

# The evolution of blood-spot newborn screening

Kaustuv Bhattacharya<sup>1,2</sup>, Tiffany Wotton<sup>1,3</sup>, Veronica Wiley<sup>1,2,3</sup>

<sup>1</sup>The Children's Hospital at Westmead, NSW, Australia; <sup>2</sup>Faculty of Paediatrics and Child Health, Sydney University, Australia; <sup>3</sup>NSW Newborn Screening Programme, The Children's Hospital at Westmead, Australia

Correspondence to: Kaustuv Bhattacharya. Department of Biochemical Genetics, The Children's Hospital at Westmead, Locked Bag 4001, Westmead, NSW 2145, Australia. Email: Kaustuv.bhattacharya@health.nsw.gov.au.

**Abstract:** Over 50 years after the introduction of a blood-spot newborn screening test using the bacterial-inhibition assay (BIA), blood-spot newborn screening has evolved into complex public service scientific programmes. For several decades, many patients with phenylketonuria (PKU), congenital hypothyroidism (CH), cystic fibrosis (CF) and hemoglobinopathy disorders have benefited from early intervention across the world. In the last 20 years, there have been great changes in laboratory techniques and high-throughput data handling meaning that a huge spectrum of disorders can be identified from an increasing population. This coupled with the fact that there are an increasing number of therapies for specific rare disorders mean that health services may become inundated with complex and expensive demands in the future. Some of these issues have been realised in the implementation of multiplex assay such as electrospray tandem mass spectrometry (MSMS) programmes but will be much more exaggerated if genomic sequencing screening becomes a reality. In this context, the core-principles for implementation of newborn screening tests remain as important today as they have in the past when new tests are considered as part of the blood-spot screening programme.

**Keywords:** Neonatal screening; tandem mass spectrometry (MSMS); massively parallel sequencing (MPS); congenital hypothyroidism (CH); cystic fibrosis (CF); phenylketonuria (PKU)

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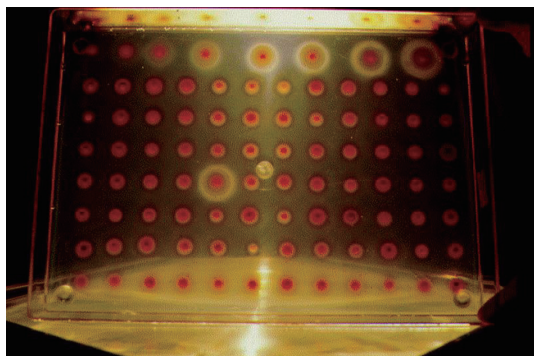
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## Introduction

The objective of medical screening tests is to identify clinically significant disorders from a target population, using a reliable method. Certain inborn errors of metabolism and other metabolic disorders can be detected pre-symptomatically in apparently healthy babies using appropriate laboratory tests. Screening is only appropriate if there is: a demonstrable benefit from early diagnosis; a suitable test with high specificity and sensitivity; a system of confirmation of results, counselling, treatment and follow-up; and the balance of harms and benefits is positive. Over the last 50 years the concept of screening asymptomatic babies to identify severe disorders has been realised. The field of newborn screening has changed dramatically over that period of time, embracing advances in technology, which have been incorporated into various programmes today.

## Phenylketonuria (PKU)

Robert Guthrie developed a simple test in 1961 that could be used for mass screening of infants for PKU (1). In 1962 he received a grant to perform a study of 400,000 infants and thus by 1963 population screening began. By 31<sup>st</sup> December 1963, 29 US states and Puerto Rico had contributed data for the study. Thus 2013 marks the 50<sup>th</sup> anniversary of the publication by Guthrie and Susi of the method used for population based screening for PKU using a bacterial inhibition assay (BIA) (2). This elegant method utilises elevated levels of phenylalanine in the blood of infants soaked into disks of filter paper, to overcome  $\beta$ -2-thienylalanine inhibition of growth of *Bacillus subtilis*, on an agar culture. As a consequence, patients with PKU can be easily recognised on gels with florid growth of these bacteria around the blood disk and approximate levels were



**Figure 1** Agar plate indicating BIA for PKU. The top line indicates control samples with increasing phenylalanine concentrations. One affected individual with PKU is identified at the centre of this gel. PKU, phenylketonuria; BIA, bacterial inhibition assay.

determined by comparison with growth obtained from a known concentration calibration curve (*Figure 1*). Although the method was used on a small scale in a single hospital to start with, with increasing success over a relatively short period of time, it was quickly adopted by all states within USA and many other countries. The success of this technique was built upon the improvement noted by an effective therapy in children as described by Bickel *et al.* (3) ten years prior to Guthrie's (2) publication. Both individuals subsequently acknowledged that both a reliable and cheap test and an effective disease modifying treatment were essential to ensure success of a PKU screening programme. The data that conclusively demonstrated that this was indeed the case took many years to come to light (4,5). It became increasingly clear with time that the management of this rare inborn error of metabolism was not just about restricting phenylalanine in the diet but also about managing all aspects of diet, ensuring effective phenylalanine restriction (by regular monitoring), and providing a healthy nutritionally complete diet, to ensure normal growth. Family support was also required in order to achieve a good outcome. These observations with the first disorder that was systematically screened for, have been replicated in different ways in disorders that have been subsequently added to screening panels. It is very clear, therefore, that the infrastructure to reliably diagnose and manage a screening result needs to be in place before a population based programme is implemented.

Following the success of PKU screening, the principle of an inhibition assay was used to develop tests for other disorders such as homocystinuria and maple syrup urine

disease (6,7). However, despite the successes of the BIA methods, disorders such as PKU are now detected using other technologies [see tandem mass spectrometry (MSMS) screening below].

### Congenital hypothyroidism (CH)

The merits for screening for CH had been realised by the early 1970s (8). Like classical PKU, this disorder has an insidious onset with permanent severe neuro-disability becoming apparent before the end of the first year of life. Initially radiological screening tests were proposed but by 1973 Dussault and Laberge determined that blood filter paper samples could be used to measure the low level of thyroxine (T4) in CH (9). Using measurement of T4 there was significant overlap between affected and unaffected and a significant percentage of infants required further testing including the measurement of a pituitary hormone, TSH, to ensure cases were identified. In 1974 and 1975, methodology using radio-immunoassay on blood filter paper samples for TSH and T4 respectively had been developed (10,11). Various programmes around the world subsequently developed these different methodologies, leading to different screening strategies around the world including T4 only, TSH only, or a combination of T4 and TSH. The physiological surge in TSH within the first 24 hours after birth led to a high rate of false positives and the need to use age specific ranges. Unlike BIA screening, these assays provided quantitative results for analytes rather than semi quantitative. This has consequently led to an ongoing debate about what cases can be identified by screening and were associated with the inclusion of quality control and proficiency testing programs. Over time, the analytical sensitivity of assays has been improved and reported missed cases from screening have also been identified. These issues have provided fuel for the debate of where to set a cut-off for either free T4 or TSH. It seems that most cases of thyroid gland aplasia would be identified by either assay but that detailed assessment of the assay and associated confirmatory testing protocols are required in order to ascertain other causes such as dyshormonogenesis and pituitary insufficiency.

The use of radioimmunoassay for other disorders was soon considered and immunoassay methods were developed for CAH and cystic fibrosis (CF) screening (12,13). The implementation of newborn screening for hypothyroidism rapidly identified several severe congenital cases with a vastly improved outcome. In this regard, the programmes

undoubtedly were a success. As the number of babies tested and the complexity of testing increased as well as the need to optimise the quality of results, programmes required increasing automation. Instruments were developed to provide consistent punched samples and automated processing for immunoassays. The automation of processes facilitated high throughput screening to a wider population.

### Cystic fibrosis (CF)

The implementation of screening for CF also required a paradigm shift in terms of the conceptual basis for screening. The improvement in health outcome was not based on a single therapy such as using thyroxine or with-holding phenylalanine. The management of CF required rigorous multi-disciplinary management incorporating improving respiratory function, prompt treatment of infections and optimising nutrition. Moreover, some patients could be relatively well over the first year of life with some severe complications only becoming apparent later in childhood. Because of this, there was some debate about the merits of a population based screening approach in terms of the effectiveness of early intervention versus the later intervention of clinically ascertained cases. This debate has led to the later implementation of CF screening programmes in countries such as the UK. The outcome in terms of survival has taken many years to ascertain, but it is now clear that cases presenting clinically with respiratory infections or steatorrhoea are very vulnerable long-term (14,15). The benefit in early diagnosis of CF prevents such presentations and delays the onset of co-morbidities and thus survival.

In the early 1960s screening newborns for CF was made possible through the measurement of meconium albumin levels (16). However, false negative results were obtained due to the samples being stored at room temperature or CF patients with normal pancreatic activity resulting in decreased albumin content (17,18). In addition, premature newborns with low pancreatic activity and those babies with intestinal atresia could produce a false positive result (17). In 1979 another conceptual shift in CF screening occurred with the development of an assay using a biomarker of disease [immunoreactive trypsin (IRT)] rather than a direct metabolite such as phenylalanine which could be quantitated from dried blood spot samples already being collected by newborn screening programmes (13).

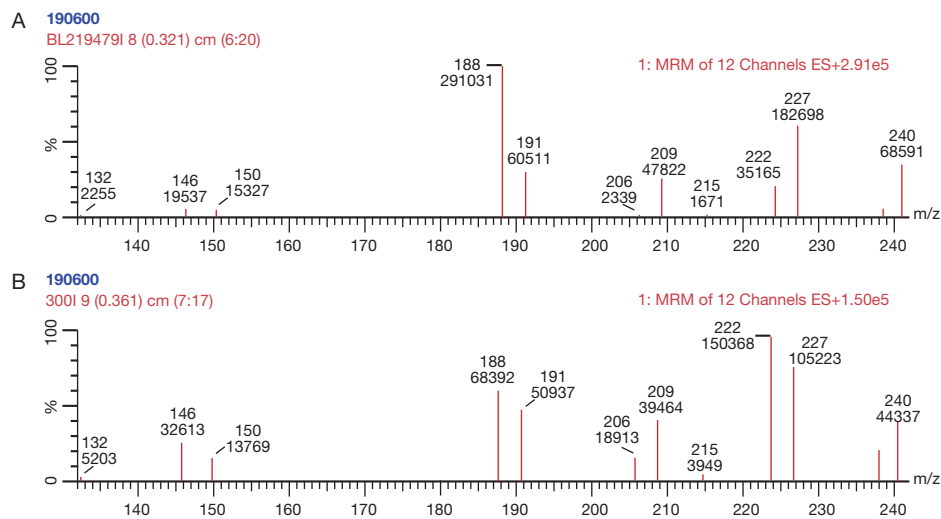
It was apparent even from early publications that a single IRT test is poorly predictive of CF. IRT levels may be increased in cases of perinatal stress, congenital infection,

renal failure or trisomy (13,18) and a falsely elevated level of IRT may be found in babies 2-5 days of age (19). It has also been demonstrated that IRT levels decline with age (13). Therefore age appropriate cut off values should be used to interpret results. To improve the specificity of CF screening protocols various approaches have been developed that use two samples collected at different ages. Furthermore, the availability of confirmatory testing, with a reliable diagnostic test, such as the sweat test was vital for the effectiveness of this test as a newborn screening tool. Since the 1990s a second tier approach of looking for cystic fibrosis transmembrane conductance regulator (CFTR) mutations has been used by several programmes. This has become more important as mutation specific therapies are developed. The use of IRT/DNA protocols for CF detection has enabled faster turnaround and minimal additional cost (20).

A more controversial problem is identification of a small number of babies who are healthy CF carriers but this seems more than balanced out by reduction of anxious families due to false-positive results obtained through employing the IRT alone protocol. In turn, this allows for informing parental reproductive decisions and information for other family members (21).

### Tandem mass-spectrometry (MSMS) screening

Until the 1990s, one test was used to provide information about one disorder. However, electrospray MSMS with automated sample injection is used to provide high throughput screening to identify a wide range of metabolites (amino acids and acyl carnitines) that are observed in a range of inborn errors of metabolism (22-25). MSMS could provide information on disorders with one significant analyte for example measurement of phenylalanine in PKU. But the strength of this technique was that a metabolic profile could be created, representing the status of various amino acids and acylcarnitines at the time the blood-spot was collected (*Figure 2*). From this blood profile, as well as PKU various pathological disorders could be reliably identified such as medium chain acyl CoA dehydrogenase deficiency, classical maple syrup urine disease, type II tyrosinaemia and mitochondrial trifunctional protein deficiencies (incorporating LCHAD). However, again the implementation of this as a screening tool was another paradigm shift as the assay was a metabolic profile that changed depending on environmental influences (including age at collection of the sample, feeding regimen, parental



**Figure 2** MSMS spectrum of amino acids. (A) MSUD—indicating elevated total leucines at position 188; (B) Elevated phenylalanine at position 222. MSMS, tandem mass spectrometry.

nutrition, temperature and time of storage). As such, the sensitivity and specificity of the screen varied depending on the analyte and condition being considered. Some analytes such as citrulline or octanoylcarnitine are elevated in different conditions and hence the utility of the test is most enhanced when the profile is considered in its entirety. As the world wide experience of this test has grown, it has become apparent that each centre needs to have rigorous algorithms that define various elements to testing:

- ❖ Age appropriate ranges (in terms of days of age and gestation)—there have been difficulties in comparison of data between centres because of high levels of certain metabolites such as tyrosine in premature babies which become normal later. There is a normal distribution of many analyte values particularly some of the fatty acid acylcarnitine species over the first few days after birth; hence a sample collected day 1 after birth cannot be compared to a sample collected day 5.
- ❖ Case definition of disease entity—a continuously distributed biochemical profile requires a specific cut-off value. For a disorder such as medium-chain acyl-CoA dehydrogenase deficiency (MCADD), there are a range of cut-off values around the world from 0.12 to 0.80  $\mu\text{mol/L}$  for octanoylcarnitine, indicating that there are different cut-offs being used to define the screen positive cases. Some institutions use various ratios of acylcarnitines for example C8/C10 carnitine to support the diagnostic definition of MCADD (26).

- ❖ Second tier test—screening laboratories have used a primary analyte as a screening test and if relevant perform a second test on the same sample, using different methodology in order to better discriminate aetiology. For example elevated methionine can be seen in liver dysfunction, methionine adenosyltransferase (MAT) deficiency and due to classical homocystinuria (cystathionine beta-synthase deficiency). All samples with elevated methionine can have total homocysteine assayed from the same blood spot in order to discriminate the biochemistry (27). Only the latter condition is treated and hence, these are the only patients that need to be contacted for clinical management. This second tier test is not a confirmatory test per se as homocysteine can be elevated in a number of circumstances.
- ❖ Confirmatory testing strategy—for some disorders a second tier test could be considered a confirmatory test such as the finding of alloisoleucine in a second-tier blood spot test after a preliminary screen of elevated total leucines. Where disorders are associated with common mutations finding two copies of known disease common mutations is diagnostic, for example *ACADM* gene. For other disorders, the confirmatory test needs to be carefully considered. In New South Wales, the unit uses specific VLCAD enzyme assay from cultures of skin fibroblasts as its diagnostic confirmation. This is an invasive test that also takes several weeks to perform. Several units around the world use mutation

testing of the *ACADVL* gene to confirm diagnoses. The result provides a difficult quandary if only one sequence variation is found or a variant of unknown clinical significance is identified. There is a temptation to call all such variants as disease-causing but this is clearly not the case. Database registries are helpful in this regard (28,29). Database registries should allow longitudinal clinical data to be included as many centres treat all positive cases and hence very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) related complications are prevented from birth. It is difficult therefore to discriminate whether the patient would have been asymptomatic or real complications have been prevented. Over time, the pathological mutations and benign polymorphisms may be inferred.

- ❖ Defining non-pathological causes of variation. Over the course of implementation of MSMS screening it is very clear that many lives have been saved with conditions such as MCADD deficiency and maple syrup urine disease. However, it is also clear that there is an over-representation of biochemical variants of analytes such as C4- and, C5OH-carnitine which may not necessarily relate to an actual disease entity. In New South Wales, we have discontinued screening for short-chain acyl-CoA dehydrogenase deficiency (SCADD) because of the over-representation of “cases” versus historical cohorts and identified cases were asymptomatic without any treatment (30,31). Similarly other programmes are considering discontinuing screening for 3-methylcrotonyl-CoA carboxylase (3-MCCC) deficiency because of the large numbers of asymptomatic mothers identified by screening programmes.

MSMS based screening protocols appear to have changed the landscape of newborn screening in the dynamic complexity of interpretation of many simultaneous results (31). However, most developed economies would consider such programmes as a necessary cost-effective tool to identify a range of severe disorders.

### Other disorders

The conditions so far described are included on all Australian bloodspot newborn screening programmes. Evaluation of test suitability should include the following assessments: there is a demonstrated benefit or likely benefit from early diagnosis; there are suitable tests and treatment; follow-up services are available. The benefit may or may not be balanced against financial and other costs depending

on the available technology, the frequency of the disorder in the region and other factors. Screening is possible for the following conditions depending on local circumstances.

- ❖ Biotinidase deficiency;
- ❖ Galactosaemias;
- ❖ Haemoglobinopathies;
- ❖ Severe combined immunodeficiency (SCID).

Screening is currently not recommended in Australia for the following conditions where screening tests are not available, or, tests are available but proof of advantage from early diagnosis is absent or uncertain, or the test is unsuitable or does not detect those cases in which there might be an advantage (32). New knowledge about screening and screening outcome in these conditions should be monitored regularly.

- ❖ Bile acid disorders;
- ❖ Cytomegalovirus;
- ❖ Duchenne muscular dystrophy;
- ❖ Familial hypercholesterolemia II;
- ❖ Fragile X;
- ❖ G6PD deficiency;
- ❖ Haemochromatosis;
- ❖ Most lysosomal storage disorders;
- ❖ Toxoplasmosis.

### Future directions

In 2006, The American College of Medical Genetics and Genomics published an evaluation scheme for assessing various disorders for consideration on the expanded newborn screening panel (33). This rigorous process systematically evaluated the indications for screening by asking a selection of specialists to assess per individual disease: the burden of untreated disease, the throughput and cost of testing strategy, the availability of confirmatory testing, the simplicity of diagnosis and treatment, the benefit of early intervention to the individual, family and society and the prevention of mortality. Using a standardised scoring system, the various disorders were ranked with MCADD, PKU and CH not surprisingly being ranked amongst the highest. There had been some discussion about screening for lysosomal storage disorders but in this paper, conditions such as Fabry disease, Krabbe disease and Pompe disease scored amongst the lowest from all the conditions being considered. It was acknowledged that the seminal publications indicating efficacy of therapy for infantile-onset Pompe disease were yet to occur and this condition in particular would score higher in future

years (34,35). However, for Fabry and Krabbe diseases, the reliability of classical case definition and the evidence base for efficacy of early intervention remained elusive (36-38). By multiplexing some of the lysosomal storage disorders together, the cost of screening can be reduced, but the difficult question of how to manage results needs to be worked through for individual disorders (39,40). SCID is the latest condition to have reached the national screening threshold for USA after a systematic approach of evaluating treatment and testing strategies was undertaken (41-44).

As more and more technologies become available, it will open up opportunities to look at all types of conditions and risk factors. Whilst it is technically possible to screen for nutritional deficiencies such as vitamin B<sub>12</sub> deficiency, or infections such as CMV or congenital disorders such as Duchene muscular dystrophy or fragile X syndrome, a strategy needs to be in place to manage the results in terms of counselling and intervention. The availability of read-through technology for certain disorders does provide a real indication for genetic targeted screening (45-47). Some are considering the panacea of whole genome screening, but this will provide risk factors for conditions yet to come and identify disorders that are yet to be described. Genomic screening may also identify risks for disease that would never manifest in the lifetime of the individual so it is imperative that any information acquired from whole genome or exome analysis is explained adequately to the end-user (48). Massively parallel sequencing (MPS) could be implemented with agreed disease targeted gene panels, lowering the risk of incidental findings and increasing depth of coverage. Countries with very large populations, spread over huge distances such as in India and China now embarking on screening programmes need to consider what conditions are prevalent, what treatments are available and how they may be delivered to the affected individual wherever they are. This may indeed be the biggest challenge.

Over the last 50 years, newborn screening using simple spots of blood has evolved using the most contemporary technology of the time, such that it now has the potential to identify a huge range of conditions. Each country needs to be aware of the potential and limitations of each technology and how they may apply these to treat their own population.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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