Int. J. Neonatal Screen. **2015**, *1*, 3-12; doi:10.3390/ijns1010003

OPEN ACCESS

International Journal of

Neonatal Screening

ISSN 2409-515X

www.mdpi.com/journal/neonatalscreening

Review

The Story of Biotinidase Deficiency and Its Introduction into Newborn Screening: The Role of Serendipity

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Academic Editor: Ralph Fingerhut

Received: 11 February 2015 / Accepted: 27 February 2015 / Published: 5 March 2015

Abstract: Today, all of the states in the United States and many countries screen their newborns for biotinidase deficiency. Biotinidase deficiency meets the major criteria for including a disorder into screening programs. However, rarely do we learn the actual story behind the discovery of a disorder where the underlying etiology was elusive or about the events leading to a disorder's incorporation into a newborn screening program. This is the story of the role that serendipity played in the story of biotinidase deficiency and the newborn screening of the disorder.

Keywords: biotinidase deficiency; biotinidase; newborn screening; screening

Before 1982, multiple carboxylase deficiency (MCD) was the descriptive name for two apparently different inherited organic acid disorders. One was called early-onset or infantile MCD because affected children first presented with symptoms within days of birth, and the other was called late-onset or juvenile MCD because symptomatic children first presented after a month of age [1,2]. Individuals with each disorder exhibited similar neurological and cutaneous symptoms and biochemical abnormalities [3]. In addition, individuals with each disorder would usually improve after being administered pharmacological oral doses of the vitamin, biotin. Although there was some overlap in the time of presentation of symptoms, the symptoms of those with early-onset MCD were subsequently shown to be due to a deficiency of the enzyme, biotin holocarboxylase synthetase, the enzyme required for covalently attaching biotin to the various apocarboxylases converting them to active holoenzymes [4,5]. However, the cause of the late-onset form of MCD was more elusive.

After completing my post-doctoral studies of propionyl-CoA carboxylase deficiency or propionic acidemia in the Department of Human Genetics at Yale University School of Medicine under the supervision of Drs. Leon Rosenberg and Ted Hsia, I became an Assistant Professor of Human Genetics and Pediatrics at the Medical College of Virginia. My first funded National Institutes of Health grant dealt with the continuation of research that I had begun at Yale. This work also included studying individuals with MCD by measuring their mitochondrial carboxylase activities in cultured skin fibroblasts or isolated peripheral lymphocytes [2].

While studying aspects of biotin metabolism and its relationship to propionic acidemia, I discovered the monograph of Dr. Jaakko Pispa of the Department of Medical Chemistry of the University of Helsinki entitled "Animal Biotinidase" [6]. This was the first serendipitous event. Dr. Pispa performed a large number of experiments characterizing the biochemical and physiological properties of the enzyme biotinidase [6,7]. This enzyme cleaved biocytin (biotinyl-ε-lysine), thereby releasing free biotin from the epsilon amine of lysine. Dr. Pispa's work was forever referred to in our laboratory as the "Bible." However, in his entire treatise, Dr. Pispa never really discussed the biological relevance of this enzyme. It soon became time to write my grant renewal to continue my studies of propionic acidemia and aspects of biotin metabolism. It was in this renewal application that I included a short section consisting of a single paragraph describing the possibility that some individuals with late-onset MCD have a primary enzyme deficiency of biotinidase activity. I speculated that these individuals could not recycle or reuse the endogenous biotin from the bound biotin (biocytin) which resulted from the proteolytic degradation of holocarboxylases. Although the carboxylases could be broken down to their constituent amino acids, some lysines remained attached to the biotin as biocytin. Only biotinidase was capable of cleaving the amide bond releasing the biotin and lysine, thereby recycling the biotin. Defective biotinidase would result in the inability to recycle the biotin and, thus, create a state of biotin deficiency, especially if inadequate amounts of exogenous biotin were ingested. This biotin deficiency would result in insufficient biotin to convert the carboxylases to their active form resulting in MCD. Although the grant was funded, I was informally told that this speculation about biotinidase being the primary defect in MCD was "a bit naïve."

At about this time, two reports were published, one in the New England Journal of Medicine by Jess Thoene's group at the University of Michigan [8] and the other in The Lancet by Dr. Arnold Munnich's group in France [9] indicating that the late-onset form of MCD was due to defective intestinal absorption of biotin. Affected children failed to absorb sufficient quantities of the exogenous vitamin from their diet, resulting in biotin deficiency and insufficient quantities of biotin to activate the holocarboxylases, resulting in MCD.

This is when the second serendipitous event occurred. Because my laboratory had been characterizing and sorting out children with both types of MCD, I knew the locations of these children and the physicians who were involved with their diagnosis and care. Although I was told the idea that biotinidase deficiency was a "naïve" idea, once we were funded, Bob Grier, a graduate student, and I set up a slightly modified version of the assay to measure biotinidase activity in serum or plasma as described by Pispa [6]. Now all that we needed were serum samples from individuals with late-onset MCD. For the first child, I called Dr. Steve Goodman at the University of Colorado School of Medicine. Steve had recently sent us fibroblast samples from a child who we subsequently showed had the late-onset form of MCD. Therefore, I asked Steve if he could get a sample of this child's serum, samples from the parents

and several samples from unrelated control individuals. Fortunately, this all occurred before we needed Institutional Research Board approval. He agreed and said he would send sera from the child, his parents and several normal controls. Several days later we received a package from Denver with five frozen serum samples labeled A through E. Bob Grier immediately performed the enzyme determinations and found that two of the samples had about 100% of mean normal activity, one had about 50% of mean normal activity and two had essentially no enzyme activity. We obviously thought we had something interesting and exciting, but we needed to "break" the code. So I called Steve's laboratory only to find out that he was en route to Montreal to visit his mother. Moreover, no one in the laboratory knew the code because Steve prepared and shipped the samples himself. I was able to obtain Steve's mother's telephone number from someone in his laboratory, I excitedly called Mrs. Goodman, introduced myself and told her that this was not an emergency, but to please ask Steve to call us as soon as he arrived in Montreal. Late that night I received a call from Steve. I told him our results and he proclaimed "you got it." I asked him "what did we have? First, he said that he sent us two control samples, hence the two samples with normal activity. Second, although he had planned to send us samples from both parents, the father was ill and could not provide his serum, so Steve sent us only the mother's sample which had 50% activity. Third, because we were expecting five samples, he split the child's sample into two; the two samples with essentially no activity. The next day we celebrated in the laboratory. Although we ran several studies to be absolutely sure of our results, I slept well knowing that we had found the primary enzymatic defect for at least some individuals with late-onset MCD. I was certain because the mother had 50% of the mean normal activity which was expected for someone who was heterozygous for this recessive enzyme deficiency. After testing additional children with MCD and finding that they also had biotinidase deficiency, in November of 1982, we proceeded to publish our results that deficiency of biotinidase activity was the primary defect many children with late-onset or juvenile MCD [10,11].

We were well aware of the findings of two reports indicating that late-onset MCD was due to an absorption defect, but perhaps there were multiple causes of the disorder.

Our third serendipitous event then occurred. Soon after we had published our results describing that biotinidase deficiency was the cause of late-onset MCD, I received a telephone call from Dr. Mark Batshaw at Johns Hopkins Hospital about a child that he thought had the disorder. He wanted to send us serum from this child to determine his biotinidase activity. We received the sample and found that, in fact, the child was deficient and was responding markedly to biotin therapy. Unbeknownst to us, Mark also sent a plasma sample to Jess Thoene to determine if the child had a low plasma biotin concentration and possibly a biotin absorption problem. I was informed that Jess did find that the child had a low concentration of biotin. I was also told that the sample that we received was obtained after the child had already received a pharmacological dose of biotin, whereas the sample sent to Jess was before the child was supplemented. Therefore, we thought it was prudent to see if Jess could send us an aliquot of his sample, just in case the supplemental biotin affected biotinidase activity. Jess agreed and sent us four samples, labeled A through D. Bob Grier immediately performed the assay on these samples and found that two had normal activity and two had almost no detectable activity. I surmised that based on our recent past experience with the samples from Denver, Jess split the sample from Johns Hopkins in two. I called Jess and told him the results. There was long pause and then came the explanation. He told me he had sent two control samples, which had normal activities, and two samples from the children. Of the children, I thought the samples were only from one child. He informed me that one sample was in fact

from the child at Johns Hopkins, but the second sample was from the child he had reported had a biotin absorption defect.

I told Jess that I thought I knew how to reconcile the results. The child he reported did not really have both biotinidase deficiency and an absorption defect, but there was a problem in how the absorption studies were performed in both his study and the one in France. In each, the children were not receiving biotin supplementation for a period of time. They were actually made biotin-depleted. Therefore, when the child was administered a low dose of oral biotin, the biotin was absorbed normally, but it was rapidly taken up by the biotin-depleted tissues, resulting in no appreciable change in her plasma biotin concentration. However, when she was given a high dose of oral biotin, there was plenty of biotin to be taken up by the tissues and enough to raise the blood concentration as expected if normally absorbed. The child actually only had biotinidase deficiency.

To be absolutely certain of this, we decided to first repeat the biotinidase activity measurement on a fresh sample from this child. Our fourth serendipitous event occurred. We received a new sample and found that instead of finding biotinidase deficiency again, the child now had activity that was considerably above normal. Bob Grier and I were astonished and dismayed. We did not know what had happened. Perhaps the sample we received was contaminated with bacteria and the bacteria were supplying the enzyme? We actually took some of the sample to our Microbiology Department to culture. However, it was soon thereafter that Bob resolved the dilemma. He put the color-developing reagents into a sample of the serum without substrate. The mauve color appeared as it did when there was "enzyme activity, but we had no substrate in the reaction. This indicated that there was something else in the serum that caused the color development. We knew that the colorimetric biotinidase activity assay was based on the Marshall-Bratton reaction [12] and that the color-developing reagents would cause color in the presence of any primary aromatic amines. Our artificial substrate is biotinyl-para-aminobenzoate, which in the presence of biotinidase, cleaves the amide bond releasing biotin and the primary aromatic amine, para-aminobenzoate. One likely candidate substance in the sample was a sulfa drug, such as a sulfonamide. I called Jess and asked if he knew if the child was being treated now or recently for an infection. He did not think so, but he would call the mother. He called back in about an hour to tell us that, indeed, she had just finished a course of Bactrim for an ear infection. It was this fifth serendipitous event that taught us that any new serum sample that comes into the laboratory must be tested with and without substrate to determine if there is a substance in the serum that could cause the development in the absence of enzyme. This protocol has become standard procedure in the testing of any sample ensuring that only biotinidase activity is determined.

I next discussed with Jess that we should repeat the biotin supplementation of the child when she was biotin replete, rather than biotin-depleted. This was done and, as predicted under these conditions, the child's plasma biotin increased even with the low dose of biotin. This reconciliation was published [13]. Moreover, we learned that the child from France who was reported as having an absorption defect was also found to have biotinidase deficiency.

Children with biotinidase deficiency, if left untreated, likely developed neurological problems, including seizures, developmental delay, eye problems, and hearing loss, which can progress to coma and/or death [14]. If an affected child is identified before developing symptoms, essentially all of these clinical problems associated with the disorder could be prevented by simply administering them biotin. Since we reported our findings, we began to receive blood samples from symptomatic children with

MCD from around the country and world. Our reports and those of others described the range and variability of symptoms of the disorder [14–17]. This included the age of onset of symptoms, types of neurological and cutaneous symptoms, and the severity of symptoms. We also found that one could not depend on finding the characteristic organic acid metabolites in the urine of symptomatic children to identify an affected child. Moreover, we found that certain features of the disorder tended to be irreversible if they occurred before treatment was initiated [18,19]. These findings made it clear to us, that biotinidase deficiency was an excellent candidate for consideration for inclusion in newborn screening programs. We also thought the disorder readily met the major criteria for inclusion in a newborn screening program. Therefore, we set out to develop a test that would be suitable for screening newborns for biotinidase deficiency and, to determine if we could perform a pilot newborn screening trial to identify newborns with the disorder, initiate therapy, and hopefully prevent the development of symptoms. I must confess that although I was a biochemical geneticist and pediatrician, I really did not have a clear understanding about how a newborn screening program functioned. I did get called to take care of children with the few disorders screened for at the time, specifically phenylketonuria, galactosemia, and maple syrup urine disease. However, my other contact with the laboratory was when I was called by my newborn son's pediatrician and informed that the state laboratory had notified him that my son needed to have his newborn screening test repeated. After the initial anxiety, I personally called the laboratory only to find out they were requesting a repeat sample because the first screening card had an insufficient quantity of blood spotted on the card to perform the testing. Now, I understood how the parents felt when they received a similar call from their pediatrician after the delivery of a healthy newborn.

To perform a pilot screening program, I realized we would have to gain the cooperation of Virginia's newborn screening laboratory. It is at this point, we encountered a series of fortunate serendipitous circumstances and events.

We had to develop a method of determining biotinidase activity in the same blood-soaked filter paper cards used for the currently performed newborn screening tests. The colorimetric method that we used to quantitatively measure biotinidase activity in serum seemed the best method for the newborn screening test [20]. We decreased the volumes of reagents, but needed a method to stop the enzymatic reaction to make an end-point assay. I was able to come up with five different strategies to stop the reaction and still permit the addition of the color-developing reagents to determine activity. A technician and a graduate student suggested a sixth alternative. They tried all six methods at the same time. After the trial, the duo showed up at my office with serious expressions on their faces. I asked about the results and they told me that all five of my suggested methods failed, but theirs worked. A huge smile came over my face, and they stared at each other wondering why I was so overjoyed at being wrong. I congratulated them and exclaimed that we now have a newborn screening test. This was the sixth serendipitous event.

Because the assay method was semi-quantitative, we subsequently refined it to provide no color development for samples with 25%–30% of mean normal serum activity so we could readily identify putative positive samples out of hundreds by merely scanning by eye for those that developed the characteristic mauve color. We now had a method that we felt was suitable to be used for newborn screening of the disorder.

We could now approach the state newborn screening laboratory about initiating a pilot screening program. Fortunately, the laboratory was using only seven of eight punches from a patient's card and was discarding the eighth spot. This was the seventh serendipitous event. The state laboratory was allowed to participate in "research" of new disorders to be considered for inclusion into the screening program. Therefore, that eighth spot obtained from each newborn's card soon became ours. This was the eighth serendipitous event.

We developed a tray to accept this blood spot. In addition, the design was based on the spacing used for the other spots and that allowed us to readily identify a specific individual if we found a putative sample with deficient activity. A technician from our laboratory, Linda Jefferson, went to the state laboratory every morning to pick up the trays with the day's eighth spots. She put the trays in a cart and wheeled them back to our laboratory about a half mile away to perform the enzyme assays. After about 37,000 tests, the state was getting concerned that we had not yet identified a baby with the disorder. We, however, were not. Those in newborn screening know this is not unusual given the incidence of these disorders; however, the state called for a meeting to address whether to discontinue the pilot program. Just before the meeting, we had our ninth serendipitous event. Katie was born. Katie was the first child with biotinidase deficiency in the world identified by newborn screening [21]. My departmental chairman, Dr. Walter Nance, and I drove to a small town in Western Virginia to visit the family and explain "why" we created so much anxiety for the family. Katie, in fact, was also found to have early hearing loss, but with biotin therapy this was resolved. Katie, is now in her thirties and has two healthy children.

In 1983, Delegate Kenneth Plum of the Virginia Legislature was in the process of bringing several other metabolic disorders into the Virginia newborn screening program. Prior to meeting us, he had already secured state funding for them. This was the tenth serendipitous event. Subsequently, he met with us and "championed" adding biotinidase deficiency to this list of disorders. We appeared before the legislature to explain why biotinidase deficiency should be added to list. We were asked what it would cost the state to add the testing for biotinidase deficiency. Because Delegate Plum had requested sufficient funding the year before to cover the cost of all other disorders, including biotinidase deficiency, our answer to question of cost was "nothing." Not unexpectedly, newborn screening for biotinidase deficiency was unanimously approved. Then our eleventh serendipitous event occurred. In fact, when biotinidase deficiency was written into the final bill, because of the physical spacing of the disorder's names on the page of the bill, the secretary put biotinidase deficiency as the first disorder listed in the final transcription of the law. In September, 1984, Virginia became the first state in the United States and the world to mandate newborn screening for biotinidase deficiency [21,22].

In 1985, I went to my first National Symposium on Newborn Screening in Columbus, Ohio, to present our experience screening newborns in Virginia [23]. I was definitely out of my element at a meeting where important topics were the uniformity of the filter paper card, which punch machine was the best, and what was the best way of reporting newborn screening results. Two major things happened at this meeting. The first occurred immediately after I presented our screening results. A negative comment came from the audience that I spent too much of my talk discussing clinical aspects of the disorder. I actually thought this group would be interested in why we were proposing that they screened for biotinidase deficiency. The second thing that occurred during the question session was when, Dr. Robert Guthrie, the "father of newborn screening" stood and publically congratulated me on our achievements

and indicated that although we had identified four children with biotinidase deficiency during the first two years of screening, we only had to reach a "magic" number of ten affected children to warrant incorporation of the disorder into a state's screening program. Although I and many in the audience thought that the incidence of the disorder would be a determining "method," clearly Dr. Guthrie had a different sense of what was important. I was concerned about the numerator and the denominator, whereas Dr. Guthrie was only concerned about the magnitude of the numerator.

Dr. Guthrie and I became good friends over the following years. We readily met his numerator expectations. He was always interested in knowing how my work was going and was very encouraging. We were both invited to be guest speakers at a symposium at the Virginia Newborn Screening laboratory.

It was purported that there were an increased number of false positive tests for galactosemia during the summer, there was speculation that galactose-1-phosphate uridyltransferase activity in the dried blood spots because of their sensitivity to heat exposure. This raised speculation that the other newborn screening test that was also enzyme based might exhibit seasonal variation. We studied this and found that although the number of false-positive tests for galactosemia did increase during the summer months, there was not a similar increase in the false positive rate for biotinidase deficiency [24]. We did find, however, that if the newborn screening cards were not thoroughly dried before placing them in a shipping envelope, enzyme activity did decrease.

Over the years, other states and countries followed Virginia's lead, and today all states in the United States and many countries screen their newborns for biotinidase deficiency [25–29]. However, there are still countries that do not screen their newborns for profound and/or partial biotinidase deficiency. I have recently presented the arguments for inclusion of both profound and partial biotinidase deficiency into newborn screening programs [30].

Our laboratory cloned and determined the cDNA that encodes biotinidase [31] and elucidated the genomic organization of the biotinidase gene [32]. We characterized common mutations causing profound biotinidase deficiency among newborns identified by screening in the United States [33]. We have shown that most children with partial biotinidase deficiency have one specific mutation that reduces enzyme activity by about half [34]. More than 150 mutations have been found to cause biotinidase deficiency [35]. Mutation analysis is now used to confirm diagnoses and help counsel families about their recurrence risks.

We have identified multiple issues related to newborn screening and the treatment of children with the disorder [19,36,37]. We have been involved with establishing the guidelines for newborn screening of biotinidase deficiency [38]. We, and others, have and are evaluating the long-term outcomes of children who were identified by newborn screening [39,40].

Besides the colorimetric method of screening, other methods have been developed and commercialized [41,42]. Recently, there has been a digital microfluidity method that allows for multiplex testing and higher throughput [43].

It is not often a scientist gets an opportunity to relate the actual story of a discovery, its incorporation into newborn screening around the world, the ups-and-downs of their research, and relating the role of serendipity in their research. This story is dedicated to all those who participated in our research, colleagues, collaborators and practitioners around the world. I thank the fellows, students and physicians who participated in the care and/or research. In particular I would like to single out the Department of General Services-Division of Consolidated Laboratory Services of Virginia which allowed us access to

the spots and professionals to pilot screening for the disorder. I especially dedicate this story to "my" children with biotinidase deficiency and their families everywhere. The photographs of literally hundreds of them fill the walls of my office. Thank you all.

Acknowledgment

This work was supported in part by the Safra Research Fund at Henry Ford Hospital.

Conflicts of Interest

The author declares no conflict of interest.

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