Letter to the Editor Regarding: "Modern diagnostic and therapeutic approaches in familial maculopathy with reference to North Carolina macular dystrophy" by Jana Nekolova et al. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2022;166(4):418-27. doi: 10.5507/bp.2021.037

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To the Editor:

I want to congratulate the authors on their interest in North Carolina Macular Dystrophy and in their exceptional efforts exerted in finding the mutation in their family. I feel the author's pain in their quest for the mutation in this family. There are three areas of research that should be considered when exploring why they did not find the causative mutation. Having looked for the original NCMD mutations for decades, I have extensively studied and personally experienced what can go "wrong" and "why" (ref.[1-21]).

One area to consider is the clinical diagnosis. Clinically there is a possibility that their family may not have the NCMD phenotype. The one clinical feature that supports that consideration to me, is the lack of choroidal excavation / lacunae / coloboma-like lesions in this family. Even the most extensive lesions in their family do not have much if any choroidal, coloboma-like defect. The "normal EOG" in their family does not automatically rule out Bests Macular dystrophy as we have shown and BVMD is a known phenocopy of NMCD (ref.[19]). Clinically, their family is not inconsistent with BVMD. Several superb retinal physicians have misdiagnosed BVMD as NCMD and visa versa. Other than that, the clinical findings are not inconsistent with NCMD. A clinical misdiagnosis can wreak havoc with interpreting the molecular data.

The second area to explore is the DNA sequencing data. In the author's method section, it is not clear what type of sequencing was performed. Was it whole exome or whole genome or targeted genomic sequencing? Particularly with NCMD having an established history of mutations in non-coding regions, it is imperative to perform WGS after doing targeted sequencing for the known mutations[20-21].

Which brings us to the third area to consider when not finding the mutation which is the bioinformatics of the sequencing data[20-21]. Again, this is important especially when searching for non-coding mutations. The tandem duplications that have been found in some NCMD families is actually surprisingly easy to miss with Nexgen sequencing. Also important to consider is that most variations found in a single small family like this will be in linkage disequilibrium making all variants in the genomic region of the mutation segregate with the disease. This will make it difficult to "prove" a variation to be causative.

Additionally, there are some statements in the manuscript that are unclear or inaccurate and could use some clarification.

In the introduction, the authors make a comment about NCMD having a "peak deterioration" without a reference. In fact, NCMD does not have a period of "peak deterioration" at all because there really is no deterioration at all. As noted in several of my sequential studies of the original family, the subjects are born with their condition and it does not significantly change with the exception of the development of CNVMs (ref.[1-13]).

In the original report by Lefler Wadsworth and Sidbury, aminoaciduria was felt to segregate with the macular dystrophy although no statistical data was ever presented[1-3]. From personal communications, Dr. Lefler explained to me that they subsequently felt that the aminoaciduria was not associated with the macular findings. These subsequent findings were never published nor has anyone tried to replicate this. I have also never gotten a good explanation as to why aminoaciduria was even tested in this original NCMD family other than it was available at the time to investigate. Aminoaciduria testing at the time was in essence a "new genetic marker" to evaluate for linkage, much like the ABO blood typing was initially.

The "traditional grading system" for NCMD was originally in "stages" meaning there was progression from one stage to the next. This is where a great deal of the confusion about the phenotype began and has been persistently reiterated mostly by authors who have never knowingly

seen a case of NCMD or have seen only a limited family with it. After I documented 30 years ago that there was no progression, I converted "stages" into "grades". Some of the "reiteration" has occurred in the authors paper such as the reference 4 by McKusick which is markedly outdated. In patient 5, Fig. 2 the authors mention that the macular lesion was "non-volatile". This terminology is confusing to me and requires further explanation or deletion.

The author's comment that "It is not difficult to trace other family members who suffer from the same disease." I have to disagree with this statement, at least in the US. Most Americans do not know their family history beyond 2 generations in part because of their high mobility and deterioration of the agrarian society. Nuclear families are now rarely in a single household. Some cultures are also more resistant to participate in research. The author did not provide a drawn pedigree of their family. That would be helpful in that some family pedigrees showing an "autosomal dominant" inheritance pattern could actually be an x-linked dominant or mitochondrial inheritance pattern.

In the discussion, the authors make several comments that are confusing and could use some clarification. The author's comment that "Currently, macular dystrophies include diseases that have Mendelian inheritance that are isolated only to the eyes and are observable in the macula." is not accurate. Even in the NCMD phenotype, there are some families with the additional hearing loss or "club hands" (ref.[22]).

The author's comment that "There is also no correlation between clinical findings in carriers of equally affected genes, and similar findings are seen in different genetic mutations." This is difficult to understand as "genes" cannot be "equally affected"? Carriers are patients, not genes, who have the mutation but lack the phenotype. Carrier states are common in autosomal recessive diseases. There are no "carrier" states in NCMD as the disease is autosomal dominant and completely penetrant. That is to say that if a subject has the mutation they have a 100% chance that they will express the disease (pathologic findings in their maculae). I believe what the authors are trying to say is that there is a great deal of variable expressivity even with the same mutation in the same family as I have shown many times in many NCMD families as pointed out by Small et on many occasions. A phenotype / genotype correlation simply does not exist in NCMD (ref.[1-16]).

The authors make the statements: "Audere and Small assign MCDR1-3 to NCMD (ref.[10,13-15]), while Michaelides and Moore assign MCDR3 to NCMD-like diseases[16]. None of the MCDR1-3 types have been attributed to any particular gene; there are only linkage data available for the selected loci." There are several issues with these statements that need to be clarified so as not to once again perpetuate errors in the literature. Firstly, Small et al. (not Audere) assigned linkage of NCMD to the MCDR1 locus on chromosome 6 in 1991. While Michaelides et al. assigned NCMD / MCDR3 by linkage to chromosome 5 over a decade later in 2003 and later confirmed by Rosenberg et al. It needs to be pointed out that Small et al. found the actual mutations first for MCDR1 (chromosome 6) AND MCDR3 (chromosome 5) in 2016. Silva et al. later found 2 additional mutations at the MCDR3 locus confirming Small et al's work. In reviewing the paper by Audere et al., they never achieved linkage at all and made no claims of assigning any genetic loci. The authors mistakenly referenced Audere et al. as that manuscript adds little to the scientific literature on the subject of NCMD. The authors made some peculiar and frankly erroneous choices of references while not including more pertinent ones such as those listed herein[4-21].

The authors make several erroneous statements that all seem to originate from the same misunderstanding. Such as, "None of the MCDR1-3 types have been attributed to any particular gene; there are only linkage data available for the selected loci." And again, "However, the pathogenic variations that cause NCMD are probably in the non-coding areas of the DNA." "The genetic locus 6q16 has been described and it most probably will contain the MCDR1 causative gene[17]". "However, the pathogenic variations that cause NCMD are probably in the non-coding areas of the DNA." None of these statements are accurate as they do not take into account that in 2016 Small et al. first found the mutations causing MCDR1 and MCDR3. These mutations we reported are single nucleotide changes in non-coding regions in DNASE 1 binding sites and tandem duplications. The mutations in the MCDR1 locus appear to affect the expression of the retinal transcription factor PRDM13 and for the MCDR3 locus likely affecting IRX1 (ref.[21-22]).

Over the decades, there have been misstatements about NCMD clinically and molecularly which have caused confusion which then gets perpetuated in the literature. My intent, with the above comments, is to try to add some clarity.

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Comment on the letter from authors:

Dear Dr. Kent W. Small,

We would like to thank you for the Letter to the Editor Regarding our manuscript Modern diagnostic and therapeutic approaches in familial maculopathy with reference to North Carolina macular dystrophy. We greatly appreciate your efforts to help us in the diagnosis of familial maculopathy, which was the cause of vision impairment in a young member of this family. Due to the uncommon occurrence of the disease in our area, we decided to publish this interesting case. We have studied many sources on familial macular dystrophies and all our statements have been supported by literary sources. It is not easy to recognize the relevance of published data, and we thank you for your valuable comments.

Since our manuscript has been already published online we are not able to make any changes to the text. We have received the information from prof. Petra Liskova, M.D., Ph.D. (Charles University and General University Hospital in Prague, Czech Republic), that since publishing our detailed clinical description, the molecular genetic cause of North Carolina Macular Dystrophy in this particular family has been refined at DNA level. A heterozygous variant g.99599064A>G (chr6,hg38) was found in hotspot-2 of PRDM13 and functionally validated in Van de Sompele et al.[1].

In any case, we take your comment on board and it will certainly help for our further scientific research.

Jana Nekolova, Alexandr Stepanov and co-authors

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