Clinical and Prognostic Importance of Serotyping *Mycobacterium avium*-*Mycobacterium intracellulare* Complex Isolates in Human Immunodeficiency Virus-Negative Patients

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We studied whether the serotypes of *Mycobacterium avium*-*Mycobacterium intracellulare* complex (MAC) isolates determine the prognosis for pulmonary MAC disease. We prospectively monitored a cohort of 68 patients with pulmonary MAC disease for whom the serotype-specific glycopeptidolipids in isolates were identified using thin-layer chromatography and fast atom bombardment mass-spectrometry in 1990 and 1995. Serovar 4 *Mycobacterium avium* was detected in 40/68 patients (58.8%). Other serotypes were serovotypes 1 (five cases), 6 (three cases), 8 (seven cases), 9 (three cases), 14 (four cases), and 16 (six cases). Patients with serovar 4 were significantly (P < 0.01) younger (63.0 ± 9.8 years) than patients with other serotypes (71.8 ± 10.3 years). Patients who failed treatment had a significantly poorer prognosis than other patients. There were no cases of MAC-related death in the cured group. Chest radiographic findings progressively worsened in 36 (90%) of patients with serotype 4, and 14/36 died from respiratory failure caused by pulmonary *Mycobacterium avium* disease. The patients with serotype 4 had a significantly poorer prognosis than patients with other serotypes. These results show that both the outcome of chemotherapy and the serotypes of MAC isolates are important for assessing the prognosis of pulmonary MAC disease.

The prevalence of nontuberculous mycobacterial (NTM) disease, especially *Mycobacterium avium*-*Mycobacterium intracellulare* complex (MAC) disease, is increasing. Among NTM, *Mycobacterium avium* and *M. intracellulare* are closely related and are usually grouped together to form the MAC. MAC organisms are ubiquitous in nature and have been isolated from water, soil, plants, house dust, and other environmental sources (6, 20). Originally, MAC was been to be the etiologic agent of avian disease and also endemic disease in porcine and poultry animals. Since the 1980s, NTM disease has become recognized as an opportunistic infections in patients with advanced AIDS (16, 18, 25, 31). In Japan, however, only a few cases of NTM disease in AIDS patients have been reported, and NTM disease in the absence of AIDS predominates. In 1968 European investigators reported that *M. avium* caused refractory advanced pulmonary *M. avium* disease in immunocompetent patients (3, 11). In Japan, Yamamoto reported in 1971 that 21% of a group of 108 patients with MAC disease died within 5 years, while only 26% had inactive disease after 5 years (36). After 1971, however, the prognosis for pulmonary MAC disease tended to be neglected, since clinical symptoms and radiographic findings could be stable for years despite persistent excretion of organisms. Beginning in 1990, we observed that some patients who were resistant to multiple-drug chemotherapy showed persistent excretion of MAC bacilli and progressive worsening of chest radiographic findings until the time of death. The cases of pulmonary disease caused by MAC occurred in immunologically healthy adults and could not be regarded as opportunistic infections. However, only a few detailed studies on the clinical course of MAC cases in immunocompetent patients have been reported (14, 28, 29, 30, 33). The American Thoracic Society announced in 1997 that the natural history of MAC lung disease is unpredictable in immunocompetent patients. Specifically, some patients had a poor prognosis despite multiple-drug chemotherapy, whereas others maintained a stable clinical and radiographic picture for years (2).

Glycopeptidolipids (GPLs) are the major and specific cell surface antigens of the MAC and *Mycobacterium scrofulaceum* group, and serospecific GPLs allow subdivision into 31 distinct serotypes (21). Our previous observations documented that most patients with pulmonary serovar 4 MAC disease had worsening chest radiographic findings (23). Therefore, in 1990 we began an investigation of the relationship between the serotypes of MAC isolates and the long-term survival of patients with pulmonary MAC disease in a prospective cohort protocol.

**MATERIALS AND METHODS**

**Subjects.** A total of 68 patients with pulmonary MAC disease were enrolled in December 1990 and July 1995 in a prospective cohort protocol study. Identification of the serotype-specific GPL by using thin-layer chromatography (TLC) and fast atom bombardment-mass spectrometry (FAB-MS) was required for enrollment. Patients were followed monthly for more than 5 years and underwent bacteriological examinations, including both smear and culture tests, in our hospital. Chest radiographs were taken every 3 months. Patients known to be human immunodeficiency virus (HIV) positive were excluded from enrollment. Subjects gave informed consent according to our institutional guidelines. All patients had been diagnosed according to the Japanese criteria. Pulmonary MAC disease indications were infiltrates, nodules or cavities on chest radiograms, multifocal bronchiectasis and/or multiple small nodules on computed tomography scans, and at least three positive cultures with 200 colonies or more using

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Ogawa's egg medium for MAC during the past year. The diagnostic criteria used in this study were in accord with those recommended by the American Thoracic Society in 1997 (2).

Initial treatment consisted of a three- or four-drug regimen that included either streptomycin or ethambutol (EB), in addition to rifampin (RFP) and isoniazid. The details of the following treatment were left entirely to the judgment of the physicians. There were no differences in the drugs used for patients between the serovar 4 group and the those with other serovars. Clarithromycin (CAM) was prescribed for 20 of 40 patients with serovar 4 (50%) and for 15 of 28 patients with other serovars (53.5%).

Causes of death. Despite treatment, some patients deteriorated from pulmonary MAC disease and progressed to respiratory failure that necessitated long-term oxygen therapy. When patients were persistently smear and/or culture positive, the cause of death was attributed to pulmonary MAC disease. Cases of hemoptysis-caused death were also categorized as MAC related. Thirteen patients who died of causes other than pulmonary MAC disease during the follow-up period were excluded from the survival prognosis analysis. Four patients transferred to other clinics when they moved to another area. These patients were included because their survival data were available.

Bacteriological outcome. Patients whose culture tests converted to negative after treatment and whose sputum remained negative on culture during the follow-up period were categorized as cured. Patients whose cultures converted to negative for more than 6 months following treatment but with culture-positive sputum during the follow-up period were classified as relapsed. Patients with sputum cultures that were continuously positive despite treatment were categorized as nonresponsive.

Sputum specimens for both smear staining and culturing were obtained every month. The sputum specimens were digested and decontaminated with a solution of 2% sodium hydroxide (NaOH) and N-acetyl-L-cysteine and then centrifuged for 15 min at 3,000 × g. The supernatant was decanted, and the sediment was mixed at 1:10 (vol/vol) with sterile water. Mycobacterial cultures were performed by inoculating 0.1 ml of the processed sample onto tubes of Ogawa medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Species of M. avium and M. intracellulare were identified with a DNA-DNA colorimetric microdilution plate hybridization kit (DDH Mycobacteria; Kyokuto Pharmaceuticals, Tokyo, Japan).

Analysis of MAC serotype. We had performed the identification of serotypes with MAC from patients with pulmonary MAC disease twice, in December 1990 and July 1995. Serotype-specific GPLs were identified by TLC and FAB-MS analyses (JEOL-SX-102; Nihonkohden Co., Tokyo, Japan) (10, 21, 32). The stains of acid-fast bacteria isolated from patients with pulmonary MAC disease were inoculated onto 1% Ogawa egg medium, cultured for 3 weeks, and then transferred to 7H9 broth medium at 37°C and cultured with shaking for an additional 3 weeks until the early stationary phase. The cells were harvested by centrifugation after being heat killed for 15 min by autoclaving. After alkali hydrolysis with 0.1 N NaOH in methanol, alkali-stable lipids were prepared and separated on TLC plates. Plates were charred after being sprayed with 50% H2SO4. Serotype-specific GPLs were identified by comparison of Rf values and coloration with those of the reference standard. Identification of serotype-specific GPLs was confirmed by the FAB-MS analysis (Fig. 1). These analyses were done at the Department of Bacteriology of Osaka City University and without prior knowledge of the patient clinical status.

MIC determinations. MICs were determined by agar dilution with mycobacterium 7H11 agar supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment. Each drug was incorporated at final concentrations of 0.05 to 100 μg/ml, along with a drug-free control. Mycobacteria were grown in 7H9 broth at 37°C and diluted with distilled water to match the turbidity of a no. 1 McFarland standard. The plates were inoculated by using a Steer's replicator with a 1/100 dilution of the suspension in distilled water. The results were read after 3 weeks of incubation at 37°C. The MIC was defined as the lowest concentration of antibiotic which completely inhibited visible bacterial growth (one colony was disregarded).

Statistical analysis. All data were analyzed using the statistical software package StatView 5.0 (SAS Institute, Cary, N.C.). The survival times were calculated by Kaplan-Meier analysis (19), and statistical significance was determined with the log rank (Mantel-Cox) test. Data were compared by the Student t and χ2 tests and are shown as means ± standard deviations.

RESULTS

Of the 110 isolates from patients with pulmonary MAC disease that were analyzed in December 1990 and July 1995, serotype-specific GPLs of 68 isolates (62%) could be identified by TLC and FAB-MS analyses. The characteristics of the enrolled patients (n = 68) with pulmonary MAC disease are shown in Table 1. Fifty patients (73.5%) had previous lung diseases. There were 42 cases of previous tuberculosis, 4 of chronic obstructive pulmonary disease, 2 of idiopathic pulmonary fibrosis, 1 of lung cancer, and 1 of pneumoconiosis. None of the 12 female patients had previous lung diseases, but they did have bronchial-ectasis lesions as shown by chest computed tomography. Six of these patients also had cavitory lesions. Five of six males without previous lung diseases had cavitory, but no bronchial-ectasis, lesions. Forty-two patients (62%) had cavitory lesions, and 6/42 also had bronchial-ectasis lesions. The lesions were moderately to far advanced in two-thirds of

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</tr>
<tr>
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</tr>
<tr>
<td>Far advanced</td>
<td>59</td>
</tr>
<tr>
<td>Moderately advanced</td>
<td>40</td>
</tr>
<tr>
<td>Minimally advanced</td>
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<td>Species and serovar</td>
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</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>9</td>
</tr>
<tr>
<td>Mean range age (yr)</td>
<td>.67 (45–91)</td>
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FIG. 1. Fast atom bombardment-mass spectra of (A) serovar 4, (B) serovar 8, (C) serovar 12, and (D) serovar 4 GPLs.
We divided the subjects into the serovar 4 group and the other-serovar group, because we had recognized by retrospective analysis at the time of enrollment that most of the serovar 4 patients had progressively worsening chest radiograms. A comparison of follow-up results between the two groups is shown in Table 2. The rates of death were the same in both groups. However, the number of deaths caused by pulmonary \textit{M. avium} disease in the serovar 4 group, despite the patients being younger, was significantly larger than that in the other-serovar group. The overall rate of sputum conversion to negative was 60.3% in this study. The rate in the serovar 4 group (52.5%) was lower than that in the other-serovar group (71.4%), despite there being no differences in the chemotherapy regimens. However, the number of relapses was similar in both groups over the long-term follow up period. The rate of nonresponsiveness to treatment in the serovar 4 group was twofold that in the other-serovar group. Ninety percent of patients with serovar 4 \textit{M. avium} disease had worsening radiographic findings, and 17/38 cases (45%) were nonresponsive to the chemotherapy.

The course of lesion progression varied in the 21 patients who had MAC-related deaths, although they could be roughly divided into two groups. Lesions in the first category progressed to giant cavities, and the second group had an association of focal emphysematous lesions with bilateral cavitary lesions (Fig. 2 to 4). Formation of giant cavities was found in 15 cases, including 5/6 cases that presented bronchial-ectasis lesions at enrollment. The causes of 13 non-MAC-related deaths that were excluded from the prognostic analysis are shown in Table 3. A comparison of survival curves (Kaplan-Meier analysis) for the serovar 4 and other-serovar groups is shown in Fig. 5. The survival period for the serovar 4 group after enrollment was significantly ($P < 0.05$) shorter than that of the other-serovar group. The survival rate in the other-serovar group stopped.
decreasing by 50 months after enrollment. However, the survival rate in the serovar 4 MAC group continued to decrease by 25% at 92 months and 50% at 175 months after the initial diagnosis (Fig. 6). The patients in the serovar 4 group were younger (58.1 ± 10.4 years old) than those in the other serovar group (68.3 ± 11.8 years old) at the time of initial diagnosis. These results reveal that the patients with serovar 4 disease have a poor long-term survival prognosis.

The outcome of chemotherapy also correlated significantly (P < 0.05) with long-term survival (Fig. 7). The prognosis of the non-treatment-responsive group was significantly (P < 0.01) worse than that of the relapsed group. There were no MAC-related deaths in the cured group. The prognosis of patients with far-advanced MAC disease was significantly (P < 0.01) worse than that of patients with less advanced lesions. However, in the long-term follow-up periods, there were deaths caused by pulmonary MAC disease in patients with only a minimally advanced lesion.

The susceptibilities of patient MAC isolates to EB, RFP, CAM, and sparofloxacin were studied in the three separate groups (M. avium serovar 4; M. avium serovars 1, 6, 8, and 9; and M. intracellulare serovars 14 and 16) (Table 4). The range of MICs of each drug for serovar 4 isolates varied widely. The RFP MICs that inhibited 50% (12.5 μg/ml) and 90% (50 μg/ml) of the isolates were significantly higher for serovar 4 than for the other groups. When the interpretation of MICs was done according to the achievable drug levels in serum, 67% of the isolates for EB (MICs of > 8μg/ml), 57% for RFP (MICs...
of >8 μg/ml), 13% for CAM (MICs of >32 μg/ml), and 17% for sparofloxacin (MICs of >4 μg/ml) were categorized as resistant in the serovar 4 group (12). On the other hand, in the M. intracellulare serovar 14 and 16 group, most isolates were categorized as susceptible or moderately susceptible to EB, RFP, CAM, or sparofloxacin. The range of MICs of each drug for isolates in the M. avium serovar 1, 6, 8, and 9 group was also found to be broad.

**DISCUSSION**

MAC diseases have attracted attention because they can develop as opportunistic infections and cause death in AIDS patients. However, the prognosis of pulmonary MAC disease in HIV-negative cases is still unclear, and therapeutic approaches had not yet been established in 1997 (2). Furthermore, when we started this prospective study in 1990, it was
probably not known that there were some pulmonary MAC patients with poor prognosis among immunocompetent patients. The evaluation of prognostic predictors and treatment outcomes for pulmonary MAC disease requires long-term follow-up of many patients. In this study, we have been able to follow 68 patients on a monthly basis for more than 5 years. Most of the patients had undergone multidrug antituberculosis chemotherapy, which was conventionally used before the American Thoracic Society recommendation of 1997 (1, 2). Half of the patients had also been treated with CAM in addition to being treated with multidrug antituberculosis chemotherapy. Consequently, the rate of sputum-negative conversion was similar to that in the previous reports (15, 22, 26, 30). At least 21 (30.9%) of 68 patients with pulmonary MAC disease experienced progression or aggravation of the disease and died, excluding non-MAC-related deaths, because the subjects were older. In considering the prognosis of MAC disease in the HIV-negative patient, the progression or aggravation of pulmonary MAC disease that was not due to an opportunistic infection was a direct cause of death.

The ratio of deaths unrelated to MAC was high in the group with serovars other than serovar 4. This was especially true for the serovar 16 patients, who were significantly older than the patients with serovar 4. The 13 cases of deaths unrelated to MAC, including six patients with worsening findings on radiograms, were excluded from the survival prognosis analysis in order to achieve accurate evaluation. Although the number of cured patients was also small, the population in our study seemed to be appropriate to evaluate the prognostic predictors in patients with pulmonary MAC disease.

Regarding the correlation between treatment outcomes and survival prognosis, it is first very important that all cured patients, including one with serovar 8 who underwent surgical therapy, were alive at the end of this study. Second, it is important that the relapsed patients had a significantly better prognosis than the nonresponders to treatment. The extent of radiographic lesions was also significantly correlated with the prognosis of survival. These results reveal that the outcomes of chemotherapy are closely associated with the survival time for patients with pulmonary MAC disease.

We also studied the relationship between the serotypes of MAC isolates and the long-term survival of patients with pul-
monary MAC disease. It was more difficult to convert sputum to negative when the disease was caused by serovar 4 than for other serovars. The serovar 4 isolates were more resistant to RFP and CAM, and the patients with serovar 4 were more frequently nonresponders to the multidrug chemotherapy than patients with other serovars. However, the sputa of most patients with pulmonary MAC disease became positive on culture during the follow-up period, and the rates were nearly equal between the serovar 4 and the other-serovar groups. This might be due to the factors involved in long-term follow-up and the higher relapse rate in the population of patients being retreated with chemotherapy than in those receiving initial treatment. The patients also may acquire subsequent infections from the living environment, and we have begun a study to investigate MAC organisms from the houses where the patients live.

The radiographic findings for the serovar 4 group had also become significantly worse in the follow-up periods compared with those in the other-serovar group. In cases of death, the radiographic findings for patients with upper-lobe cavitary disease or with nodular bronchiectasis at enrollment had progressed to bilateral cavitary lesions with sclerofibrotic and emphysematous changes (Fig. 2 to 4). Although radiographic findings alone, whether cavitary or bronchiectasis, cannot be a prognostic tool, the progress of the disease should be closely observed by means of radiographic changes.

In conclusion, the survival time of the serovar 4 group was significantly shorter than that of the other-serovar group. At an

![FIG. 7. Kaplan-Meier survival analysis of patients with pulmonary MAC disease divided by (a) bacteriological outcome and (b) the extent of radiographic lesions. (a) Log rank test, \( P < 0.05 \) (#); (b) log rank (Mantel-Cox) test, \( P < 0.01 \).]

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<th>No. of isolates with MIC (( \mu \text{g/ml} )) of:</th>
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<th>3.13</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>( \geq 100 )</th>
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<tbody>
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<td>1</td>
<td>8</td>
<td>11</td>
<td>7</td>
<td>2</td>
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<tr>
<td></td>
<td>M. avium serovars 1, 6, 8, 9</td>
<td></td>
<td>1</td>
<td>3</td>
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<tr>
<td></td>
<td>M. intracellular serovars 14, 16</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1</td>
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**TABLE 4. Susceptibilities of patient isolates to ethambutol, rifampin, clarithromycin, and sparofloxacin**
early stage of infection, it is difficult to judge the prognosis of patients with pulmonary MAC disease. Although further studies with large numbers of patients are required, the identification of serotype-specific GPLs may be helpful for prognosis. However, there were MAC-related deaths in patients with pulmonary MAC disease caused by serovar 8 or serovar 14. The importance of other serovars as prognostic predictors will be analyzed when the number of cases with each serovar increases to a level sufficient for statistical evaluation. There were also a number of nonserotypeable patients, including some of the cases with poor prognoses. Thus, it could be argued that the sensitivity of serotyping should be improved. Also, the laborious and time-consuming serotype identification of isolates used in this study needs to be replaced with simple and rapid diagnostic methods, and serotyping is not routinely performed. We have developed enzyme-linked immunosorbent assay serodiagnostic tests that use various serotypes of glycopeptidolipids as antigens and a new serotyping method which uses high-performance liquid chromatography/mass spectrometry analyses with a small-scale preparation of GPLs from MAC isolates for 2 days (21, 27).

The number of serovars identified was lower than in previous reports (4, 9, 13, 35). This might be due to either the effects of the preceding multidrug chemotherapy or to problems of identification, because only 14 serovar-specific polar GPLs from MAC have been structurally characterized at this time (5, 8, 24). Also, neither the GPL antigen-based enzyme-linked immunosorbent assay nor the original type-specific rabbit antisera were available during our study. However, the serotype specificity could be accurately identified using TLC and FAB-MS analyses. *M. avium* and serotype 4 predominated in this study because of the high geographical distribution of *M. avium* in the Kinki area, in which our institute is located (32). The skewed distribution may also relate to the previous multidrug chemotherapy that most of the enrolled patients had undergone, because the disease caused by *M. avium* was more difficult to cure than *M. intracellulare*-related disease (37). Furthermore, in cases where the serotypes were analyzed twice, both isolates were identified as serovar 4.

The mechanisms underlying resistance to MAC organisms are likely to be highly informative in regard to both host defense against these infections and the basis of the more virulent MAC relatives. It was recognized early in the HIV pandemic that the decline of CD4+ T lymphocyte levels to below 100 cells/mm3 was a profound risk for the development of disseminated MAC disease on the part of both the patient and the physician. The virulence of the organism, and resistance to chemotherapy, a problem regarding the worsening of pulmonary MAC disease that also needs to be addressed is patient compliance issues, which may include a lack of knowledge of the gravity of the disease on the part of both the patient and the physician.

REFERENCES


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The mechanisms underlying resistance to MAC organisms are likely to be highly informative in regard to both host defense against these infections and the basis of the more virulent MAC relatives. It was recognized early in the HIV pandemic that the decline of CD4+ T lymphocyte levels to below 100 cells/mm3 was a profound risk for the development of disseminated MAC (7, 17). Since the CD4+ T lymphocyte is a major producer of gamma interferon as well as other cytokines, it was speculated that gamma interferon might be important in the control of MAC disease. Despite the wealth of information regarding disseminated infections with MAC, the underlying immune defects have yet to be convincingly demonstrated for pulmonary MAC infections, except in the case of HIV infection. Specific serotypes such as 1, 4, and 8 can be frequently isolated from humans infected with HIV, and the prognosis after infection differs depending on the serotype. Serovar 4 shows an unfavorable prognosis, whereas serovar 16 is associated with rapid recovery (8, 13).

No information dealing on the virulence factor of MAC that is directly related to the intracellular bactericidal activity has been available to date. We have previously reported effects of (i) various GPLs purified from MAC on the phagocytic processes of human polymorphonuclear leukocytes, (ii) GPL-coated heat-killed staphylococcal cells that were phagocytosed by polymorphonuclear leukocytes, and (iii) the phagosome-lysosome (P-L) fusion (34). Phagocytosis was strongly promoted and the P-L fusion was markedly inhibited by serovar 4, but not serovar 16, GPLs. Serotype 8 GPLs showed concomitant stimulation of both phagocytosis and P-L fusion. These effects may be due to an unknown interaction between specific carbohydrate chains and host phagocytic cell membranes.

The pulmonary MAC disease caused by serovar 4 had a poor long-term survival prognosis. For the treatment of pulmonary MAC disease, including surgery, diagnosis and treatment at an early stage are important. In cases of patients with pulmonary MAC disease caused by serovar 4, it was argued that multichemotherapy including CAM (one of the newer macrolides) and sparofloxacin (one of the newer fluoroquinolones) should be prescribed, because the results of MIC determinations showed that CAM and sparofloxacin were more effective against serovar 4 than EB or RFP. Furthermore, lung resections should be performed in patients with adequate pulmonary reserve for whom medical therapy has been unsuccessful. In addition to the defense mechanism in the patient’s lung, the virulence of the organism, and resistance to chemotherapy, a problem regarding the worsening of pulmonary MAC disease that also needs to be addressed is patient compliance issues, which may include a lack of knowledge of the gravity of the disease on the part of both the patient and the physician.