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RESEARCH ARTICLE

Epigenetic Changes Associated with Early Life Experiences: Saliva, A Bio-specimen for DNA Methylation Signatures

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Abstract: ***Background*:** Adverse Childhood Experiences (ACEs), which include traumatic injury, are associated with poor health outcomes in later life, yet the biological mechanisms mediating this association are unknown. Neurocircuitry, immune system and hormone regulation differ from normal in adults reporting ACEs. These systems could be affected by epigenetic changes, including methylation of cytosine (5mC) in genomic DNA, activated by ACEs. Since 5mC levels influence gene expression and can be long-lasting, altered 5mC status at specific sites or throughout the genome is hypothesized to influence mental and physical outcomes after ACE(s). Human and animal studies support this, with animal models allowing experiments for attributing causality. Here we provide a lengthy introduction and background on 5mC and the impact of early life adversity.

***Objective*:** Next we address the issue of a mixture of cell types in saliva, the most accessible bio-specimen for 5mC analysis. Typical human bio-specimens for 5mC analysis include saliva or buccal swabs, whole blood or types of blood cells, tumors and post-mortem brain. In children saliva is the most accessible biospecimen, but contains a mixture of keratinocytes and white blood cells, as do buccal swabs. Even in saliva from the same individual at different time points, cell composition may differ widely. Similar issues affect analysis in blood, where nucleated cells represent a wide array of white blood cell types. Unless variations in ratios of these cells between each sample are included in the analysis, results can be unreliable.

***Methods*:** Several different biochemical assays are available to test for site-specific methylation levels genome-wide, each producing different information, with high-density arrays being the easiest to use, and bisulfite whole genome sequencing the most comprehensive. We compare results from different assays and use high-throughput computational processing to deconvolve cell composition in saliva samples.

***Results*:** Here we present examples demonstrating the critical importance of determining the relative contribution of blood cells *versus* keratinocytes to the 5mC profile found in saliva. We further describe a strategy to perform a reference-based computational correction for cell composition, and therefore to identify differential methylation patterns due to experience, or for the diagnosis of phenotypes that correlate between traits, such as hormone levels, trauma status and various mental health outcomes.

***Conclusion*:** Specific sites that respond to adversity with altered methylation levels in either blood cells, keratinocytes or both can be identified by this rigorous approach, which will then be useful as diagnostic biomarkers and therapeutic targets.

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1. INTRODUCTION

The Adverse Childhood Experience study (ACE) demonstrated that many types of childhood adverse experiences correlate with a multitude of chronic diseases in the adult leading to early death [1-4] (Fig. **1**). ACEs include various forms of child abuse and household dysfunction, including intentional physical harm. Another form of negative early life experience is accidental traumatic injury. Traumatic injury in children is common, the leading cause of death, and accounts for 34% to 39% of all deaths in children ages 1 to 14 years [5]. Like ACE, survivors of accidental trauma can have dramatically altered mental and physical health throughout the lifespan [6]. Thus, abuse-inflicted trauma has particularly egregious effects, but injury regardless of intention may also lead to many types of mental disorders, such as Post-Traumatic Stress Disorder (PTSD) during childhood [6, 7], or increased risk of PTSD as an adult [8, 9].

The biological basis for the long-term consequences of ACEs and traumatic injury is unknown, yet it seems logical to propose adverse experiences activate a biological program that plays out over the lifespan [10], such as the epigenetic

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program, one component of which is DNA methylation. As proposed by Bruce McEwen in his "allostatic load" model [11], physiological systems likely to be impacted by early trauma are: brain circuitry, hormonal balance, and immune system (Fig. **2**). Biomarkers have been sought for each of the systems involved [12], yet the fundamental underlying mechanism for trauma's impact is unknown. In this paper, we will focus on one specific epigenetic event, methylation of cytosine (5mC), or DNA methylation, in the genome.

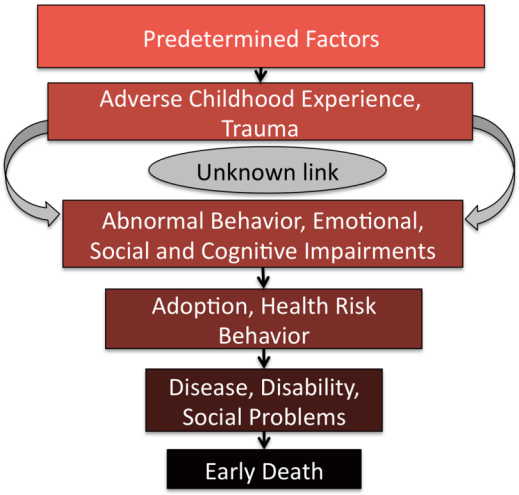


Fig. (1). The ACE pyramid. This diagram shows relationships between Adverse Childhood Experience (ACE) and diseases, discovered by Felitti *et al.* in their landmark 1998 paper [3], modified in Bearer *et al.* [10] and further re-designed here. These associations have been confirmed by 100s of subsequent studies. More information is posted on the website for the U.S. Centers for Disease Control (https://www.cdc.gov/violenceprevention/acestudy/). The biological basis by which ACE increases risk for disease across the lifespan is unknown (indicated by unknown link and gray arrows), but epigenetic events, such as changes in DNA methylation levels of specific genes involved in immunity, hormone regulation and neurosystem development, are expected to occur.

During childhood, brain circuitry, hormonal balance and the immune system are still developing. This development involves the unfurling of a discrete program of epigenetic events, a fundamental element of which is DNA methylation. "Epigenetic" refers to chemical modifications of the genome that alter gene expression without changing the DNA sequence. This chemistry includes histone modifications, chromatin packaging alterations, transcription factor activation and repression, microRNA dynamics and DNA methylation levels. Here we focus on DNA methylation as it is proximal to the other epigenetic processes and is among the most accessible of the epigenetic events responsive to experience which influence gene expression [13, 14]. DNA methylation involves the enzymatic addition or removal of a covalently bound methyl group on the 5 position of the pyrimidine ring in cytosine within the genomic DNA sequence (Fig. **3**; described in more detail below).

This epigenetic program occurs in all systems, with a common event being enzymatic methylation and demethylation of cytosines in nuclear DNA. Methylation is most dynamic during development, and changes may endure throughout the lifespan [15]. Dynamics during childhood makes DNA methylation particularly vulnerable to abnormal childhood experiences, such as ACEs or traumatic injury, with potentially different outcomes depending on the developmental stage of the epigenetic program at the time of insult. Thus, such experience could interfere with the normal epigenetic program, and this interference may underlie the lifelong impact of traumatic experience in childhood.

In 2004 we proposed that a complex interplay between experience and genetics created a dynamical system: experience would trigger altered gene expression, which then affects neurocircuitry formation, which then reciprocally influences both gene expression and behavior [16-18]. These ideas were inspired by work of Stephen Suomi [19, 20] and of Gilbert Gottlieb, reviewed in Bearer 2010 [21]. In the early 21st century, exploration of these ideas was empowered by the emerging sequence of the complete human genome,

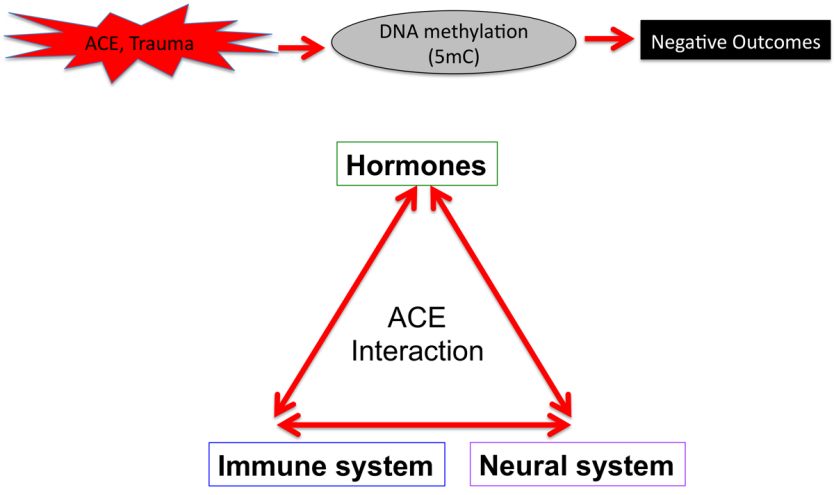


Fig. (2). Diagram of biological system interconnectivity. Adverse childhood experience including physical injury (trauma) are hypothesized to alter DNA methylation and lead to negative outcomes. Biological systems most likely to be impacted by trauma include hormonal regulation and the immune and neural systems, all of which are inter-related and affect each other; and all of which are developing in early childhood.

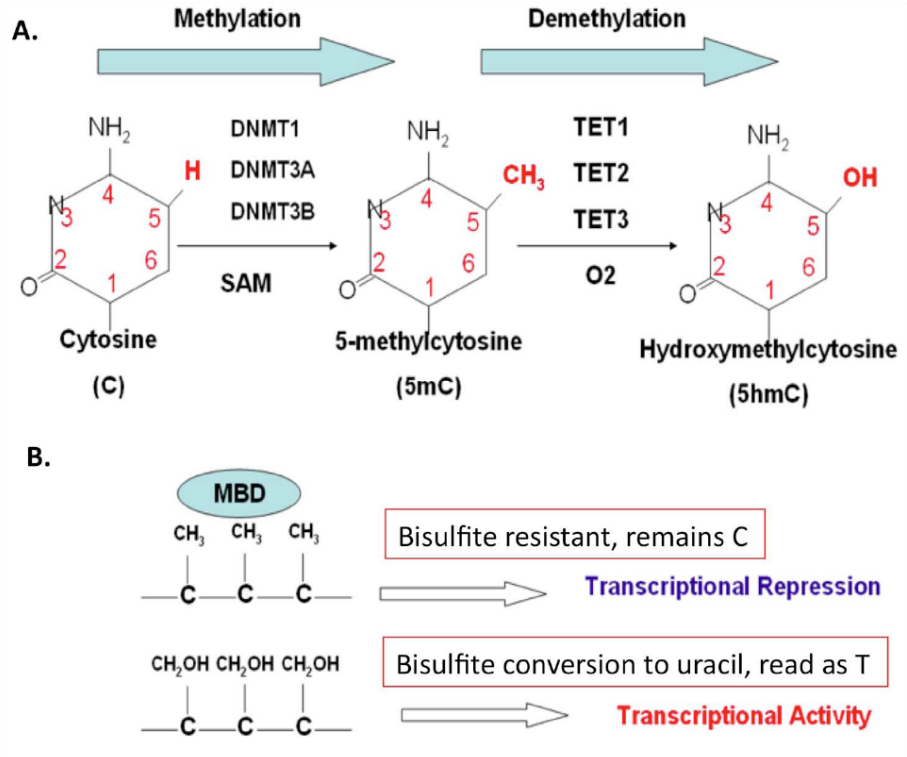


Fig. (3). Biochemical basis of DNA Methylation. DNA Methylation is one of the biochemical events that are changed by experience and affect gene expression. **A.** Methyl groups are added to the 5' position on cytosine in DNA from a methyl donor (*i.e.* SAM) by at least three enzymes, DNMT 1, 2 and 3. Removal of methyl groups is initiated by oxidation mediated by any of the TET enzymes converting the 5' methyl cytosine to 5' hydroxyl-cytosine. The hydroxyl-cytosine is subsequently removed by other enzymes. Some investigators argue that additional enzymes may initiate the removal process, or that removal may involve excision of the methylated cytosine from the DNA strand and replacement with an un-methylated cytosine *via* DNA repair enzymes. Importantly, methylation for some sites appears to be dynamic. This dynamic reversibility makes DNA methylation sites promising biomarkers of experience, and as targets for theoretical interventions. **B**. Methylation in promoter and enhancer regions influences gene expression by recruiting methyl-binding proteins that may interfere with transcription. Methyl-binding proteins may also recruit histones and lead to other mechanisms of transcriptional repression, including chromatin packaging. Methyl-binding domains from these proteins are useful in isolating methylated DNA from a mixture of DNA fragments useful for reduced representation whole genome methylation analysis.

its large amount of un-transcribed regions, and new information about how gene expression is regulated by epigenetic events [22-24]. Another technical advance in the last 20 years is the ability to image activity and track circuitry in the living brain with magnetic resonance imaging (MRI) in human, non-human primates and rodents. We demonstrated structural alterations of neuro-circuitry in the mesocortical limbic system in adult mice after life-long deregulation of the monoamine systems (serotonergic, dopaminergic and noradrenergic) through genetic manipulation of their reuptake transporters, targets of cocaine and of drugs for depression, such as serotonin reuptake inhibitors [25-27]. These circuits and neuronal activity within the limbic system altered by early life stress in mouse experimental systems (Barto and Bearer *et al.,* MS in preparation). In human, alterations in limbic system activity and volume of brain regions have been found in adults who report abuse in childhood, reviewed in Hart [28]. Correlations between early life stress, brain activity patterns and epigenetic changes are an exciting frontier.

Studies in humans of the biological impact of childhood adversity are complicated by three major considerations: First, different types of adversity, such as neglect, abuse and trauma, are not well defined. As an example, for childhood maltreatment/abuse, there are at least 5 different types, although the more abused children often suffer from more than one type [29]. Sexual abuse probably activates a different set of biological events than neglect [30, 31], the age of the child, the child's relationship to the person inflicting harm, the timing and chronicity of abuse all influence outcomes in complicated ways, but many of these types of influence are difficult to document. Pediatric traumatic injury may be accidental or non-accidental (NAT) yet produce similar types of injuries, and conversely, different injuries may activate different processes thereby producing different outcomes. Abuse may be chronic, with multiple events occurring repeatedly over time, and thus resulting in a complex outcome dissimilar from a one-time accidental injury. ACEs include domestic violence and criminality, not often considered in the list of types of child abuse. Witnessing traumatic events or experiencing natural disasters is also traumatic. If there are common biological pathways leading to responses to trauma regardless of type of insult is unknown. Mitigating influences on the degree of a child's response to trauma, including natural resiliency or context of the event, are just beginning to be defined, and often not included in reports of traumatic events.

Second, the timing of the trauma probably influences the type and extent of the biological impact, yet often these are poorly reported and frequently rely on self-reports and anecdotal evidence rather than objective measures obtained at the time of trauma. We need better recording of traumatic events and biological measures at the time of injury.

Third, we still know very little about the time course of biological responses, *i.e.* how soon after the event(s) do they occur, how long do they last, do they resolve to the pre-existing state or evolve to some other state. Little is known either about the impact of trauma on the methylome in the acute phase, or about the dynamics of 5mC status during recovery.

2. BACKGROUND

2.1. Evidence that 5mC is Altered by Early Life Experience

In this section, we will not attempt to provide a comprehensive literature review, but rather focus on a subset of studies that serve to exemplify the questions being addressed and ideas for next steps. Please refer to Table **S1** in Supplemental Materials for a list of examples of human and animal studies that report 5mC changes with experience. Until recently, studies performed with saliva as source have not been corrected for cell composition and thus may be unreliable [32]. In some cases, 5mC patterns identified in saliva were also found in separate, validation experiments performed with blood, and further confirmed in brain samples from a new cohort [33, 34], and thus these results are more likely to report reliable information. Altered methylation is expected to affect gene function based on many other studies [35]. While methylated genes are expected to be "turned off" and un-methylated to be expressed, this may not always be the case. In a few studies, correlation of methylated sites with RNA expression has been examined, and the degree of methylation in mammals does not consistently correlate with expression levels [36]. Rigorous verification of the effect of methylation of specific sites reportedly affected by early life adversity is lacking. Such verification would include cell culture experiments with methylated and un-methylated expression vectors, as has been done for methylation sites correlating with diabetes metabolic memory [37].

With these caveats in mind, much evidence supports the notion that child maltreatment alters 5mC profiles in children [38-42]. Early studies focused on individual candidate genes, while the recent explosion of NexGen sequencing and high-density arrays is now allowing Epigenome-Wide Association Studies (EWAS) of 5mC sites throughout the genome. An initial focus was on promoters for genes involved in stress responses, particularly the glucocorticoid system and the hypothalamic-adrenal-pituitary axis (the glucocorticoid receptor (GR/NR3C1), the glucocorticoid releasing factor/hormone (CRF/CRH), and FKBP5, the FK506 binding protein 5, implicated in feedback regulation of GR with mutations associated with PTSD and other mental health outcomes after stress, including childhood trauma) and the serotonergic system (serotonin transporter: SERT /5HTT), since stress is thought to be a major factor in outcomes of ACE.

A "conserved transcriptional response to adversity" in circulating leukocytes has been identified [43], which is likely to be regulated by 5mC. Methylation of the promoter for cortisol releasing factor/hormone (CRF/CRH) alters the expression of this peptide hormone and influences circulating cortisol levels-and thereby all of its downstream effects, including immune suppression and cognitive functioning in rodent models [44-46]. Increased methylation of the GR/NR3C1 gene is associated with child maltreatment [41, 47-49] and with suicide victims with a history of child abuse [50].

Variations in mental health outcomes after adversity may in part be mediated by individual-specific single nucleotide polymorphisms in the genome, which may occur as far as 5 genes away from the methylation site [51]. Such effect has been found for the glucocorticoid response elements and the FKBP5 gene. In children from the Bucharest Early Intervention Project who were institutionalized at a very early age, buccal cells collected at age 12 displayed altered methylation of the serotonin transporter gene (*5htt/sert/slc6a4*) and *Fkpb5* [52]. In a recent report of 5mC tested in saliva from maltreated children, altered levels of methylation were found in a large number of sites with significant p-values [38]. Among these were 5 sites in the first exon of *Aldh2* (an enzyme that protects against oxidative stress), *Ankk1* (a kinase associated with the dopamine system and mutated in some cases of schizophrenia) and glucocorticoid receptor gene, *Nr3c1*. Abnormal DNA methylation in the serotonin transporter gene, *5htt/sert/slc6a4*, has been associated with sexual abuse [31], and this could influence the brain as well as the GI tract, coagulation, the immune system and the brain. Another recent study suggests that some aspects of early life adversity, including 5mC levels in *Fkbp4, Fkpb5, Nr3c1 and Nr3c2*, may improve stress management in the adult [53], an idea reminiscent of Homberg and Lesch's proposal that the long or short promoter alleles in the serotonin transporter may confer resilience [54]. This finding suggests that methylation patterns may contain clues to resilience.

Socio-Economic Status (SES), which is suspected of producing high rates of stress in children [55, 56], has been controversially linked to altered 5mC patterns in whole blood [57, 58], although in many of the early studies using high-throughput-whole genome epigenetic (Epigenome-Wide Association Study) (EWAS) analysis the importance of accounting for cell composition of the biospecimen is not always recognized [32]. These studies showed altered transcriptional activity for cortisol-related and inflammatory genes in low *versus* high SES groups [55] and some of these changes appear to be correlated with methylation of a Glucocorticoid Response Element (GRE) in the gene for *Kitlg* [33]. These changes may underlie the findings of altered immunity in abused children where various serologic markers of immune activation, such a C-reactive protein [59], and depressed immune reactivity [60-64] are reported.

Finally, incarcerated male adults reporting early life adversity have a consistent pattern of changes in methylation levels in EWAS studies [39]. Internationally adopted adolescents, who were adopted as young children from conditions of poverty and deprivation, demonstrate altered 5mC profiles in 30 of 413,000 CpG sites as compared to adolescents raised in affluent American families [42].

Early life trauma heightens the risk for PTSD in adulthood, thus methylation changes identified in adults with

PTSD may have occurred in childhood, although evidence for this remains to be found. For example changes in expression of the pituitary adenyl cyclase-activating peptide, PACP [65, 66] found in adults with PTSD could be mediated by 5mC alterations, possibly stimulated by corticoid response elements in response to cortisol levels [33]. Such changes may begin in childhood. An example of increased susceptibility to PTSD from a single nucleotide polymorphism (SNP), rs717947, was found to associate with the gold-standard diagnostic measure for PTSD in combat veterans, and with a methylation quantitative trait locus (meQTL) in female subjects from the Grady Trauma Project [67]. It is expected that such SNP-conferred susceptibility would also be found in children but has yet to be sought.

Retrospective reports of adversity have been important in the study of DNA methylation. However, the time between the event and the study leaves several questions to be answered: How do early life events correlate with adult 5mC patterns? Is the impact direct or indirect, do changes evolve over time, and what is the role of resiliency and 5mC in those who report adversity and have normal (non-ACE) 5mC patterns?

Factors that could influence results from these studies include

- baseline methylation pattern of the child before the injury/event(s)

- type of biospecimen used and the cellular composition of each sample (saliva, blood, brain, other tissue)

- type of methodology used to assay for methylation levels (whole genome bisulfite sequencing, (RRBS) representation sequencing, methylated DNA immunoprecipitation (aka, methyl-binding pull-down (MeDIP) sequencing), or high density arrays, such as the Illumina BeadChip arrays, *etc*.

- age of the child when events occurred and the normal biological timing of 5mC changes during childhood

- temporal relationship between the traumatic event and sample collection

Currently, the 5mC baseline for “normal” in any given child is unknown, making detection of a change after trauma difficult. This unknown baseline may be influenced by prenatal experience, the methylation patterns in the parents, and the age of the child. Studies of the Dutch famine, which occurred towards the end of World War II, reported that individuals who were prenatally exposed to famine during the Dutch Hunger Winter in 1944-45 had, 6 decades later, less DNA methylation of the imprinted *Igf2* gene compared with their unexposed, same-sex siblings [68]. Other examples of prenatal impact on methylation levels include the finding that maternal stress links to abnormal infant responses to acute stress [69], possibly mediated in part by altered methylation of the glucocorticoid receptor gene in the newborn, which is modified in babies of mothers with depression [70]. Moreover, some evidence suggests that there may be trans-generational transmission of methylation levels either directly or indirectly through microRNA inheritance from both sperm and egg [71]. Intergenerational transmission of violence is recognized [72] and attributed to the behavior of parents, but could also be transmitted through inherited epigenetic processes, or acquired during intra-uterine development [73]. Finally, methylation patterns in the brain dramatically change during normal childhood [74], and continue to change across the lifespan. An epigenetic "clock" of aging based on evolving methylation profiles has been identified that is independent of experience, ethnicity, or sex [15]. Most exciting, changes in global methylation of regulatory regions for the DNA methyltransferase (*Dnmt*) genes, whose protein products add methyl groups to DNA, correlate with maternal care in the rat hippocampus [75], suggesting that early life experience affects the very machinery of developmental methylation. Expression of these genes appears to be highly regulated during development, both pre- and postnatal [76]. Thus early life stress may impact the normal "clock" and alter the development of immune, hormonal, and neural systems globally.

2.2. DNA Methylation as a Biomarker

In this review, we are focusing on altered 5mC patterns as a major indicator and mediator of the impact of early life trauma on the lifespan. In this section, more detailed information on the biochemical process of methylation is provided.

Covalent modification of DNA by addition of methyl groups was initially recognized in X-chromosome inactivation [77, 78]. The power of DNA methylation analysis as a method to detect gene expression is now being realized for cancer diagnosis and treatment [79, 80], and technology is being developed for that application that can be re-directed for studies of other disorders where epigenetic dynamics are suspected, such as brain development and the impact of early life experience [81]. DNA methylation in promoter and enhancer regions of genes regulates expression of their gene products (Fig. **3**) [35], although some methylated sites may have other functions [82]. Methylated promoters are often repressed and may be activated by de-methylation [83]. Hence, alterations in DNA methylation levels are expected to influence expression levels of proteins or other gene products. Enzymes involved in methylating and demethylating DNA include the DNMTs and the Ten-eleven translocation methylcytosine dioxygenases (TETs) [84-86]. The TET enzymes convert the methyl to a hydroxyl group, and additional enzymes are needed to complete the process of demethylation once the methyl group is converted. These additional factors include Gadd45 [87] among others. Other mechanisms for 5mC removal include excision-repair processes by which the methylated nucleotide may be removed and replaced.

How these enzymes are activated and whether that activity is selective for specific genes or global across the genome are areas of intense investigation. One proposal is that breaks in DNA trigger activation of the TET enzymes, possibly through Gadd45 [88] and others suggest that an Activation-Induced Deaminase (AID) is responsible for gene-specific rather than whole genome demethylation [89]. In the brain, a neuronal activity-induced DNA demethylase in the dentate gyrus of the adult mouse hippocampus may represent TET1 activity [88, 90]. Studies in the *Tet1*-knockout mouse have shown that TET1 is critical for neuronal activity-regulated gene expression and memory extinction [91], that hydroxylation of 5mC by TET1 promotes active DNA demethylation

[90], and epigenetic priming of memory updating during reconsolidation attenuates remote fear memories [92]. Although these studies were performed in the adult, they suggest that 5mC changes in early life would promote adult vulnerability. Indeed evidence for a two-hit model for impact of early life stress on the epigenome has been found in a mouse experimental system for depression [93]. In this case, early life stress was induced by limited bedding in the post-natal period, which increased susceptibility to depressive behaviour after adult social defeat stress *via* long-lasting transcriptional programming mediated by *Otx2* [93].

DNA methylation/demethylation regulates neuronal arborization during post-natal development, as shown in the *Dnmt3b* knockout mouse [94]. In the absence of DNMT3b the normal stochastic expression of the protocadherin gene cluster, that regulates single neuron dendritic diversity in the cerebellum, is lacking. This loss results in abnormal dendritic trees in Purkinje cells [94]. Intriguingly, alternative splicing of the protocadherin cluster may also regulate axonal tiling and global arborizations of the neuromodulatory serotonergic and noradrenergic systems that develop in early life. Conditional deletion of a domain in mouse protocadherin α (*Pcdhα*) gene cluster disrupts normal distribution of these systems in the hippocampus and ventral pallidum [95]. This coincides with changes in methylation of the protocadherin gene cluster in humans reporting early life adversity [96]. *Dnmt* promoter methylation and DNMT expression are altered in response to maternal care in the hippocampus of rats [75]. Taken together these observations suggest that if early life stress alters 5mC methylation, it could interfere with the development of the normal anatomy of neuromodulatory systems, impacting emotional experience and response.

Critical to our ability to understand a DNA methylation (5mC) response to trauma is to determine when methylation changes occur in relation to the traumatic event, whether persistence leads to future pathology, whether any sites are unique to the type of trauma, and whether alterations persist or resolve to normal levels. These questions may best be resolved in animal experimental systems, where many of the variables that remain either unknown quantities or are uncontrollable confounders in human studies can be experimentally determined.

2.3. Animal Experimental Systems Provide Cause-effect Evidence for Childhood Adversity and Alterations in 5mC Levels

Primate studies pioneered the relationships between environment, genes and behavior with the work of Stephen Suomi, who applied Harry Harlow's maternal deprivation protocol in rhesus monkeys to show an association between outcomes and allelic variation in the serotonin transporter gene [19, 97-100]. Even though primate research is under increased scrutiny, some studies are still being done. In particular now that the genomes have been reported for the vervet [101] and the marmoset (https://www.hgsc.bcm.edu/non-human-primates/marmoset-genome-project) behavioral studies are on-going and will yield new insights into gene x environment interactions that may be more relevant to the human condition than current rodent systems [102, 103].

Several types of experimental systems that test for the impact of early life stress in rodents have emerged [104], a few of which are naturalistic [105] while others are dramatic and abnormal. Perhaps the most naturalistic is the spontaneous behavior of mother rats who naturally, without experimental manipulation, have either a low or high degree of maternal licking and grooming [106]. Pups of mothers with low grooming have lower cortisol levels and altered methylation of the promoter in the gene encoding CRF/CRH compared to high-groomed pups [44, 49, 50]. Other rodent protocols have been developed to replicate maternal neglect by depriving the dam of adequate bedding [107]. These offspring display elevated cortisol as adults and altered transcriptional activity for the gene encoding CRF/CRH in the hypothalamus [107, 108]. These mice also show disrupted hippocampal microstructure by diffusion-weighted Magnetic Resonance Imaging (MRI) post-mortem [109].

Since ACE is a risk factor for vulnerability to PTSD, a number of studies have been done in animals towards discovering the biological basis of that association. Rodent models of depression and anxiety with good face-, construct, and predictive validity when compared to the human condition include: Two rat lines (Finders Sensitive (FSL) and High Anxiety-Like Behavior (HAB) lines); two transgenic models (*5-Htt* knockout in mouse and rat), and the experimentally provoked PTSD-like response induced by exposure to predator odor [110]. The *5-Htt* (*Sert*) knockout mouse has been found to have altered limbic system circuitry in adolescence in imaging studies using manganese-enhanced MRI [25]. Circuits from the prefrontal cortex into the deeper limbic system are also altered in mice with life-long disruption of dopamine or noradrenergic systems [26, 27]. New work shows that these systems are also altered by exposure to innate fear (predator odor) and by Early Life Stress (ELS), and that this alteration is greater in *Sert* knockout mice than in wild type (Barto, Bearer and Jacobs, MS in progress). Changes in circuitry of ELS-exposed mice replicate areas of altered neural activity induced by predator odor or by *Sert* knockout.

In rodents it is difficult to develop naturalistic experiments that mimic all types of human child abuse, which include emotional abuse and neglect, as well as physical or sexual abuse. Perhaps the most successful are systems for neglect and for physical abuse. Methods to mimic maternal neglect in rodents include fragmented care systems, in which insufficient bedding is provided to the dam during nursing [107, 108] and other types of maternal deprivation as described above. For physical harm, the adoptive dam model may prove the most promising model to harm by a parent on the child [111-114]. When rodent dams are given a pup from a mother of a different strain, the dam turns physically abusive to the adopted pup [112, 115, 116]. These manipulations alter 5mC levels in the promoter of the *Bdnf* gene [111, 113, 117, 118] that encodes the Brain-Derived Neurotrophic Factor (BDNF). Site-specific decrease in methylation in the exon IV of *Bdnf* correlates with prenatal exposure to predator odor, which presumably activated a fear response in the pregnant dam.

Life-long impacts of ACE on the rodent brain include altered anatomy of limbic system circuitry. These anatomical changes could be a consequence of fear-activated neuronal activity, which could abnormally stimulate neuronal cell

migrations, axonal sprouting and/or synaptic strength during brain development in young children. Any or all of these consequences could collude to alter the neuronal cell methylome, thereby changing neuronal expression patterns and perpetuating circuitry abnormalities. Iteratively, these changes could beget one another in a vicious cycle.

Some evidence exists to support this idea of a vicious cycle. Notably mice carrying knockout of *Tet1*, one of the three hydroxylation enzymes, do not have changed expression levels of other gene products after fear conditioning as do wild-type mice [91], suggesting that stress-activated expression changes are mediated in part by the DNA methylation machinery; and in the case of hormonal regulation, altered cortisol levels [29, 119-121], or other neural growth factors, such as BDNF, correlate with maltreatment [111]. For the hippocampus, the cold swim test in rodents induces alterations of *c-Fos*, *Per1* and *Sgk1* genes [122], which may be dependent on adrenalin rather than cortisol [123], and would influence neural activity, circuitry and secondarily behavior.

Animal experimental systems suffice for testing causal impact of some but not all experience-driven epigenetic events in human. Hence investigators must be prudent to choose the system carefully and compare results to human earlier rather than later. Because of the high evolutionary conservation of the limbic and monoaminergic systems, both at the molecular and structural levels, rodents may best be deployed to study conserved molecular mechanisms and anatomy rather than behavioral responses and outcomes, which are highly variable even in genetically identical mice, and differ greatly between rodents and humans.

2.4. Methods to Assay for DNA Methylation

DNA methylation is perhaps the most biochemically accessible epigenetic event. Methylation is thought to influence transcriptional activity and chromosome packaging through recruitment of methyl-binding proteins. Methylation is quite stable *in vitro* and can be identified either by methyl-pull-downs (MeDIP), by bisulfite conversion followed by DNA sequencing or by high-density array hybridization as in the Illumina BeadChips. MeDIP takes advantage of methyl-binding proteins to pull-down methylated DNA fragments. A new method, recently reported, using targeted selection of highly differentially methylated sites across 25 different cell types shows promise [124] and could potentially be deployed to identify methylation dynamics in pre-clinical animal systems for which no array is available. These various assay methods can produce widely differing results, as will be discussed below.

The most common methylated nucleotide is cytosine, and cytosine followed by a guanine (CpG) is thought to be even more commonly methylated, although CA, CC and CT may also be methylated [74]. Conversion of the methyl group to a hydroxyl also occurs and may persist, and other nucleotides may also be methylated/hydroxylated. The human genome is 47% CG, and 60-80% of these are methylated in mammals. Early work focused on analysis of methylated promoter regions for candidate genes in genomic DNA, primarily using bisulfite conversion followed by Polymerase-Chain Reaction (PCR) amplification and sequencing of the products [125]. Bisulfite treatment converts un-methylated cytosines in the DNA to uracil but leaves methylcytosine unconverted (Fig. **3**). During polymerase-chain reaction amplification (PCR) the uracil is read as thymine (T), thus altering the sequence of the DNA. Hence when sequenced after bisulfite conversion, methylated cytosines are preserved as C, and un-methylated are converted to T. Pyrosequencing, while useful for short DNA segments (<200bp) of known sequences for clinical tests, is too intensive for efficient analysis of whole genome methylation levels [10]. One caveat to the bisulfite approach is the rare event of the C being already mutated to a T at particular sites. In this case, the "T" in the sequence would not evidence a methylated C. Thus, sequencing of the DNA without bisulfite treatment is necessary.

Since genomic DNA samples include material from a mixture of diploid human cells, both methylated and un-methylated sites are present in resulting sequences. Saliva, buccal swabs, blood and post-mortem brain are all useful biospecimens for DNA extraction and all contain varying mixtures and differing ratios of cell types. Saliva is emerging as an especially accessible biospecimen from living children [126], but contains white blood cells as well as buccal keratinocytes in different proportions depending on the child's oral physiology at the time of collection. Microbes give little to no signal when alignments or probes are human-specific. However, as will be shown below, accounting for the relative contributions to the methylation pattern of keratinocytes *versus* that of blood cells is critical in the analysis [32]. While a reference-free cell composition deconvolution algorithm is reported and incorporated into analysis software [127, 128], depending on the size and variability of the dataset, the algorithm may inaccurately estimate the relative components of the samples. As will be shown below, the emerging wealth of the Genome Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) databank provides cell-specific 5mC measurements, which may be used for a reference-based approach. Care must be taken to use data from the same detection approach (bisulfite sequencing, Illumina BeadChip, *etc*.) when using GEO reference data.

Methylation levels are typically reported as percent methylated, *i.e.* what proportion of the DNA at each site was C (methylated) *versus* T (un-methylated) in bisulfite sequencing. A serious drawback for whole genome methylation analysis by bisulfite sequencing is that the converted DNA sequence is challenging to align with standard genomic sequence databases, especially as it contains a mixture of sequences, and the number of potential sites is enormous. Often the DNA fragments obtained (75-200bp) are long enough to have multiple methylation sites, all with partially converted sequences. Similarly, methyl-binding pull-downs result in enrichment for heavily methylated sites, especially those in centromeres, telomeres and gene bodies, and not in the low-methylated sites in the more dynamic promoter-enhancer and transcriptional regulatory regions. An alternative method to MeDIP is reduced RRBS [23]. In this case, the genomic DNA is digested using a methylation-insensitive enzyme, such as Msp1, which targets 5'CCGG3' sequences. After addition of methylated adaptors to each end of the sized fragments, the DNA is bisulfite-converted and PCR amplified. Resultant fragments can be sequenced. Simi-

lar to MeDIP or whole-genome bisulfite sequencing, alignments of the resulting sequences to the standardized human genome may be complicated by the sequence conversions [129].

The lack of a high density BeadChip array for rodent genomes has limited whole genome methylation analysis for the ACE models described above. These models have been tested for impact on 5mC in four main ways: 1) Bisulfite pyrosequencing or PCR of candidate loci/genes; 2) RRBS; 3) MeDIP followed by either sequencing or Affymetrix mouse genome promoter chip array. Examples of sites in candidate genes are *Bdnf* and *Crf/Crh* (Table **S1** in Supplemental Material); 4) transgenic animals lacking methylation machinery, such as *Tet* and *Dnmt* knockouts.

RRBS has been applied to identify differentially methylated sites in mouse [130], although this approach has not been applied to early life adversity to our knowledge. In addition to the difficulty aligning bisulfite-converted sequences to template DNA, there is also the drawback that bisulfite treatment poses problems for sequencing machines, which produce orders of magnitude fewer sequences than expected for each run of bisulfite-converted genomic DNA.

Others have used Affymetrix mouse promoter arrays to identify DNA sequences after MeDIP [36]. For the *5-htt*(*sert/slc6a4*)-knockout mouse, prenatal stress results in a re-programming of promoter DNA methylation in at least 25 genes genome-wide in the hippocampus at 3 months of age. This discovery was made using the MeDIP procedure followed by GeneChip Mouse Promoter tiling arrays [36]. These findings will prove exciting for future explorations where the power of mouse genetics, tools for manipulation of the mouse genome, and a vast array of transgenic lines can be coupled with robust experimental procedures to induce early life stress.

The large number of potentially methylated sites has one huge advantage: statistical power. If a single site is unmethylated reproducibly in even as few as 3 individuals who share a trait or an experience, as compared to individuals lacking that trait, the chance of this happening by chance is extremely low. Of course this power is only possible when the trait is clearly defined across individuals, often not currently possible using self-reports and other psychosocial measures which can be highly variable and unreliable determinants of a trait. This statistical advantage for conserved sequences could be exploited to obtain trait-methylation correlations without the enormous sample sizes typically required for SNP-trait correlations.

In addition to discovery of sites, new methodology is needed to implement methylation testing of multiple sites for diagnosis. Smaller platforms that test for a limited number of sites confirmed to be clinically relevant would be useful in clinical laboratories or even at the bedside. Promising devices are already in development, and include nanofluidic channels that accommodate single DNA strands [131], which can then be probed for specific methylated sites with labeled methyl-binding proteins [132]. Decrease in the channel size and transparency of the silica using lithography may increase multiplexing to hundreds of sites within a few microns detectible by fluorescence labeling and high-density high-sensitivity cameras [133, 134].

3. MATERIALS AND METHODS

De-identified saliva samples (n=45) from children were obtained from other studies. All samples were de-identified and numerically labeled. This study was approved by the UNM IRB. DNA was extracted using the Qiagen kit and submitted for testing on the Illumina 450k BeadChip. For 4 samples, DNA was analyzed by three different methods in parallel: MeDIP followed by bisulfite conversion and then sequencing by either the IonProton at the ATG facility at UNM, or the Illumina MiSeq in the lab of Darrell Dinwiddie at UNM. Parallel aliquots of the same DNA were submitted for Illumina BeadChip. Resulting sequences were aligned using Interactive Genome Viewer (GV; (http://software.broadinstitute.org/software/igv/). Results from the Illumina BeadChip were further analyzed using RNBeads. Methylation patterns of brain, keratinocytes and whole blood for cell type deconvolution were from the Genome Omnibus (GEO). Please see figure legends for more detail on methods.

4. RESULTS

4.1. Example Comparing DNA Methylation Assays in Humans

To compare two of these methods, we isolated DNA from saliva collected from four normal children and prepared the genomic DNA in parallel for either MeDIP or Illumina HM450 BeadChip in parallel according to the flowchart shown in Fig. (**4**). While some reports suggest that either method produces equivalent results based on number of sites obtained [135], we took a closer look at what specific sites were identified and found large differences between the two methods. We compared resulting methylation sites by aligning both sets of results with a standard hg38 human genome in Integrative Genome Viewer (IGV) (http://software.broadinstitute.org/software/igv/) [136, 137] (Fig. **5**). Note that MeDIP produces a high density of hits in the centromeric location of the chromosome, a region known to be highly methylated, while high density array identifies individual sites in regulatory and promoter regions not found in the results from MeDIP. Alignments of results from the MeDIP-sequencing are complicated by the bisulfite conversion, which alters sequence, and the low yield from sequencers-we used both IonTorrent and Illumina MiSeq on the same genomic DNA with similar low yields for either machine. These sequencing platforms should have theoretically yielded over 100 million reads. We obtained about 13 million reads, only half of which were usable- *i.e.* they had few sequencing errors and could be aligned. Because of this we decided to use the Illumina BeadChip arrays. The HM450 has more than 450,000 pre-selected methylation sites across the genome [114, 138], and the EPIC, 850,000 [139].

High-density arrays provide an alternative approach that overcomes both of the drawbacks of bisulfite sequencing (low yield and difficulty of alignment), although the number of sites is currently limited by chip capacity. By using synthetic DNA oligomers as the target on the array, the precise genomic location of the methylated cytosine is known, and sites can be selected for their relevance to transcriptional regulation and chromatin packaging. Single nucleotide

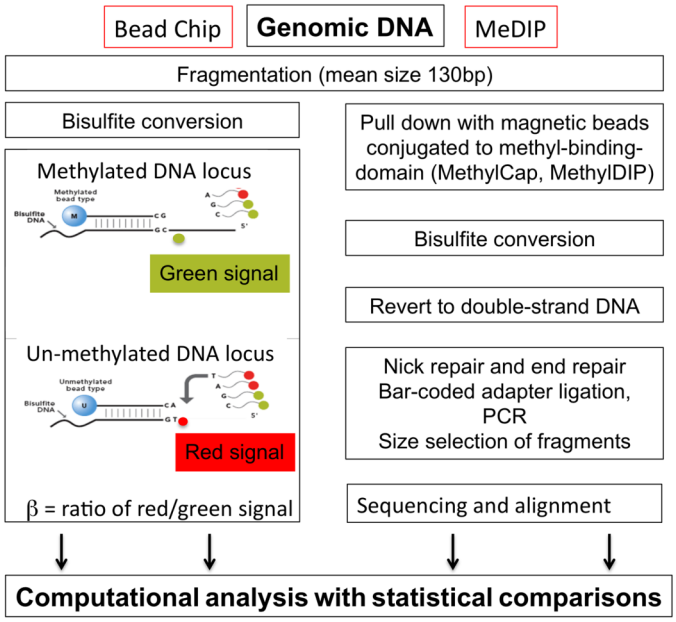


Fig. (4). Diagram for comparing assay methods for detecting genome-wide methylations. Diagram comparing two methods for identifying differences in methylation levels at specific sites across the genome, with high-density array on the left and MeDIP on the right. In both cases the DNA is fragmented, the fragments are sized and adaptors ligated. Both protocols use bisulfite conversion. Fragmentation may occur before or after bisulfite conversion for the BeadChip analysis but must be done before conversion when using MeDIP since this method depends on the 5mC to pull-down fragments. For the BeadChip, single-stranded DNA is hybridized to known sequences on the chip that encode the unconverted or converted cytosine methylation site. Fluorescently labeled nucleotides are added for single nucleotide extension [140], and the ratio of red or green fluorescent methylated probes intensity gives the ratio of methylated to un-methylated fragments at that site. For MeDIP, many more steps are required and computational analysis is complicated by the multiple methylation sites in many of the fragments that are altered after bisulfite conversion is performed. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

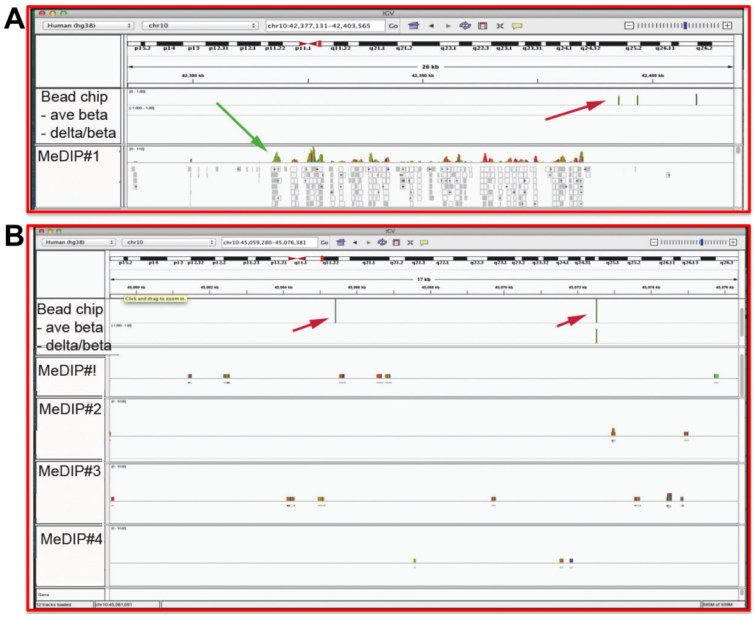


Fig. (5). Comparison of sites identified by MeDIP or Illumina BeadChip aligned on a template human genome. Shown are screen shots from the Integrative genomics viewer (IGV) showing alignments for four children at two loci on chromosome 10 analyzed by two different methods: Illumina BeadChip HG450 and methyl-binding pulldown (MeDIP) with bisulfite sequencing. In (**A**) the centromeric region of ch10 is shown. Note that alignment of the Illumina BeadChip data shows no hits over this centromeric region and only a few sites adjacent to it (red arrow). In contrast, sequences from the MeDIP in the centromeric region are numerous (green arrow). Only one of the four children is shown for the MeDIP, but all showed the same pattern of hits. In **(B)** the Illumina BeadChip identified two sites with significant differences between the children (red arrows). These sites are not detected in the MeDIP for any of the four children, even when results for each of the four children are separately aligned. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

addition during amplification is used for detection of methylation sites [140] (Fig. **4**). An Illumina BeadChip, EPIC, is currently available for 850,000 sites on the human genome [139], and previous versions with smaller numbers of sites have already produced a wealth of information about DNA methylation across the whole human genome. Although we predict 1 billion cytosines in the human genome, less than 20% are likely to have dynamic methylation and play regulatory roles.

The methylation level at each site is quantified by a color-coding scheme for either the T or the C sequence at that site (Fig. **4**). As the number of sites on these chips increases, the reference cell-type data must be updated so that cell type deconvolution may be performed, and this step is lagging behind chip manufacture. Also older chips with fewer and slightly different sites, are not available, making it difficult for continuity in longer time-span studies, and impossible to validate results on the same chip type. No chip is currently available for other species, although one for rodents has been in planning stages.

Types of tissue that can be used for studies of childhood trauma include saliva or whole blood or post-mortem brain. While changes in neural methylation will be very interesting, in human these require autopsy studies, which are possible but do not allow follow-up analysis for persistence or resolution. Autopsy studies will be useful to determine the relevance of 5mC changes for brain development and function. Additionally, post-mortem brain analyses in experimental animals may be extremely enlightening.

4.2. Saliva as a Bio-specimen to Test 5mC in Children

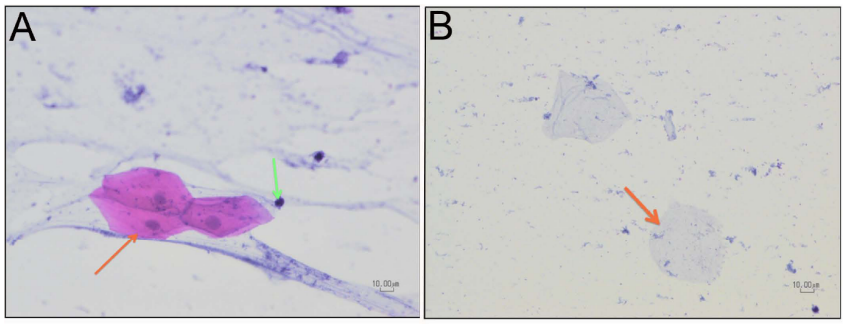
While saliva is the most accessible biospecimen from children, care must be taken to control for cell composition. Saliva contains both white blood cells and buccal keratinocytes, in different proportions in different samples even when taken from the same child (Fig. **6**). Stressed children may have scant saliva since fear and anxiety in children produce a severe dry mouth, possibly due to catecholamines, which alters the cellular composition of saliva [141]. Hence the cell composition of the saliva must be accounted for in any analysis of methylation sites, since keratinocytes and white blood cells, being differentiated down divergent pathways, will have widely different methylation patterns, as has been shown for mouse [129], and discussed for human [32]. There are two ways to manage this: purify each cell type from the sample, or de-convolve the contribution of each cell type within the methylation results. Purification of cell types from saliva seems daunting, as saliva contains enzymes and microbes as well as thick mucus that would interfere with standard cell purification approaches. Hence de-convolution of the contribution of each cell type to the 5mC patterns is currently the method of choice. In some cases, the cell type may not matter, as a subset of sites appear to respond to specific conditions similarly in blood, saliva and brain [33, 34, 142], which gives further confidence that saliva may serve as a proxy for brain for some subgroup of 5mC changes.

While comparisons of the methylation levels at different sites between tissue types in the same group of subjects are useful, even better would be to have data on methylation patterns from purified cell types such as neurons, glia, keratinocytes and the various cell types found in blood. A limited number of these are available for the 450K Chip. New data to meet this need are being posted daily onto the US National Center for Biotechnology Information site, the Gene Expression Omnibus (GEO), yet much more is needed. In particular, 5mC patterns from many different cell types obtained from the 850k EPIC Chip are not yet available as of this writing. Since we are unlikely to perform brain biopsies on children to detect 5mC changes, saliva may prove the most useful proxy in living children [34], but normal standards of keratinocytes and, at minimum, whole blood, for cell de-convolution must be available to utilize this most accessible biospecimen. Saliva is more easily obtained than peripheral blood from children-spitting is both more acceptable to a child, and easier for investigators to obtain permission from offices for protection of human subjects, such as US Federally required Institutional Review Boards (IRB). Buccal swabs are less acceptable to children as these involve scrapping the inner cheek and are somewhat painful. In our experience, children enjoy spitting into a cup, especially when given a sugar-free candy to suck during the collection. While some reference-free deconvolution computational approaches have been described for whole blood [127, 143, 144], these have not been proven in saliva. Hence methylation data for purified buccal keratinocytes and blood cells would greatly advance our ability to identify interesting methylated sites in DNA from saliva specimens.

4.3. Example of Methylation Pattern Analysis Useful for Saliva Analysis: The Case of Keratinocytes and White Blood Cells

As an example for this paper, we chose to perform an analysis of cultured human keratinocytes and whole blood cells using methylation data posted on GEO from 4 different DNA preparations of human keratinocytes [145, 146] and 4 of whole blood [147] that had been analyzed with the 450k BeadChip from Illumina [114, 138]. Since these two cells are the major cellular components of saliva, data from comparisons of methylation levels in these two cell populations are useful for subsequent determination of the relative cell composition in individual saliva samples, with a reference-based deconvolution.

The computational steps from this analysis are diagrammed in a flowchart (Fig. **7**). We use an R package, RnBeads, to perform analysis including quality control and normalization with data obtained from the Illumina HM450 BeadChip, preliminarily quality controlled in the Illumina GenomeStudio environment (http://rnbeads.mpi-inf.mpg.de) [128]. First, all data from the Illumina assay is best when in the same format to upload into R (IDAT or .csv files). Since the cellular data came from various studies posted in GEO, we performed all steps on all data from the 8 different analyses from 4 keratinocyte primarily cell cultures and 4 whole blood samples from healthy volunteers. Thus this data is processed at the same time in batch to eliminate any variance introduced by differences in quality controls and preprocessing. Due to the variation in results from different methylation assays, we only use data produced by Illumina chips for this strategy. Initial normalization and quality controls are performed during finalization of the Illumina BeadChip assay.



Reported Experience	Avg % Skin Cells
Trauma	68.7(± 3.3)
Non-trauma	36.4(± 5.7)

Fig. (6). Micrographs of saliva smears. To determine the types of cells in saliva, we first performed histopathology on saliva smears collected from 6 healthy volunteers either directly onto a glass slide (**A**) or first into Oragene DNA collection cups which contain a buffer to preserve the DNA and then onto a glass slide (**B**). Slides were stained with haematoxylin and eosin according to normal pathology procedures and cover-slipped in mounting media. Slides were reviewed and types of cells counted. Keratinocytes (an example indicated by the orange arrow) and WBCs (example indicated by green arrow) were present. Preparations differed in the relative number of each type of cell. After treatment with the Oragene buffer (**B**), keratinocytes appeared ghosted and lacked nuclear staining, consistent with DNA extraction (orange arrow). Some small round blue dots remaining apparently represent un-extracted nuclei or non-human microbes. These results convinced us that methylation patterns obtained from saliva need to be corrected for relative amounts of DNA from keratinocytes *versus* white blood cells. Analysis of our 5mC data demonstrated that children with no stress-related cortisol elevation had a lower ratio of keratinocytes ("skin cells", aka cheek or buccal cells) to blood cells than children with a history of trauma (lower table). Because 5mC levels at specific sites differs between cell types [162], 5mC levels can be used as a surrogate for quantifying cell types in saliva samples [151]. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

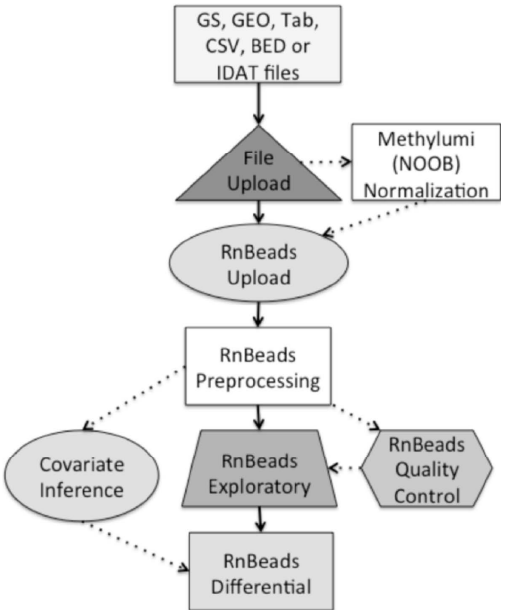


Fig. (7). Flow chart of computational processing of Illumina BeadChip data. Workflow showing data sourcing/acquisition through site by site differential methylation assessment, starting with data acquisition and formatting, pre-processing steps, quality control, exploratory analysis, including heatmaps, and differential methylation tables with statistically significant values reported. Output from intermediate steps allows for global data trends to be reviewed, corrected and re-assessed along the way. Differential methylation output may be used to identify gene ontology with other software.

We begin with the IDAT Illumina HM450 output and repeat quality controls, using RnBeads, to ensure equivalent signal from all wells on the chip and elimination of sex chromosomes and ancestry-related SNPs. Further normalization steps are also taken to account for the differences between different chips and collections. The HM450 contains a large number of control probes to assist with these controls that can be applied for determining and equalizing variance in stain intensities, hybridization, extension, target removal, bisulfite conversion, specificity and non-polymorphic hybridizations. Also included on the chip are negative controls (8 probes) that provide information about background levels for both red and green probes, which is useful later for intensity scaling. Single Nucleotide Polymorphisms (SNPs) on the chip can be used to identify sample mix-ups (Fig. **8**). The read-out is filtered by removing the following probe sites: unreliable probes defined as having a β value with corresponding detection p-values below the threshold we set at $p > T = 0.05$ using programs such as GreedyCut in R [148]; then all sites on sex chromosomes because these will create differentials based on gender; all -enriched probes where the SNP may influence the methylation independent of the experimental condition; and probes that include regions where an ethnically determined SNP site may interfere with methylation status. DNA context-specific probes (CC, CAG, CAH, CTG, CTH and others) are also removed. In this case, SNP analysis reveals that two of the cultured cell samples were from the same individual, and suggests that the genetic backgrounds of the samples separate into four groups, which may indicate that four ethnic groups are represented (Fig. **8**).

When .csvs are used as input format, background is subtracted using the methylumi package (method, *NOOB*,

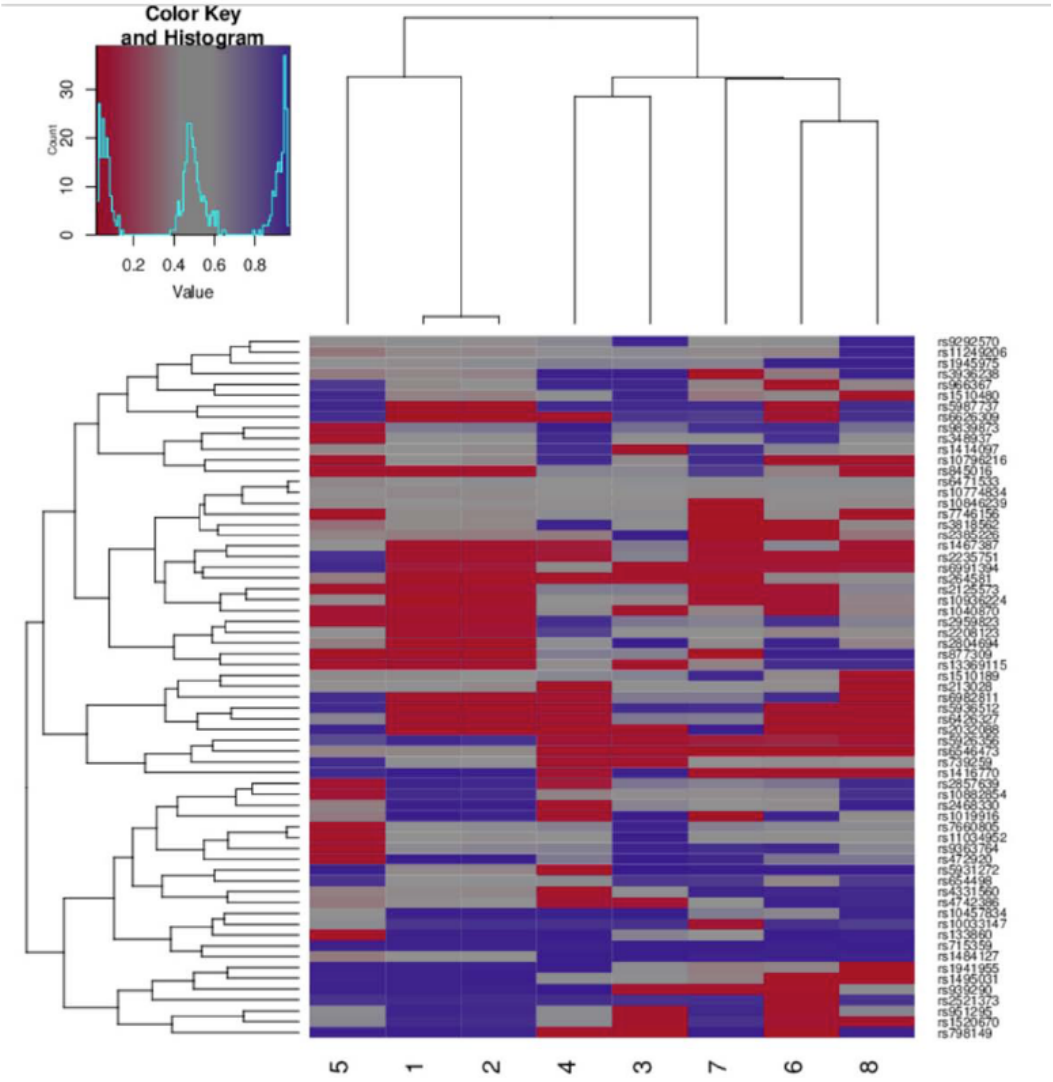


Fig. (8). SNP distance heatmap. We obtained 8 IDAT files of methylation data from GEO, four for keratinocytes and four for whole blood, performed on the Illumina 450k chip. The Illumina 450k chip contains 65 genotyping probes that determine ancestry, and that can take one of three possible β-value levels: low (homozygous for one allele), high (homozygous for the other allele), or intermediate (heterozygous for both alleles). An individual should have the same β -values at all sites regardless of cell type, since this is based on somatic genomic sequence. The heatmap is produced by unsupervised hierarchical clustering of the intensity signals for 65 SNPs. The dendrogram above the heatmap gives a global picture of genotype-related sample grouping and similarities. Shown is a heatmap for methylation of the 8 different cell lines posted on GEO (GSM2260732; GSM2260731; GSM2260730; GSM2260729; GSM1936951; GSM1936939; GSM2071075; GSM2071074) [124, 145-147]. Cell lines numerically coded (1-8) and indicated below the heatmap. The SNP heatmap shown here demonstrates that two of these cell lines numbers 1 and 2, were obtained from the same individual, known to have been performed as technical duplicates. As more buccal keratinocyte data is obtained, the database for cell composition will expand. Heatmaps for the other samples demonstrated different individual SNPs. Such clustering of SNP heatmaps can also be used to detect sample mix-ups.

"Normal Exponential Out Of Bounds") (https://www.rdocumentation.org/packages/methylumi/versions/2.18.2) [149], and in both cases methylation β values are normalized using the BMIQ normalization method [150]. Once these quality controls are completed, RnBeads produces figures demonstrating the success of the processing-a subset is shown in Fig. (**9**). A methylation value densities plot shows similar distribution of methylation values for each cell type before and after quality controls, filtering and normalization steps, and similar distribution of β values for probe categories (Fig. **9A-C**). The types of sites retained or removed can be compared between sample sets. For example, whether promoters or CpG Islands were preferentially removed in one set compared to the other, which would lead us to review all steps in the procedure. The final outcome of these filtering steps may remove as many as 150,000 probes from a typical 850,000-site bead chip. In this case, only ~13,000 probes were removed (Fig. **9C**).

Following these steps, a principal component analysis reveals the source of variation in the dataset, which is important in order to control for batch effects, such as different chips, different dates of harvest or other technical variations (Fig. **9D**). In this case, only three principal components explained 95% of the variance: cell type being the most important, with the anatomic location of the keratinocyte (buccal

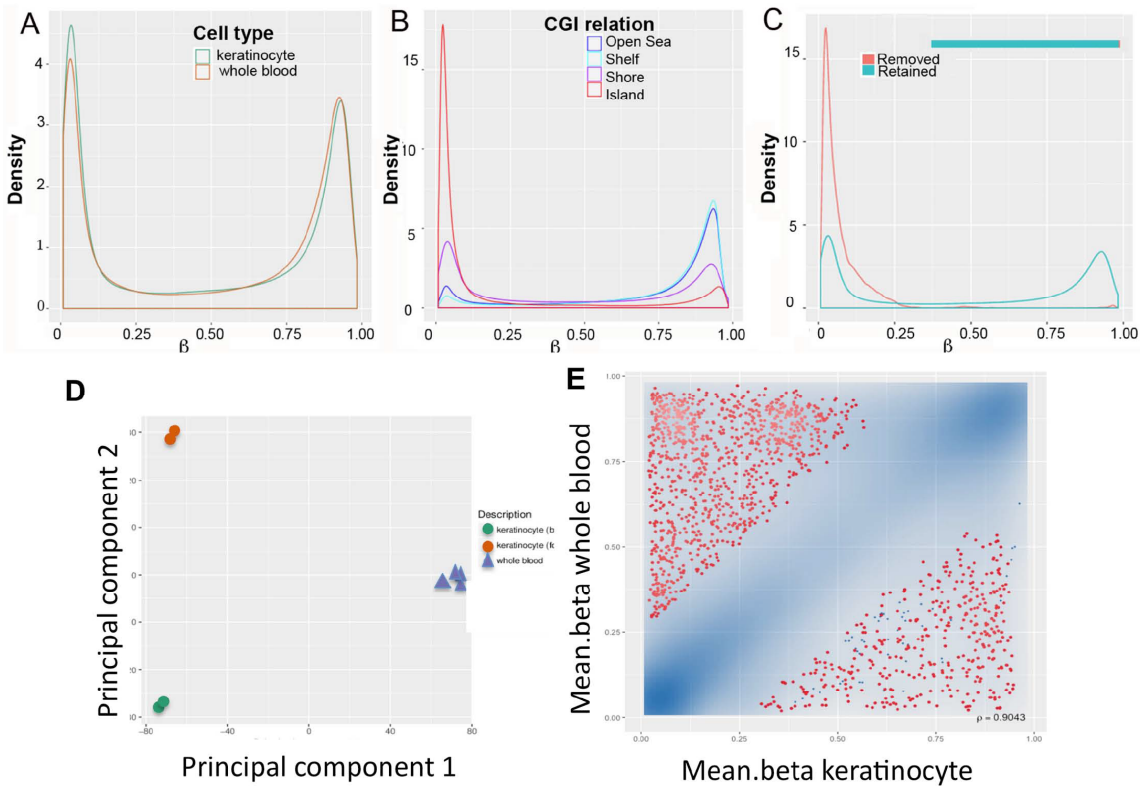


Fig. (9). Quality controls of cell type methylation data from keratinocytes and whole blood. A. Original probe distribution. Note similar methylation level distribution in both cell types. **B**. Distribution of probes in genomic locations: Open sea (between genes); Shelf (2kb flanking genes); Shore (region where methylation levels are highly variable, usually close to promoters, likely to encode enhancers); Island (CpG island, typically at least 200 bp with a CpG ratio greater than 50%). Note sites in all genomic locations. **C**. Histogram comparing the removed and retained sites after quality controls and filtering. Note the relatively sparse numbers of sites removed for both datasets. **D.** Principal Component Analysis (PCA) of data from three cell types: Buccal keratinocytes, foreskin keratinocytes and whole blood from 8 different cell culture samples. Shown is a graph of principal components 1 and 2. Three components were identified that predict >95% of all variance as based on all sites remaining after filtering. The keratinocytes (orange and green circles) are widely separated from the whole blood, as has also been shown for mouse cell types assayed by reduced representation bisulfite sequencing (RRSS) [128, 162]. The range of the x-axis is -80 to +80, and the range for the y-axis is -30 to +30. DNA was from primary cultures of keratinocytes from foreskin (green, Kerat.f) or from buccal (red, Kerat.b) and whole blood (purple, WB). **E**. Scatter plot of group-wise mean DNA methylation levels for all keratinocyte and blood samples across all promoter sites. Sites with significant differences between cell types are colored red, as determined by RnBeads *via* a three-part metric. Sites with similar methylation levels in both samples lie on the diagonal and are colored blue. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

versus foreskin) also contributing. An expected result for samples of mixed cell composition that validates the dataset is to find that the trait being analyzed is responsible for at least 50% of the variance in the first principal component. If the first three principal component's (PCA) variance is primarily attributed to the chip ID, gender, or ethnicity, all of which should have been corrected for during the filtering and normalization steps, then filtering or normalization were not performed sufficiently, or the phenotypic trait in question did not contribute statistically enough to dictate differences within the PCA. If chip ID, gender or ethnicity arise as a statistically relevant trait after normalization, this suggests that the phenotypic trait does not explain the basis of the differences between the groups.

A scatter plot of group-wise-mean DNA methylation levels between the two cell types shows a wide degree of variance (Fig. **9E**). In this example, all promoter sites for each cell type are shown, with those sites, shown in red, that meet the RnBead's analysis-specific ("arbitrary") rank cut-off, which is based on mean difference between the two groups, mean quotient, and t-test p-values (https://rdrr.io/bioc/RnBeads/f/inst/doc/RnBeads.pdf). The plot reveals both lower and higher methylation level differences across multiple sites between keratinocytes and blood cells. These sites are useful for the development of automated adjustment for cell composition distributions between samples.

For a global assessment of whether the data have been properly prepared for subsequent differential methylation analysis, hierarchical clustering of percent methylation by specimen visualized as a heatmap can be useful (Fig. **10**). Clustering by trait, in this case cell type, rather than by chip or other possible batch effects is expected after good filtering from a reliable dataset (Fig. **10A**). Clusters are indicated by the dendrogram above the heatmap, with traits (*i.e.* cell types) color-coded in boxes above each heatmap column. Heatmaps for all 5mC levels at each site identified in keratinocytes and whole blood by sample neatly separate into two clusters, demonstrated by density of methylation levels (Fig. **10A**) or by sites (Fig. **10B**). Higher resolution analysis of 1000 promoter sites reveals that the buccal and foreskin cultured keratinocytes have slightly different patterns of methylation levels, but both types cluster separately than whole

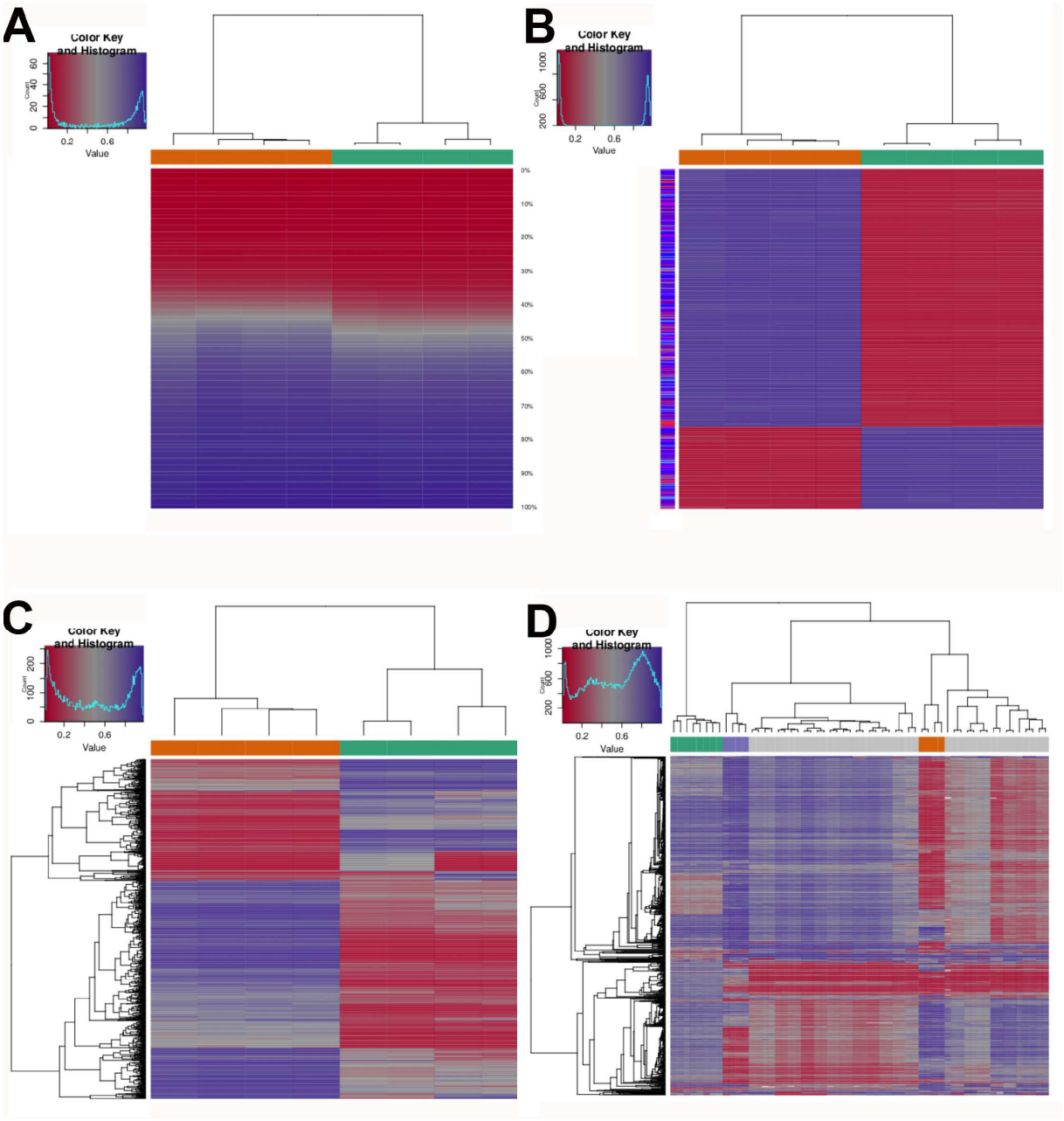


Fig. (10). Exploratory analyses of methylation patterns. A. The degree of methylation across all sites in each sample of keratinocyte and whole blood is displayed as a heatmap of the number of sites in each sample with that percent of methylation, ranging from 0 to 100% (see color key in upper left). Pure red is 0% and blue is 100% methylated, with blended red/blue for partial methylation and no color for equally methylation/demethylated (50%). Note that cell types cluster together according to the dendrogram above the heatmap, with blood samples indicated by orange and keratinocyte samples by green bars above the heatmap. Note that even at this low level of analysis, the samples cluster according to cell type. **B.** Heatmaps and unsupervised hierarchical clustering for all sites across all samples of both cell-types, correlation-based, with complete agglomeration strategy (linkage), visualizing the 1000 most variable loci, demonstrates large differences between cell types, where some sites are highly methylated in blood and not much methylated in keratinocytes and others are *vice versa*. These dramatic differences represent the outer corners of the mean difference plot shown in Fig. **(9E)**. The column to the left is color coded for the 5mC location, whether in open sea (blue), shelf, (turquoise), shore (purple), or CpG island, (red) for each site. **C**. Heatmap from the same analysis visualizing only promoter sites in the 1000 most variable loci, with the dissimilarity metric set for correlation-based and agglomeration strategy (linkage) average. Note the large difference in 5mC patterns between the two cell types. **D**. Children's 5mC patterns from saliva clusters with either skin or blood cells. Heatmaps of hierarchical clustering of methylation data from brain (green), blood (purple) and keratinocytes (orange) together with 45 saliva samples from healthy children (color coded in gray above the heatmap). Some children were sampled at two different time points, and some were run in duplicate. Note that these saliva samples fall into two clusters as shown in the dendrogram above the heatmap. One group clusters with blood and the other with keratinocytes. Brain clusters with all samples at a greater distance [163]. Examination of the samples revealed that a few children sampled at two different time points clustered differently: one time point with keratinocytes and the other time point with whole blood. This result demonstrates the critical need to adjust 5mC results for cell composition. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

blood, which surprisingly shows little variance between samples in this analysis (Fig. **10C**).

Next we obtained 45 different saliva specimens from children, isolated DNA, and obtained the methylation patterns on HM450 or EPIC Illumina BeadChips. We converted IDAT files to .csv format with NOOB and loaded all into R. Data were merged such that only sites in common between HM450 and EPIC were included. We then loaded the methylation data into RnBeads and processed our saliva data together with both cell type and additional human brain methylation data obtained from GEO. This exploratory analysis demonstrated that each saliva sample clustered either with whole blood or with keratinocytes and by individual, nor by study, or phenotypic trait. The brain data clustered separately from either children's saliva, or the other two cell types (Fig. **10D**). These samples did not cluster by age, sex, or ethnicity to any statistically significant degree (African American, Latina/o, and Caucasian individuals were represented). This result argues that cell composition in saliva is the primary variable governing methylation patterns and must be corrected for in any analysis comparing between samples from the same child over time or between different children.

To account for cell composition, we performed a reference-based differential methylation analysis in RnBeads (Figs. **11** and **12**). To begin we submitted the data to covariate inference in RnBeads, which assesses the data in light of the differences between samples' cell type concentration. We selected Houseman's reference-based linear approach [151]. Once the samples and references are submitted to the deconvolution process, the methylation markers of the references are found. These markers are used in building a model of both keratinocytes and whole blood, to which the samples are compared. The 50,000 CpGs with the most variance across all samples was compared to these models (Fig. **11A**) and, by solving for the vectors of the samples, the program then assesses how much each cell type contributed to each methylation site in each individual sample (Fig. **11B**). The projections of these samples are stored as covariates and later used to perform an association analysis, which theoretically equalizes the cell type composition among samples. This is then used in the differential methylation analysis to adjust the methylation levels at all sites based on the contribution from its unique cell composition.

A scatter plot of methylation results comparing ASD with non-ASD shows few sites surviving cell composition correction (Fig. **11C**). These samples represent a subset of the 45 samples described above, and are part of a study comparing saliva methylation patterns in children with and without ASD (Mulligan and Bearer *et al.*, MS in preparation). Since the genetics of ASD remain complex and not well understood (https://www.sfari.org/resource/simons-simplex-collection/), epigenetics may hold the answer.

5. DISCUSSION

Prior to deconvolution, the differences between cell types overwhelmed the signal from sites that correlated with ASD. Thus deconvolution using reference samples for the types of cells known to be present in the biospecimen enables saliva to be used as a painless, meaningful and reproducible assessment for global epigenetic changes after defined experience or with specific diagnoses.

Once significant 5mC differences in particular sites are identified, these sites can be located on the human genome and their relationship to adjacent genes determined. Gene names are then submitted to gene ontology programs. In the example shown here (Fig. **12**) we submitted the list of gene names to DAVID (https://david.ncifcrf.gov), a bioinformatics resource from NIAID at NIH [152, 153]. Surprisingly, although the cell types in saliva are skin and blood, the largest number of associations was found with neural genes (Fig. **12**). Smith *et al.* also reported that saliva is more similar to brain than to blood in a study comparing saliva, blood and brain without cell composition corrections [34]. Of note is the large proportion of sites associating with each function that cluster with neural-specific components, processes and functions. For example, post-synaptic density and post-synaptic membrane represent 50% of the associations with cellular components. Almost all of the associations with biological processes are neural, including long-term memory, axon guidance, neuron differentiation, neurogenesis and neural system development. And the sites in genes associated with metabolic function are dominated by binding factors, with contributions from transcription factors and co-factors. What these associations may mean in terms of brain function will be an exciting area for future exploration.

5.1. Confirmation, Replication and Validation of 5mC Sites, Methylation Levels and Associations with Childhood Adversity and/or with Mental Health Outcomes

Ideas for how best to confirm, replicate and validate 5mC in childhood adversity are often drawn from the cancer 5mC literature, or from genetic findings in RNA transcriptomics, genomic mutations, and SNP correlations. Confirmation of 5mC sites identified by Chip is typically performed *via* pyrosequencing or bisulfite PCR sequencing across the high probability sites from the original DNA. Replication is tested by repeating, either by the whole genome analysis, individual site confirmation by bisulfite pyrosequencing or PCR sequencing of select high probability sites in another, different, cohort recruited with the same intake parameters and exclusion criteria as the original samples. Alternatively, some studies simply search for sites already known to be associated with adversity, such as the *Nr3c1, Nr3c2, Fkbp5, Crf/Crh, 5Htt/Sert/Slc6a4, Bdnf* sites, to show reproducibility of the altered methylation in a new cohort. Some investigators search for mutations or SNPs already shown to be associated with a given mental health outcome to discover whether these may impact methylation levels at high probability sites in their analysis [67], or are in genes whose expression levels have been shown to influence a mental health condition [154]. As the public databanks grow, more opportunities to compare results between studies will become increasingly available. Validation that the methylation has an effect on expression is done by expressing mutated constructs in tissue culture cells and measuring gene product [37]. Validation of mental health associations with outcomes in humans may require huge subject numbers for statistical power. Both the long delay between insult and outcome and the cost may make this unfeasible for most labs if EWAS is attempted, although statistics are stronger, and thus fewer subjects are needed, for methylation sites than for DNA SNPs. Animal systems have also been used to confirm and extend results in human with experimental testing, but better platforms for animal

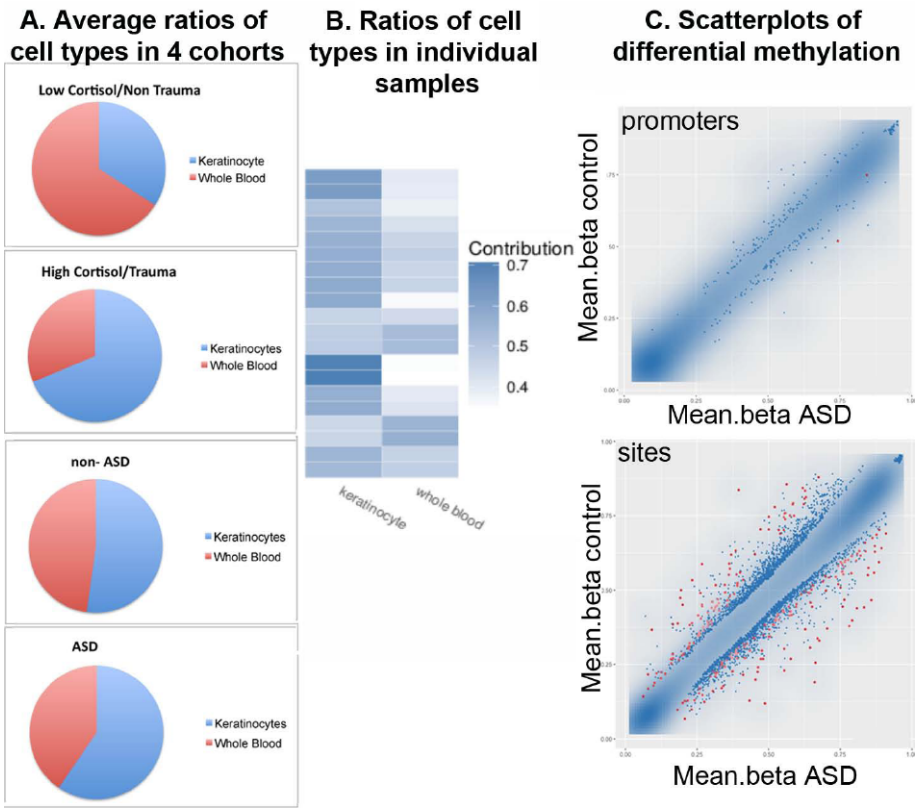


Fig. (11). Cell composition de-convolution: Adjusting methylation data for varying contributions of keratinocytes and nucleated blood cells. (**A**) The average ratios of cell types in four cohorts of children's saliva. The top two pie-charts are for a cohort of children ages 4-8 years old, and the bottom two from children ages 18 months to 4 years old. Red indicates blood cells and blue keratinocytes. Two of the pie-charts (the 1st and 3rd down) are children without reported trauma and the other two are from children with high cortisol levels and reported traumatic experience (2nd) or an autism spectrum diagnosis (4th down). (**B**) Comparing profiles for individual children in the datasets, even though the pie-charts for the cohorts appear relatively similar, there is wide variation between individual samples of cell composition as detected by covariate inference [151]. In the first step the reference methylomes were used to estimate the association of each CpG position to each of the cell types. The strength of association was measured using an F-test. To decrease the computational load, only 50,000 most variable CpGs were considered. Finally, only 500 CpGs with the lowest F-test p-value were used in the contribution estimation. Selecting the most informative CpGs is equivalent to applying an F statistic cut-off of 1.788. (**C**) Scatterplots showing the significant sites between ASD and non-ASD (control) after cell composition de-convolution for promoters (top) or over all sites (bottom). Non-significantly different sites are colored blue, and those that met the automatically generated rank cutoff are red. Compare these scatterplots with the one shown in Fig. **(9E)** comparing 5mC patterns from cultured keratinocytes and whole blood, where many more significantly different sites appear. De-convolution decreases the complexity of the sample, removes the confounder of variation in cell type ratios, and improves identification of trait-related differences. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

methylation patterns analogous to the Illumina BeadChips are needed. Especially exciting will be the new gene-editing technology by which DNA in the brain of living animals can be altered, generating new transgenic mice regulating expression of methylation/demethylation machinery [76, 155-161]. Critically important in human data, where such experimentation is challenging, is the need for quantifying the cell-type composition of the specimen, particularly for saliva but also for blood and brain, and deconvolution of the contributions to 5mC patterns from differing cell types, either experimentally before DNA extraction, or computationally during processing preferably using reference-based methodology. More precise information about the type of trauma and the temporal relationship between adverse experience and 5mC changes will improve the chances of finding significant targets, of replicating them and validating them in new cohorts of subjects.

CONCLUSION

Much progress has been made and many questions remain as to the biological impact of ACE on the child and its propagation throughout the lifespan. Most importantly, how pediatric trauma acutely affects the child's methylome will require longitudinal studies of the same child over time. Beginning at birth with a comparison to the parents may help to know what that child's baseline methylation levels were prior to traumatic experiences, and knowledge of the prenatal events may be also important. Then, whether alterations occur at the time of trauma or emerge later must be determined also in longitudinal studies in which the time of trauma can be precisely documented. Finally, 5mC analysis longitudinally after a traumatic event is key to understanding the persistence or evolution of 5mC changes, with demographic and psychosocial information necessary to understand the contextual basis for resolution, evolution or persistence. Cross-sectional correlations between different study groups are

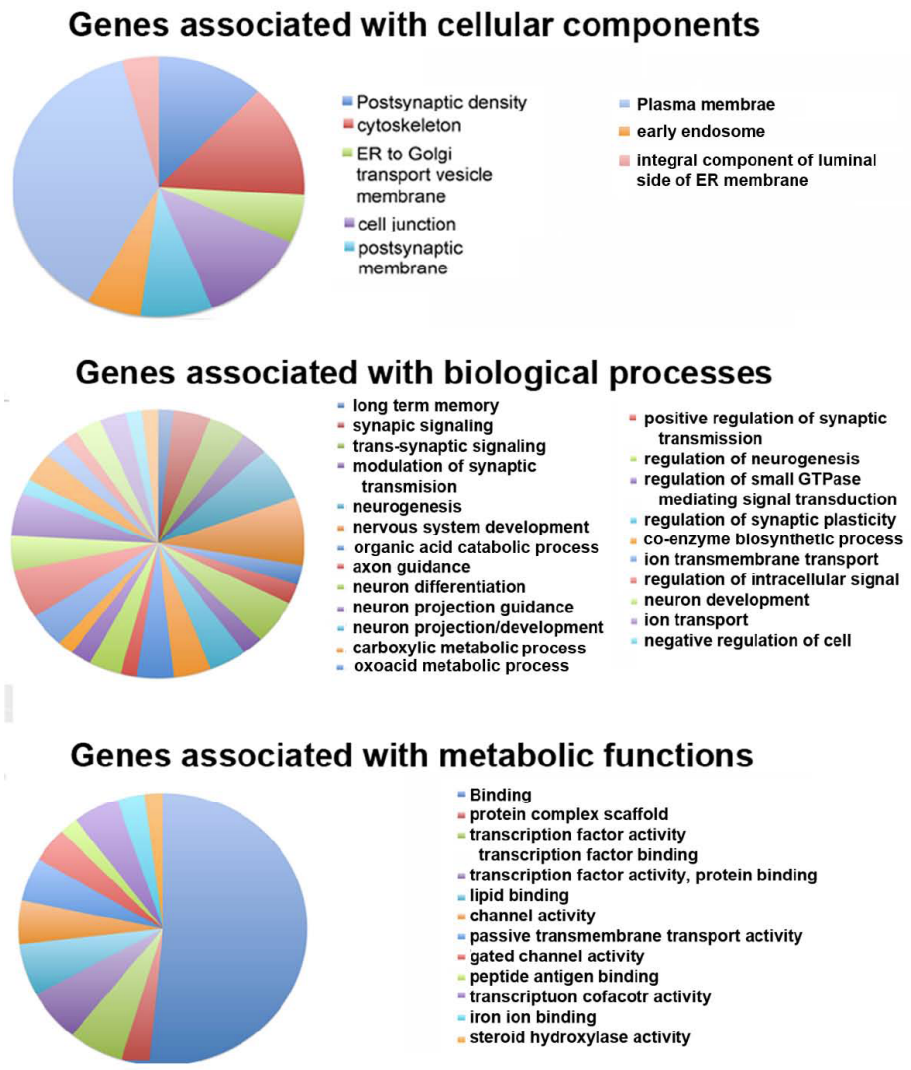


Fig. (12). Gene Ontologies of significant differentially methylated sites in saliva DNA after cell type deconvolution. Differential methylation was analyzed in RnBeads after adjusting the methylation level for each site in each sample according to the estimated cell composition of that sample, using as references the keratinocyte and whole blood methylation patterns shown in Figs. **(8-10)**. Gene names of regions associated with each gene surviving statistically significant differentially methylated site between non-ASD and ASD cohorts (237 sites) were updated in DAVID (https://david.ncifcrf.gov) and submitted for associations in the DAVID v6.7 database in November 2017. Results of associations with cellular components, biological processes, and metabolic functions are shown in pie charts generated in Excel from the DAVID output. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

only useful if the altered sites are consistent between groups. Hence without experimental animals with shorter lifespans in which to test for impact under experimentally controlled conditions we will not achieve a thorough understanding of the mechanism of activation of 5mC changes, of the time course, nor of causal relationships between pediatric trauma and 5mC profiles. Such animal experimentation must be carefully controlled and targeted at those questions most difficult to address in human association studies. In the end, the functional impact of 5mC changes across the lifespan will be critical in understanding when, how, and whether to intervene, and will provide biomarkers and biotargets for objective diagnosis and effective interventions.

LIST OF ABBREVIATIONS

5mC	=	5' methylcytosine
ACE	=	Adverse Childhood Experience
AID	=	Activation-Induced Deaminase
BDNF	=	Brain-Derived Neurotrophic Factor
CpG	=	Cyotsine nucleotide followed by a guanine
CRF,CRH	=	Glucocorticoid release factor/hormone
DNMT	=	DNA Methyl Transferase
ELS	=	Early Life Stress
EWAS	=	Epigenome-Wide Association Studies
FSL	=	Finders Sensitive Line
GEO	=	Genome Expression Omnibus
GR/NR3C1	=	Glucocorticoid receptor
GRE	=	Glucocorticoid Response Element
HAB	=	High Anxiety-like Behavior
ID	=	Identity
meDIP	=	Methylated DNA immunoprecipitation, methyl-binding pull-down
meQTL	=	Methylation Quantitative Trait Locus
MRI	=	Magnetic Resonance Imaging

NAT = Non-Accidental Trauma
PCA = Principal Component Analysis
Pcdha = Protocadherin alpha gene cluster
PCR = Polymerase Chain Reaction
PTSD = Post-Traumatic Stress Disorder
SERT/5HTT = Serotonin transporter
SES = Socio-Economic Status
SNP = Single Nucleotide Polymorphism
TET = Ten-eleven translocation methylcytosine dioxygenases

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study is approved by the University of New Mexico Health Sciences Internal Review Board (approval number HRP 503 14-030).

HUMAN AND ANIMAL RIGHTS

No animals were involved in this study. The study was performed according to US Code of Regulations 45 CFR part 46, known as the "Common Rule" (https://www.hhs.gov/ohrp/regulations-and-policy/regulations/45-cfr-46/index.html). All reported experiments are in accordance with the ethical standards of the committee responsible for human experimentation at UNM-HSC with the US CFR Common Rule and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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