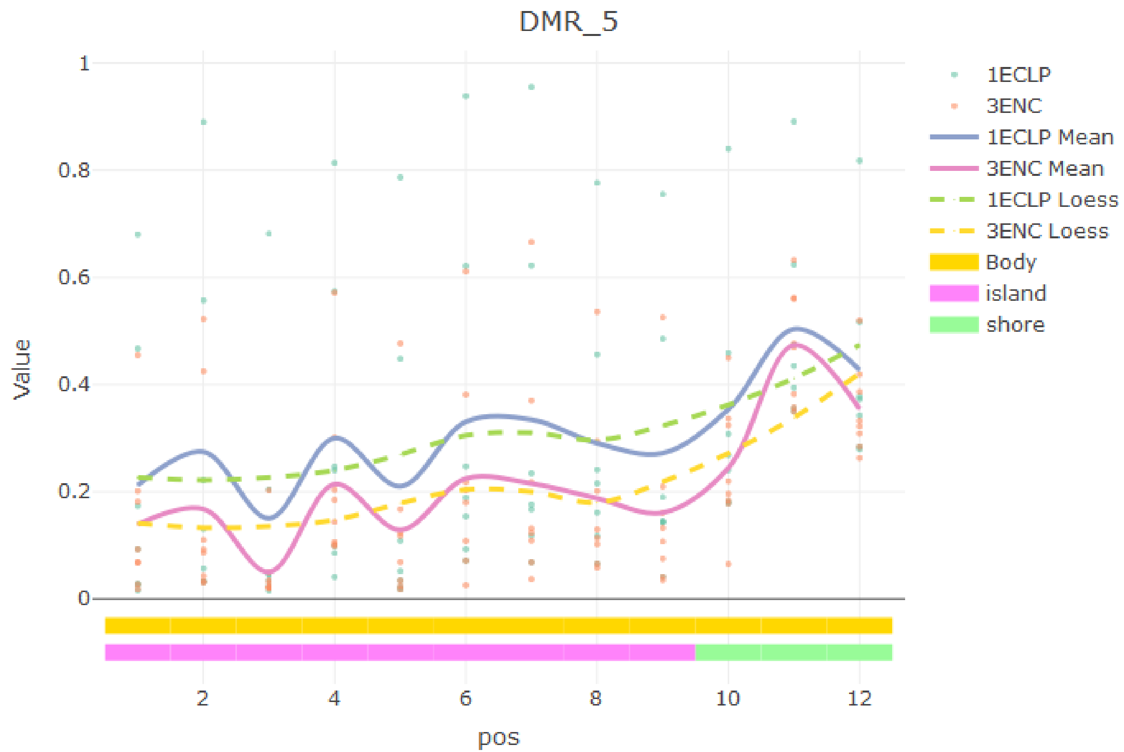

Figure 9: ChAMP Map of DMR 5 for exposed cleft groups with genome features. 1ECLP=Exposed with CLP, 3ENC=Exposed No CLP. Loess = local regression curves based on methylation means.

2.3.3 GSEA

The GSEA analysis on CLP exposure groups generated a list of 246 significant gene sets. Sorting by the Benjamini-Hotchberg adjusted p-value the top ten most significant pathways are described in table 4.

Table 5: Top 10 GSEA results for CLP exposure groups by Benjamini – Hotchberg adjusted p-value.

Pathway	# of genes	BH adj. p-value
MARTENS_BOUND_BY_PML_RARA_FUSION	**415**	**1.14E-11**
MODULE_45	**532**	**3.99E-09**
MODULE_60	**388**	**7.45E-08**
CHEN_METABOLIC_SYNDROM_NETWORK	**1083**	**1.76E-07**
BLALOCK_ALZHEIMERS_DISEASE_UP	**1546**	**1.31E-06**
MODULE_38	**424**	**1.31E-06**
MULLIGHAN_MLL_SIGNATURE_2_UP	**378**	**2.28E-06**
MULLIGHAN_MLL_SIGNATURE_1_UP	**340**	**2.82E-06**
RYTTCCTG_V$ETS2_B	**971**	**3.55E-06**
MODULE_84	**504**	**4.81E-06**

The top pathway is the MARTENS_BOUND_BY_PML_RARA_FUSION gene set (Martens et al., 2010). These genes have promoters that are occupied by the PML-RARA fusion protein. It has been suggested these genes have a role in histone deacetylase recruitment in genes involved in retinoic acid receptor (RAR) signaling. Excess levels of retinoic acid have been implicated in non-syndromic CLP (Cong et al., 2014).

MODULE_45 are whole blood genes. MODULE_60 are heart genes. The CHEN_METABOLIC_SYNDROM_NETWORK pathway is composed of genes implicated in general metabolic syndrome and obesity phenotypes (Chen et al., 2008). The BLALOCK_ALZHEIMERS_DISEASE_UP pathway is made up of genes upregulated in Alzheimer's disease. It includes genes involved in cell proliferation and differentiation, adhesion, apoptosis, and cell metabolism (Blalock et al., 2004). MODULE_38 are placenta genes. MULLIGHAN_MLL_SIGNATURE_2_UP and

MULLIGHAN_MLL_SIGNATURE_1_UP are genes that are upregulated in acute myeloid leukemia. The RYTTCCTG_V$ETS2_B pathway is uncatalogued. MODULE_84 are genes involved in the inflammatory response.

2.3.4 EpiMod results

There were no significant EpiMod results for this comparison.

2.4 Non-clefting groups (Gps 2 and 4)

There were no significant methylation differences between children without clefts whose mothers were exposed to the war (2) and children without clefts whose mothers were not exposed the war (4). This may be a function of small sample size.

2.5 CLP vs. non-CLP groups

In removing sites significantly associated with clefting from the exposure test, a list of 77 CpG sites associated with clefts in this Vietnamese population was generated (Table 4). The list was sorted using the same functional methodology as above. The top functionally significant probe was associated with the *ELANE* gene. Loss of function of the ELANE gene is associated with neutropenia (Donadieu, et al., 2017). The gene's main functions are host defense, local immunological response and tissue remodeling (Chua and Laurent, 2006). *DTX1* regulates cell fate during development and is part of the Notch

signaling pathway that has been implicated in palatogenesis (Lan et al., 2015). None of the top 10 genes have been associated with clefting in the literature and may represent novel associations may identify new loci to be explore for CLP.

Table 6: Top 10 probes significantly associated with clefts.

Probe ID	Average exp.	adj.P.Val	Cleft Avg.	Control Avg.	Chromosome	Gene	Feature	Cgi
cg06100973	0.425631605	0.004923682	0.552985014	0.306237785	19	*ELANE*	TSS200	shore
cg06745030	0.222635983	0.005139374	0.281582676	0.167373459	2	*B3GNT7*	TSS200	island
cg10283505	0.424819376	0.005438327	0.528432128	0.327682422	11	*FUT4*	1stExon	island
cg25467652	0.251274585	006112151	0.321073345	0.185838248	1	*AGTRAP*	TSS200	island
cg19697725	0.408946391	0.006284339	0.458448714	0.362537963	17	*SLC25A35*	TSS200	shore
cg06320982	0.105871992	0.006284339	0.132704761	0.080716271	16	*SLC38A7*	TSS200	shore
cg21842274	0.451508236	0.006643455	0.52352396	0.383993495	5	*CRHBP*	TSS200	shore
cg17718377	0.636160681	0.007484059	0.580521058	0.688322827	12	*DTX1*	TSS200	shore
cg15871206	0.705049662	0.007607122	0.75571786	0.657548226	2	*ESPNL*	1stExon	island
cg23433370	0.235345338	0.007739194	0.328141296	0.148349127	12	*STK38L*	TSS200	shore

2.6 Discussion

In this study we found multiple differentially methylated DMPs and DMRs in children associated with a mother's exposure to the stress of the Vietnam War. While some sample groups were likely too small to return results, this preliminary study lays the groundwork for a larger analysis.

Methylation status of two genes, *VTRNA2 (alias MIR886)* and *RNF39,* was significantly associated with both exposure and with clefts. *VTRNA2-1* is hypomethylated in both the exposed and exposed with clefts groups. Its function is the regulation of cell growth. This gene is classified as a metastable epiallele, which is methylated early in development, before the onset of gastrulation (Rakyan et al., 2002). Metastable epialleles have consistent methylation status in all tissues, while being highly variable between individuals. Methylation status of this gene has previously been associated with maternal nutritional status and stress (Dominguez-Salas et al., 2014, Silver et al., 2015). A recent study found that *VTRNA2-1* was hypomethylated in a population of Gambian children whose mothers experienced nutritional stress periconceptionally (Kessler et al., 2018). The authors link hypomethylation at this site with increased risk of some cancers as it acts as a tumor suppressor. In the current study *VTRNA2-1* was significantly hypomethylated in children whose mothers were exposed to war stress and in children whose mothers were exposed and have CL/P. The methylation status of the DMR at this site and its link to stress and clefts, may offer a mechanism that affects both the risk of clefting and the risk of cancer in those with a clefting phenotype.

The second gene, *RNF39,* which is significant in both the exposed and exposed with clefts groups, is associated with synaptic plasticity in rats (Matsuo et al., 2001). This is a meaningful association because clefts have also been associated with developmental abnormalities in the brain (Nopoulos et al., 2007). In humans certain SNPs in *RNF39* are significantly associated with allergic rhinitis (Morin et al., 2017).

Methylation status associated with three other genes was significantly associated with exposure only. These three genes are also involved in brain processes. *PPP3CC* encodes calcineurin, a neuron-enriched phosphatase. Certain polymorphisms in this gene have been associated with an increased risk of bipolar disorder (Mathieu et al., 2008) and schizophrenia (Gerber et al., 2003). *HS3ST2* is expressed highly in neurons and may be involved in the formation of tau plaques in Alzheimer's disease (Sepulveda-Diaz et al., 2015). *GRM2,* a glutamate receptor, is involved in synaptic regulation (Morishima et al., 2005). In mice, knockdowns of similar glutamate receptors increase susceptibility to addiction. These associations indicate that future studies of the epigenetics effects of stress should include a cognitive assessment in data collection.

Genes involved in craniofacial development are of particular interest for this study. One craniofacial gene, *LOXL3,* was significantly hypermethylated in the exposed groups. This gene is involved in the cross linking of fibrillar collagen types. This gene has been associated with cleft palate in mice (Zhang et al., 2015). Mice with heterozygous deletions of this gene have impaired palatal development and abnormalities in the vertebral column.

Genes involved in epigenetic processes were significantly associated with exposure in both the GSEA results and the single DMP probes. *SINA3*, which encodes a protein that forms a scaffold for histone deacetylase complexes, was hypermethylated in the exposed groups. *SINA3* is also involved in the response to hypoxia (Tiana et al., 2018). The

MARTENS_TRETINOIN_RESPONSE_UP gene set was significantly associated with exposure. This set of genes is responsive to retinoic acid (Martens et al., 2010). The mechanism of action is binding of PML and RARa, a translocation fusion protein, can cause changes to H3 acetylation. Both of these pathways could lead to system wide regulatory changes.

The Epimod analysis returned one protein interaction pathway significantly associated with exposure. It included the genes: *MEGF10* (seed gene) *TAOK2*, *BAHD1*, *TMEM132A*, *CUL7*, *ALMS1* and *FBXW8.* The genes in this cluster are involved in basic developmental processes, craniofacial, and brain development. This cluster may represent a mechanism for maternal stress that alters both mental function and craniofacial development in offspring.

As part of the methodology, DMPs significantly associated with clefting were removed from the exposure groups. This generated a list of genes that represent associations with clefting in this population. The most significant gene based on functional methodology was *ELANE,* which encodes a precursor to neutrophil elastase. This gene was also the seed gene of an Epimod pathway. This interaction network also includes *CSF3* and *CSF3R*, which regulate granulocyte function. The hypomethylation of these three genes in an immune pathway could represent an association between clefting and immune function in this population. The results for clefting also produced another protein interaction network with the seed gene *ELN*. *ELN* encodes a protein that forms the building blocks of elastin. This network also includes the following: *SPINK1*, involved in pancreatic function; *FCN1*, 2, and 3, in the lecithin pathway; *LYZ*, associated with renal disease; and *LTF*, involved in immune regulation.

For some group comparisons, statistically significant results were not obtained. This may be the result of the small sample size. Sample size in epigenetic studies is a fraught subject. The exact variance of methylation marks in populations is not yet well characterized.

A methylation odds ratio (methOR) (Rakyan et al., 2011) or 0.9649 was calculated based on the data in this study. According to Tsai and Bell (2015) this corresponds to an effect size of 3-5 percent. An a priori effect size of 10-15 percent, based on similarity of the magnitude of exposure to extreme stress to smoking (Joehanes et al., 2016), was used to calculate power for this study. This likely means this study is likely underpowered, but is still useful as a. exploratory data analysis. A total of 505 samples exist for this population, with approximately 100 in each of the 4 cohorts. This would put the power of this study between 90 and 100 percent (Tsai and Bell 2015).

Maternal stress caused by a war exposure can be said to affect several core biological systems: developmental processes, cell differentiation, proliferation, growth, adhesion and apoptosis, each of which were represented in the significant findings of this study. Additionally, genes involved in brain development were represented. Since clefting has been linked to abnormalities in the brain, differentially methylated genes involved in brain faction may represent altered pathways leading to the morphological changes seen in the brains of those with clefts.

2.6.1 Wild(ish) speculation

Extreme maternal stress of any kind may act through a pathway wherein offspring have methylation changes in many genes, especially those involved in cognition and brain development. These changes are then set as the result of changes to epigenetic regulation, particularly histone acetylation. Clefts may be only the visible manifestation of these changes. Brain abnormalities, increased susceptibility to schizophrenia, Alzheimer's disease and addiction may all be part of the cluster of conditions associated with extreme maternal stress. To better understand these myriad effects that affect those with clefting via maternal stress exposure, a broader data collection effort is needed.

3.0 Summary

The significant results obtained at the FDR .05 level confirm that overall methylation patterns in children born to mothers who were exposed to war stress and children born to mothers who were not exposed to war stress are different. The affected genes represent an array of core biological functions from cell growth and proliferation to neurological and craniofacial development. The results for groups 1(exposed with clefts) and 3(exposed without clefts) are less conclusive, likely because of the small sample size. Additionally, the results for group two (not exposed with clefts) vs. group four (not exposed, no clefts) were inconclusive for the same reason. One of the affected DMRs is associated with a gene, *VTRNA1-2* that is involved in the general process of cell growth. The results for the clefts vs. non-cleft groups may have identified novel loci that are associated clefts in this population.

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