Development of epigenetic biomarkers for complex disease

There is growing evidence that non-communicable diseases such as cardiovascular disease (CVD), stroke, hypertension and type 2 diabetes may originate in early life, a paradigm known as the Developmental Origins of Health and Disease (DOHaD) [1]. Investigations into mechanisms underpinning DOHaD using animal models and human specimens have suggested the involvement of epigenetics: mitotically heritable changes in gene expression controlled by chemical modifications to chromosomes without altering the DNA sequence. One striking example implicates a role for the epigenetic mark of DNA methylation in the long-term effects of the Dutch famine during the Second World War. By middle age, offspring previously exposed to maternal malnutrition during early gestation had a higher incidence of CVD than their unexposed siblings, together with differences in DNA methylation in metabolic and CVD-related genes [2]. Animal studies indicate that epigenetic modifications may be reversible by pharmacological or dietary interventions, suggesting approaches for future targeted interventions in humans.

To accelerate progress towards this goal, EWAS have been advocated. These studies use techniques varying in sensitivity, coverage, sequence bias and amounts of DNA required. A handful of EWAS have already been published and many more are in progress. An important question raised by these studies is that of causality: when disease-associated epigenetic differences are identified, do they reflect causal pathological pathways of disease or a subsequent effect of disease? Furthermore, false positives can be captured in such screens. Thus, ideally, EWAS should be conducted longitudinally where possible. To this end, recent EWAS have found common epigenetic changes in pre- and post-symptomatic children with type 1 diabetes (T1D) [3], and differences in fresh cord blood at gene loci whose expression was associated with body mass index in late childhood [4].

The utility of Guthrie cards in epigenetic research

Around the world, newborn babies are routinely screened for inborn errors of metabolism and other congenital disorders through testing of neonatal blood spot cards, a technique pioneered in the early 1960s by Robert Guthrie, after whom the cards are named. Collected within a few days of birth from heel pricks, Guthrie cards usually contain four blood spots 6 to 10 mm in diameter. The duration of Guthrie card archiving varies between and within countries, ranging from a few months to indefinitely. Thus, in many countries, Guthries represent a near-perfect national biorepository. In addition to serum analytes, DNA has been extracted from Guthries and has been used for such purposes as carrier screening for cystic fibrosis, detection of HIV and, more recently, genome-wide association studies. Modest degradation of DNA occurs during storage and extraction that could affect data quality [5], although the quality appears sufficient for genomic assays. Remarkably, gene expression has also been analyzed in RNA from 20-year-old
Guthries [6]. As for the epigenetic regulators of gene expression, it has been shown that DNA methylation can be analyzed at individual genes using the widely used technique of bisulfite conversion, which converts methylation differences to sequence differences [7]; this has been applied to Guthrie-based methylation screening of the FMR1 (fragile X mental retardation 1) gene to predict cognitive impairment in individuals with fragile X syndrome [5].

A recent paper by Beyan and colleagues [8] has now taken this one stage further, conducting a proof-of-principle epigenome-wide pilot study using Guthrie card methylomics. On average, 200 ng of DNA from each 6 mm-diameter Guthrie spot was extracted and used in two methods of genome-scale methylation profiling: one array-based, the other based on immunoprecipitation of methylated DNA followed by high-throughput sequencing. For the array-based method, DNA was extracted from 10-year-old Guthries and compared with fresh blood and sperm from unrelated individuals. This approach identified tissue-specific differentially methylated regions between sperm and blood. There was an excellent genome-wide correlation between archived Guthrie DNA and fresh blood, but a weaker correlation for the subset of regions showing small (<20%) differences in methylation between the two tissues. No comparisons were reported between fresh and aged DNA from the same individual, which would have been an ideal control for the effect of storage of Guthries on measurement of DNA methylation. This represents an important caveat of the present study. For the immunoprecipitation-based method, which usually requires 2 µg of DNA, the method was adapted to work with 200 ng. The team then attempted to define the regions of the genome that differed between individuals but remained constant from birth to 3 years of age. This was an important comparison because it has been proposed that such 'metastable epialleles' are influenced by environmental and stochastic factors in utero, remain constant thereafter, and can act as stable biomarkers for disease risk [9]. For this, the team was careful to exclude genomic regions for which genetic heterogeneity could influence epigenetic variation and focused on clusters of variable, stable regions. Unfortunately, due to the low DNA yields, the longitudinal comparisons were limited to the array-based technique at birth and the immunoprecipitation-based technique at 3 years of age. Nevertheless, up to a dozen metastable epialleles were identified, two of which had previously been associated with human disease.

Where do EWAS go from here?

The study of Beyan et al. highlights the utility of Guthries for longitudinal EWAS in which retrospective case-control studies can produce data more quickly and cheaply than birth cohort studies. However, the latter are designed to collect data on maternal exposures with minimal recall bias, which is not possible in retrospective studies. Furthermore, it is likely that a portion of the epigenome is still susceptible to environmental and stochastic influences in early postnatal life, making a case for repeat sampling. One disadvantage of using whole blood is that methylation levels represent an average of the levels in each of its component cell types, the proportion of which may change over time. However, if the early environment leaves an epigenetic legacy in multiple tissues, this will be a minor issue.

Future longitudinal EWAS (Figure 1) will need to be sufficiently powered to detect disease-associated epialleles in contrast to the pilot study of Beyan and colleagues, which was designed to detect large methylation differences (>20%) in a small number of comparisons (n = 3 individuals). The group previously compared methylation profiles of 15 monozygotic twin (MZ) pairs discordant for T1D, identifying 132 T1D-associated methylation variants with within-pair methylation discordance of 0.1 to 6.6%. Cross-cohort validation was performed with four additional T1D-discordant MZ twins and the temporal origin of T1D-associated methylation differences were assessed with blood sampled from seven children with T1D before and after presentation, the latter using profiles from the same individuals positive for diabetes-associated auto-antibodies but negative for typical symptoms of T1D [3]. Another recent study looked for methylation events that co-varied with body mass index at two time points in 74 individuals [9]. Although different techniques were used, importantly, both of these studies used the same array platform at each time point.

It is also worth noting that there may be ethical barriers to longitudinal EWAS, as the use of Guthries without consent has been a major issue in some locations [10]. Currently, cards can be used for limited forensic purposes and de-identified in research. But should consent always be sought for use of Guthries in epigenetic research? Research studies using small numbers of samples are not generally problematic because it is easy to get consent from the individuals or parents. However, studies requiring large numbers of samples, such as well-powered EWAS, are a problem because it may not be practical to obtain consent from all the individuals involved. Newborn infant screening programs have recognized these ethical issues and parents are presently better informed about potential uses of stored Guthries, with some programs having introduced a consent process for future de-identified research.

Conclusions

Beyan and colleagues have shown that it is possible to perform longitudinal EWAS starting with blood samples
from cases and controls after disease presentation and adding in blood samples obtained from Guthries at birth (Figure 1), a feat only previously achievable (in reverse order) through large birth cohort studies. Limitations that still need to be overcome include optimization of the amount and quality of DNA extracted from Guthries, identification of any technical artifacts associated with long term storage, an increase in study power and overcoming ethical barriers. In addition, longitudinal birth cohort studies should aim to sample at multiple time points to determine which disease-related epigenetic changes are present at birth and which develop after birth in response to postnatal environmental exposures. Nevertheless, the central message of the paper by Beyan and colleagues is that we now have another arrow in our quiver with which to reach the ultimate target of EWAS: to discover early, reversible biomarkers for human disease. We should move forward in an ethically responsible manner.

**Abbreviations**
CVD, cardiovascular disease; DOHaD, Developmental Origins of Health and Disease; EWAS, epigenome-wide association study; MZ, monozygotic twins.

**Competing interests**
MC and JMC are currently performing EWAS with DNA from Guthrie cards.

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