DNA methylation as a potential mediator of environmental risks in the development of childhood acute lymphoblastic leukemia

5-year survival rate for childhood acute lymphoblastic leukemia (ALL) has risen to approximately 90%, yet the causal disease pathway is still poorly understood. Evidence suggests multiple 'hits' are required for disease progression; an initial genetic abnormality followed by additional secondary 'hits'. It is plausible that environmental influences may trigger these secondary hits, and with the peak incidence of diagnosis between 2 and 5 years of age, early life exposures are likely to be key. DNA methylation can be modified by many environmental exposures and is dramatically altered in cancers, including childhood ALL. Here we explore the potential that DNA methylation may be involved in the causal pathway toward disease by acting as a mediator between established environmental factors and childhood ALL development.

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Genetics of acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common form of childhood leukemia. ALL is a cancer of the blood and bone marrow which arises from genetic abnormalities which can occur *in utero*. These abnormalities lead to the malignant transformation of lymphocyte progenitor cells into leukemic cells of the B-cell and T-cell lineages [1]. Childhood ALL is a heterogeneous disease. Most cases are definable by large-scale chromosomal translocations/aberrations, resulting in distinct biological subtypes, each with individual characteristics. Patients are usually organized into subtypes depending on their cellular immunophenotype and recurrent cytogenetic aberrations. The most prognostically important subtypes include T-ALL, high hyperdiploidy (51–65 chromosomes), *E2A-PBX1*, *BCR-ABL*, *ETV6-RUNX1* (*TEL-AML1*) and *MLL* rearrangements [2]. The time frame between the emergence of

genetic abnormalities and disease onset, and the frequency of children born with abnormalities compared with the number of children who go on to develop ALL, suggests multiple 'hits' are required for the manifestation of disease [3]. A recognized leukemic clone with the *TEL-AML1* fusion gene has been found in 1% of newborn babies by screening neonatal blood spots [4]. This frequency is a 100-times higher than the incidence of ALL defined with this fusion gene later in childhood [5]. Diagnosis of ALL is variable with peak incidences between 2 and 5 years of age [4], suggesting an undefined period of latency whereby additional abnormalities are acquired for malignant transformation to occur. Furthermore, identical twins with the *TEL-AML1* fusion gene showed only a 10% concordance rate further supporting the concept that additional events or 'hits' are needed for the full transformation to leukemia, or specifically ALL development [4].

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DNA methylation & ALL

DNA methylation was first reported as a regulatory mechanism influencing gene expression in 1975 by Holliday and Pugh, and Riggs [6]. Methylation of cytosine residues located within CpG dinucleotides across the genome is an epigenetic modification which plays a crucial role in the creation of cellular identity by influencing gene expression during early development [7]. Methyl groups are added to DNA by *DNMTs*. This group of enzymes was responsible for the establishment of methylation patterns during development (*DNMT3A*, *DNMT3B* and their co-factor *DNMT3L*-like) and the maintenance of methylation during replication (*DNMT1*) [8]. The majority of mammalian CpG sites are methylated but CpG islands (CGIs) significantly deviate from the regular genomic pattern; CGIs are GC and CpG rich, and predominantly non-methylated [9]. Hyper-methylation of CpG sites occurs in a nonrandom tissue specific manner, and when this occurs at promoter sites it can block gene transcription. This mechanism of gene regulation plays an important role in tissue differentiation, X-chromosome inactivation, genomic imprinting and suppression of transposable elements [1].

Cancer is now recognized as an epigenetic disease [10]. The cancer cell genome undergoes dramatic shifts in the pattern of genomic methylation, including genome wide hypomethylation in conjunction with local areas of hypermethylation, often centered on promoter associated CGIs. One known disruptive mechanism in cancer is the silencing of tumor suppressor genes, such as p16^{INK4a}, via hypermethylation of their promoter associated CGIs [10]. Aberrant CpG methylation in cancer cells can have extensive effects on gene regulation, which can affect cell function and lead to adverse outcomes if genes such as tumor suppressors and others involved in important cellular processes (DNA repair, apoptosis, drug-detoxification and many more critical genes) are inactivated.

One of the first studies to investigate the DNA methylome of childhood ALL focused on the most common subtypes, *ETV6/RUNX1* and high hyperdiploidy [11]. A microarray platform comprising 28226 CGIs, cDNA microarrays and array comprehensive genomic hybridization was used to investigate altered global methylation, correlation between hypermethylation and gene expression, and detection of genomic imbalance, respectively. The three most common groups of genes which displayed hypermethylation and resultant under-expression were transcription regulators, regulators of apoptosis and cell signaling genes, all possible targets for future treatments.

An array designed to investigate DNA methylation patterns of 416 genes in cells from 401 diagnostic ALL

samples included CpG sites 2 kb upstream and 1 kb downstream of transcription start sites [12]. CpG sites located outside of CGIs showed a greater variation and overall methylation compared with CpG sites located within CGIs. These sites may offer an alternate transcription start site, and therefore alterations in DNA methylation at these CpG sites may affect gene expression. This notion was supported by a notable inverse correlation between CpG site methylation and gene expression. Methylation profiles of forty genes were also identified as consistently and accurately discriminating between four subtypes of B-cell precursor ALL, and DNA methylation levels of 20 individual genes predicted relapse.

Integrated genetic and epigenetic analysis of childhood ALL has provided evidence for alterations in DNA methylation playing a key role in leukemogenesis [13]. Figueroa *et al.* [13] analyzed 137 B-cell and 30 T-cell lineage childhood ALL samples, and 19 B-cell samples from healthy children. Genome-wide cytosine methylation profiling analysis was carried out using the HpaII tiny fragments enrichment by ligation-mediated PCR (HELP) assay. A high degree of agreement was found between epigenetic subtypes and genetic subtypes. Furthermore a core set of epigenetically deregulated genes were common to all cases, suggesting their involvement in a central role in the initiation or maintenance of lymphoid transformation.

Nordlund *et al.* [1] used the Infinium HumanMethylation450 Beadchip (450K BeadChip) to investigate genome wide DNA methylation signatures in pediatric ALL, and were able to characterize different groups through methylation pattern changes and these corresponded closely to the known cytogenetic subtypes. They found that the methylomes of ALL cells shared 9402 predominantly hypermethylated CpG sites compared with controls, these observations were seen across all ALL subtypes. A unique set of hypo/hyper-methylated CpGs were identified for each cytogenetic subtype. Subtype specific altered methylation strongly associated to gene expression in the promoter and enhancer regions. Six thousand six hundred and twelve CpG sites were also found predominantly hypermethylated in ALL cells at relapse compared with matched samples at diagnosis. Although a significant association between methylation at diagnosis and subsequent relapse was only seen in the *ETV6/RUNX1* subtype. These findings were followed up by developing methodology for DNA methylation profiling for pediatric ALL [14], enabling the clarification of a heterogeneous group of cytogenetically undefined ALL patients. Gabriel *et al.* [15] were unable to predict ALL patient relapse using the 450K BeadChip or to replicate the *ETV6/RUNX1* relapse signature identified

by Nordlund *et al.* However, this study did reproduce the correlation Nordlund *et al.* [1] identified between genome wide DNA methylation pattern and the different cytogenetic subgroups, and validated many of the cytogenetic specific markers. This indicates that subtype specific patterns of altered methylation are consistent across different cohorts.

Epigenetic remodeling of pediatric B-cell ALL (B-ALL) has been investigated in reference to normal B-cell precursors [16] using whole-genome bisulfite sequencing and 450K BeadChips. The analysis of 227 B-ALL patients from the California Childhood Cancer Study revealed two tracks of epigenetic alterations. *De novo* methylation occurred at small functional compartments, for example, CGIs, promoters, TF-binding sites and DNase hyper sensitive sites. While demethylation in subsets of leukemia was apparent in large inter-compartmental backbones, although this change was subtle. CGIs were *de novo* methylated throughout promoters and bodies rather than gene bodies, a suggested crude yet potent way of gene silencing. In regions with hypermethylation there was an enrichment of *CTBP2* sites, suggesting that *CTBP2* may recruit factors which drive the observed hypermethylation.

MIRA-seq has also been utilized to identify differentially methylation regions (DMR) in ALL compared with healthy precursor B cells isolated from cord blood, with a total of 15,492 regions losing methylation, and 9790 regions gaining methylation [17]. The majority of DMRs associated with a CGI were hypermethylated, although roughly 80% of the total DMRs were identified in intronic or intergenic regions. Functional gene analysis revealed that 70% of the intergenic DMRs were associated with functional regulatory elements. Genes which are regulated by DNA methylation and provide a selective growth advantage to cancer have been named epi-driver genes [18], and provide insight into the progression of cancer as well as being valuable therapeutic targets. These findings elaborate and strengthen previous research proving evidence for alterations in DNA methylation in ALL and their possible implications in the causal pathway, disease progression and relapse [1,12,19,20]. These alterations in DNA methylation are an invariable feature of development of ALL and environmental factors that disrupt DNA methylation patterns could lead to increased risk to all subtypes of ALL, or increased risk of specific subtypes depending on the genomic regions affected.

DNA methylation & the environment

DNA methylation is susceptible to change through environmental influences [9], and it has been suggested that it may provide a lifetime record of a person's exposure to environmental exposures [21]. Reports from the

literature provide evidence that a range of environmental exposures can influence DNA methylation [22,23]. There are several avenues through which environment has the potential to influence methylation patterns, illustrated in Figure 1.

In the early stages of life, epigenetic marks undergo radical changes, and there appears to be two main cycles of reprogramming in mammalian embryos. The first cycle occurs following fertilization, when the DNA methylation marks of the parental gametes are erased in two waves of demethylation. This is followed by a second cycle of remethylation during germ cell development, creating a more developmentally restricted epiblast [24,25]. As this developmental stage is therefore key in the establishment of epigenetic marks that will be passed on through the life course during mitosis, early life environmental exposures which may influence the establishment of these marks have the potential to affect gene expression in later life [26]. Indeed mouse studies which have utilized the *Agouti variable yellow* (*A^{vy}*) metastable epiallele as an epigenetic biosensor for environmental effects on the fetus have demonstrated that maternal supplementation during pregnancy with methyl donors, genistein and ethanol can influence methylation at this gene locus. This change in methylation was concomitant with altered phenotypic outcomes [27–29], providing evidence supporting the hypothesis that environmental influences on the epigenome during early life can affect adult phenotype. Waterland *et al.* [30] identified metastable epiallele loci in the human genome and subsequently observed elevated DNA methylation of those loci in individuals conceived during the nutritionally challenged rainy season in rural Gambia. Maternal aflatoxin B1 exposure has also been observed to alter DNA methylation of 71 loci when measured utilizing 450K BeadChip platform in white blood cells from infants in the Gambia [31], which the authors suggest may be relevant to aflatoxin-related child stunting. Furthermore, the effect of dietary exposure during gestation was evaluated using a quasi-experimental setting of the Dutch Famine of 1944–1945 [32]. Methylation levels were measured using the 450K BeadChip on whole blood samples from 422 individuals roughly aged 59 years. Famine exposure during the first 10 weeks of gestation was associated with altered methylation at various CpG sites linked to genes which are involved in growth, development and metabolism. Within the Dutch Famine cohort increased coronary heart disease, raised lipids, altered clotting and more obesity have been reported in association with exposure to famine in early gestation [33]. Taken together this may suggest a critical time window of susceptibility to change in DNA methylation via environmental influences.

Figure 1. Overview of pathways by which environmental factors may influence DNA methylation. Environment may influence DNA methylation at any time in the life course, however, critical windows exist (i.e., during early development *in utero* and early life known as developmental programming) whereby these factors may have a more profound influence. Environmental factors may affect DNA methylation directly (white arrows) and indirectly (grey arrows). Direct pathways include altered substrate availability, in other words, of the universal methyl donor, SAM which is a substrate used to methylate DNA; altering the expression of genes responsible for maintaining or establishing methylation patterns (i.e., *DNMTs* enzymes); altering other regulatory epigenetic mechanisms which influence methylation patterns. Indirect pathways include altering expression of genes responsible for substrate availability (i.e., genes involved in OCM which is responsible for the generation of SAM); altering other epigenetic factors which may influence substrate availability through further gene expression changes. Genetic factors (indicated by dashed black arrows) are also likely to affect substrate availability, gene expression and other epigenetic factors, and may interact with environment to influence DNA methylation levels. OCM: One carbon metabolism; SAM: S-adenosylmethionine.

Identification of exposures associated with risk of developing childhood ALL

Literature searches were carried out to identify exposures associated with ALL risk (listed in Table 1) using the PubMed and ScienceDirect databases (1987–2015). An initial search was performed to identify reviews discussing ALL and possible causes or associations with an increased risk. This created a list of possible risk factors for ALL and key words (Box 1) were used for a more rigorous investigation of the literature to confirm the association between risk factor and leukemia development. There were no language restrictions imposed. In addition, the lists of references in previous studies (including reviews) were also screened to identify additional relevant studies. We subsequently investigated the literature for evidence of variation in DNA methylation in response to the exposures identified by the above analysis as having published evidence linking them to ALL risk. These further literature searches were carried out using the PubMed and ScienceDirect databases (1987–2015). The key words used were ‘DNA methylation’ along with key words stated for each of the exposures identified (Box 1). Each individual search produced varying amounts of literature supporting an association between an exposure and

changes in methylation patterns. Duplicate publications retrieved from different databases were removed. Where large numbers of publications were found, human epidemiological studies using array based technologies to analyze DNA methylation were discussed as a priority over animal or cell line studies using site specific methods to measure DNA methylation.

Environmental factors associated with ALL risk

A number of *in utero* and early childhood exposures have been implicated in the etiology of childhood ALL [110]. These include birth weight, breast feeding, infection history, childcare/day care attendance, smoking, alcohol, caffeine, folic acid, iron, radiation, household chemicals, paints, pesticides and herbicides (see Table 1). While these listed exposures have all been observationally associated with increased risk of ALL, the weight of supporting evidence for the role of each exposure in the etiology of childhood ALL varies. While there is fairly strong evidence in support of the role of some exposures (such as day care attendance, radiation, folic acid, smoking and alcohol) for ALL risk through replication, biomarker and genetic studies, for other exposures (i.e., iron, caffeine,

pesticides/herbicides, paints and chemicals) the evidence base is much weaker. One reason for this is the lack of accurate exposure data available for such studies, as most often this is collected retrospectively and exposure measurements are often not optimal. Furthermore, given the relative rarity of the occurrence of ALL, estimating the effect of what could potentially be subtle changes in environmental exposure on risk is difficult as large numbers of cases are required to accurately assess the impact of such factors on disease risk. Therefore alternative approaches are required to strengthen the evidence for a role of environmental factors in risk of childhood ALL. As discussed, DNA methylation can be influenced by environmental exposures and is also aberrant in leukemic cells, and therefore may act as a mediator between environment and disease outcome, and as a secondary event in the multiple hit pathway to ALL (see Figure 2). The following sections summarize evidence from the current literature regarding environmental exposures and ALL risk and, to what extent these exposures have also been associated with variation in DNA methylation.

Prenatal exposures

Smoking

The knowledge of the carcinogenic properties of cigarette smoke, with an estimated 7000 chemicals affecting the body, led to studies to investigate the effect maternal smoking on ALL risk. Studies have offered conflicting evidence surrounding the association between ALL and prenatal/maternal smoking. John *et al.* [44] were one of the first studies to find an association between maternal smoking and ALL risk. A case-control study was used, and smoking data were attained by 1:1 interviews of parents from 223 cases of childhood cancer (diagnosed in Denver, Colorado 1976–1983). An increased risk of childhood cancer including ALL was observed in mothers who smoked during their first trimester of pregnancy. However, subsequent studies have found no association between maternal smoking and ALL [35–36,42]. It is pertinent to indicate that most studies utilize data collected from telephone interviews, questionnaires and 1:1 interviews with mothers. A recent review confirmed the supposition of reporting bias on self-reported smoking [111]. Previous literature was systematically reviewed and trends of underestimation were shown when the evidence is based on self-reports compared with using biomarkers. A recent meta-analysis investigated the association between childhood ALL and maternal smoking during pregnancy [43]. Data were analyzed from 21 individual studies conducted between 1999 and 2014. An association between maternal smoking and ALL was found, but the authors were not able to

Table 1. Literature providing evidence for positive and negative associations between environmental exposures and acute lymphoblastic leukemia risk.

Environmental exposure	Ref.
Prenatal exposures	
Smoking	[34–44]
Alcohol	[35,36,45–52]
Folic acid	[40,52–61]
Caffeine	[36,42,62–65]
Iron	[66–71]
Pesticides and herbicides	[72–80]
Paints and chemicals (home/occupational use)	[76,81]
Post-natal exposures	
Infection history	[3,82–89]
Childcare and day care attendance	[3,49,84,87,88,90–104]
Radiation	[105–107]
Breast feeding	[88,94,104,105]
Birth weight	[88,108,109]

evaluate the effect of quantiles of cigarettes used by the women during pregnancy.

Associations have also been found between paternal smoking at home, parental smoking after birth, and number of smokers in the household and increased risk of ALL [38]. More recently, paternal smoking pre-conception reported through retrospective telephone questionnaires was found to be significantly associated with ALL [42]. These data were consistent with a previously published meta-analysis which used data from 18 epidemiological studies and analyzed dose-response relationship between ALL risk and smoking, finding associations with paternal smoking preconception and during pregnancy [112]. The possibility remains that this association may be confounded due to the concordance observed between maternal and paternal smoking, and thus the null associations observed between maternal smoking and ALL may be due to maternal self-report bias. Conversely, evidence from animal models suggests that paternal environmental factors can influence the sperm epigenome and pregnancy outcome [113], and influence methylation patterns of the offspring [114,115], which may suggest that paternal, as well as maternal smoking may play a role in offspring ALL risk.

While smoking clearly impacts DNA damage which is important in carcinogenesis, and therefore may influence risk of ALL [37], DNA methylation, which is also altered through smoking [34,116], may be an additional mechanism involved in the causal pathway leading to ALL. The possible long-term effects that either mater-

Box 1. Key words used to identify literature on specific environmental factors associated with acute lymphoblastic leukemia risk.

Leukemia, acute lymphoblastic, radiation, smoking, alcohol, folate, folic acid, iron, coffee, caffeine, herbicides, pesticides, household chemicals, chemicals, household paints, paints, childcare, day care, breast feeding, infection history, birth weight, infection, virus, and bacterial.

nal or paternal smoking may have on offspring DNA methylation patterns may lead to the development and progression of disease through altered gene expression.

A large epigenome-wide association study (EWAS) recently investigated extensive genome-wide changes in DNA methylation in association with current, former and never tobacco smoking in the KORA cohort (Cooperative Health Research in the Region Augsburg) [116]. Significant site-specific differences were observed in each of the 22 autosomes, identifying 187 CpG sites with differential methylation associated with smoking. Importantly it was also noted that even after participants stopped smoking there were still measured differences in methylation, showing the long term impact that smoking can have on DNA methylation [116]. While this study does not measure the effects of smoking during pregnancy and possible effect on offspring, it demonstrates that the effect of tobacco smoking on DNA methylation is evident, even long after cessation.

There is, however, evidence from the literature suggesting that maternal smoking can influence DNA methylation in offspring. Joubert *et al.* [34] used the 450K BeadChip to measure differential methylation related to maternal smoking during pregnancy in 1062 newborn cord blood samples from the Norwegian Mother and Child Cohort Study (MoBa). Maternal plasma cotinine, an objective biomarker of smoking, was measured during pregnancy and related to offspring cord blood DNA methylation [34]. In addition, maternal self-report of smoking during pregnancy was also related to offspring methylation. Twenty-six CpGs mapped to ten genes were found to be differentially methylated in association with maternal smoking, assessed by measuring plasma cotinine levels in cord blood. These included *AHRR*, *CYP1A1* and *RUNX1* (aka *AML1*). *AHRR* and *CYP1A1* are of particular interest as they encode proteins known to be involved in the detoxification of compounds from tobacco smoke (polycyclic aromatic hydrocarbons) [39]. *AHRR* codes for an evolutionary conserved bHLH-PAS (basic helix-loop-helix/Per-AHR nuclear translocator [ARNT]-Sim) protein. This protein mediates toxicity via the

aryl hydrocarbon receptor signal cascade, which is also responsible for regulation of cell growth, cell differentiation and the modulation of the immune system [116]. Also of interest to this review, *RUNX1* which is involved in the development of normal hematopoiesis and leukemia. Differential methylation at the 26 CpG sites found to be related to smoking in the discovery study (MoBa) was further investigated in a replication study (using maternal self-reports). The replication population which consisted of 18 children born to smoking mothers and 18 children born to nonsmoking mothers from the US Newborn Epigenetics Study (NEST). Of the 26 CpGs measured, 21 were found to have altered methylation in association with maternal smoking in the replication cohort. In the same cohort (MoBa), Joubert *et al.* [41] also evaluated the impact of timing of the mothers smoking. A significant association was only found with sustained smoking exposure (through at least 18 weeks gestation). *AHRR* and *RUNX1* were again highlighted as genes which had altered methylation due to smoking exposure. The most recent EWAS to date analyzing the effect of smoking on DNA methylation observed dose-response associations for 15 CpG sites in seven genes (including previously mentioned *AHRR* and *CYP1A1*) in cord blood [117]. Longitudinal analysis of the effects of smoking on DNA methylation at age 7 and 17 years demonstrated that some CpG sites methylation changes were reversed, while others such as *AHRR* and *CYP1A1* showed persistent altered patterns of methylation. This demonstrates that offspring methylation differences induced by prenatal smoking exposure persist during childhood, providing a potential mechanistic link between *in utero* exposure and later disease risk.

Altered methylation has been documented in fetal liver samples (from elective terminations between 11 and 21 weeks gestation) from mothers who smoked compared with controls [118]. For accurate classification of smoking status cotinine concentrations were measured. DNA methylation was measured at a number of regions known to be important in controlling the *IGF2*, which has been previously described as susceptible to the *in utero* environment. The male fetal liver samples showed an increase in DNA methylation at one CpG site within the *H19* imprinting control region, whereas female fetal liver samples showed a decrease in methylation at multiple CpG sites within the *IGF2* DMR associated with smoke exposure [118].

Alcohol

Studies have investigated the association between maternal alcohol drinking as a nongenetic risk factor for ALL [35]. Alcohol is recognized as being carcinogenic for humans and can affect the fetus via ethanol crossing

the placental barrier. Acetaldehyde has the ability to initiate mutagenic activity within the fetus and can be directly ingested through alcohol consumption by the mother or as a result of alcohol metabolism [48]. There is conflicting evidence with respect to the relationship of alcohol and ALL risk, some in support of an association [35,36,47], some reporting no association between alcohol and ALL risk [48,49]. In an early study, Petridou *et al.* [49] assessed the association between alcohol and childhood leukemia in a case–control study comprising 153 confirmed cases of childhood leukemia in Greece, with interviewer-administered questionnaires for exposure data collection. They found an inverse association with maternal alcohol consumption (small or moderate intake) and ALL. In another study, data collected via telephone interviews highlighted specific time frames of maternal alcohol intake that were shown to increase the risk of ALL [35], with the susceptible period being in the second or third trimester of pregnancy. Menegaux *et al.* [36] carried out a multicenter case–control study (280 incident cases and 288 hospitalized controls) in which data were collected during 1:1 interviews. Their study showed significant associations with alcoholic beverage consumption (wine, beer, spirits) during pregnancy and ALL, with a higher odds ratio for children diagnosed at less than 2 years of age. A review of data published on the effect of parental alcohol consumption and childhood cancer observed that roughly a third of the epidemiological evidence evaluated (published between 1982 and 2003) found at least one statistically significant risk increase in relation to parental drinking [47]. The first meta-analysis investigating *in utero* exposure to alcohol and its relationship with ALL found no significant association, although this report was deemed inconclu-

sive due to the lack of appropriate published data [48]. When interpreting these data we must consider the potential problems with the method of data collection used. Reporting bias may influence the accuracy of the data as the information regarding alcohol intake during pregnancy was collected retrospectively which may introduce recall bias. Furthermore, there is also the possibility of under reporting intake during 1:1 or telephone interviews due to the stigma of alcohol drinking while pregnant.

Interactions between alcohol consumption and maternal folate intake have been reported to influence ALL risk. Several associations between genetic variations of folate pathway genes and risk of ALL have been recorded, which varied depending on levels of maternal folate and alcohol intake [52]. This provides evidence for a possible cumulative effect; in other words, being exposed to multiple ALL associated environmental exposures could create a higher risk, further strengthening the hypothesis that ALL requires multiple ‘hits’ to reach full disease state.

There is evidence from the literature to suggest that DNA methylation may be influenced by alcohol intake. A significant increase in DNA methylation was found in the *HERP* promoter in the blood of patients with alcohol dependence compared with controls. This was significantly associated with elevated homocysteine levels [50] (raised homocysteine levels have been found in social drinkers [51]). Since elevated homocysteine concentrations can influence genomic and gene-specific DNA methylation in peripheral blood cells [119], the elevation of homocysteine may account for the observed associations between DNA methylation and alcohol intake. Therefore alcohol consumption may induce epigenetic modification via ethanol-related dysfunction

Figure 2. Plausible causal pathway to acute lymphoblastic leukemia: an initial genetic abnormality, followed by an alteration in DNA methylation influenced by an environmental exposure.

to one carbon metabolism (OCM) [45]. Furthermore, neonatal exposure to ethanol in rats resulted in global disruptions in DNA methylation [120], suggesting that maternal alcohol intake may affect epigenetic programming of offspring. Although the mechanism by which alcohol-induced methylation changes occur is unclear, one suggested pathway maybe via altered one-carbon metabolism.

Folic acid

Epidemiological studies have provided evidence of the importance of folic acid in the maternal diet for fetal development [121], and suggest that it may have a protective role against some childhood cancers [122]. The protective role of folate is considered to be due to its ability to influence DNA synthesis, repair and methylation through the OCM pathway [123].

There are a number of studies which support the protective effects of maternal folic acid supplementation [54,56], and specifically for ALL [52,55,60]. One of the largest studies to date investigating maternal folic acid supplementation suggested that prenatal use of folic acid supplements reduces the risk of ALL [60]. Maternal supplementation data were obtained on 6963 ALL cases (and controls) from multiple case–control studies participating in the Childhood Leukemia International Consortium (CLIC). It was also acknowledged that the observed association varied by parental education which was used as a proxy for lifestyle and socio-demographic characteristics. Amigou *et al.* [55] also found that childhood acute leukemia was inversely associated with maternal folic acid supplements before and during pregnancy. Correlations between genetic polymorphisms of the folate metabolism pathway with ALL susceptibility were also investigated. A positive association with ALL was found with carriers of both *MTRR* variant alleles measured while being homozygous for any variant allele of the *MTHFR* polymorphisms measured. There have also been associations reported between childhood ALL and single SNPs found in *CBS* and *TYMS*, as well as haplotype blocks within *CBS*, *MTHFD1*, *MTRR* and *MTHFR*, and haplotype blocks found just outside *CBS* and *TYMS* [52]. All of these genes code for enzymes or co-factors that are involved in the OCM pathway.

Due to its important role in the biosynthesis of the universal methyl donor, S-adenosylmethionine, altered folate intake has been associated with altered DNA methylation. Indeed, DNA methylation has been reported to be decreased in cells grown in the absence of folate [53], and moderate restrictions in folate intake in human intervention studies displayed reduced genome-wide DNA methylation for women [57,58]. Amarasekera *et al.* [59] specifically investigated the effect of folic

acid on regulation of fetal DNA methylation. Folate status was measured in blood samples collected in the third trimester, and mothers were split into high folate (HF) and low folate (LF) groups. The DNA methylation profiles of the offspring were analyzed in neonatal immune cells (CD4⁺ and antigen-presenting cells) to identify the effects of folic acid. Seven folate sensitive regions were found. Hypomethylation of a CpG dense region upstream of the *ZFP57* was associated with HF. *ZFP57* controls DNA methylation during early multicellular stages of development and is required to regulate and maintain imprinting of genes [59], and has also been suggested as a novel oncogene [124]. Interestingly, Silver *et al.* [61] found that season of conception in rural Gambia affected methylation at *ZFP57*, and identified the genomic region (~3 kb upstream of *ZFP57*) as a potential metastable epiallele. This points the effect that maternal nutrition, including folate, may have on systemic methylation in offspring.

Caffeine

Caffeine has been associated with the risk of low birth weight when consumed daily by pregnant women [62]. However, the relationship between maternal caffeine intake and ALL risk has not been extensively investigated, some studies suggest that increased caffeine consumption may increase ALL risk [63], while others found no association [49]. There are a number of possible mechanisms by which caffeine could increase ALL risk. Caffeine may act as an inhibitor for DNA topoisomerase II, DNA repair or carcinogen metabolism [63]. Via the inhibition of these important cellular processes it could induce chromosomal aberrations and translocations, such as abnormalities of chromosome 11q23 which is one potential cause of ALL [63]. Caffeine may also increase ALL risk via alteration in DNA methylation.

A recent meta-analysis carried out by Cheng *et al.* [63] investigating the risk of maternal coffee consumption and risk of childhood leukemia included seven case–control studies, with a collective total of 2090 cases (AL, ALL and AML) and 3630 controls. The odds ratio for maternal coffee intake increased linearly with the amount of coffee consumed daily; compared with non/lower drinkers (≤ 3 cups/day) of coffee there was a 22% increased risk for ever drinkers (4–8 cups/day), and 72% increased risk for higher level coffee drinkers (> 8 cups/day) and offspring acute leukemia. Findings suggest a significant association between maternal coffee drinking and childhood ALL; this association has also been found in other studies [36].

Some evidence from animal model studies suggests maternal caffeine consumption can alter DNA methylation. Ping *et al.* [62] intra-gastrically treated pregnant

rats with caffeine from gestational day 11–20. Caffeine treatment enhanced the expression of *DNMT1*, *DNMT3a* and *DNMT3b* genes responsible for methylating DNA. It was also associated with a notable increase in total methylation within the *SF-1* promoter, as well as increased methylation frequency at single CpG sites within the *SF-1* promoter. Buscariollo *et al.* [65] treated pregnant A1AR knockout mice with 20 mg/kg caffeine at embryonic day 8.5 and identified altered DNA methylation patterns using DNA methylation arrays. An overall decreased methylation of 26% with 7719 DMRs was observed in adult hearts of offspring exposed to caffeine during pregnancy. These data suggest that further investigations are warranted to understand the role of maternal caffeine consumption on offspring health in human studies.

Iron

Iron is an essential micronutrient required to maintain metabolic homeostasis and genome stability, it partakes in oxygen transport, mitochondrial respiration and metabolizing nucleic acids as well as being an antioxidant. Increased volemia and fetal requirement means pregnant women require more iron. The current recommended daily allowance for pregnant women is 27 mg/day. As for most nutrients, both iron deficiencies and overload are associated with health risks. Iron has the ability to damage biomolecules, which leads to the production of hydroxyl radicals and other reactive oxygen species. Iron possesses the ability to induce a wide array of DNA lesions, from base modifications to strand breaks and adducts [66].

The *HFE* gene has been identified as being associated with cancer susceptibility, including an increased risk of ALL [68,71], and these findings have been replicated [70]. Polymorphisms of the *HFE* gene are now known to have correlations with altered iron status; C282Y polymorphisms impair β 2-microglobulin association and cell surface expression of the *HFE* protein, H63D leads to a loss of ability to reduce transferrin receptor affinity for its ligand. The *HFE* C282Y polymorphism has previously been reported to elevate the risk for ALL [67]. In two independent groups of patients the C282Y mutation has been shown to be associated with altered ALL risk in males specifically [68], suggesting a gender-specific increased risk for hematological malignancies according to genotype. An association with *HFE* and ALL risk appears to be heightened through an interaction with a polymorphism in the transferrin receptor gene (*TFRC*). This increased risk effect may be due to the biological interaction between *HFE* and *TFRC* genes, and iron transfer across membranes such as the placenta and intestinal mucosa [70].

Although there is limited evidence, iron has been reported to have the ability to alter DNA methylation. While iron accumulation/overload has been shown to induce DNA hypermethylation [66], a small positive association was found between *LINE-1* methylation levels in leukocyte DNA and chronic iron exposure measured in toenail clippings [125]. As there is currently very little evidence to support the relationship between iron and DNA methylation further investigations are warranted to better understand this possible interaction.

Pesticides & herbicides

Despite several pesticides and herbicides being classified as probably, possibly or carcinogenic to humans [126], we do not have a good understanding of any long-term health effects of exposures to such agents, particularly in pregnant women and their children. Past studies have revealed weak associations at best between the effects of herbicides and ALL risk [77]. However, over the last decade, a number of case–control studies have shown positive associations with home/garden use of pesticides/herbicides and an increased ALL risk in children [72–76]. All studies reported relationships between exposure and ALL outcome, with some studies observing that timing of exposure was also important. Exposure to pesticides pre-pregnancy, during pregnancy and early childhood, appeared to confer an increased risk of ALL compared with exposures later in life [77].

Environment, home, school and dietary intake are all possible forms of exposure to pesticides/herbicides for mothers and their children, making it hard to avoid exposure, as well as having implications for the measurement and evaluation of exposure [78]. Furthermore, for most studies data were collected through the use of self-reports which are subject to recall bias, as well as there being a lack of available information on the full range of potential active ingredients in products used. Rudant *et al.* [72] specifically investigated the effect of selection bias in previous studies of household exposure to pesticides, and found that even though selection bias was likely within these studies this still did not explain the positive correlation between the use of pesticides and increased risk of ALL. The NCCLS used several different methods to overcome previous limitations by including: quality control of self-reports; a home pesticide inventory and linkage to the Environmental Protection Agency (to obtain active ingredients data); collection and analysis of home dust samples (~600); a geographical environmental database (agricultural pesticides); and large-scale genotyping to assess the role of genes in xenobiotic pathways (transport and metabolism of pesticides) [78]. Findings from a subset (162 leukemia patients and matched controls) of

the NCCLS suggested an amplified risk of childhood leukemia when exposed to household pesticides, and further indicated that timing of exposure appeared to be important (during pregnancy and early childhood significantly increased risk) [77].

Although the evidence is limited, some animal models suggest that pesticide exposure may alter DNA methylation. An alteration in methylation patterns in the hypothalamus of rats was shown after exposure to the agricultural insecticide dichlorodiphenyltrichloroethane, whereby six CGIs were hypomethylated in dichlorodiphenyltrichloroethane exposed rats compared with controls [127]. Furthermore, Desaulniers *et al.* [79] reported that offspring DNA methylation can be altered due to maternal exposure to pesticides. Pregnant rats were exposed to high doses of organochlorine pesticides, methylmercury chloride (MeHg) or polychlorinated biphenyls (PCB) and offspring livers were collected at postnatal day 29. Gene expression levels of *DNMT1*, *DNMT3A* and *DNMT3B* were reduced with high doses of PCB, and for mRNA for *DNMT1* and *DNMT3B* with high doses of MeHg. Pyrosequencing methylation analysis revealed that high doses of PCB and MeHg were also associated with decreased methylation of the *p16* promoter region.

Home & occupational use of chemicals & paints

Evaluating the effect of household chemicals and paints used by parent's pre- and post-natally encounters the same problems as estimating the exposure of pesticides and herbicides, with the potential for recall bias and insufficient data on active ingredients in products used. Despite these difficulties, some studies have provided evidence of an association between the uses of household chemicals and paints, and an increased risk of ALL. Prolonged exposures such as occupational contact have been investigated pre and postnatally [76], with an increased risk of ALL observed in children whose fathers worked with spray paints while the mothers were pregnant, as well as working with spray paints, chlorinated solvents, dyes/pigments, methyl ethyl ketone and cutting oil once the child was born [76]. Research findings vary, with some studies finding strong associations with postnatal exposure to paints, while others, such as solvent exposure, yield inconsistent results and warrant further investigation [81]. There is also evidence that paint exposure appears to be specifically related to the ALL subtype with t(12;21)*TEL-AML1* translocations, although exactly how the exposure influences disease progression is still unclear [81].

One study investigated the influence of several chemicals in relation to epigenetic regulation and established alterations in total DNA methylation as well as at specific gene loci [128]. Chemicals investigated

included bromodichloromethane, dibromochloromethane, chloroform, hydrazine, trichloroethylene, benzidine, trichloroacetic acid and di(2-ethylhexyl) (DEHP). Points of departure for cancer incidence, and change in DNA methylation were studied in laboratory animals (mice, rats and hamsters). A high degree of correlation was found between points of departure for cancer incidence and DNA methylation changes following exposure to environmental chemical carcinogens [87]. The administration of DEHP to pregnant rats and subsequent analysis of male offspring exposed a notably vulnerable epigenome during early developmental periods. This study provides evidence which supports the theory that DNA methylation may mediate the influence of chemical exposures on later cancer development. It also offers a method of testing the potentially harmful effect of chemicals on the epigenome, which could lead to better guidelines for chemical use.

Post-natal exposures

Infection, the immune system & breast feeding

Infection was one of the first suggested risk factors for ALL [82]. There are currently two main infection-based hypotheses for ALL. The Kinlen population mixing hypothesis, which states that the association is due to unusual demographic mixing of susceptible and infected individuals, this happens perinatally and is probably caused by a single novel virus. Current evidence for this hypothesis is the transiently increased incidence of ALL in several situations of population mixing or clustering [3]. The Greaves delayed infection hypothesis suggests that an abnormal immune response to delayed exposure to common infections in childhood due to a lack of early life exposure as infants increases risk of ALL. This hypothesis is supported by studies providing evidence that there is a reduced risk of ALL associated with day care attendance [3].

Greaves hypothesis concentrates on the importance of timing of exposure rather than focusing on specific agents as suggested by the Kinlen hypothesis [83]. Children with a delayed or reduced exposure to common infections at an early age will develop a less adaptive immune system. This could lead to an increased cell proliferation when later confronted with a common infection, thus an increased risk of a second mutation and the development of ALL [84]. The UK Childhood Cancer Study (UKCCS) findings support this hypothesis, showing that a dysregulated immune response to infection in the first few months of life promotes progression to ALL disease later in childhood. A higher frequency of clinically diagnosed infectious episodes was also found to be correlated to an earlier onset of

ALL [89]. Breast feeding is another proxy of early stimulation of the immune system, promoting adequate maturation of the immune system in infants, and has also been inversely associated with ALL [88].

The effect of infections on their hosts epigenetic landscape is becoming more widely discussed, with bacterial [129] and viral infections [130–132] being shown to alter the epigenetics of infected cells. The Epstein–Barr virus (EBV) has already been associated with multiple human malignancies, as well as being shown to have been of high incidence in pediatric ALL patients [133]. This virus can cause lifelong infection of resident epithelial and B cells, resulting in a distinct pattern of EBV gene expression in infected cells which is regulated via epigenetic modifications [132]. The epigenetic effect of EBV has been analyzed in an immortalized keratinocyte cell line. Global DNA methylation analysis showed over 13,000 differentially methylated CpG sites compared with controls, from this 65 genes which acquired methylation presented altered transcript levels. Birdwell *et al.* [132] suggested that the EBV virus may leave a lasting epigenetic imprint that could enhance the tumorigenic phenotype of infected cells. Parvovirus B19 (PVB19) has previously been associated with ALL [131], and a link between PVB19, DNA methylation and ALL has now also been observed. Bone marrow samples of B-cell ALL taken at diagnosis were serologically tested, revealing that samples were positive for PVB19 IgM and IgG. DNA methylation was found to be associated with a history of PVB19 infection, indicated by IgG ($p = 0.02$). This may cause increased leukemogenic potential in susceptible B-precursor cells via PVB19 driven epigenetic alterations [131].

Childcare & day care attendance

Since Greaves hypothesized that delayed exposure to common infections leads to an increased risk of ALL, a number of studies have attempted to provide evidence for this hypothesis including analysis of time spent in day care at a young age, and thus exposure time to common infections in relation to ALL onset [3]. Theoretically, attendance of day care at a young age should mean that a child is confronted with common infections at an early age. This would allow them to build a more sophisticated immune system and reduce the chances of an increased proliferation and risk of mutation if confronted with common infections at a later date [84]. A large body of evidence suggests that there is a connection between early or increased day care attendance and a reduction in the risk of ALL [49,90–103].

A recent study using the findings from the NCCLS evaluated a summarized measure of ‘child-hours of

exposure’, allowing them to capture the variance which can be contributed by individual day care variables [84]. Non-Hispanic white children who attended more than 5000 day care hours during infancy compared with children who did not attend day care had a reduced risk of ALL [84]. Children had a 58% reduced risk for ALL (95% CI: 0.18–0.99) and a 67% reduced risk for precursor-B ALL (Burkitt’s lymphoma/leukemia; 95% CI: 0.11–1.01). The trend was also observed, supporting the theory that there is a dose-response relationship. Also, as Greaves suggested, timing is important and reduction of risk was associated with attending day care during infancy, showing the importance of early life exposure [3].

When considering the effect childcare and day care attendance may have on DNA methylation one must first consider the Greaves delayed infection hypotheses [3], in other words, that childcare and day care attendance act as a proxy for exposure to infection. This appears to provide a protective effect against ALL, with timing and number of hours of care also affecting the risk. On the other hand as mentioned in the previous section infection also appears to increase the risk of ALL. It may be that more serious infections have the ability to influence the child’s epigenome (discussed above) [131]. Consequently delayed or limited childcare and day care attendance could leave a child’s immune system insufficiently matured and more susceptible to more serious infections.

Radiation

Potential cancer risk for children exposed to radiation is much higher than in adults as they are more radio sensitive [105]. A retrospective study found a positive association between radiation dose from CT scans and leukemia, with an almost triple risk of leukemia when children had cumulative doses of roughly 50 mGy [105]. Furthermore, while radiotherapy treatment has contributed to the improved survival rates of childhood cancer over recent decades (30–80%) [106], an investigation into secondary malignant neoplasm occurrences post-radiotherapy in children and adults revealed leukemia as one of the most prevalent secondary malignant neoplasms [107].

Radiation has been observed to induce changes in DNA methylation and there is evidence to suggest that this may lead to an altered cell response to subsequent radiation exposure [134]. In nuclear power plant workers, low dose radiation was associated with DNA methylation levels [89] whereby *LINE-1* methylation levels were higher in radiation exposed-workers than controls. Associations between chromosome aberrations and radiation-induced DNA methylation were also suggested.

Birth weight

The association between birth weight and childhood cancer was originally suggested by MacMahon and Newill over 50 years ago [108]. Paltiel *et al.* [109] pooled data from six cohorts to investigate cancer incidence in relation to infant and parental characteristics, reporting a 26% increased risk of childhood cancer (including ALL) for every kilogram increment in birth weight. No association was found with prenatal overweight or pregnancy weight gain, suggesting that the component of childhood ALL risk explained by higher birth weight is not a consequence of maternal overweight or obesity but likely due to another pathway leading to fetal (over) growth.

The first large EWAS investigating the relationship between birth weight and methylation revealed methylation at 19 CpG sites to be associated with birth weight. Some of the identified CpG sites were located within genes responsible for adipogenesis and DNA repair [135]. A more recent study confirmed two CpG sites identified in the MoBa cohort as well as a further 21 CpG sites were associated with birth weight [136]. Both studies found that several of their birthweight related CpG sites were linked to genes which played an important role in development. Simpkin *et al.* [136] also acknowledged that the effect of birth weight on methylation was predominant in cord blood, and this highlights a potential critical window for the effects of prenatal and early life exposures on DNA methylation, which may impact on future disease risk.

Conclusion

ALL is the most common cancer in children [110], but the causes of this disease are still largely unknown. However, a growing amount of literature now supports the contribution of various environmental factors to risk of ALL development. Roughly 80% of cases are of precursor-B cell origin (CD19⁺, CD10⁺), and the incidence of this specific immuno-phenotype has increased in the Western world over the past several decades [110]. This increase may be due to changes in exposures pregnant women and young children are confronted with in modern everyday life. Indeed, this review suggests that there are a number of environmental exposures which increase the risk of ALL, and therefore warrant further investigation. Although survival rates have improved dramatically over the past few decades ALL survival is associated with a greatly increased ill health in adulthood [137]. This is due to the impact of treatment, thus prevention strategies are desirable. Therefore understanding exactly how the exposures discussed in this review are increasing the chance of ALL will

be critical in understanding the causal pathway to disease. Furthermore, this will provide potential predictive disease biomarkers and plausibly may help determine appropriate and effective preventative intervention strategies.

As an epigenetic modification, DNA methylation, which plays a crucial role in forming cellular identity by influencing gene expression, is likely to be involved in the causal disease pathway of ALL. Indeed, there is a body of evidence to indicate that DNA methylation is altered in childhood ALL [1,14,138–140], but knowledge of how these alterations occur and if they could be prevented will be important in improving understanding of the underlying mechanisms of the disease. Furthermore, since DNA methylation patterns can be environmentally orchestrated, knowledge of the involvement of this mechanism in disease etiology may provide plausible and implementable intervention strategies for high risk individuals such as those with Down's syndrome or Fanconi Anemia. Here we have explored the supposition that environmental exposures associated with ALL risk have the potential to alter DNA methylation thus making DNA methylation a plausible mediator of environmental influences in the pathogenesis of ALL. We have reviewed evidence in support of this hypothesis, and conclude that evidence from the literature could suggest that several environmental exposures associated with increased childhood ALL risk, in other words, alcohol, smoking and folate are able to alter DNA methylation and therefore this may be one mechanism by which these exposures are involved in the causal pathway to disease. *AHRR* and *CYP1A1* are the two clearest genes which exhibit methylation change due to an environmental exposure (smoking), and have similarly been shown to be frequently abnormally methylated in ALL [1]. The review of the literature and crossover between aberrant methylation in response to environmental factors and in ALL is not exhaustive in the context of this review. Further rigorous investigation of the available data is required to explore further connexions, these examples add weight to the hypothesis that DNA methylation may act as a mediating mechanism in this context. Further consideration should be given to the likelihood of a cumulative effect of exposures, whereby exposure to multiple ALL risk associated factors could further increase the chance of disease development through cumulative epigenetic aberrations. Further research is therefore warranted to investigate this hypothesis in order to aid understanding of the causal pathway to disease, which is vital in facilitating new treatments, initiating preventative strategies and screening for disease.

Future perspective

To understand the pathway from the initial genetic ‘hit’ to a child being diagnosed with ALL, future studies will need to combine multiple investigations toward realization of the multifactorial etiology of ALL. The literature examined in this review provides evidence for a potential role of DNA methylation in ALL development through environmental exposures. Given the rarity of childhood cancers, the availability of robust exposure data and patient samples prior to diagnosis is limited, multiple complementing strategies will be required to further explore this concept. Initially, clearer evidence is required to show that ALL-associated risk exposures result in disease-associated DNA methylation changes. Global DNA methylation alterations seen in ALL are mostly seen across all subtypes [1], and thus appear to be early events in ALL development. However, the importance of these methylation changes in inducing or contributing to disease development is less clear. As environmental exposures can potentially drive these changes in methylation it is important to understand if these changes can in turn drive ALL pathogenesis. Confirmation of the establishment of aberrant methylation patterns in ALL patients prior to diagnosis will be important in determining the role of these events in the causal pathway, however, such studies may be challenging given the lack of biological material available. The utilization of neonatal blood spot samples or collective cases from multiple large cohort studies may be feasible avenues of pursuit for case–control studies in this area. However, while this may estab-

lish proof of aberrant methylation prior to diagnosis, such studies are likely to be unable (through lack of data) or underpowered to detect changes in methylation associated with environmental factors which may be associated with disease outcome. In addition to using the ‘meet in the middle’ approach [141] to link early initiating epigenetic events in disease to environment, Mendelian randomization approaches utilizing genetic instruments as proxy markers for environment [142], will be key where environmental data are lacking but genetic material or data are available. Data from these combined approaches would further support the evidence that environmental factors are drivers in disease progression, and provide a mechanism by which they are part of the multiple hit pathway for ALL development. Such findings may provide predictive disease biomarkers and offer insights into how preventative strategies may be introduced.

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Executive summary**DNA methylation & acute lymphoblastic leukemia**

- Altered DNA methylation has been observed between acute lymphoblastic leukemia (ALL) cells and nonleukemic bone marrow, and as well as between ALL subtypes.
- Nine thousand four hundred and six CpG sites were found to be predominantly hypermethylated across all subtypes in a large scale epigenome-wide association study of ALL cells compared with controls, demonstrating a genome wide disruption of the epigenome.

Environmental exposures relationship with DNA methylation & ALL risk

- Environmental exposures have been associated with an increased risk of ALL.
- Currently there is fairly strong evidence supporting the association with exposures such as day care attendance, radiation, folic acid, smoking and alcohol.
- There is also evidence, although weaker for other exposures associated with ALL risk, in other words, iron, caffeine, pesticides/herbicides, paints and chemicals.
- There is a growing amount of supporting evidence for alterations in methylation caused due to environmental exposures that are also linked to ALL risk, especially for smoking, folic acid and infection.

Future perspective

- Further studies are warranted in order to support and strengthen the evidence for a potential mediating role of DNA methylation between risk exposures and ALL development. Multiple integrated and complementing strategies, in other words, ‘meet in the middle’ approaches will be required to provide evidence of this concept.

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