Crystals and Fatty Acid Abnormalities Are Not Present in Circulating Cells From Choroideremia Patients

Alina Radziwon,1 Woo Jung Cho,2 Artur Szkotak,3 Miyoung Suh,4 and Ian M. MacDonald1

1Department of Ophthalmology and Visual Sciences, University of Alberta, Edmonton, Alberta, Canada
2Imaging Core Facility, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada
3Department of Laboratory Medicine & Pathology, University of Alberta, Edmonton, Alberta, Canada
4Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Correspondence: Ian M. MacDonald, Department of Ophthalmology and Visual Sciences, Royal Alexandra Hospital, Office 2319 - 10240, Kingsway Avenue, Edmonton AB T6H 3V9, Alberta, Canada; macdonal@ualberta.ca.

Submitted: June 25, 2018
Accepted: August 1, 2018

Citation: Radziwon A, Cho WJ, Szkotak A, Suh M, MacDonald IM. Crystals and fatty acid abnormalities are not present in circulating cells from choroideremia patients. Invest Ophthal-mol Vis Sci. 2018;59:4464-4470. https://doi.org/10.1167/iovs.18-25112

PURPOSE. To confirm whether choroideremia (CHM) is a systemic disease characterized by blood lipid abnormalities and crystals found in, or associated with, circulating peripheral blood cells of patients.

METHODS. Peripheral blood samples obtained from three subjects with confirmed mutations in the CHM gene and three age-matched normal controls were processed for transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Fatty acids from plasma of nine male CHM subjects were analyzed and compared to reference values for a sample from a Canadian population.

RESULTS. Intracellular crystals were not observed in the cells from choroideremia-affected males. No crystals were found adherent to the external plasma membrane of red blood cells. Fatty acid profiles of patients were similar to reference values, with the exception of lower levels of nervonic acid.

CONCLUSIONS. This investigation failed to observe crystals previously reported in peripheral circulating blood cells derived from CHM subjects, and showed no significant fatty acid abnormalities, not supporting the view of CHM as a systemic disease.

Keywords: choroideremia, CHM, REP-1, retinal dystrophy

Choroideremia (CHM; Online Mendelian Inheritance in Man [OMIM] 303100) is an X-linked retinopathy caused by mutations in the CHM gene that encodes Rab escort protein-1, REP-1. Progressive degeneration of photoreceptors, retinal pigment epithelium (RPE), and the choroid causes affected hemizygous males to develop nystagmus in the first or second decade of life, followed by a decrease in visual peripheral fields and an eventual loss of central visual acuity in advanced stages of the disease.1 Carrier females, while usually asymptomatic, may exhibit signs of retinal degeneration upon fundoscopic examination, and (more rarely) have impaired dark adaptation and peripheral vision.2,3 The most common pathogenic variants in CHM include nonsense, large deletions, splice defects, and indels. No genotype-phenotype correlation has been found, with age at onset of symptoms, the progression of visual acuity and visual field loss being unrelated to mutation type, supported by a near-ubiquitous lack of functional REP-1 protein in cells of patients of different genotypes.4 As a monogenic disease with a phenotype that is compartmentalized to the eye, CHM presents an attractive target for gene therapy; treatment by subfoveal injection of adeno-associated viral vector carrying the CHM gene is currently under investigation.5

REP-1 acts in all cells to enable the lipid modification (geranylgeranylation, a form of prenylation) of the Ras-related GTases known as Rabs, allowing for their function as regulators of intracellular trafficking pathways and phagosome fusion and maturation.6,7 Rabs require prenylation by Rab geranylgeranyltransferase (RabGGTase) for their activity, but unlike the other prenyltransferases, RabGGTase must complex with REPs, which perform the role of substrate recognition.7 A related protein, REP-2, encoded by the autosomal gene CHML (choroideremia-like), shares over 75% identity with REP-1, and also functions as a chaperone in the same prenylation pathway.8 The ubiquitous expression profile of REP-1 and the essential role of Rab prenylation could suggest that CHM may also result in systemic abnormalities.9,10 While certain Rabs have indeed been associated with syndromic diseases such as Griscelli syndrome type 2 (Rab27) and Charcot-Marie-Tooth disease type 2B (Rab7),11,12 CHM has been viewed as a retinal disease, with no significant nonocular symptoms reported in males or carrier females. Previous work has demonstrated that a loss of REP-1 in fibroblasts and monocytes affected intracellular transport, increased pH levels in lysosomes, impaired proteolytic degradation, and altered secretion of cytokines, but these effects did not appear to translate to wider systemic effects.13 Patients’ apparent lack of nonocular symptoms or signs has been understood to result not from tissue-specific expression of REP-1, but from the differing affinities of REP-1 and REP-2 for target Rabs, such as Rab27 and Rab38, which may themselves be differentially expressed or possess tissue- or cell-specific activity.14,15 Research proposes that a lack of REP-1 leads to defects in opsin transport to photoreceptor outer segments, the movement of RPE melanosomes, and the ability of the RPE to phagocytose photoreceptor outer segments.16 These data suggest a compensatory mechanism by REP-2 in systems other than the eye.

In a 2015 publication by Zhang and colleagues,17 putative crystalline material was identified in electron microscopic (EM)
images of cells derived from four patients with CHM. Additionally, based on data from lipid analyses of affected males, the authors hypothesized that altered serum and red blood cell membrane lipids “ultimately result in crystalline inclusion deposition” (p. 8164) and provided evidence that CHM is a systemic disorder.17 In our experience, “crystalline material” is not seen in images of the retina from CHM patients or within their cells, and as the possible presence of crystals may be relevant to patients’ health, we chose to independently confirm this finding.

**METHODS**

**Recruitment of Subjects**

The study was approved by the Health Research Ethics Committee, Panel B, of the University of Alberta, and performed in accordance with the tenets of the Declaration of Helsinki. Informed written consent was obtained prior to the individuals’ participation in this study. Three male patients (P1–3) with a confirmed molecular diagnosis of CHM and three male age-matched unaffected controls (C1–3) were recruited for microscopic analysis of blood cells. Nine males (P4–P12) with molecularly confirmed CHM were recruited for plasma phospholipid analysis (Table 1).

**Transmission and Scanning Electron Microscopy**

Peripheral blood was drawn into EDTA blood collection tubes and immediately separated into a buffy coat and red cell mass by centrifugation at 400g through a density gradient. Isolated peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs) were washed twice with PBS, and the resulting pellet prefixed by addition of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). For transmission electron microscopic (TEM) study, PBMCs were embedded in 3% agarose, dehydrated in 1% OsO4, dehydrated in the same ethanol series as for TEM study, and postfixed in 1% OsO4, dehydrated in 1% OsO4, dehydrated in the same ethanol series as for TEM study. Upon blood collection, plasma lipids were extracted using the described procedure.19 Phospholipids were visualized with 0.1% methanol. FAME was separated on a polar BPX-70 capillary column using 14% (wt/wt) boron trifluoride in methanol with UV light. Fatty acid in phospholipid was transformed into methyl esters (FAME) using 1% OsO4, dehydrated in 1% OsO4, dehydrated in the same ethanol series as for TEM sample preparation and dried in hexamethyl-disilazane. The dried RBCs were coated with a 5-mm-thick platinum by a high vacuum sputter coater (Leica EM ACE600). The coated RBCs were imaged by a secondary electron detector of a Hitachi S-4800 field emission gun SEM (Hitachi High-Technologies Canada, Inc., Rexdale, ON, Canada) at 5-kV accelerating voltage. The images were taken from ×22,000 to ×55,000 magnification for TEM and ×2500 magnification for SEM. Forty fields of view for each affected sample (P1, P2, and P3) and 30 fields of view for each control sample (C1, C2, and C3) were observed under TEM, of which a random 15 (affected) and 10 (control) were captured as digital images (Table 2). Each field of view contained on average 3 cells, therefore a total of over 100 cells from patients were observed. These images were subsequently analyzed by a hematopathologist (AS) who blinded as to whether the cells came from a patient or control.

Ten fields of view for each affected and unaffected sample were observed under SEM, of which three were randomly captured as digital images (Table 2). Each field of view contained 11 cells on average, for a total of over 100 cells observed.

**Phospholipid Fatty Acid (FA) Analysis**

Upon blood collection, plasma lipids were extracted using the Folch method18 and phospholipids were separated on silica gel G plates with a developing system composed of petroleum ether:diethyl ether:acetic acid (80:20:1 by volume) as described previously.19 Phospholipids were visualized with 0.1% aniline naphthalene sulfonic acid in water (wt/vol) under UV light. Fatty acid in phospholipid was transformed into methyl esters (FAME) using 5% (wt/wt) boron trifluoride in methanol. FAME was separated on a polar BPX-70 capillary column (25 m × 0.22-mm ID) using a Hewlett Packard 5890
GLC equipped with a Vista 654 data system, as previously described. All FAs were compared with a commercial standard (Nu-Chek Prep 461; Elysian, MN, USA). Each sample was analyzed in duplicate. Data were expressed as mean with standard deviation (SD) and as a percentage for an individual FA relative to all FAs (wt/wt %) and μg/mL plasma with nonadecanoic acid (C19:0) as an internal standard. Reference values from a similar-sized sample of a Canadian population were obtained from the literature.

RESULTS

Peripheral Buffy Coat TEM

One representative image from each subject is presented in Figure 1. For CHM patients P1, P2, and P3, no cells from the buffy coat contained crystals among the images recorded of cells examined under TEM among 15 fields of view. Additionally, no crystals were observed in an additional 25 fields of view.

![Figure 1](https://www.arvojournals.org/)
for each sample that was visually inspected without image capture. No crystals were observed among controls C1, C2, and C3. Table 2 summarizes the findings.

A board-certified hematopathologist (AS) evaluating the images assessed them to be of high quality and resolution, allowing for a detailed identification and localization of ultrastructural elements. He did not see any structures that suggested intracytoplasmic crystal formation, or note any appreciable difference between cells of patients or controls.

**Red Blood Cells SEM**

A representative image from P3 and C3 is shown in Figure 2. SEM imaging revealed that no cells (10 fields of view) from the red cell mass of CHM subjects or age-matched controls had crystals associated with the external limiting membrane (Fig. 2). The red blood cell morphology of both groups appeared to be identical.

**Lipid and Fatty Acid Analysis**

Analysis of a full profile of common FAs revealed no major abnormalities. Saturated, monounsaturated, and polyunsaturated FAs, as a weight percentage relative to all FAs, were similar to baseline measures of a Canadian population sample found in the literature. Only nervonic acid varied from reference values by more than two standard deviations, but in contrast to Zhang et al., it was lower at 0.57 ± 0.33% wt/wt rather than elevated, in our disease cohort. Erucic and gondoic acid, previously documented as lowered in CHM patients, were elevated, in our disease cohort. Erucic and gondoic acid, therefore intrigued by the study of Zhang and colleagues, reporting CHM to be a systemic disease with FA profiles of patients that differed from controls. Further, the authors reported that crystals were detected by TEM in the lymphocytes and platelets of all CHM patients they examined.

In contrast, our data indicate no evidence of crystals within the cytoplasm of the buffy coat cells from CHM subjects or age-matched controls when analyzed with TEM (Fig. 1; Table 2). Further, we found no crystals in association with the external plasma membrane of red blood cells when examined by SEM (Fig. 2; Table 2). All patient cells had a normal appearance indistinguishable from that of controls. Significantly, prior histopathologic and EM studies of the retina from CHM carriers and affected males, as well as an examination of macrophages, revealed no crystals.

The hematopathologist (AS) noted that two of the “lymphocytes” reported by Zhang et al. in their Figures 3E and 3F as containing “striking intracytoplasmic crystalline structures” or separately, “rod-like crystals,” are in fact eosinophils. Highlighted in those images are eosinophils’ characteristic secretory crystalloid granules, whose cores consist of highly concentrated crystallized protein and appear intensely electron-dense upon imaging TEM. The presence of this cell type and the crystals contained therein is therefore completely unrelated to a diagnosis of CHM and not indicative of any pathologic process. Moreover, the cells were not lymphocytes as is implied in the legend, as the granules they contained are too large and numerous. Ultrastructural characteristics of lymphocytes are a large nucleus, developed mitochondria, and abundant free ribosomes, yet none of these characteristics appear in the cells presented in Figures 3 and 6 of Zhang et al.’s article.

From examination of Zhang’s figures, our faculty’s senior lead of EM, WJC, independently concluded that structures in parts E and F of Figure 3 feature the dense and lamellated crystalloid core contained by normal eosinophilic granules. His opinion of the “needle-like” crystals presented in parts A through D of Figure 3 was that they were typical uranyl acetate artifacts. Sample Preparation Handbook for Transmission Electron Microscopy references similar needle-shaped uranyl acetate precipitates in Figure 6.40, which closely resemble what is seen in Zhang et al.’s Figure 3, A and D. Uranyl acetate tends to crystallize when it is dried during poststaining; however, the method presented by Zhang et al. for EM and preparation in the publication shows incomplete conventional TEM sample preparation.
Systemic Findings in Choroideremia

**Table 3. Plasma Phospholipid Fatty Acid Profile of CHM Patients**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>CHM P4–P12, µg/mL</th>
<th>CHM P4–P12 %, wt/wt</th>
<th>Reference Value %, wt/wt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated FAs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0 Myristic acid</td>
<td>6.47 (1.75)</td>
<td>0.47 (0.06)</td>
<td>0.55 (0.10)</td>
</tr>
<tr>
<td>15:0 Pentadecanoic acid</td>
<td>2.74 (0.85)</td>
<td>0.19 (0.05)</td>
<td>Not reported</td>
</tr>
<tr>
<td>16:0 Palmitic acid</td>
<td>394.85 (89.34)</td>
<td>29.13 (4.47)</td>
<td>28.44 (0.99)</td>
</tr>
<tr>
<td>17:0 Margaric acid</td>
<td>6.03 (1.76)</td>
<td>0.44 (0.08)</td>
<td>Not detected</td>
</tr>
<tr>
<td>18:0 Stearic acid</td>
<td>229.21 (56.64)</td>
<td>16.57 (0.87)</td>
<td>16.14 (1.97)</td>
</tr>
<tr>
<td>20:0 Arachidic acid</td>
<td>5.79 (1.41)</td>
<td>0.29 (0.07)</td>
<td>0.40 (0.06)</td>
</tr>
<tr>
<td>22:0 Behenic acid</td>
<td>9.63 (3.72)</td>
<td>0.67 (0.16)</td>
<td>1.10 (0.24)</td>
</tr>
<tr>
<td>24:0 Lignoceric acid</td>
<td>12.84 (5.58)</td>
<td>0.80 (0.17)</td>
<td>1.06 (0.26)</td>
</tr>
<tr>
<td>Total saturates</td>
<td>655.58 (145.90)</td>
<td>47.91 (4.27)</td>
<td>48.25 (2.39)</td>
</tr>
<tr>
<td><strong>Monounsaturated FAs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:1n-9 Myristoleic acid</td>
<td>1.67 (0.82)</td>
<td>0.13 (0.07)</td>
<td>Not detected</td>
</tr>
<tr>
<td>16:1n-7 Palmitoleic acid</td>
<td>7.10 (2.91)</td>
<td>0.50 (0.11)</td>
<td>0.46 (0.15)</td>
</tr>
<tr>
<td>18:1n-7 Vaccenic acid</td>
<td>31.66 (7.56)</td>
<td>2.28 (0.36)</td>
<td>1.69 (0.30)</td>
</tr>
<tr>
<td>18:1n-9 Oleic acid</td>
<td>117.28 (28.19)</td>
<td>8.58 (1.03)</td>
<td>8.05 (1.09)</td>
</tr>
<tr>
<td>20:1n-9 Gondoic acid</td>
<td>1.79 (1.22)</td>
<td>0.12 (0.07)</td>
<td>0.16 (0.04)</td>
</tr>
<tr>
<td>22:1n-9 Erucic acid</td>
<td>1.86 (2.05)</td>
<td>0.13 (0.14)</td>
<td>0.10 (0.02)</td>
</tr>
<tr>
<td>24:1n-9 Nervonic acid</td>
<td>7.76 (4.10)</td>
<td>0.57 (0.33)</td>
<td>1.39 (0.35)</td>
</tr>
<tr>
<td>Total monounsaturates</td>
<td>168.74 (38.16)</td>
<td>12.29 (1.19)</td>
<td>11.85 (1.42)</td>
</tr>
<tr>
<td><strong>Polyunsaturated FAs, n-6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6 Linoleic acid</td>
<td>278.10 (73.39)</td>
<td>20.14 (2.74)</td>
<td>19.33 (1.62)</td>
</tr>
<tr>
<td>20:3n-6 DGLA</td>
<td>36.18 (21.08)</td>
<td>2.48 (0.88)</td>
<td>2.73 (0.51)</td>
</tr>
<tr>
<td>20:4n-6 Arachidonic acid</td>
<td>164.42 (81.83)</td>
<td>11.33 (3.31)</td>
<td>9.89 (1.35)</td>
</tr>
<tr>
<td>22:4n-6 Adrenic acid</td>
<td>6.21 (3.36)</td>
<td>0.42 (0.17)</td>
<td>0.37 (0.04)</td>
</tr>
<tr>
<td>22:5n-6 Osbond acid</td>
<td>4.24 (2.75)</td>
<td>0.29 (0.15)</td>
<td>0.40 (0.10)</td>
</tr>
<tr>
<td>Total n-6 polyunsaturates</td>
<td>489.15 (169.09)</td>
<td>34.66 (4.59)</td>
<td>33.16 (1.82)</td>
</tr>
<tr>
<td><strong>Polyunsaturated FAs, n-3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n-3 -linolenic acid</td>
<td>1.72 (0.90)</td>
<td>0.11 (0.07)</td>
<td>0.20 (0.05)</td>
</tr>
<tr>
<td>20:3n-3 Eicosatetraenoic acid</td>
<td>2.65 (0.38)</td>
<td>0.25 (0.10)</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>20:5n-3 EPA</td>
<td>6.90 (4.64)</td>
<td>0.50 (0.39)</td>
<td>0.73 (0.33)</td>
</tr>
<tr>
<td>22:5n-3 DPA</td>
<td>9.68 (5.45)</td>
<td>0.68 (0.14)</td>
<td>0.84 (0.26)</td>
</tr>
<tr>
<td>22:6n-3 DHA</td>
<td>54.57 (13.75)</td>
<td>2.55 (1.11)</td>
<td>2.75 (0.65)</td>
</tr>
<tr>
<td>Total n-3 polyunsaturates</td>
<td>55.05 (19.64)</td>
<td>4.02 (1.51)</td>
<td>4.57 (0.78)</td>
</tr>
<tr>
<td>20:3n-9 Mead acid</td>
<td>3.95 (2.07)</td>
<td>0.27 (0.09)</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

Values given are means (SD). The age ranges were 9 to 70 years. The only FA to differ (% wt/wt) by more than two standard deviations from the reference value is nervonic acid (seen in bold), which is lower in our CHM cohort. DGLA, dihomo-γ-linoleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Reference values were obtained from Metherel et al.20 Surprisingly, our cohort of nine males showed levels that were lower than those measured in a normal population, as opposed to those reported by Zhang et al., who found elevated nervonic acid in patients. We also observed that gondoic acid C20:1(n-9) and erucic acid C22:1(n-9) were within the expected range for our affected subjects, and not significantly decreased as found by Zhang et al.17 We did not quantify some of the unusual FAs found at extremely low concentrations with unknown significance to health as they are rarely included in FA profiling. Capric acid C10:0, tridecaenoic acid C13:1, myristolenic acid C14:2, octacosanoic acid C28:0, and dodecaenoic acid C12:1 were provided with normal values ranging from 0.006% to 0.00001%, and we would argue that as these numbers approach the detection threshold one may be cautious in drawing conclusions from the changes detected. Evaluation by a second method, such as gas chromatography, would help strengthen the previously reported findings. Zhang et al.’s report of elevated and lowered levels of docosadienoic

preparation. No description is given of a dehydration step (whether a series of ethanol or acetone) between the postfixation step and the Epon resin infiltration step. Additionally, the exact nature of the fixation compound is believed to be osmium tetroxide, but is not clear. No description of ultramicrotome sectioning thickness and post-staining (i.e., uranyl acetate and lead citrate) after the ultramicrotome sectioning is provided. Electron contrast from ultrathin section (i.e., 60–90 nm in thickness) itself without the poststaining is insufficient to identify ultrastructure at high magnification; poststaining (with uranyl acetate and lead citrate) is essential to increase contrast at over ×20,000 magnification of TEM. All of the TEM images in Figures 3 and 6 in Zhang et al.17 show over ×20,000 magnification. As Zhang et al. did not specify how frequently crystals occur in the cells surveyed, we suggest that selection bias likely led to their identification in patient cells but absence in controls. A random sampling of fields of view and accurate quantification of the proportion of crystal-bearing cells would serve to strengthen the reliability of such findings.

Our analysis of common plasma phospholipids, while not as broad as in previous studies, does reveal discrepancies with earlier reports (Table 3). When compared to a reference sample from the Canadian population, the only FA to differ by more than two standard deviations was nervonic acid, C24:1(n-9).20 Surprisingly, our cohort of nine males showed levels that were lower than those measured in a normal population, as opposed to those reported by Zhang et al., who found elevated nervonic acid in patients. We also observed that gondoic acid C20:1(n-9) and erucic acid C22:1(n-9) were within the expected range for our affected subjects, and not significantly decreased as found by Zhang et al.17 We did not quantify some of the unusual FAs found at extremely low concentrations with unknown significance to health as they are rarely included in FA profiling. Capric acid C10:0, tridecaenoic acid C13:1, myristolenic acid C14:2, octacosanoic acid C28:0, and dodecaenoic acid C12:1 were provided with normal values ranging from 0.006% to 0.00001%, and we would argue that as these numbers approach the detection threshold one may be cautious in drawing conclusions from the changes detected. Evaluation by a second method, such as gas chromatography, would help strengthen the previously reported findings. Zhang et al.’s report of elevated and lowered levels of docosadienoic...
Systemic Findings in Choroideremia

acid C22:2(n-6) and dimethyl acetal acid C16:0, respectively, remains to be independently corroborated..Multiline limit in establishing relationships between FAs and disease is the lack of accepted normal ranges representing healthy individuals and the substantive effect of diet on FA levels, which poses a limitation on the conjectures that can be drawn from small-scale analysis. We therefore hesitate to draw inferences from the lower levels of nervonic acid found among our cohort. Further validation using larger sample sizes, the use of a food questionnaire, and inclusion of age- and ethnicity-matched controls may therefore help clarify the discrepancies between the two studies. Further studies of this nature could shed additional light on the possibility that FA levels are altered in CHM. Our studies have failed to recapitulate the finding that CHM is a systemic condition, and supports the widely held view that it is primarily an ocular disorder.

Acknowledgments

The authors thank Piotr Radziwon, MD, PhD, of the Department of Hematology, Medical Academy in Białystok, Poland, for his comments and additional insight provided with regard to blood cell ultrastructure. We would like to thank MT Clandinin, PhD, and YK Goh, PhD, Alberta Institute for Human Nutrition, Edmonton, Alberta, for their advice and technical expertise on the DHA studies. Supported by the Foundation Fighting Blindness Canada, and the Canadian Institutes for Health Research Grant 119190.

Disclosure: A. Radziwon, None; W.J. Cho, None; Z. Szkotak, None; M. Suh, None; I.M. MacDonald, None

References


Downloaded From: https://www.arvojournals.org/ on 09/07/2018


