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Sequencing from Dried Blood Spots in Infants with “False Positive” Newborn Screen for MCAD Deficiency

Shawn E. McCandless^{a,b}, Ram Chandrasekar^c, Sharon Linard^c, Sandra Kikano^a, and Lorrie Rice^a

^aDepartment of Genetics and Genome Sciences, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA

^bCenter for Human Genetics, Rainbow Babies and Children's Hospital/University Hospitals Case Medical Center, 11100 Euclid Avenue, Cleveland, OH 44106, USA

^cOhio Department of Health, Public Health Laboratory, Newborn Screening Program, 8955 East Main Street, Reynoldsburg, OH 43068, USA

Abstract

Background—Newborn screening (NBS) for medium chain acyl-CoA dehydrogenase deficiency (MCADD), one of the most common disorders identified, uses measurement of octanoylcarnitine (C8) from dried blood spots. In the state of Ohio, as in many places, primary care providers, with or without consultation from a metabolic specialist, may perform “confirmatory testing”, with the final diagnostic decision returned to the state. Confirmatory testing may involve measurement of metabolites, enzyme analysis, mutation screening, or sequencing. We now report sequencing results for infants said to have “false positive” NBS results for MCAD deficiency, or who died before confirmatory testing could be performed.

Methods—Dried blood spots (DBS) were obtained from all 18 available NBS cards identified as “false positive” by NBS for the 3 year period after screening began in Ohio in 2003 (N=20, thus 2 had no DBS available), and from all 6 infants with abnormal screens who died before confirmatory testing could be obtained. DNA extracted from DBS was screened for the common c.985A>G mutation in exon 11 of the ACADM gene, using a specific restriction digest method, followed by sequencing of the 12 exons, intron-exon junctions, and several hundred base pairs of the 5′ untranslated region.

Results—The NBS cut-off value for C8 used was 0.7 μmol/L. Sequencing of ACADM in six neonates with elevated C8 on NBS who died before confirmatory testing was obtained did not identify any significant variants in the coding region of the gene, suggesting that MCADD was not a contributing factor in these deaths. The mean C8 for the 18 surviving infants labeled as “False Positives” was 0.90 (95%CI 0.77-1.15), much lower than the mean value for confirmed cases. Ten of the 18 were premature births weighing <1200 g, the rest were normal sized and full term. Eight infants, mostly full term with appropriate birth weight, were heterozygous for the common c.985A>G mutation; one of those also has a novel sequence change identified in exon 9 that predicts

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Corresponding author: Shawn E. McCandless, Case Western Reserve University School of Medicine, 2109 Adelbert, Room 622, Cleveland, OH 44106 Tel: +1 216 844-7236, Fax: +1 216 844-7497, shawn.mccandless@case.edu, shawn.mccandless@case.edu (SE McCandless), ram.chandrasekar (R Chandrasekar), sandra.kikano@case.edu (S Kikano); sharon.linard@odh.ohio.gov (S Linard), lorrie.rice@case.edu (L Rice).

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a PRO to LEU change at residue 258 of the protein. Both the phase and any possible clinical significance of the variant are unknown, but several lines of evidence suggest that it could lead to protein malfunction. That child had an NBS C8 of 2.2, more than double the mean for the False Positive group. Unfortunately, the study design did not provide clinical outcome data, but the child is not known to have presented clinically by age 7 years.

Conclusions—These results suggest that sequencing of ACADM from dried blood spots can be one useful follow-up tool to provide accurate genetic counseling in the situation of an infant with elevated C8 on NBS who dies before confirmatory testing is obtained. Of surviving neonates, there appear to be two populations of infants with false positive NBS C8 values: 1) term AGA infants who are heterozygous for the common c.985A>G mutation, and, 2) premature infants, regardless of carrier status. The finding of two sequence variants in an infant reported to the state as not affected suggests the possibility that some infants with two mutations may be reported as normal at follow-up. State registries may wish to consider asking that metabolic specialists, who are most familiar with the variability of these rare disorders, be involved in the final diagnostic evaluation. Finally, providers may wish to consider ACADM sequencing, or other diagnostic testing, as part of the confirmatory evaluation for infants with NBS C8 concentrations that are significantly above the cut-off value, even if plasma and urine metabolites are not strikingly increased.

Keywords

Medium-chain acyl-CoA dehydrogenase deficiency; fatty acid oxidation disorders; newborn screening; dried blood spots

1. Introduction

Newborn screening for medium chain acyl-CoA dehydrogenase (MCAD) deficiency, the most common inborn error of metabolism identified by newborn screening (NBS) [1] has been a true public health success, doubtlessly saving lives. The newborn screening test itself, using octanoylcarnitine (C8) as the marker, is both reasonably specific and highly sensitive, meaning that there are relatively few false positive results, and, to date, no reports in the literature of missed cases [2]. Typically, confirmatory testing includes one or more metabolic measures, often repeating the acylcarnitine profile on plasma, measurement of urinary dicarboxylic acids and acylglycines, and sometimes enzyme assay. Sequencing is also available as a diagnostic test, but may be limited because of the expense and perceived lack of added clinical benefit. In fact, the American College of Medical Genetics has published an algorithm for diagnostic testing for MCAD deficiency that specifically shows DNA analysis only if there is persistence of the metabolic abnormalities. On the other hand, clinical experience with other defects of fatty acid oxidation disorders, particularly very long chain acyl-CoA dehydrogenase deficiency [3, 4], suggests the possibility that reliance on metabolic measurements alone may be misleading. We therefore undertook a study to evaluate the possibility of missed cases of MCADD in two groups of subjects with abnormal newborn screen results, those who died before confirmatory testing could be obtained and those reported as false positives. We hypothesized that we would find, at least, heterozygous mutations for ACADM (the gene coding the MCAD protein, NCBI accession number **NM_000016.4**) in the infants who died before confirmatory testing. We further hypothesized that we would not find any “false positive” subjects with two mutations. Both hypotheses turned out to be incorrect.

2. Materials and methods

Human subjects protection approval for this work was obtained both from the University Hospitals Case Medical Center and the State of Ohio, Department of Health Human Subjects Protection Review committees. Minimal de-identified clinical information (NBS C8 value, gender, reported race, gestational age, birth weight, final diagnosis used to close the case, and method of confirmatory testing) was provided along with two 3mm punches from the dried blood spots (DBS) of subjects with NBS C8 measurement of greater than 0.7 mol/L, the cut-off value used in Ohio. All subjects screen positive for C8 from the inception of the expanded NBS in 2003 through the beginning of the study were included.

DNA was extracted from the DBS using a Qiagen QIAamp DNA Micro Kit (#56304) kit optimized for extracting DNA from DBS and other very small samples. Each sample was screened for the common c.985A>G mutation in exon 11 of the ACADM gene by PCR amplification of the region using nested primers followed by restriction enzyme analysis using NcoI as previously described [5]. As none of the false positive samples was homozygous for the c.985A>G mutation by the screening assay, each exon of the ACADM gene was amplified from the DNA obtained from the DBS by PCR (primers shown in supplemental Table 1) and sequenced using an ABI 3730 instrument in the Department of Genetics and Genome Sciences Genomics Core Facility of the Case Western Reserve University School of Medicine, Cleveland, OH. Bioedit software (Thomas Hall, <http://www.mbio.ncsu.edu/bioedit/bioedit.html>) was used for sequence alignment, and aligned sequences were manually compared to the reference sequence from the NCBI (mRNA -- NCBI Reference Sequence: NP_000007.1; genomic sequence -- NCBI Reference Sequence: NG_007045.1).

Statistical analyses were performed in Excel for Macintosh (*Part of Microsoft Office Professional Edition*. Microsoft; 2008). The student's t-test was used for comparison of continuous data.

2.2 Definitions

The following terms are defined for this study. When capitalized, the term refers to the group of subjects meeting that criteria in this study; when uncapitalized, the term refers to an individual subject:

- “Screen Positive (SP)” is defined as an individual with a positive NBS for, in this case, C8, suggestive of increased risk for MCAD deficiency.
- “Normal (NL)” refers to an individual who is not suspected to have MCAD deficiency, either because the NBS C8 value was below the cut-off, or they were screen positive but had normal (negative) confirmatory testing.
- “Affected” refers to an individual with a confirmed diagnosis of MCAD deficiency.
- “True Positive (TP)” refers to an individual who was screen positive and was determined to be affected on confirmatory testing.
- “False Positive (FP)” refers to an individual who was screen positive but determined to be normal on confirmatory testing.
- “False Negative (FN)” refers to an individual whose NBS C8 was below the cut-off but was later determined to be affected by confirmatory testing.
- “Heterozygote” refers to an individual who has a single copy of the mutation in question, with the normal sequence on the other allele.

- “Carrier” is defined as an individual who is not affected with MCAD deficiency, but who has one heterozygous mutation in the ACADM gene.

3. Results and Discussion

During the study period 53 individuals were identified with abnormal NBS C8 values suggestive of MCAD deficiency (Screen Positive). Of those, 29 were reported to the NBS Laboratory to be affected with MCAD deficiency, 6 died before confirmatory testing was obtained (NDR, or “no diagnosis recorded”) and 18 cases were reported to the Ohio NBS Laboratory as normal and are considered to have a false positive NBS for C8. The mean NBS C8 values (Figure 1) for the NDR group and the False Positive group were similar to each other (mean 1.00 vs 0.90, $p=.46$), and both were strikingly different from the Affected (MCAD deficient) group (mean C8 13.26, $p=.005$ and $p=.00001$ compared to NDR group and the False Positive group, respectively).

3.1 “Affected” individuals

To confirm the method, 4 of 29 MCAD affected patients, 2 of whom were reported to be homozygous for c.985A>G, and 2 heterozygous for c.985A>G, had DNA extracted from the DBS and full sequencing was performed. The remaining 25 affected individuals did not have molecular testing done as part of this study, thus their genotype is not known. The clinical results were confirmed in both of the reported c.985A>G homozygotes in whom sequencing was performed.

One of the MCAD deficient children heterozygous for c.985A>G (NBS C8 value = 2.5 mol/L) had confirmatory plasma acylcarnitine analysis showing typical findings of MCAD deficiency (C6=1.25 mol/L, C8=4.25 mol/L, C10=0.85 mol/L, 3-, 8- and 2-fold above the upper limit of normal for the clinical lab that performed the analysis, respectively). Clinical testing had not identified a second mutation, however a novel heterozygous sequence variant was identified by full sequencing at position c.1118T>C (p.V373A). This amino acid residue, immediately adjacent to the FAD binding site in the protein, is highly conserved in the protein, and is predicted to be deleterious by both Polyphen [6] and SIFT [7]. The study design using completely de-identified samples did not allow for contacting the family to obtain parental samples to identify the phase of the mutations or to determine clinical outcome.

A second heterozygous individual, reported by the primary care provider to the NBS lab with a final diagnosis of “variant MCAD”, did not have a second mutation identified in this study. The NBS C8 in that infant was 0.84 mol/L, which is in the range seen for MCAD carriers who are heterozygous for the common c.985A>G mutation, but well below the range of values seen in confirmed affected individuals (Figure 1). While we cannot rule out an intronic variant or some other mutation outside of the coding regions examined, in this case the data suggest a misleading classification by the provider giving feedback to the NBS laboratory. This case points out the need for NBS laboratories to specifically and precisely define terms used for closing out cases, and it also suggests that experts in the diagnosis of inborn errors of metabolism should be involved during the diagnostic process to ensure accurate interpretation of results, both to the state tracking mechanism as well as to the family of the infant.

3.2 Infants who died before confirmatory testing

Details regarding the 6 subjects with screen positive NBS C8 who died before confirmatory testing was obtained (NDR = “no diagnosis reported”) are shown in Table 1. The entire coding region of the ACADM gene was sequenced in all 6 subjects. No copies of the

common c.985A>G mutation were found in these individuals nor were any other deleterious mutations or variants of unknown significance identified. Several studies demonstrate that in the case of confirmed MCAD deficiency 96% or more of individual alleles are identified by exon sequencing [8, 9] but that only a tiny fraction of patients, ranging from 0% [10] to ~1.5% [8], have no recognized mutation. Therefore, while DNA sequencing cannot rule out the condition with 100% confidence, the probability that an affected infant would have no identifiable mutation is quite low.

The distribution of NBS C8 values in this group was similar to that of the False Positive group (Figure 1), but clearly different than the Affected (MCAD deficient) group of subjects. Care must be taken in comparing these two groups, though, because the timing of the sample collection was very different. In spite of that caveat, these findings suggest that MCAD deficiency was not a factor in the neonatal deaths in these mostly premature infants.

As noted, the samples in the NDR group were obtained at an average of 18 days of age, with the latest collected at 38 days, consistent with the fact that 4 of the 6 samples were repeats obtained for other reasons: either the first sample was drawn before 24 hours of age (n=2) or there was different abnormality on the initial screen (n=2, elevated 17-hydroxyprogesterone and low thyroid stimulating hormone). The policy in Ohio is that all children, regardless of gestational age, be screened at 24 to 72 hours, with the law requiring that testing be obtained by 120 hours for all infants born in the hospital. Some experts believe that a low-risk initial screen for C8 in the 2 infants with initial NBS obtained in the recommended time frame would be sufficient to rule out MCAD deficiency and that no further testing would be required in the case of a screen positive NBS from a repeat sample. It should be noted that this approach has not been evaluated in the literature. Our data suggest that elevated C8 may be a non-specific marker for infant distress, particularly in very low birth weight infants.

It is worth noting that one of the samples shown in Table 1 had a C8 concentration of 1.47, which is within the range reported as suggestive of MCAD deficiency in DBS from post-mortem samples of infants with unexplained death [11]. The sample in the current study was obtained at 38 days of life. While not a post-mortem sample, this finding suggests that elevated C8 at this age is not always due to MCAD deficiency. Chace, et al. [11], described a variety of other tools, including the use of acylcarnitine ratios and DNA sequencing, to confirm the diagnosis when the post-mortem screening test is above the cut-off range. In any such case the data must be interpreted in their entirety to make an accurate diagnosis, but the results of this study suggest that sequencing the ACADM gene can be one of several useful tools to confirm that MCAD deficiency is not a concern in infants with abnormal NBS who die before confirmatory testing can be obtained. This approach provides for the most accurate genetic counseling for the family after the death of their child.

At the time the data were being collected in the NBS laboratory C10 values were not measured. In an attempt to evaluate the potential utility of using ratios, for example C8/C10, from NBS data to give added information in these NDR cases we re-analyzed the acylcarnitines in four of the six samples that had remaining blood spots. The original samples had all been destroyed by the state NBS lab following a new policy instituted several years ago, and 2 of the 6 samples had been entirely consumed in the sequencing process. The remaining 4 samples had been stored at room temperature without special packaging from collection to the time of this analysis, which in some cases may have been as much as 8 years.

The DBS were assayed in our clinical laboratory using liquid chromatography-mass spectrometry (LC/MS), which is able to resolve individual isomers of the 8 carbon acylcarnitines, including valproylcarnitine. The assay performed uses standard curves of

valproylcarnitine and octanoylcarnitine, and a heavy-labeled stable isotope (D3-octanoylcarnitine) internal standard, to allow true quantitation. In the four samples from which data could be obtained, the C8 value (sum of all of the acylcarnitines with a molecular weight matching octanoylcarnitine) was well below that originally measured by MS/MS. This almost certainly reflects deterioration of the sample due to inadequate storage, however, another interesting finding was observed. All four samples had a modest quantity of a second compound, with the same molecular weight as C8, which eluted just before the valproylcarnitine standard (see supplemental figure 1). In all four samples that compound accounted for the majority of the C8 species still present in the sample. We have observed the same peak in other plasma and urine samples in the clinical laboratory from infants in whom valproic acid had not been given therapeutically, suggesting that there is another organic compound, possibly a drug, a dietary additive, or a product of gut bacterial metabolism of a longer branched chain compound, that has a molecular weight identical to octanoylcarnitine (and valproylcarnitine) and elutes close to, but not completely with, valproylcarnitine in the LC/MS system. It is impossible to be certain from these data whether this represents a degradation product from sample storage; however, it does suggest a possible cause of interference with the MS/MS acylcarnitine interpretation in newborn screening.

3.3 False positive NBS results

The remaining 18 subjects were reported to the state laboratory as false positives for NBS C8 because confirmatory testing was within normal limits. The entire coding region of the ACADM gene was sequenced in all 18. Ten of the 18 had no deleterious variant identified by sequencing. Eight of 18 were found to be heterozygous for the common mutation (c.985A>G); all 8 carriers with the common mutation were born at >36 weeks gestation and birth weight was appropriate for gestational age (mean 3.22 kg). Figure 2 shows that subjects in the False Positive group who were heterozygous for the common mutation (c.985A>G) were born closer to term than those who had no mutation (mean 37.8 vs. 27.5 weeks, $p=.0006$) and, consistent with the gestation, they had higher birth weight (mean 3.24 kg vs. 1.13 kg, $p=4\times 10^{-5}$). This suggests that there are two major groups of false positive NBS C8 results, likely with different causes; namely, carriers of the common mutation (and, conceivably carriers of other deleterious mutations) and premature infants. Only two of the ten in the False Positive group without identified mutations were born at full term and had normal birth weight.

One of the 8 common mutation carriers was also found to carry a previously undescribed sequence change in exon 9, c.873C>T (Supplemental Figure 2), predicted to change a proline residue to leucine at amino acid residue 258 (p.P258L). This child had a significantly elevated NBS C8 (2.2), which was more than 4 standard deviations above the mean of all the False Positives, including this sample, (mean C8 0.90, s.d., 0.29, 97th %ile 1.52), with a value that falls within the range seen in those subjects confirmed to be MCAD deficient. While the use of predictive algorithms to determine the potential for deleterious effects on protein function must be interpreted with caution, the novel nucleotide change identified is predicted by both SIFT [7] and PolyPhen [6] to be deleterious (data not shown), which is supported by the strong pattern of evolutionary conservation of the nucleotide (Supplemental Figure 3). Computer generated modeling of the protein (Supplemental Figure 4), and of the homotetramer complex of the active protein (data not shown) suggest the potential for a modest affect on folding and that the location of the variant in the mature protein is distant from the catalytic centers and binding sites for FAD and the electron transport flavoprotein. Together, these findings suggest that the variant likely leads to some functional change, but could imply a less severe biochemical effect of the alteration.

Unfortunately, the phase of the two sequence variants (c.985A>G and c.873C>T) could not be definitively resolved without parental samples.

The study design, using anonymized samples, did not allow follow-up contact with the subject from whom the sample was taken. As a result, the clinical outcome is not known, other than that the child was reported to the Ohio NBS Laboratory as “normal”. The NBS laboratory follow-up staff members are not aware of any child having been clinically diagnosed with MCAD deficiency after NBS since the MS/MS expanded program began, nor have any of the metabolic centers in the state identified such a patient (personal communication). Therefore, it is likely that the variant has not lead to clinically recognized disease in that child, although we cannot rule out the possibility that there have been unrecognized consequences.

In retrospect, several aspects of this study would have been stronger had their been more complete clinical information and long-term follow-up data available. Unfortunately, at the time this project was designed and approved the Ohio Department of Health IRB committee would not approve a project that required contacting families after newborn screening was completed. Further, it was felt that the option of contacting primary care providers to ask them to contact families would have resulted in incomplete data collection to the degree that the study would have little, if any, value. This issue raised significant bioethical questions and challenges, some of which are, to our minds, still unresolved. A separate manuscript discussing some of the bioethical issues related to this study is currently in preparation. “Normalization” of the metabolic profile in several reported cases of VLCAD deficiency identified by NBS has led most centers to require more than evidence of normal metabolites to rule out that deficiency [4, 8]. On the other hand, prior to this report, there were no data published to support a similar approach for MCAD. An important distinction may be that there appears to be a clear difference in NBS results between typically affected MCAD patients and those that are heterozygous carriers, or have other reasons for slightly elevated screening results. Specifically, as seen in these data (Figure 1), the NBS C8 value may indicate more, or less, probability that the child is actually affected. When the NBS value is in the range generally associated with affected status, it is conceivable, although not to our knowledge previously reported, that follow-up analyte studies obtained in a non—fasted state may be misleading. The lesson for clinicians from this case is certainly that the final diagnosis should be assigned based on interpretation of all of the data available, including the NBS data. In this case, recognition that the NBS result was strongly suggestive of a true affected may have lead to additional molecular testing, or, at the least, additional analyte testing during periods of metabolic stress. It is important to point out that, because of the de-identified nature of the samples in this study, we do not have any information about the subject other than the minimal amount supplied to the newborn screening laboratory. Therefore, additional studies and data from clinical follow-up are needed to determine if there are, in fact, cases where a newborn with MCAD deficiency may have normal follow-up studies of biochemical markers.

The state of Ohio, like most states, routinely and regularly assesses its newborn screening program, including follow-up. Findings such as those reported here have lead to tightening of definitions for reporting positive cases back to the state lab follow-up program in Ohio, and they have contributed to on-going discussion about the role of primary care providers and metabolic specialists in confirmatory testing and case definition. As is common in the area of NBS, there are many opinions, and each state has a relatively unique set of circumstances that they must address. The findings of this study should provide useful information for those discussions across the many states.

4. Conclusions

This study reports the results of sequencing the exons of the ACADM gene in infants with abnormal NBS C8 values who were not later confirmed to have MCAD deficiency. It supports the impression, which many clinicians have recognized, that slight increases above the C8 cut-off value of 0.7 mol/L, similar to the cut-off value in many states, can identify heterozygous carriers of the common c.985A>G mutation in ACADM. These infants are generally full-term AGA infants. False positive results are also seen in premature and low-birth weight infants and are unrelated to carrier status, suggesting that C8 is not a highly specific marker for MCAD deficiency in that population. In the group of infants with abnormal C8 NBS results who die before confirmatory testing, sequencing the gene from DNA extracted from the dried blood spot is both feasible and helpful in determining that MCAD deficiency was not present. Finally, these data suggest that for infants with NBS C8 values that are in the range typically seen in affected individuals, care must be taken in interpreting follow-up metabolite testing results to ensure that rare cases are not missed. The final diagnosis following any NBS test requires correlation of all the available data to the clinical situation. State newborn screening programs, and their advisory councils, benefit from regular review of their outcome, follow-up protocols and standardization of the definitions used for case reporting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

We sequenced the ACADM gene in infants with “false positive” NBS C8 values

Slight increases above the C8 cut-off value identify carriers of the common 985A>G mutation in full-term AGA infants

False positive results are also seen in non-carrier premature infants

We identified 1 out of 18 cases diagnosed as “false positive” with 2 ACADM mutations, but phase could not be confirmed

Final diagnosis following any NBS test requires correlation of all the available data to the clinical situation

Figure 1.

Box and whisker plot showing the NBS C8 values for three groups of subjects, false positives, those that died before confirmatory testing, and those with confirmed MCADD. The boxes bound the 25th and 75th quartiles; the whiskers (vertical lines) show the range, and the horizontal line represents the mean value for the group. The “NL” or normal group represents those infants with a positive screening test in whom the confirmatory testing did not support the diagnosis of MCAD deficiency; “NDR” (no diagnosis recorded) refers to those infants who died before confirmatory testing for MCAD deficiency was obtained; “MCAD affected” indicates those infants with a positive NBS who were confirmed to be MCAD deficient. The insert changes the scale for the NL and NDR groups for the sake of clarity.

Figure 2.

Box and whisker plot showing gestational age for the for different diagnosis and mutation status groups. The boxes bound the 25th and 75th quartiles, and the whiskers (vertical lines) show the range for the group. All subjects screened positive for elevated C8, indicating increased risk of MCAD deficiency. The groups are the same as in Figure 1, with the “NL” being further subdivided into “NL 985A>G carrier” that indicates infants whose final diagnosis was unaffected who were found to have a single copy of 985A>G; “NL no mutation” refers to those whose final diagnosis was unaffected and no mutation was identified. The child in the NL group with one copy of 985A>G in whom a second mutation was identified is included in that group since that was the official diagnosis.

Clinical information available for the de-identified samples obtained from individuals who died before confirmatory testing was obtained.

Table 1

Gender	Gestation (weeks)	Birthweight (g)	Race	NBS C8	Age at collection (hours)	On TPN	Initial C8	Age at collection (hours)
M	23	625	EA	1.02	234	NR	0.15	1
M	24	620	EA	0.84	318	Yes	0.34	1
M	24	800	EA	0.71	151	Yes	ND	ND
M	27	685	EA	0.80	238	NR	0.13	28
M	28	680	AA	1.47	910	Yes	0.14	39
M	38	3200	EA	1.14	773	Yes	ND	ND

NDR = No diagnosis recorded; EA = European American; AA = African American; NR = Not reported; ND = Not done;