

A causal mechanism for childhood acute lymphoblastic leukaemia

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Abstract

In this Review, I present evidence supporting a multi-factorial causation of childhood acute lymphoblastic leukaemia (ALL), a major subtype of paediatric cancer. ALL evolves in two discrete steps. First, *in utero* initiation by fusion gene formation or hyperdiploidy generates a covert, pre-leukaemic clone. Second, in a small fraction of these cases, the post-natal acquisition of secondary genetic changes (primarily V(D)J recombination-activating protein (RAG) and activation-induced cytidine deaminase (AID)-driven copy number alterations in the case of *ETV6-RUNX1*⁺ ALL) drives conversion to overt leukaemia. Epidemiological and modelling studies endorse a dual role for common infections. Microbial exposures earlier in life are protective but, in their absence, later infections trigger the critical secondary mutations. Risk is further modified by inherited genetics, chance and, probably, diet. Childhood ALL can be viewed as a paradoxical consequence of progress in modern societies where behavioural changes have restrained early microbial exposure. This engenders an evolutionary mismatch between historical adaptations of the immune system and contemporary lifestyles. Childhood ALL may be a preventable cancer.

[H1] Introduction

Childhood acute leukaemia is the most common paediatric cancer in developed societies, accounting for one third of all cases with a variable incidence rate of 10–45 cases per 10⁶ children per year and a cumulative risk of ~1 in 2000 up to the age of 15 years ¹. The most common paediatric leukaemia, acute lymphoblastic leukaemia (ALL), is an intrinsically lethal cancer, as evidenced by a universally adverse clinical outcome before effective therapy was developed ². Currently, however, cure rates for ALL using combination chemotherapy are around 90% ³, making this one of the real success stories of oncology.

Whilst this is a cause for celebration, the current treatment remains toxic, traumatic for young patients and their families and with some long term health consequences ^{4,5}. It is unfortunate that we have remained ignorant as to the cause of ALL. The open question as to whether this cancer is potentially preventable is therefore important.

Environmental exposures possibly linked to ALL are legion ⁶ but in many cases these associations are weak, inconsistent or lacking in biological plausibility ⁷. Large and multi-disciplinary nationwide studies or international consortia ^{8,9} have provided a more enabling framework for addressing this question but, to date, the only accepted causal agent for ALL, albeit under exceptional circumstances, is ionising radiation ¹⁰⁻¹². The causes of ALL might best be understood by using biological insights into the cancer itself as the foundation for designing, testing and validating hypotheses.

Childhood ALL includes a number of subtypes defined by cell lineage (B or T), differentiation status and genetics (Fig 1a). These differ by age distribution (Fig 1b), clinical outcome (see Fig 1 legend) and could have distinctive aetiologies. In this Review, I focus on the body of evidence – epidemiological, biological and genetic, that has accumulated, particularly over the past decade, supporting a causal mechanism that is selective for the common, or B cell precursor, subtype of childhood ALL (designated here as BCP-ALL) ⁷. This is suggested to be a multi-factorial mix of infectious exposure, inherited or constitutive genetics plus chance, with patterns or timing of common infection in early life identified as the critical component and a potential route for preventative intervention.

[H1] Infection hypotheses

The idea that infections might play a causal role in childhood ALL is around 100 years old ¹³. When it became clear that leukaemia in a number of animal species – chickens, mice, cattle and cats, were viral in origin ¹⁴, there was an expectation that a similar transforming virus might

be responsible for childhood ALL, as well as for other blood cell cancers. To date, all attempts to molecularly identify or otherwise incriminate a leukaemogenic virus in ALL have failed ⁷.

In 1988, two hypotheses were presented that suggested a new perspective on this problem. The two models are sometimes considered as alternative or competing explanations. I believe they portray the same picture, through different lenses. Both propose that childhood leukaemia may arise as a consequence of an abnormal immune response to common infection(s). One model, advanced by epidemiologist Leo Kinlen, was based on transient and localised increases in the incidence of childhood leukaemia that could be ascribed, epidemiologically, to population mixing ^{15,16} (Box 1).

The other model that I proposed was dubbed the ‘delayed infection’ hypothesis, the focus of this article, and was more biological than epidemiological in its origins and was applied specifically to BCP-ALL ^{7,17}. Central to this were two propositions.

First, that the immune system, in both its innate and adaptive arms, had evolved to both anticipate and require microbial infectious exposure peri-natally or in infancy ¹⁸. The dynamics and composition of the microbiome and virome of infants is highly variable ¹⁹ and early, microbial exposures have lasting impacts on immune function and health ^{20,21}. Metabolites of commensal bacteria promote regulatory T cells and impact on subsequent inflammatory signalling pathways ²². Deficits of this ‘natural’ microbial experience, especially in modern societies, result in an unmodulated or distorted immune network ²³.

A consequence of an under-exposed immune network in infancy was predicted to be subsequent dysregulated responses to common infections that could promote or trigger BCP-ALL. The increased incidence in childhood ALL in developed societies was therefore considered to be a paradox of progress, and the link to infection inverted: the problem might be lack of infection. An equivalent mismatch of evolutionary adaptations and modern lifestyles may underlie the causation of several common adult cancers in the developed world ²⁴.

The second proposition related to the natural history of the disease. The speculation was that ALL most likely developed by two critical steps: first, an initiating event *in utero* and second, a post-natal mutational event that promotes clinical leukaemia development. The prediction was then that an abnormal immune response to infection(s) indirectly triggered the requisite secondary mutational events. No specific infection was proposed in relation to either protection in early life or post-natal promotion and the immunological mechanism was considered to be indirect and therefore not akin to a transforming virus.

Common infections were therefore proposed to have two opposing impacts on risk of ALL, depending upon timing – antagonistic (early) or promotional (late). A parallel would be with the divergent roles of microbial infections and chronic inflammation in gastro-intestinal and other common cancers in adults ^{25,26}.

[H1] The two-hit model of childhood ALL

With a few informative exceptions (Supplementary Information S1 (Box)), ALL has a clinically silent natural history prior to diagnosis. My colleagues and I developed three different tactics for back-tracking the origins of this covert process to before birth. These exploited the fact that common fusion genes in ALL (e.g. *ETV6-RUNX1* (also known as *TEL-AML1*), *MLL-AF4* (also known as *KMT2A-AFF1*, *BCR-ABL1*) have uniquely variable or idiosyncratic breakpoints within the intronic, breakpoint cluster regions of the two partner fusion genes involved. The genomic sequences at the gene fusion junction provide stable, sensitive and clone-specific markers ^{27,28}.

[H2] Comparative genomics of concordant ALL in monozygotic twins

Studies on monozygotic twins have been especially informative ²⁹. The possibility that concordance of leukaemia in identical twins might be attributable, not to co-inheritance of

genetic susceptibility, but to an *in utero* origin in one twin was first proposed in 1962³⁰ and elaborated on in 1971³¹. This idea was based on prior understanding that monochorionic or single placentas have vascular anastomoses permitting twin-twin blood transfusion with consequent blood cell chimaerism³².

The prediction was then that ALL in both twins should arise in one twin but be monoclonal. Unambiguous evidence that this was the case derived from the finding of identical, but non-constitutive, clone-specific fusion gene breakpoints and sequences in a series of twin pairs^{29,33} (Fig 2). That evidence is strengthened by the observation of shared, clone-specific immunoglobulin heavy chain (*IGH*) diversity-joining (*DJ*) or variable-diversity-joining (*V(D)J*) genomic sequences of concordant BCP-ALL in twins³⁴.

With further exploration of ALL genomes in twins, it has become clear that whilst cases with concordant ALL share the identical and singular fusion gene event, other genetic alterations present, including copy number alterations (CNAs) and single nucleotide variants (SNVs), were different in twin pairs^{35,36} (Fig 2). This then suggested that such distinctive mutational events reflected independent and divergent sub-clonal evolution, post-natally. Similarly, the majority of ongoing *V(D)J* rearrangements in *IGH* are sub-clonal and distinctive in twin pairs³⁴. In one twin pair with concordant *ETV6-RUNX1*⁺ ALL, whole genome sequencing revealed that the fusion gene was the only shared or clonal genetic lesion³⁷. These data endorsed the likelihood that *ETV6-RUNX1* fusion was an initiating event or founder mutation for ALL.

The concordance rate in monozygotic twins varies according to age and ALL subtype. In infants (<18 months) with pro-B ALL and *MLL* fusions, the rate approximates to 100% for those with a monochorionic or single placenta²⁹. This suggested that *MLL* fusion driven leukaemogenesis in such infants was essentially completed *in utero* and that the fusion gene, or a single mutation, was sufficient for leukaemogenesis. Subsequent genomic sequencing of

such cases is compatible with this possibility even though other sub-clonal mutations, in *RAS* family genes for example, do occur^{38,39}.

[H2] Pre-leukaemic clones in healthy co-twins

The concordance rate in older children with BCP-ALL was calculated to be around 10-15%, lower than that in infants²⁹. A prediction for those pairs of twins with a monochorionic placenta, where only one twin develops ALL, is that the healthy co-twin should have a population of covert pre-leukaemic cells harbouring the same initiating lesion as his/her co-twin with ALL, i.e. the twins are discordant for the critical post-natal secondary, genetic event. This has been confirmed in three twin pairs with BCP-ALL with hyperdiploidy⁴⁰, *BCR-ABL1* fusion³⁶ or *ETV6-RUNX1* fusion^{35,41}. In this context, the healthy co-twin provides a rare ‘experiment of nature’ and unique access to the pre-leukaemic clone. Putative pre-leukaemic cells from the blood of one healthy co-twin and propagation *in vitro* and *in vivo* (in NOD/SCID mice) established that these cells have both self-renewal capacity and intact B cell differentiation capacity⁴¹, features commensurate with a pre-leukaemic status. Equivalent pre-leukaemic stem cells for acute myeloid leukaemia (AML) have now been identified in patients with AML⁴² and normal adults⁴³.

[H2] Backtracking early genetic events in ALL to neonatal blood spots

Less than 1% of childhood BCP-ALL cases occur in twins. However, ALL in twins is no different, in its biological and clinical features or age incidence, to that in singletons. This suggests that many or most childhood ALL cases in singletons might also be initiated *in utero*.

To validate this proposition, my colleagues and I exploited the fact that neonatal blood spots, or Guthrie cards, contain reasonably intact DNA. Archived blood spots from patients with ALL were screened for clone-specific fusion gene sequences identified at diagnosis. This

was first carried out in three cases of infant ALL with *MLL-AF4* fusion and blood spots from all three cases evaluated were positive⁴⁴. Subsequent studies with samples from children with *ETV6-RUNX1*⁺ ALL found that around 75% were positive⁴⁵. These results have been independently confirmed^{46,47}. Negative blood spot results are uninterpretable as this could reflect either a post-natal origin or an inadequate number of leukaemic cells in the sample. The conclusion drawn from these screens was therefore that the majority of childhood ALL cases were pre-natal in origin, though possibly not all.

The twin and blood spot studies also provided insight into persistence of pre-leukaemic stem cells and post-natal latency in ALL. The oldest twin with concordant ALL originating *in utero* was 14 years at diagnosis, her twin sibling having been diagnosed with ALL some nine years earlier⁴⁸. The oldest non-twin case with ALL and a positive neonatal blood spot to date was diagnosed at 9 years, 4 months old⁴⁹.

[H2] Frequency of ALL initiation in utero

The data on discordant, monozygotic twins suggested that some or possibly most individuals harbouring a pre-natally generated, covert pre-leukaemic clone never progress to overt ALL. This begs the important question, relevant to aetiology, of the frequency of initiation of ALL *in utero* and the frequency of its transition to overt leukaemia.

To address this issue, my colleagues and I screened a large cohort of unselected cord blood samples for *ETV6-RUNX1* fusion mRNA (data summarised in Supplementary Information S2 (Fig)). The striking result was that approximately 1% of newborns (6/567) had a covert and modest sized, putative pre-leukaemic population at around 10^{-4} of B lineage cells⁵⁰. This result, initially challenged⁵¹, has been independently confirmed⁵²⁻⁵⁴. A ~1% incidence for *ETV6-RUNX1* in relation to incidence of the leukaemia itself reflects a low transition probability of around 1% with 99% of pre-leukaemic clones initiated during foetal

development never progressing to clinical ALL. This low transition probability could reflect either lack of persistence of the pre-leukaemic stem cells after birth or a severe bottleneck in acquisition of the necessary secondary genetic changes.

These data suggest that initiation of leukaemia *in utero* may be far more common than indicated by the incidence of disease, with implications for causation. The same may apply to some other paediatric cancers. Histological evidence and some genetic data suggest that the frequency of precursor lesions for neuroblastoma ⁵⁵ and Wilms tumour ⁵⁶ may also be some 100 times the incidence of clinical cancer ⁵⁰.

[H2] Other subtypes of ALL

These lines of investigation were pursued using fusion genes as the predominant clonal markers of early genetic events in ALL. The most frequent subtype of BCP-ALL is, however, characterised by chromosomal hyperdiploidy, which is harder to track than fusion genes. There is evidence however that the key findings described above for the *ETV6-RUNX1* subset are likely to apply to hyperdiploid ALL. Monozygotic, monochorionic twin pairs concordant for hyperdiploid ALL are described with identical karyotypes ⁴⁰ and neonatal blood spots of children with hyperdiploid ALL have clone-specific *IGH* sequences ⁵⁷⁻⁵⁹. In one case of hyperdiploid ALL, the child's cord blood had been frozen at birth. Retrieval of this sample led to the identification of putative pre-leukaemic cells in the cord blood with the same triploid chromosomes as in the child's subsequent ALL ⁶⁰. Hyperdiploidy, generated by a one-off abnormal mitosis resulting in trisomies ⁶¹, can therefore occur *in utero* as an alternative initiating event to gene fusion for BCP-ALL.

[H1] Further genomic exploration of ALL

Whole genome sequencing of a cohort of 57 cases of *ETV6-RUNX1*⁺ BCP-ALL provided an audit of all mutational changes ⁶². This confirmed the previous finding that the most common

recurrent events were CNAs, primarily gene deletions⁶³. SNVs were also present but with low or undetectable recurrency⁶². Genomic sequencing in cancer can reveal mutational signatures of relevance to aetiology⁶⁴. Almost 50% of CNAs in *ETV6-RUNX1*⁺ BCP-ALL had partial or complete V(D)J recombination-activating protein (RAG) heptamer-nonamer recognition motifs within 20 base pairs of the breakpoints⁶². This finding may explain the observation that highly recurrent CNAs in BCP-ALL are reiteratively present in subclones of individual patients^{65,66}. A comparison with CNAs in around 14,000 cases of breast, prostate and pancreatic cancer revealed none with RAG motifs⁶². SNVs in *ETV6-RUNX1*⁺ BCP-ALL had two main mutated signatures: one was C>T transitions at CpGs and C>G and C>T mutations at TpCs, and a second was transitions and transversions in a TpC context at NpCpG trinucleotides⁶². These second signatures are very common in cancer generally reflecting APOBEC cytidine deaminase activity^{64,67}.

These genomic studies indicate that BCP-ALL has very restricted but informative mutational signatures and a low level of background or neutral genetic alterations⁶². This makes it less likely that BCP-ALL is caused by genotoxic exposures, which generally precipitate more widespread genomic instability with multiple distinctive signatures⁶⁴.

The other genetic subtype of BCP-ALL – hyperdiploid ALL, also has recurrent CNAs that may be RAG-mediated in genes including *PAX5*, *IKZF1* and *ETV6*. In contrast to *ETV6-RUNX1*⁺ ALL however, hyperdiploid ALL have recurrent mutations in receptor tyrosine kinase (RTK)-RAS pathways and histone modifiers^{61,63}.

Collectively, these data provide a firm cellular, genetic and mechanistic framework for the two step model for BCP-ALL and highlight both critical time windows, pre- and post-natally, and mutational mechanisms. Any proposed causative mechanism in ALL should accommodate this natural history profile. The initiating role of *ETV6-RUNX1* and the postulated sequence of events in ALL is endorsed by modelling within both human and animal

cells (Box 2 and further below). The timing and tissue site of B cell precursor ALL initiated by *ETV6-RUNX1* or hyperdiploidy *in utero* is uncertain but may involve transformation of a unique, foetal liver progenitor cell (Box 3).

[H1] Inherited susceptibility

Childhood ALL only very rarely runs in families but this observation may underplay inherited genetic risk since the disease itself is rare. Twin concordance is unhelpful in this respect since the risk has a mostly non-genetic basis – blood cell chimaerism *in utero*. The risk of ALL in non-identical twins is unknown but sibling risk has been estimated to be around 3.0x compared to the risk in the general population, which provides evidence of a modest but definite contribution of constitutive, genetic variation to risk ⁶⁸.

Earlier, targeted gene screening approaches suggested that inherited allelic variants encoding proteins involved in DNA repair, carcinogen metabolism or the folic acid pathway might be linked to risk of childhood ALL ^{69,70}. Unfortunately most of these studies were under-powered to detect small effects or have not been consistently replicated, so their significance remains uncertain ⁷⁰.

Genome wide association studies (GWAS) have provided unambiguous evidence for multiple gene variants impacting on risk of ALL (Table 1) ^{70,71}. The individual alleles described to date have a significant but relatively modest impact (see odds ratio in Table 1) and appear to be additive rather than synergistic, functionally. The functional logic of these associations is unclear but as most of the relevant single nucleotide polymorphisms (SNPs) lie outside coding regions, they are likely to be regulatory, impacting on levels of protein ^{72,73}.

It is striking that in ALL, as in many other cancers, most of the candidate risk genes implicated in GWAS (Table 1) are the same genes that have acquired (non-constitutive) mutations in the same cancer type. One interpretation of this is that the inherited allelic variants

interact functionally (or epistatically) with the mutated alleles to increase vulnerability of cells to transformation. A low functioning inherited allele for example would render a deletion in the other allele functionally homozygous with a potentially increased impact on cellular fitness. A prediction that follows from this is that there should be a preferential loss, by deletion, of the non-risk allele (in heterozygotes for that allele) as only that deletion would increase clonal fitness. Evidence for this has been presented with respect to risk variants of *CDKN2A*^{74,75}. However, for *ARID5B* there is preferential gain of the risk allele (via trisomy 10) in heterozygotes⁷².

To date GWAS have provided no evidence implicating immune response gene variants, as might have been anticipated from infection-based hypotheses for the aetiology of ALL. However, prior studies examining major histocompatibility complex (HLA) genes⁷⁶⁻⁷⁸, interferon- γ (*IFNG*)⁷⁹, Toll-like receptor 6 (*TLR6*)⁸⁰ or the presence and/or absence of specific killer-cell immunoglobulin-like receptor (KIR) family genes⁸¹ did record significant associations with particular allelic variants. Notably, *TLR6* variants and KIR genes were associated with decreased risk of all childhood ALL. It remains unclear if the large GWAS multi-cohort studies invalidate these data or whether the SNP screens in GWAS adequately detect the relevant variants. This is an important discrepancy to resolve.

Childhood ALL can also arise in a rare familial syndrome context with inherited mutations in genes also implicated as acquired mutations in leukaemia including *PAX5*⁸² and *ETV6*⁸³. The relatively infrequent low hypodiploid subset of BCP-ALL is strongly associated with inherited *TP53* mutations or Li Fraumeni syndrome⁸⁴. Further rare risk alleles, but with intermediate to high penetrance, are likely to be uncovered in ongoing, large scale studies. Children with Down's syndrome have an approximately 20-30 fold increased risk of BCP-ALL⁸⁵. Trisomy 21 in Down's syndrome is associated with over-expression of a nucleosome remodelling protein HMGN1 and enhanced self-renewal of B cell progenitors⁸⁶. In patients

with ALL, this is complimented by secondary genetic changes including in *CLRF2*, *JAK2*, *NRAS* and *KRAS* ⁸⁵. All told, however, familial syndromes and Down's-associated ALL are likely to account for only a small fraction of cases of childhood ALL.

The general conclusion to be drawn from these genetic studies is that inherited susceptibility does contribute to risk of BCP-ALL. The attributable risk or quantitative contribution is, however, unclear. A sibling risk of three fold for ALL, seen against a background risk of 1:2000, suggest the genetic component, though real, is minor; at least compared to some other common adults cancers (prostate, breast) ⁸⁷. On the other hand, there could be a complex interplay between genes and environmental exposures ^{73,88} in which genetic background makes a more substantial difference. This has yet to be fully explored.

[H1] Possible causes of initiating events No epidemiological studies to date have clearly implicated exposures during pregnancy, linked to risk of ALL, that might explain how the initiating mutations for BCP-ALL arise. *ETV6-RUNX1*⁺ BCP-ALL has no mutational signatures that might implicate any particular type of aetiological exposure. There is no evidence for RAG involvement but, in common with *IGH* rearrangements ⁸⁹, the recombination event appears to involve non-homologous end joining via micro-homologies ²⁷.

If around 1% of unselected newborns have an in frame *ETV6-RUNX1* fusion in an expanded clone derived from the appropriate cell type for BCP-ALL (B lineage progenitor), then considerably more newborns should have acquired this or other fusion genes in the wrong cell types or out of frame for a functional protein. It therefore seems likely that whatever causes this genetic alteration, it could be very common or possibly ubiquitous.

The original proposition ¹⁷ was that ALL was initiated, *in utero*, by a spontaneous mutation, or with no external exposure involvement. Developmental, endogenous factors such as proliferative and oxidative stress or the profound apoptotic signalling in early lymphopoiesis

could be involved. Spontaneous mutations or mutations caused by endogenous processes are common during foetal development ⁹⁰. Endogenously-driven double-strand breaks, required for fusion gene recombinants, occur at around 50 per cell cycle in human cells ⁹¹. It has been suggested that most paediatric cancers arise during embryonic or foetal life and can similarly be considered as developmental errors ⁹². In the absence of evidence to the contrary, this remains the most plausible explanation for initiation of BCP-ALL and focuses attention on the post-natal triggering of promotional events which are required for clinical disease. There is clearly scope for more research on the origins and mechanisms involved in fusion gene formation, and hyperdiploidy, *in utero*.

[H1] Epidemiological evidence

Epidemiological evidence suggests that patterns of infection after birth have a causal role in triggering ALL. The ‘delayed infection’ hypothesis lends itself to epidemiological evaluation in a case-control setting. A prediction of the model was that common infections in infancy should be protective against BCP-ALL. There is no prior reason to implicate any particular infectious agent (eg, bacteria, virus or parasite), and the relevant infections need not be symptomatic or pathological. A longstanding need for microbial, immune network modulation might reflect common, commensal, or ‘old friends’ organisms, such as gut microbiota, soil mycobacteria or helminth parasites ⁹³. In this context, a surrogate of overall infectious exposure during infancy could be considered an appropriate variable. Quantifiable surrogates include social exposures of infants in the home, related to the number of siblings and birth order, or in day care centres, and breast feeding. These variables have been investigated in epidemiological case/control or cohort studies for risk associations with ALL overall and, in some instances, selectively for the major BCP-ALL subset.

[H2] Impact of day care attendance in infancy

In the 1990's, the UK Children's Cancer Study Group (UKCCS) was set up to test the delayed infection hypothesis, in a case-control context, in addition to an analysis of other exposures including ionising and non-ionising (EMF) radiation and chemicals⁸. Day care attendance was chosen as one surrogate for infectious exposure since this was well documented as a context for enhanced social contacts facilitating spread of common infections⁹⁴. The UKCCS involved almost 1300 cases of ALL (all subtypes) and over 6000 matched controls. Although only a relatively small number of controls experienced day care in the first twelve months of life, the data showed a significant protective impact on risk of ALL overall and on BCP-ALL⁹⁵.

This association has been documented in additional studies in California⁹⁶, Scandinavia⁹⁷, France⁹⁸ and an international consortium⁹⁹, and has been endorsed by a meta-analysis¹⁰⁰. The meta-analysis noted significant, between study heterogeneity and one early, large scale study failed to detect any impact of day care on risk of ALL¹⁰¹. No protection is afforded against childhood AML by day care attendance or, to date, any other paediatric cancer, which increases confidence that the associations seen in ALL was not confounded by social or other variables.

It was anticipated that assessing, in a case-control fashion, actual infections in infancy would be informative. This however is fraught with difficulties and has provided mixed results. Parental recall is known to be suspect or inaccurate in this respect¹⁰². Medical records are more reliable, particularly in the UK with nationwide registration of children with general practitioners (GPs) and a free health service. One such analysis found more, rather than less, infections reported for children who subsequently developed ALL, compared with controls¹⁰². The main difficulty here, other than possible bias in use of GP services, is that we do not know if the relevant 'modulating' infectious exposures in infancy are necessarily symptomatic; they might well not be. In this sense the surrogate of day care could be considered preferable.

Several studies have however reported, in accord with the hypothesis, an inverse relationship between common infections in early life, including inner ear infections, and risk of ALL^{98,101,103-106}.

[H2] Birth order and risk of ALL

A further surrogate measure of infectious exposures in infancy is the number of siblings cohabiting and, in particular, birth order. The prediction was that first-borns would be more at risk compared to later-borns who would, as infants, benefit from protective exposures via older siblings. One large UK-based study (>3000 cases of ALL and the same number of matched controls) found a striking association of birth order with risk for ALL, but not for AML¹⁰⁷. Other case-control studies in France⁹⁸ and California¹⁰⁰ also found a significantly increased risk for first (versus third) born children, as does a recent international cohort analysis (Ora Paltiel; personal communication 2018)

If natural infections early in life reduce risk of ALL, then it might be expected that some vaccinations would have an effect. The data on vaccination histories have produced null or inconsistent results. However, there is one exception: immunisation against *Haemophilus influenzae B* in infancy appears to confer a degree of protection against ALL¹⁰⁸.

If the ‘natural’ microbiota is part of a longstanding and critical interaction with the developing immune system, then antibiotic use in infancy might increase risk of ALL. This has not been systematically evaluated to date, though an earlier report from China did suggest an increased risk associated with exposure to chloramphenicol¹⁰⁹.

[H2] Mode of delivery, breastfeeding and risk of ALL

Mode of delivery at birth influences the early exposure of newborns to benign microbiota¹¹⁰, as caesarean delivery deprives newborns of the microbial exposures associated with vaginal

passage. Cohort and case/control studies have reported a significantly increased risk of ALL associated with caesarean delivery ¹¹¹⁻¹¹⁴. No such increased risk was observed for brain cancer or lymphoma ¹¹¹.

Breastfeeding during infancy provides nutritional support, maternal antibodies, anti-inflammatory molecules, some maternal cells, microbes (lactobacilli) and oligosaccharides that nourish the infant's intestinal microbiome (bifidobacteria) ¹¹⁵. It might be anticipated that prolonged breastfeeding would have a modulating effect on the immune system of infants and reduce risk of ALL. Seventeen case/control studies of the impact of breastfeeding on ALL risk have been published ¹¹⁶. In the largest of these, from the USA ¹¹⁷ and UK ¹¹⁸, there was a reduced risk for ALL (10-20%) associated with breastfeeding of six months or more. Five meta-analyses have now been published with concordant conclusions and the latest of these indicated a reduced ALL risk of around 20% for breastfeeding of six months or more ¹¹⁶.

[H2] Clusters of ALL

Although no specific microbial agent or a unique transforming virus is suspected in ALL, there might be one circumstance where a single type of infection is involved. This is in the very rare cases of space-time clusters. A prediction of our hypothesis would be that a single cluster of cases might be associated with a single infection or microbe species but independent, space/time clusters could involve different infectious triggers.

Many putative clusters of childhood leukaemia have been reported but two stand out. The first was in of Niles, a suburb of Chicago in 1957-60 – where there were eight cases (relative risk (R.R.) 4.3) diagnosed as ALL or 'stem cell' leukaemia ¹¹⁹. All patients and/or their older siblings attended the same school. The second cluster involved 13 cases of BCP-ALL over four years (2000-2004; R.R. = 12.0), but ten cases in just ten months in 2001 in the small town of Fallon, Nevada ¹²⁰.

A neglected aspect of these two clusters is that the cases, though resident in the cluster area at the time of diagnosis, were mostly born outside of that area⁷. Plus the clusters involved children diagnosed with ALL at different ages (2-11 years) and a narrow time frame of diagnoses. Given what we now know of the natural history of ALL, these data then indicate any causal exposure, linked to the cluster, would have to be proximal in time to diagnosis (rather than pre-natal) and therefore promotional. The Niles cluster was linked, observationally, to an outbreak of streptococcal fever¹¹⁹. The cause(s) of the Fallon cluster of ALL remain unresolved and contentious though a possible role of adenovirus was hypothesized¹²⁰.

A significant space-time cluster of BCP-ALL in Milan has recently been recorded¹²¹. Seven cases were diagnosed in a four week period; four of these lived within one small residential area and three of these four attended a single school. The Kulldorff scan method¹²² identified this as a significant space/time cluster ($p = 0.017$) Given the narrow time window of the diagnoses (four weeks) and the age range of the cases (2-11 years), any causal exposure, as in Niles and Fallon, would be proximal to diagnosis, promoting overt ALL evolution from a prior and covert pre-leukaemic state. The Milan cases sparked substantial public anxiety, particularly in relation to the school, and a detailed epidemiological investigation was launched. No link was found with ionising radiation, non-ionising radiation or chemicals. There was, however, an association with a particular, common infection. All seven cases had been infected with endemic AH1N1 swine flu virus during the epidemic that preceded the ALL cluster by three to six months. The infection frequency in children in Milan during the same period was relatively high, at around one third, but this still indicated the link with cases was significantly different from expected ($p = 0.01$)¹²¹. Six of the seven cases were first born and none attended day care in the first year of life.

Proof of a causal role for infections in these situations is not possible and clustering of cases by chance cannot be excluded. But the observations accord with predictions of the

infection hypothesis and highlight that influenza viruses are potential promoting agents for ALL. A prior study in the UK observed peaks in incidence of childhood ALL ~6 months after seasonal influenza epidemics¹²³. A final piece of epidemiological evidence indirectly supporting a role of common infection in childhood ALL comes from anecdotal but striking observations on rapid changes in the incidence of ALL that were preceded by major social changes in Germany and Hong Kong (Supplementary Information S3 (Box)).

There is no compelling reason for postulating an exclusive role for influenza viruses or indeed for viruses. A role for cytomegalovirus (CMV) in ALL has been postulated, but as an early, *in utero*, modulator of immunity, rather than as a proximal trigger¹²⁴.

In some respects it is surprising that the epidemiological data is as consistent as it is on individual factors related to infection in ALL since many variables will compound to influence patterns of microbial exposures in early life (Supplementary Information S4 (Box)).

[H1] Modelling the ‘missing link’ in ALL

There are limits to what epidemiological studies can achieve and to the robustness of the findings. Nevertheless, the associations described are compatible with the infection model proposed and their selectivity for BCP-ALL, versus AML, is striking. But associations are not necessarily causal. Functional components of the infection hypothesis are best addressed by modelling studies in mice (Box 2). These have proven very informative. One inflammatory cytokine - TGF β , was found to induce preferential expansion of *ETV6-RUNX1*-driven pre-leukaemic cells of both mouse and human origin¹²⁵. Normal B cell precursor proliferation is inhibited by TGF β via activation of the cell cycle inhibitor p27. *ETV6-RUNX1* blocks this activity giving pre-leukaemic cells a fitness advantage in the presence of TGF β ¹²⁵.

The missing link in the chain of events between infection, inflammatory responses and promotion of BCP-ALL may be activation-induced cytidine deaminase (AID)^{126,127}. As noted

above, genomic sequencing in BCP-ALL revealed that the recurrent CNAs have signatures of RAG activity⁶². Physiological RAG activity in germinal centre Ig class switching or hypermutation requires AID,¹²⁸ as does illegitimate recombination between the *IGH* locus and oncogenes^{129,130}. In B cell precursors, AID is not normally co-expressed with RAGs but is inducible by infection-driven cytokine signals¹³¹. This suggested that one route by which infection or chronic inflammation might trigger RAG-mediated CNAs and ALL is via activation of AID expression in pre-leukaemic stem cells.

A mouse model has tested the requirement for RAGs and AID in the transition from *ETV6-RUNX1* pre-leukaemia to overt ALL¹²⁶. The data revealed that lentiviral transfection of *ETV6-RUNX1* into progenitor cells leads to B cell precursor ALL when those cells are treated with a surrogate inflammatory signal (bacterial lipopolysaccharide binding to TLR4) that activates AID. Critically, mice did not develop ALL if the same experiment was conducted in a *RAG1*^{-/-} genetic background. More recently, my colleagues and I have screened a series of inflammatory cytokines for their ability to trigger AID expression in human B cell precursors. The most potent was TGFβ (V Cazzaniga, AM Ford, M Greaves, unpublished data). TGFβ is known to promote other cancers, often in the context of chronic inflammation¹³². In ALL, its role may include not only selective expansion of pre-leukaemic cells¹²⁵ and activation of AID but compromise of natural killer (NK) cell-based immune-surveillance¹³³.

If the aetiological hypothesis is correct, then it should be possible to influence risk or penetrance of ALL in murine models by timed exposure of the immune system to natural infections. Using a model of BCP-ALL it was shown that ALL developed if *Pax5*^{+/-} mice were switched from a germ-free environment to one providing exposure to common microbial pathogens¹³⁴. Similarly, another study found that ~10% of mice with *ETV6-RUNX1* developed BCP-ALL after exposure to common pathogens¹³⁵. The experiments provide evidence, albeit in murine models, that common infections can have, as predicted, a promotional role in ALL.

Another mouse model has provided evidence that early stimulation of the immune system can be protective. Exposure of mice transgenic for *Eμ-Ret* or *E2A-PBX1* to oligodeoxynucleotides (which bind to TLR7, TLR8 and TLR9) at four weeks depleted both normal and pre-leukaemic precursors and both delayed and diminished risk of progression to ALL¹³⁶. This effect was dependent upon IFN γ . In contrast, binding of polyI:C, a TLR3 ligand which does not induce IFN γ , resulted in an expansion of the pre-leukaemic cell pool. These data hint that the nature of infectious exposures in infancy and responses of the innate immune system may influence not only subsequent immune responses but the fate of pre-leukaemic cells.

[H1] Conclusions: paradoxes of progress

“We incline on our evidence to the belief that the solution of the problem of leukaemia lies rather in some peculiar reaction to infection than in the existence of some specific infective agent.”

F J Poynton, H Thursfield and D Paterson

(Great Ormond Street Hospital for Sick Children), 1922¹³⁷

Collectively, the accumulated evidence derived from epidemiological studies, GWAS, genome sequencing, biological scrutiny of the natural history and molecular pathogenesis of BCP-ALL, plus mechanistic and modelling studies, provide us with a more substantive and credible version of the original^{7,17} two hit model for childhood ALL, as summarised in Fig 3. The model applies selectively to the common, B cell precursor subset of ALL, although the evidence is,

currently, more compelling for the *ETV6-RUNX1*⁺ subset of BCP-ALL than for hyperdiploid subset. The rarer pro-B ALL in infants appears likely to have a different causation and molecular pathogenesis, as does childhood AML and childhood lymphoma. There is insufficient data for T-ALL in this respect. Other causal associations in leukaemia and cancer in general might be revealed or strengthened by a focus on well-defined subtypes, as suggested for breast cancer ¹³⁸.

The causal mechanism proposed here is multifactorial, involving patterns of infection, inherited genetics and other modulators of risk including chance and, probably, diet (Box 4). It has a logical coherence ¹³⁹ and is grounded in the fundamental biology of leukaemia and evolutionary logic of the immune system network functions. The central thesis posits BCP-ALL as a paradox of progress in developed societies contingent upon a mismatch between the historical or evolutionary programming of the immune system and contemporary lifestyles that restrain opportunities for early life microbial exposures. Childhood ALL is probably not the only unanticipated, deleterious health consequence of diminished infectious exposure in infancy ⁹³. Similar epidemiological associations exist for Hodgkin lymphoma in young adults ¹⁴⁰ as well as for childhood allergies and autoimmune disease ¹⁴¹ (Supplementary Information S5 (Box)). In all these clinical situations, the common theme is that acquisition of common microbial infections in early life has an impact on later responses of the immune system to challenge and the subsequent presence or absence of pathology ^{24,93,142}. Diminished exposure early in life to microbes that are pathological has been highly beneficial, reducing infant mortality, but it seems plausible that a suite of illnesses prevalent now in young people in more developed societies, including BCP-ALL, could be due to an unanticipated consequence of this advance ⁹³.

The infection hypothesis would benefit from further scrutiny including validation and extension of the animal modelling but its public health implication is clear. Most cases of

childhood ALL are potentially preventable. But how? Promotion of lifestyle changes including day care attendance or protracted breastfeeding in the first year of life can be advocated but would be difficult to achieve. A more realistic prospect might be to design a prophylactic ‘vaccine’ that mimics the protective impact of natural infections in infancy, correcting the deficit in modern societies. Reconstitution or manipulation of the natural microbiome¹⁴³⁻¹⁴⁶ or Helminth injections^{147,148} are strategies under consideration for early life immune disorders in modern societies including autoimmune and allergic conditions. Oral administration of benign synbiotics (bacteria species such as lactobacillus and oligosaccharides) can have profound, and beneficial, modulating effects on the developing immune system¹⁴⁹. The results of those endeavours might inform approaches for preventing BCP-ALL. Cross collaboration of scientists working in disparate fields of early life immune dysfunction – allergy, autoimmune disease and ALL – would be beneficial.

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M. Greaves researched data for the article and wrote, reviewed and edited the manuscript.

Competing interests

The author declares no competing interests.

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Box 1**The population mixing hypothesis**

The Kinlen hypothesis^{15,16} was prompted by public concerns over apparent increases in childhood leukaemia in the vicinity of nuclear power plants in the UK. Kinlen's model proposed that childhood leukaemia was caused by a rare or abnormal reaction to a common infection of low pathogenicity in a population at risk because of migration and mixing in the context of lack of herd immunity¹⁵⁰. By analogy with animal leukaemias, Kinlen favoured a specific virus. The model was also considered to apply across the board to childhood blood cell malignancy, irrespective of subtype and was not prescriptive with respect to timing of infection in the life of a child.

The population mixing hypothesis for childhood leukaemia has been explored by Kinlen and others in a variety of geographic and demographic settings. The data suggests that where an influx of adults and families occurs, particularly into relatively isolated or rural areas, a transient increase, in the order of twofold, occurs in incidence rates, compatible with an infectious aetiology^{16,106,151-153}. These studies have not been informative with respect to timing of infection in relation to the natural history of disease, the nature of the infection(s) involved or mechanistic aspects. Nevertheless, they provide an important tranche of evidence supporting a role for common infection(s) in childhood leukaemia.

Box 2

Insights from modelling

The initiating role of *ETV6-RUNX1* in ALL has been modelled in zebra fish ¹⁵⁴, mice ¹⁵⁵⁻¹⁶² and with human cells ⁴¹. A consensus view is that the ETV6-RUNX1 protein functions as a weak oncogene imparting only a modest proliferative or survival advantage on pro-B or pre-B cells. This accords with the small clone size of *ETV6-RUNX1* pre-leukaemic cells in cord blood and in the blood of co-twins of patients with ALL ⁵⁰. *ETV6-RUNX1*⁺ ALL cells ectopically express erythropoietin (EPO) receptors and modelling with both human and mouse cells suggest that the receptors are functional and that EPO may provide a survival signal to *ETV6-RUNX1*-expressing pre-leukaemic cells ^{163,164}.

The observations on identical twins and cord blood suggest that *ETV6-RUNX1* fusion is insufficient by itself to induce overt or clinically evident ALL. This is endorsed by the various models (see table below). However, as anticipated, *ETV6-RUNX1*-expressing clones in mice do progress to BCP-ALL if additional, co-operative mutations are introduced by cross-breeding with mice heterozygous or homozygous null for the genes recurrently deleted in *ETV6-RUNX1*⁺ ALL, including *Cdkn2a* and *Pax5* ^{161,162}. ALL also develops in some of these models if mice expressing *ETV6-RUNX1* are subjected to insertional mutagenesis ¹⁶⁰ or chemical exposure ¹⁵⁹.

The impact of these additional genetic lesions that complement *ETV6-RUNX1* is to impede or block differentiation, mirroring the early B lineage phenotypes observed in the clinical disease. This is in accord with the normal function of the transcription factors ETV6 and RUNX1, which is primarily to promote differentiation, and their loss of function or deletion in ALL ⁶³. This interpretation is further endorsed by modelling studies in which experimental regulation of *Pax5* expression and gene dosage govern both early B lineage differentiation and leukaemogenesis ¹⁶⁵.

These murine models support the ‘two hit’ hypothesis for BCP-ALL and have also provided evidence for the role of common infections in the development of BCP-ALL (table below) ¹³⁴⁻¹³⁶.

Initiating, transgenic lesion		Manipulation	Outcome	References
	<i>ETV6-RUNX1</i>	None	Covert pre-leukaemia only	125,157
	<i>ETV6-RUNX1</i>	Activation of AID, <i>in vitro</i> , with LPS, transplant to RAG1 ^{+/+} or ^{-/-} mice	BCP- ALL in RAG1 ^{+/+} background	126
	<i>ETV6-RUNX1</i>	Switch to non-germ-free housing	BCP- ALL	135
	<i>Pax5^{+/-}</i>	Switch to non-germ-free housing	BCP- ALL	134
	<i>Eμ-Ret</i> or <i>E2A-PBX1</i>	CpG ODN oligodinucleotide (at 4-8 weeks)	Delay and reduced penetrance of BCP-ALL	136

Box 3

Is there a unique foetal target cell for childhood BCP-ALL?

Adults do develop BCP-ALL. But it is striking that hyperdiploid ALL and *ETV6-RUNX1*⁺ BCP-ALL decline in incidence in teenage years and are infrequent after 20 years of age. Adult BCP-ALL has a higher frequency of *BCR-ABL1* fusion or a *BCR-ABL1*-like signalling phenotype, perhaps explaining their poorer prognosis¹⁶⁶. Childhood ALL in this respect appears to be a different cancer. Why should *ETV6-RUNX1* or hyperdiploidy initiate BCP-ALL preferentially in early life, and especially *in utero*? *ETV6-RUNX1* expression, driven by the endogenous *Tel* promoter in mice, increases quiescence and persistence of foetal liver stem cells¹⁵⁹. Moreover in this mouse model, foetal haemopoietic stem cells expressing *ETV6-RUNX1* had a protracted but still limited lifespan as self-renewing cells and did not contribute to the early B cell progenitor pool of adults. This suggests that the low penetrance of pre-leukaemia in children and the declining incidence of *ETV6-RUNX1*⁺ (and hyperdiploid) ALL with age may be, at least in part, be due to natural attrition of the foetal pre-leukaemic clone.

One possibility is that foetal B lymphopoiesis is distinctive, in cell type and/or microenvironment, and provides a selective context in which *ETV6-RUNX1* and hyperdiploidy have effective transforming functions. This appears to be the explanation for *GATA1* mutations in Downs syndrome-associated acute megakaryoblastic leukaemia (AMKL) being restricted to foetal liver¹⁶⁷. Recently a strong candidate target cell for childhood BCP-ALL has been identified¹⁶⁸. This cell type is developmentally restricted to foetal lymphopoiesis, with a mixed myeloid/B lineage phenotype. In common with the putative target cell suggested by our studies in identical twins^{34,41}, this foetal liver myeloid/B progenitor also has *DJ* rearrangements of *IGH*¹⁶⁸.

Box 4**Other modulators of risk in childhood ALL**

In addition to patterns of infectious exposure and inherited genetics, other factors are likely to contribute to multi-factorial risk including diet and chance. For ALL as well as AML and most other paediatric cancers, risk is significantly and consistently elevated in association with higher birth weights ^{169,170} or, possibly, accelerated foetal growth ¹⁷¹. A plausible interpretation of this link is that higher weight, possibly orchestrated via IGF1 levels ^{172,173}, may provide greater number of cells at risk. IGF1 potentiates expansion of B lineage progenitors ¹⁷⁴. Recently, evidence has been presented, using mouse models of ALL, that a restricted diet can have a risk-reducing impact ¹⁷⁵. Intermittent fasting was shown to block expansion of leukaemic cell populations and progression of disease. The effect operated via attenuation of leptin-receptor expression on leukaemic cells, possibly enforcing differentiation. Diet or calorie intake may, therefore, have a modulating impact on risk of ALL, reinforcing the likely multi-functional nature of causation of ALL, as in cancer in general.

Random events or chance get short shrift in cancer epidemiology but it has long been recognised that contingency and chance pervades all of biology ¹⁷⁶. Some posit that a substantial fraction of cancers are due to chance alone ¹⁷⁷ but this has been contentious. Chance is likely to be an ingredient in each and every cancer including childhood ALL. This is because inheritance of risk alleles is a lottery at conception, exposures including infections, at particular times, may or may not happen and mutational mechanisms alter genes independently of their function ¹⁷⁸.

Figures

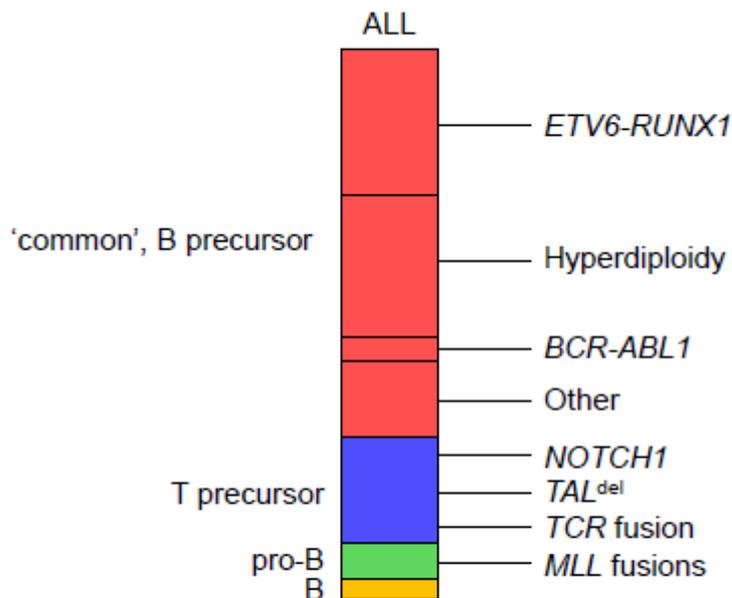


Figure 1a. Immunophenotype screens in the 1970's and early 1980's established that ALL could be divided into subsets corresponding to early developmental compartments of the B and T cell lineages, as indicated in the key Common or B cell precursor ALL (BCP-ALL) is genetically diverse (as illustrated) with the two most prevalent alterations being *ETV6-RUNX1* fusion and hyperdiploidy. The rare (~2%) subtype with a mature B cell immunophenotype (and frequent *IGH-MYC* rearrangements) was subsequently recognised (and treated) not as ALL but as B lymphoblastic lymphoma. For more detailed descriptions of genomic diversity in ALL, see references ¹⁷⁹⁻¹⁸¹.

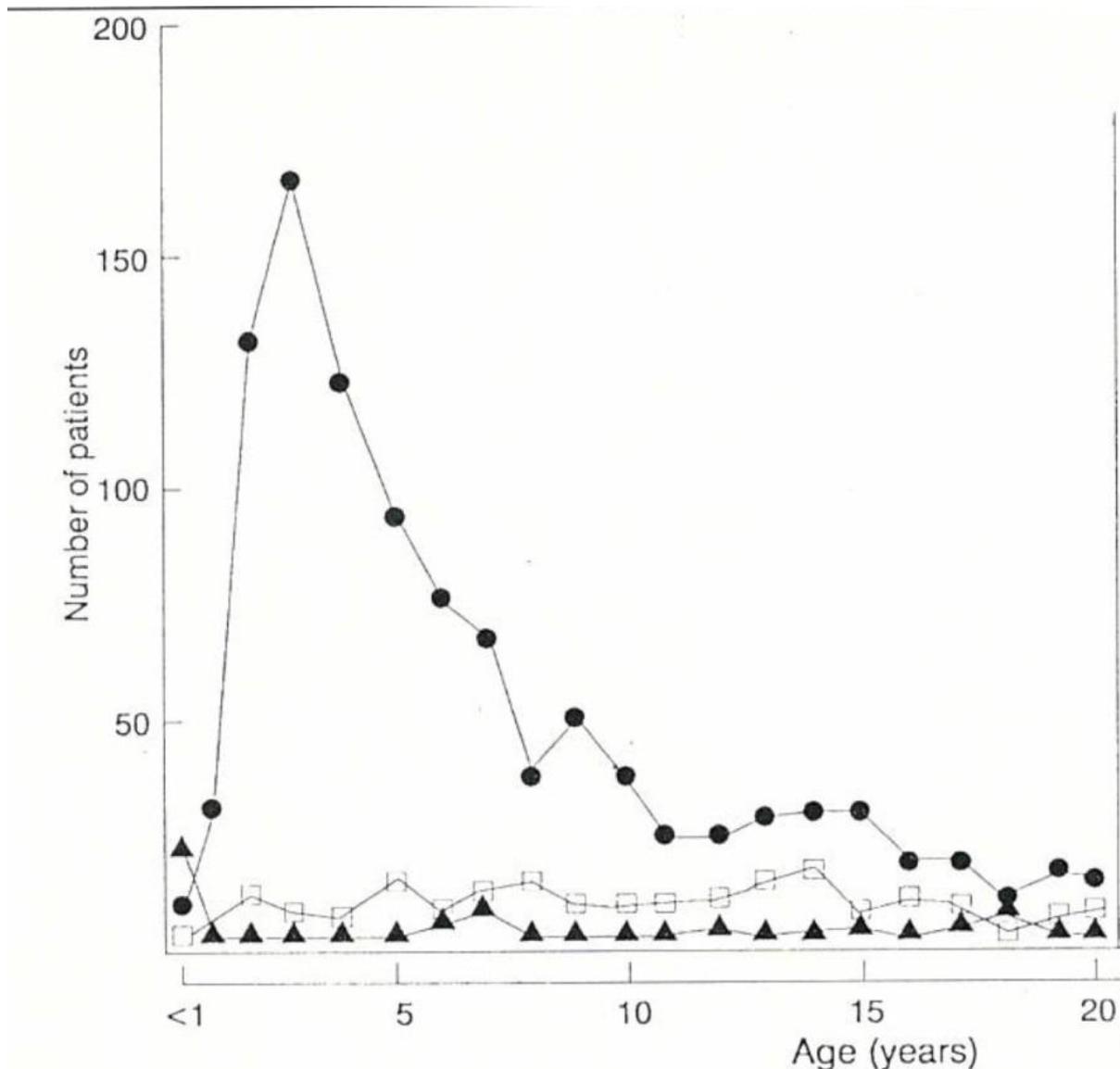


Figure 1b. Age distribution of ALL subtypes from a cohort of 1184 patients with ALL entered into MRC-UKALL clinical trials¹⁸² (1975-1984). This pattern of age-associated ALL subtypes was validated in a later cohort of MRC-UKALL trials (1991-1996; 1088 patients up to 14 years of age)⁸ It had been known that childhood ALL had a very marked age incidence peak at 2-5 years throughout the developed world. But this peak appeared to be diminished or absent in less developed societies and appeared in particular countries and ethnic groups at different times¹⁸². Immunophenotypic screens, linked to clinical trials in the UK¹⁸³, and an international collaborative group study¹⁸² documented that the peak in incidence was selectively common or BCP-ALL. Recent epidemiological data indicates that the incidence of this subtype of leukaemia in Europe has continued to increase at around 1% per year¹⁸⁴⁻¹⁸⁶. This suggested that the increase over time could be real, rather than ascertainment bias, and that BCP-ALL might have had a distinctive aetiology. The BCP-ALL subtype was also found to have a much more favourable clinical outcome^{183,187,188}, emphasizing its distinct biology. Modified with permission from ref¹⁸².

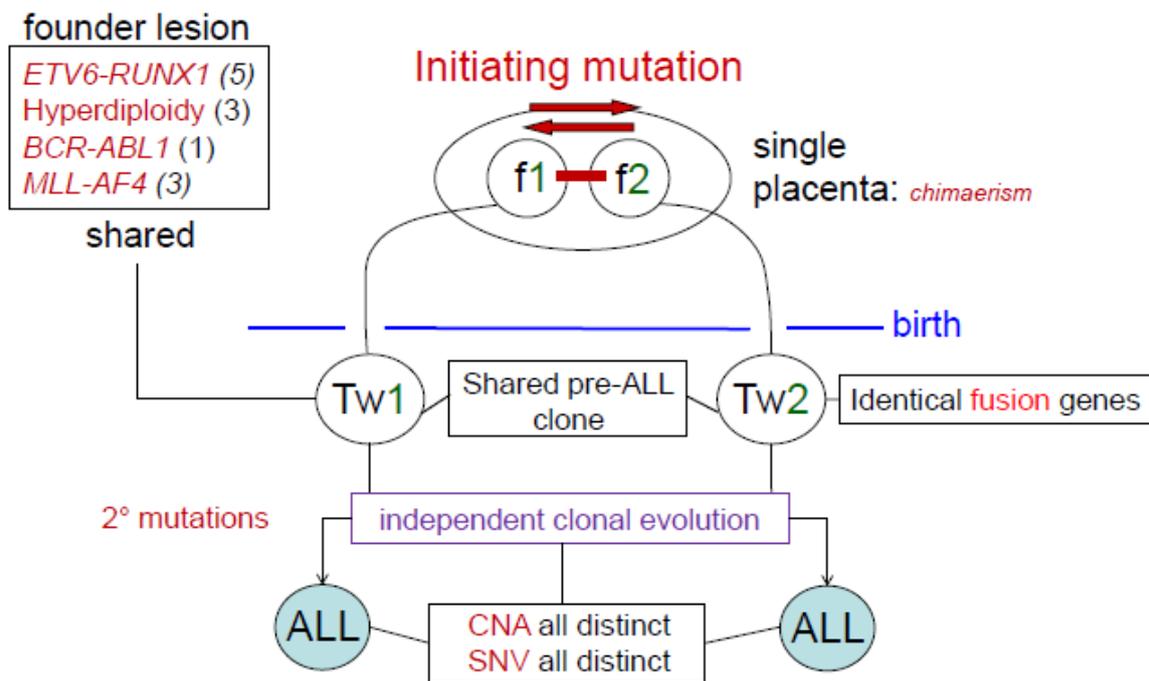


Figure 2. Summary of comparative genomics of ALL in identical twin pairs. The figure is based on analysis of 12 monozygotic twin pairs (the number of pairs with each founder lesion is noted in parentheses) with concordant ALL^{29,33,37,40,48}. The sharing of a patient- and clone-specific fusion gene that is not inherited in the twins indicates that in such cases of concordant ALL, the leukaemia must have been initiated in a single cell, in one twin of the pair *in utero*, the clonal progeny of that cell then disseminating to the co-twin, via intra-placental anastomoses. In further support of this notion, it was noted that concordance of ALL only occurred where the twins shared a single or monochorionic placenta, providing a route for cellular transmission²⁹. Figure adapted from reference¹⁸⁹. CNAs, copy number alterations. SNVs, single nucleotide variants.

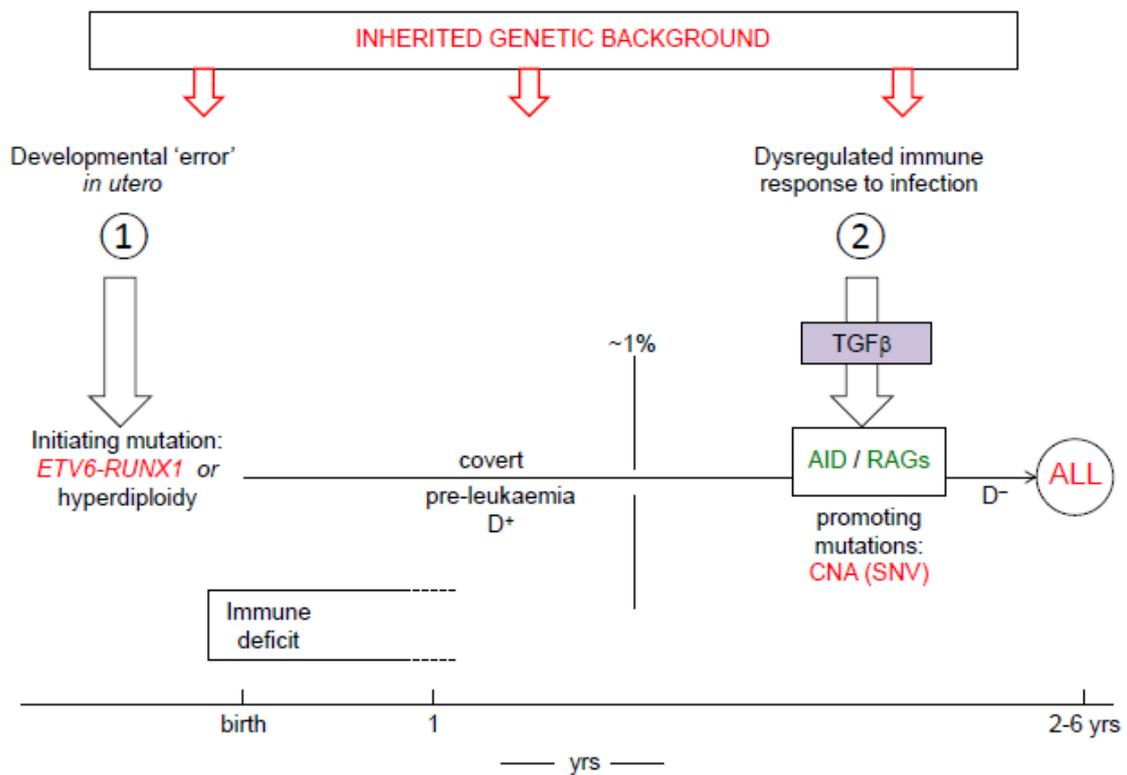


Figure 3. Summary of 2 hit model for role of infections in B cell precursor ALL.

Genetic, inherited risk alleles are depicted (top of figure) as impacting at any or several stages of the step-wise process of ALL.

Table 1. Inherited alleles and risk of childhood B cell precursor ALL identified in GWAS^a

Candidate gene (chromosome) ^b		ALL subset bias	Odds ratio ^c (95% CI)		References
	<i>IKZF1</i> (7p12.2)	None detected	1.69 (1.58 – 1.81) 1.69 (1.4 – 1.9)		190 191
	<i>ARID5B</i> (10q21.2)	Hyperdiploid	1.65 (1.54 – 1.36) 1.91 (1.6 – 2.2)		190 191
	<i>CDKN2A</i> and <i>CDKN2B</i> (9p21.3)	None detected	1.36 (1.18 – 1.56) 1.72 (1.50 – 1.97) 2.41 (1.99 – 2.92) ^b		75 192 193
	<i>CEBPE</i> (14q11.2)	Hyperdiploid	1.34 (1.22 – 1.45) 1.45 (1.30 – 1.62)		190 194
	<i>GATA3</i> (10p14)	<i>BCR-ABL</i> -like	1.31 (1.21 – 1.41) 3.85 (2.7 – 5.4)		195 196
	<i>PIP 4K2A</i> (10p12.2)	None detected	1.40 (1.4 – 1.53)		195 75
	<i>TP63</i> (3q28)	<i>ETV6-RUNX1</i>	0.63 (0.52 – 0.75)		197
	<i>PTPRJ</i>	<i>ETV6-RUNX1</i>	0.72 (0.68 – 0.89)		197
	<i>LHBP</i> (10q26.13)	None detected	1.20 (1.15 – 1.28)		198
	<i>ELK3</i> (12q23.1)	None detected	1.19 (1.12 – 1.26)		198
	8q24.1	None detected	1.34 (1.21 – 1.47)		199,200
	2q22.3	<i>ETV6-RUNX1</i>	1.32 (1.17 – 1.49)		199
	<i>IKZF3</i> 17q12	None detected	1.18 (1.11 – 1.25)		200

^a Cohort sizes in these studies were between ~400 and 3500 cases.

^b More low risk alleles are likely to be uncovered as larger cooperative group studies are pursued and other populations considered. Rare allelic variants, missed in prior studies, may also be identified which have a stronger impact on risk of ALL as described for *CDKN2A* (OR 2.4) ¹⁹³. ^c p values in these studies were between 10⁻⁵ to 10⁻¹⁹. All cases are white, European descent in which the alleles exist at relatively high frequency (10-50%).