# Toxic Effects of Nucleoside Reverse Transcriptase Inhibitors on the Liver

## Value of Electron Microscopy Analysis for the Diagnosis of Mitochondrial Cytopathy

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## Abstract

*Nucleoside reverse transcriptase inhibitors (NRTIs) induce mitochondrial toxic effects resulting in multiple organ disorders. Liver involvement has been associated mainly with severe lactic acidosis and massive steatosis. However, patients with HIV infection who are receiving antiretroviral treatment frequently have mildly abnormal liver test results that, to date, have not been linked unambiguously to the toxic effects of NRTIs.*

*Thirteen patients with HIV infection treated with NRTI-based regimens had low-grade abnormal liver test results associated with digestive and nonspecific general symptoms. Histologic examination of liver samples showed diffuse steatosis in only 6 cases and mild steatosis in the remaining cases, associated with megamitochondria, mild lobular inflammation and necrosis, Mallory bodies, and perisinusoidal fibrosis. In all cases, ultrastructural study disclosed mitochondrial abnormalities.*

*Our work demonstrates that NRTI-induced toxic effects in the liver may occur as indolent nonspecific disease with variable histologic features and emphasizes the diagnostic value of electron microscopy, particularly when diffuse steatosis is absent.*

The nucleoside reverse transcriptase inhibitor (NRTI) class of drugs, including zidovudine (AZT), didanosine (ddI), lamivudine (also known as 3TC), zalcitabine (also known as ddC, or 2',3'-dideoxycytidine), and stavudine (also known as d4T), is used widely in combination therapy with nonnucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) in HIV-infected patients.[1] Thus, the benefits of so-called highly active antiretroviral therapy (HAART) have been well established.[2] However, the major limitation of HAART is a broad range of side and toxic effects that are the main reason for discontinuing or modifying antiretroviral therapy.[3-5] Among the different classes of antiretroviral drugs, the toxic effects induced by NRTIs seem to be of particular importance.

Long-term treatment with NRTIs gives rise to a broad spectrum of tissue involvement, including hematologic disorders, myopathy, cardiotoxic effects, peripheral neuropathies, and hepatotoxic effects.[6] These heterogeneous adverse effects of NRTIs are related to defective mitochondrial DNA replication secondary to the NRTI-induced deleterious inhibition of the mitochondrial DNA polymerase gamma.[7] NRTIs also have been clearly associated with peripheral lipodystrophy, which is characterized by lipoatrophy of the face, legs, and arms. To date, reports of liver involvement associated with NRTI-based therapy are rare compared with the wide use of NRTIs.[8-14] In most of these cases, severe toxic effects on the liver of AZT, ddI, or zalcitabine were associated with dramatic and potentially lethal lactic acidosis. However, abnormalities of liver test results frequently are observed during HAART. Thus, elevated aminotransferase levels are present in 6% of

patients undergoing an NRTI-based regimen.[15] Fortgang et al[16] reported an incidence of 1.3 patients per 1,000 person-years for symptomatic NRTI-related hepatotoxic effects. This progressive and indolent liver injury is certainly important in the presence of a preexisting liver disease such as chronic viral hepatitis or alcoholic liver disease.[3] Interestingly, in people receiving NRTI-based regimens, these mild hepatic abnormalities have not been linked clearly to mitochondrial toxic effects. Furthermore, little is known about the diagnosis, outcome, and therapeutic implications of such low-grade toxic effects on the liver.

We report the clinical, histologic, and ultrastructural evaluation of 13 HIV-infected patients treated with NRTIs. These patients were mildly symptomatic and had only mildly abnormal liver test results. Our observations highlight the limitations of standard liver histologic examination and the value of electron microscopy (EM) in the diagnosis of NRTI-induced hepatotoxic effects.

## Materials and Methods

### Patients

Thirteen HIV-seropositive patients followed up in the department of Clinical Immunology of the Georges Pompidou European Hospital, Paris, France, underwent liver biopsy for histologic and ultrastructural assessment. All patients had mildly specific symptoms and mildly abnormal liver test results that raised the question of the diagnosis of NRTI-induced toxic effects. We excluded from our series 2 patients with HIV infection who had liver insufficiency, lactic acidosis, and massive steatosis. For these patients, the diagnosis of NRTI-induced toxic effects was obvious even without ultrastructural confirmation, and antiretroviral treatment was stopped immediately.[17]

Our series included 12 men and 1 woman with a median age of 48 years (range, 36-56 years). The median CD4 cell and HIV RNA counts at the time of the liver biopsy were 401/µL (401 × $10^6$/L; range, 210-613/µL [210-613 × $10^6$/L]) and 3.1 log copies per milliliter (range, 1.69-4.99 log copies per milliliter), respectively. No patient had a history of alcohol abuse. Circulating hepatitis B surface antigen was absent in all cases. Patients coinfected with HIV and the hepatitis C virus (HCV) were excluded from our series.

All patients had received NRTI-based therapy for a median time of 65.5 months (range, 16-96 months). The antiretroviral therapy being received at the time of the liver biopsy is summarized in **Table 1**. Five patients were treated with NRTIs alone. NNRTIs and PIs were associated with NRTIs in 5 and 2 cases, respectively. One patient received a regimen combining NRTI, NNRTI, and PI drugs.

### Control Patients

To assess the specificity of EM findings, we selected 2 patients coinfected with HIV and HCV as control patients who underwent a liver biopsy during the follow-up of chronic viral hepatitis. These 2 patients, who had not received any antiretroviral therapy, were men aged 37 and 34 years. At the time of the liver biopsy, their respective CD4 counts were 1,198/µL (1,198 × $10^6$/L) and 398/µL

**Table 1**
**Biologic Characteristics of Patients***

| Case No. | Antiretroviral Therapy | AST (U/L) | ALT (U/L) | AP (U/L) | GGT (U/L) | Total Bilirubin (mg/dL) | Amylase (U/L) | Lipase (mIU/mL) | LDH (U/L) | CK (U/L) | Lactate (mg/dL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | d4T, 3TC, EFV | 110 | 151 | 58 | 151 | 0.9 | 122 | 23 | 548 | 165 | 35 |
| 2 | d4T, 3TC, RTV | 110 | 201 | 112 | 19 | 0.5 | 127 | ND | 560 | 110 | ND |
| 3 | d4T, ddI, EFV, NFV | 84 | 144 | 49 | 55 | 0.5 | 26 | 44 | 592 | 93 | 15 |
| 4 | d4T, ddI, HU | 126 | 160 | 44 | 84 | 1.5 | 218 | 37 | 686 | 285 | 26 |
| 5 | ddI, d4T, NVP | 68 | 64 | 124 | 536 | 1.5 | 293 | 101 | 798 | 58 | 45 |
| 6 | AZT, ddC, NVP | 45 | 85 | 79 | 236 | 0.8 | 64 | 17 | 527 | 73 | ND |
| 7 | d4T, ddI | 87 | 98 | 111 | 735 | 0.6 | 126 | 77 | 609 | 642 | 25 |
| 8 | d4T, 3TC, NFV | 75 | 178 | 39 | 92 | 1.0 | ND | ND | 346 | 57 | 11 |
| 9 | d4T, ddI, EFV | 19 | 15 | 102 | 71 | 0.5 | 47 | 27 | 336 | 31 | 28 |
| 10 | d4T, 3TC | 59 | 99 | 15 | 33 | 0.9 | ND | 47 | 229 | 150 | ND |
| 11 | d4T, ddI | 102 | 143 | 105 | 720 | 2.3 | 74 | 41 | 564 | 386 | 44 |
| 12 | d4T, ddI, EFV | 76 | 81 | 101 | 1,101 | 0.5 | 144 | 59 | 401 | 73 | 22 |
| 13 | d4T, ddI | 126 | 141 | 72 | 158 | 0.9 | 85 | 112 | 847 | 180 | 37 |

ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; AZT, zidovudine; CK, creatine kinase; ddC, zalcitabine; ddI, didanosine; d4T, stavudine; EFV, efavirenz; GGT, gamma-glutamyltransferase; HU, hydroxyurea; LDH, serum lactate dehydrogenase; ND, not done; NFV, nelfinavir; NVP, nevirapine; RTV, ritonavir; 3TC, lamivudine.

* Antiretroviral therapy at the time of liver biopsy. Reference values for the analytes, in conventional (Système International [SI]) units, are as follows: ALT, 7-35 U/L (7-35 U/L); amylase, 15-100 U/L (15-100 U/L); AP, 30-80 U/L (30-80 U/L); AST, 9-36 U/L (9-36 U/L); CK, 15-140 U/L (15-140 U/L); GGT, 10-45 U/L (10-45 U/L); lactate, <20 mg/dL (<2.2 mmol/L ); LDH, 280-500 U/L (280-500 U/L ); lipase, 7-60 mIU/mL (7-60 U/L); total bilirubin, <1 mg/dL (<17 µmol/L). The values in the table are given in conventional units. For all except lactate and total bilirubin, the conversion factor for SI units is 1.0; for lactate, multiply the value by 0.1110; for bilirubin, multiply by 17.1.




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($398 \times 10^6$/L), and the HIV RNA counts were 3.22 and 5.57 log copies per milliliter, respectively. One patient had normal liver test results. The other patient had a mild elevation of aminotransferase levels that was less than 2 times the upper limit of the reference range. Neither had an elevation of the lactate level. For obvious ethical reasons, no liver biopsy was performed in patients with HIV infection who were receiving antiretroviral treatment but who had no abnormal liver test results and were without associated liver disease, ie, viral hepatitis that motivated the liver histologic assessment.

### Histopathologic and EM Studies

Liver percutaneous needle biopsy specimens were cut into 2 pieces: one piece (>2 mm long) was fixed in 2.5% glutaraldehyde for EM and the other (>10 mm long) in 10% buffered formalin for standard histologic examination. Paraffin sections were stained with H&E, picrosirius red, and the Perl method. Sections were evaluated by 2 observers (J.-P.D.V.H. and P.B.) for microvesicular and macrovesicular steatosis, cholestasis, necrosis, inflammation, fibrosis, and the presence of Mallory bodies. Glutaraldehyde-fixed samples were available for all patients and were processed following standard EM technique. Mitochondrial abnormalities were defined by the loss of mitochondrial profiles and the presence of mitochondrial inclusions. They were evaluated semiquantitatively as mild (incomplete loss of mitochondrial cristae in less than 50% of mitochondria), intermediate (incomplete or total loss of mitochondrial cristae in 50% or more but less than 100% of mitochondria), or severe (incomplete or total loss of mitochondrial profiles in all mitochondria). Hyperplasia of the endoplasmic reticulum, steatosis, cholestasis, and perisinusoidal fibrosis also were assessed.

## Results

### Clinical and Laboratory Findings

Clinical and laboratory findings are given in Table 1. All patients had nonspecific symptoms, including asthenia, anorexia, and weight loss. Additional digestive symptoms (ie, dyspepsia, nausea, vomiting, and diarrhea) were present in 4 cases. Case 5 exhibited dysesthesia, areflexia, and distal sensory loss suggesting a sensitive polyneuropathy that was confirmed by electromyography. Case 4 had myalgia with an increased creatine kinase level. Five additional cases showed an elevation of the creatine kinase level. In all cases, liver test results were abnormal. Mean ± SEM serum aminotransferase levels were mildly elevated (aspartate aminotransferase, 83.6 ± 8.8 U/L; alanine aminotransferase, 120 ± 14.3 U/L; n = 13). Alkaline phosphatase and gamma-glutamyltransferase levels were abnormal in 6 and 11 cases, respectively. Amylase and lipase levels were elevated mildly in 6 and 3 cases, respectively, without clinical signs of pancreatitis. The serum lactate dehydrogenase level was abnormal in 10 cases. None of the patients had liver failure. A mild increase in serum lactate levels was found in 6 patients without any acidosis. The level of serum creatinine was normal in all cases. Lipid abnormalities were observed in 6 cases. In addition, physical examination revealed lipoatrophy in 8 cases.

NRTI treatment was discontinued in 9 patients; 4 of them then received an NRTI-sparing regimen. In addition, 4 patients received L-carnitine and polyvitamin therapy, including thiamine, riboflavin, nicotinamide, pyridoxine, and calcium pantothenate. Within 7 months, mean ± SEM aminotransferase levels were reduced (aspartate aminotransferase, 39.2 ± 5.4 U/L vs 82 ± 11.2 U/L; alanine aminotransferase, 43.2 ± 8.6 U/L vs 108.8 ± 17.2 U/L; n = 9). This normalization of aminotransferase levels was associated with an improvement in clinical conditions (ie, polyneuropathy, digestive symptoms, and general state). NRTI-based treatment was continued in 3 of the 4 remaining patients (1 was lost to follow-up). In these 3 patients, no improvement of aminotransferase levels was observed after 6 months.

### Histologic Findings

Pathologic findings are illustrated in **Image 1** and **Image 2** and summarized in **Table 2**. Histologic examination of the liver revealed diffuse steatosis (50% or more of involved hepatocytes with occasional fat granulomas) in only 6 cases (Image 1A). Mild steatosis (occasional vacuoles or steatosis <20%) was observed in the 7 remaining cases (Images 1B and 1C). Macrovesicular steatosis was the prominent pattern of steatosis associated with a limited area of microvesicular steatosis in 5 cases (Image 1D). Additional foci of clarified hepatocytes were present in 10 cases (Image 2A). Megamitochondria were disclosed in only 2 cases (Image 2B). Centrolobular cholestasis was present in 5 cases. Mild lobular necrosis, including occasional Councilman bodies (Image 2C) or ballooned hepatocytes was found frequently, with occasional Mallory bodies (Image 2D) and small foci of mononuclear cells. Picrosirius red stain revealed perisinusoidal fibrosis in the centrolobular area in 7 cases. Overall, standard histologic examination alone, mainly based on the occurrence of severe steatosis (involving at least 50% of the hepatocytes) supported NRTI-induced toxic effects in 6 of 13 patients. In the 2 control patients, the histologic examination revealed chronic hepatitis with mild activity and occasional vacuoles of steatosis.


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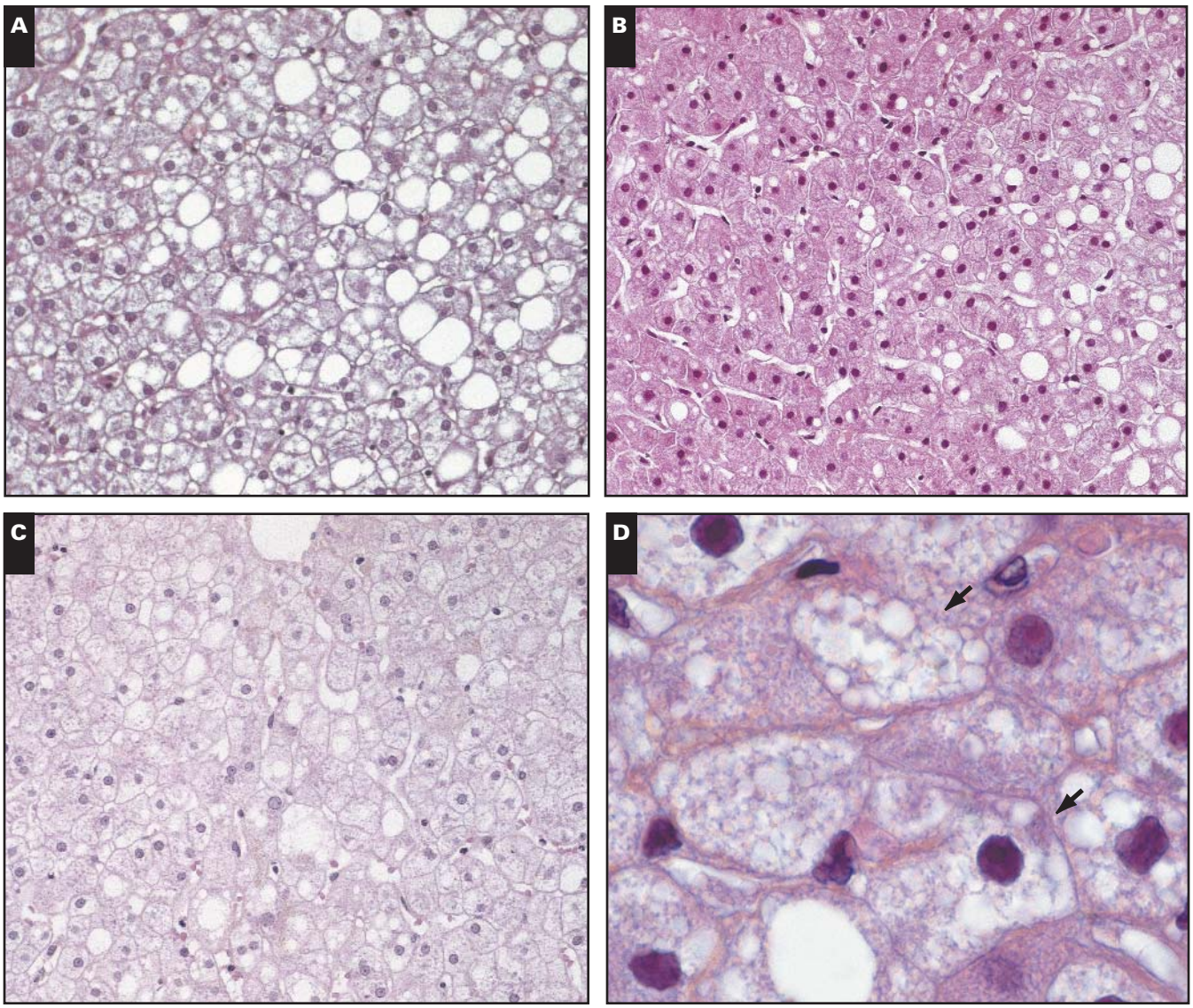


**Image 1** Histopathologic examination reveals heterogeneous liver steatosis. **A** (Case 10), Diffuse macrovesicular steatosis involving 80% of the hepatocytes (H&E, ×200). **B** (Case 11), Moderate macrovesicular steatosis located in the centrolobular zone (H&E, ×200). **C** (Case 9), Macrovacuolar steatosis involving less than 10% of the hepatocytes (H&E, ×200). **D** (Case 7), Foci of microvesicular steatosis (arrows) (H&E, ×2,000).

### Ultrastructural Findings

Ultrastructural findings are illustrated in **Image 3**, **Image 4**, and **Image 5** and summarized in Table 2. Mitochondrial abnormalities were observed in all 13 cases and in neither of the control patients (Images 3A and 3B). The mitochondria showed a reduction in or complete loss of cristae and an amorphous or granular matrix (Images 3C and 3D). Crystalline inclusions were found in 4 patients, sometimes in megamitochondria (Images 4A and 4B). In 1 case, intramitochondrial dense and hyalin inclusions were observed. Autophagolysosomes containing remnants of degenerative mitochondria also were present (Images 4C and 4D). Severe mitochondrial damage with alteration of all mitochondria was present in only 1 case, associated with mitochondrial crystalline inclusions in megamitochondria. Intermediate and mild alterations of mitochondria were present in 10 and 2 cases, respectively. There was no correlation between the severity of steatosis found by standard histologic examination and the intensity of mitochondrial alteration found by EM. In addition, cholestasis and sinusoidal fibrosis (Image 5A) and lipid droplets (Image 5B) were observed frequently. In all the liver biopsy samples, the endoplasmic reticulum was prominent and sometimes markedly dilated (Images 5C and 5D). Interestingly, this hyperplasia of the endoplasmic reticulum also was observed in the untreated control patients.




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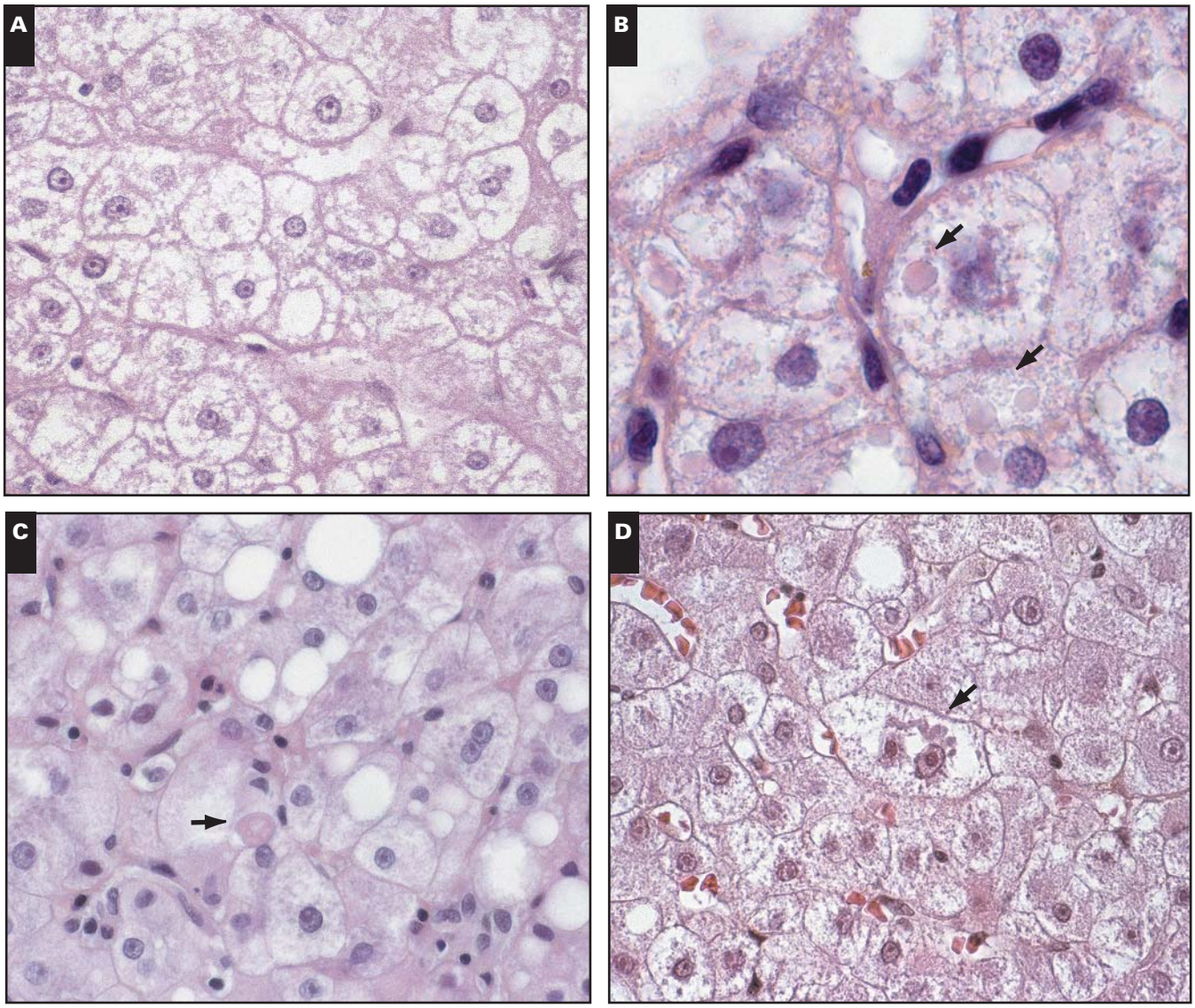


**Image 2** Hepatocyte abnormalities associated with steatosis. **A** (Case 7), Cytoplasmic clarification (H&E, ×1,000). **B** (Case 6), Giant mitochondria (arrows) (H&E, ×2,000). **C** (Case 5), Hepatocyte death. Councilman body (arrow) (H&E, ×1,000). **D** (Case 6), Mallory body (arrow) (H&E, ×1,000).

## Discussion

We have described NRTI-induced hepatotoxic effects in 13 HIV-infected patients with proof of the mitochondrial toxic effects by liver biopsy. Excluding the classic but rare dramatic hepatic failure with lactic acidosis,[8-11,18] our major finding is that NRTI-induced hepatotoxic effects may be present as a mildly symptomatic and nonspecific disease.

In the present study, although liver biologic features always were abnormal, NRTI-induced toxic effects were clinically suspected in fewer than half of the cases, based on elevated serum lactate levels or extrahepatic symptoms (ie, neuromuscular abnormalities). The remaining patients were mildly symptomatic, complaining of asthenia, weight loss, or nausea. In patients with HIV infection in whom the liver test results were abnormal, the differential diagnosis of drug-induced toxic effects includes opportunistic infections, hepatic neoplasia, alcoholic liver disease, and the toxic effects of intravenous drug abuse. Concerning the hepatotoxic effects of antiretroviral drugs, most patients are treated simultaneously with several potentially toxic drugs. Liver toxic effects have been well described with NNRTIs and PIs.[19-21] Nevirapine and efavirenz have been associated with hepatotoxic effects.[20] The clinical and histologic features of the toxic effects of PIs seem to be quite variable and have been described mainly with ritonavir and indinavir.[19,21] Moreover, an assessment of synergistic toxic


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**Table 2**
**Histologic and Ultrastructural Findings***

| Case No. | Histopathologic Findings | | | | Electron Microscopic Findings |
|---|---|---|---|---|---|
| | Steatosis (%) | Cholestasis | Megamitochondria | Lobular Necrosis | Mitochondrial Cytopathy |
| 1 | 70 | – | – | – | + |
| 2 | <5 | – | – | – | ++ |
| 3 | 60 | + | – | – | ++ |
| 4 | 70 | – | – | + | ++ |
| 5 | 20 | + | – | + | ++ |
| 6 | <5 | + | – | – | + |
| 7 | 50 | – | – | + | ++ |
| 8 | 10 | – | – | – | ++ |
| 9 | <5 | + | – | – | ++ |
| 10 | 80 | + | – | – | ++ |
| 11 | 50 | – | – | + | +++ |
| 12 | 15 | – | + | – | ++ |
| 13 | 20 | – | + | – | ++ |

* Steatosis was assessed as a percentage of involved hepatocytes. Other histopathologic findings were judged as present (+) or absent (–). Mitochondrial cytopathy was evaluated as mild (+), intermediate (++), or severe (+++), as described in the "Materials and Methods" section.

effects of the combination of multiple NRTIs, NNRTIs, and PIs is particularly difficult.

Concerning NRTI class, mildly abnormal serum liver test results are well-known side effects of HAART and of regimens containing AZT, ddI, or stavudine.[1,15] A study based on abnormalities of liver test results and not on liver biopsies found an incidence of hepatic toxic effects during NRTI-containing regimens in 21 cases per 1,000 treated person-years.[22] Several studies have evaluated the hepatotoxic properties of different drugs of the NRTI class.[6,23] Among these drugs, stavudine alone or associated with didanosine (9 patients from our series were treated with this combination) has been involved frequently in hepatotoxic effects.[24]

Diffuse macrovesicular steatosis, megamitochondria, and cholestasis that strongly suggest NRTI-induced toxic effects were not a constant feature in our series. Severe microvacuolar steatosis was not observed in our series, probably owing to the case selection that excluded patients with liver failure. Additional lobular inflammation and acidophilic or ballooning hepatocyte necrosis with Mallory bodies also were observed frequently in our series. These findings may support an associated drug-induced steatohepatitis.[10,25,26] Perisinusoidal fibrosis is a common finding in liver biopsy specimens of HIV-infected patients and patients coinfected with HIV and HCV.[27] Several physiopathologic hypotheses have been proposed to explain such a fibrosis. A direct toxic effect of the NRTI via mitochondrial defects cannot be excluded.[3]

To date, ultrastructural studies and biochemical mitochondrial tests are available to confirm the diagnosis of NRTI-induced mitochondrial toxicity.[23,28,29] We confirmed the ultrastructural mitochondrial abnormalities, mainly reported in clinical and experimental studies in the liver[30] and in muscular tissue.[7] These mitochondrial alterations include megamitochondria, loss of the cristae, autophagic vacuoles, and electron-dense and crystalline inclusions. The absence of mitochondrial abnormalities in the 2 control patients supports that these ultrastructural defects are specific to NRTI-induced mitochondrial cytopathy. Only a few studies have dealt with liver ultrastructural findings and mitochondrial pathology in HIV-infected patients.[31,32] Megamitochondria and mitochondrial inclusions (without any other features reported in the present study) have been reported, but these studies were limited strongly by the absence of clinical and biologic data.

Based on these mitochondrial defects, electron microscopic study permitted the diagnosis of NRTI-induced hepatotoxic effects in all cases without typical diffuse steatosis. However, and in view of the limited number of cases in our study, the severity of mitochondrial damage did not correlate with the severity of steatosis and with the lactate level. Our EM findings in mildly symptomatic patients are similar to those reported during dramatic lactic acidosis and severe liver damage.[9,11,17] This absence of correlation among histologic findings, electron microscopic findings, and liver biologic test results suggests that EM assessment is valuable in the positive diagnosis of NRTI-induced mitochondrial cytopathy. However, EM findings cannot be considered a prognostic factor of liver involvement.

The clinical management of patients with NRTI-induced hepatotoxic effects is still a matter of debate. Although discontinuation of the NRTI is required in cases of life-threatening lactic acidosis, there still is insufficient information on the follow-up of patients with only mild hepatic abnormalities. In the present study, discontinuation of the



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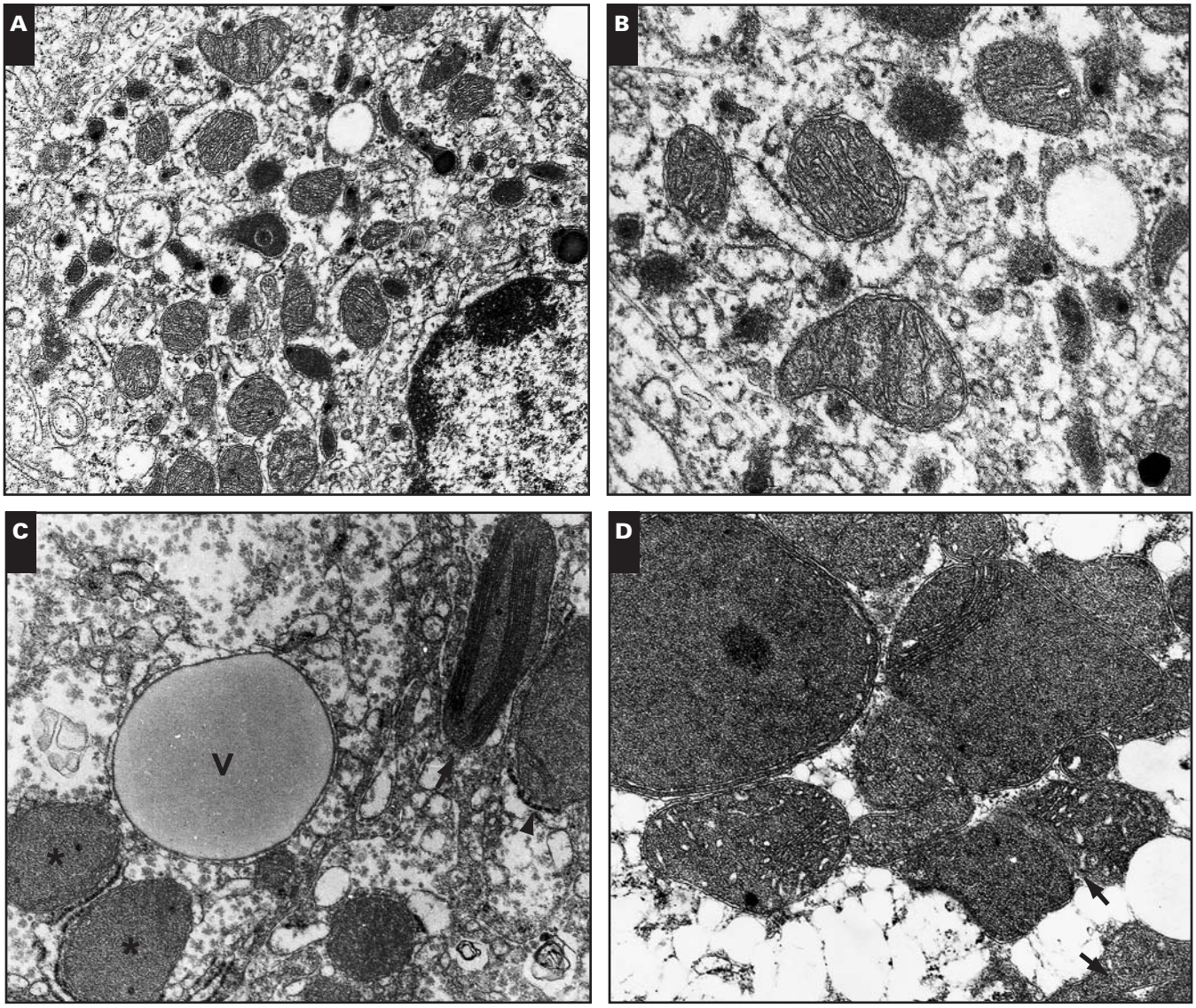


**Image 3** Transmission electron microscopy reveals mitochondrial cytopathy. **A** and **B**, Normal mitochondria in liver biopsy specimens from control patients (**A**, ×16,000; **B**, ×30,000). **C** (Case 5), Intermediate mitochondrial cytopathy with complete loss of the cristae and amorphous matrix (*), partial loss of the cristae (arrowhead), and a crystalline inclusion (arrow). Note the lipid vacuole (V) (×20,000). **D** (Case 12), Intermediate mitochondrial cytopathy with persistence of the cristae in some mitochondria (arrows) (×16,000).

NRTI was associated with improved liver test results. Some authors have proposed regular monitoring of liver enzyme levels without discontinuation of the HAART regimen if the aminotransferase levels remain less than 5 times the upper limit of the reference range.[3,24,33]

Liver biopsy is necessary for the initial evaluation of liver dysfunction in patients with HIV infection who are treated by HAART and with NRTI-based regimens. The diagnosis of adverse events associated with NRTIs is important because of the virologic implications of discontinuing antiretroviral treatment. The ultrastructural assessment of mitochondrial abnormalities is a valuable tool for the diagnosis of NRTI-induced toxic effects because standard histologic examination may be nonspecific in about 50% of cases.

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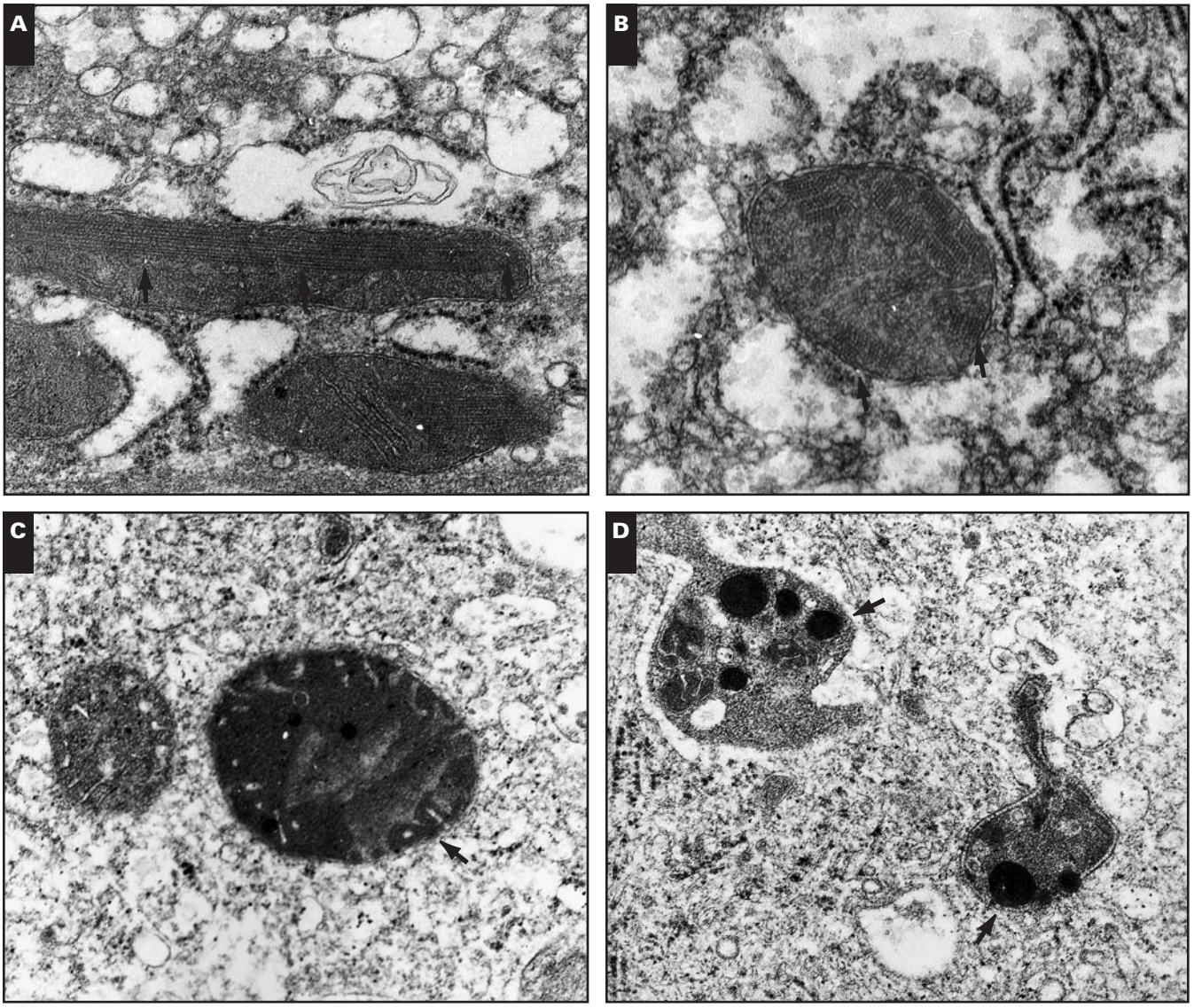


**Image 4** Electron microscopy reveals mitochondrial inclusions and alterations. **A** and **B** (Case 6), Crystalline inclusions (arrows) (×7,500). **C** and **D** (Case 10), Autophagolysosomes containing remnants of mitochondrial cristae (**C**, arrow) and electron dense inclusions (**D**, arrows) (×30,000).

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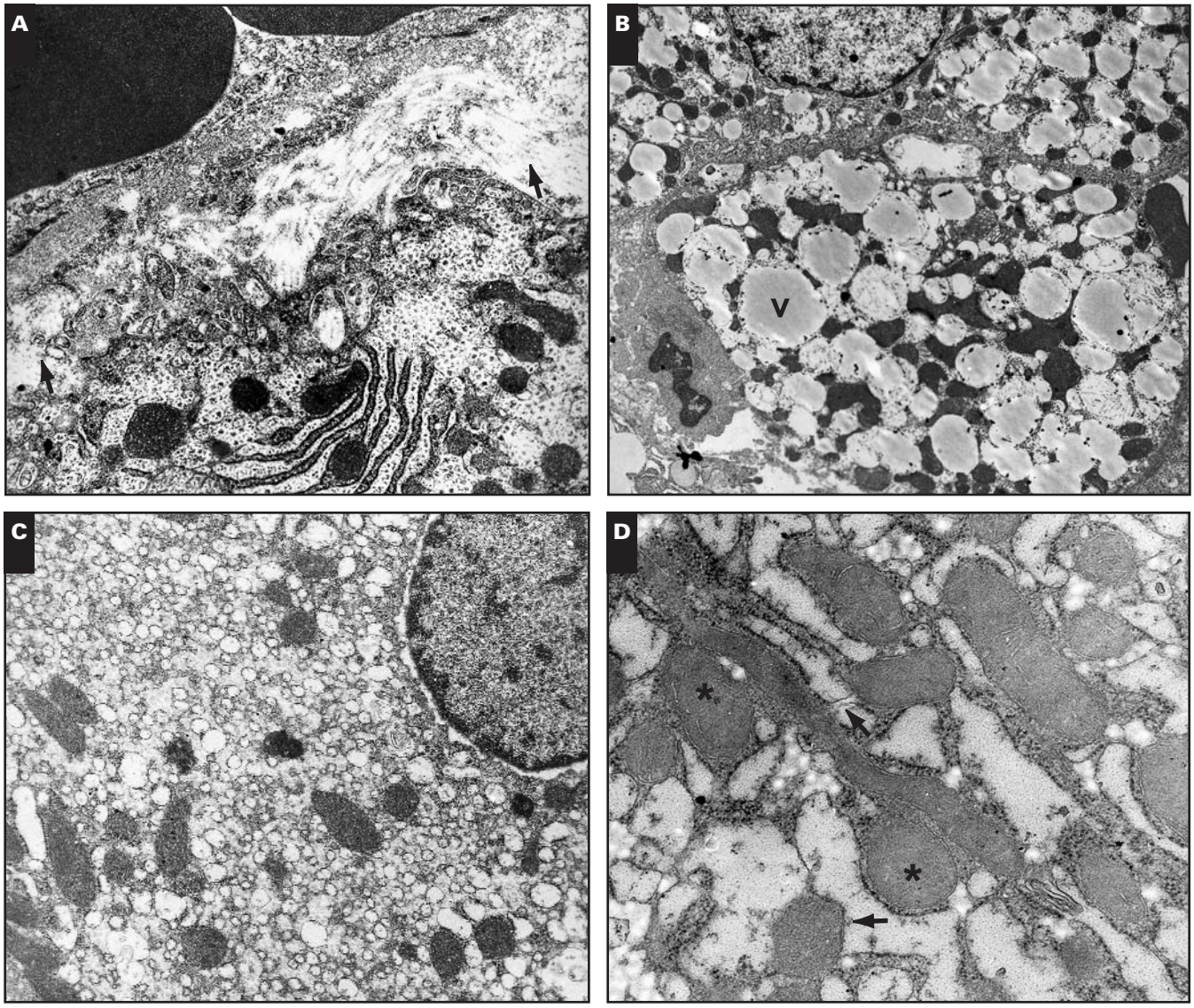


**Image 5** Electron microscopy reveals ultrastructural abnormalities associated with mitochondrial cytopathy. **A** (Case 7), Sinusoidal fibrosis (arrows) (×7,500). **B** (Case 13), Microvesicular steatosis (V) (×10,000). **C** (Case 6), Hyperplasia of the endoplasmic reticulum (×7,500). **D** (Case 2), Dilatation of the reticulum profiles (arrows). Note the alteration of the mitochondria (*) (×20,000).

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