2004

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Journal of Clinical Microbiology

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J. Clin. Microbiol. **2004, 42(9):4199. DOI: 10.1128/JCM.42.9.4199-4203.2004.**

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2004, p. 4199–4203 Vol. 42, No. 9
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.9.4199–4203.2004

Correlation of Cerebrospinal Fluid (CSF) Cell Counts and Elevated CSF Protein Levels with Enterovirus Reverse Transcription-PCR Results in Pediatric and Adult Patients

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Received 30 March 2004/Returned for modification 15 April 2004/Accepted 28 May 2004

During the 2001, 2002, and 2003 enterovirus seasons, we investigated the correlations between cerebrospinal fluid (CSF) nucleated cell counts and elevated CSF protein levels and the detection of enteroviral RNA by reverse transcription (RT)-PCR. Our objective was to determine if pleocytosis and/or elevated protein levels were predictive of positive RT-PCR results for enterovirus. We were also interested in determining if the presence of West Nile virus during the 2002 enteroviral season contributed to a change in these correlations. We found that in the group of patients aged >2 months, the absence of pleocytosis was highly predictive of a negative RT-PCR result. Elevated CSF protein level was not a good predictor of RT-PCR positivity for enterovirus and did not add to the diagnostic sensitivity or specificity of pleocytosis.

Enteroviruses (EVs) cause annual seasonal epidemics of aseptic meningitis in temperate climates and are the most common cause of this clinical syndrome in the United States. The infections are most clinically noticeable in children, but they are also clearly present in adults (9). The viruses are maintained within the human population in tropical climates year-round and at low but detectable levels in both children and adults during the cold months in temperate climates (3).

Reverse transcription (RT)-PCR has become the method of choice for the rapid and efficient diagnosis of EV infections from cerebrospinal fluid (CSF). The use of commercially available (6, 11, 12, 20) and in-house assay methods (6) is currently common in molecular diagnostic virology laboratories and often results in fewer tests being performed, less medication being used, and earlier release from the hospital than with previous diagnostic methods for patients with CSF samples with positive RT-PCR results for EV (EV RT-PCR) and negative Gram stains (4, 8, 10, 14, 15).

Whether CSF pleocytosis plays an important role in defining aseptic meningitis or in prioritizing the differential diagnosis has been investigated but is still not completely clear (7, 14, 17). In this era of rapidly rising health care costs, it is important, if possible, to limit laboratory-based testing by encouraging syndrome-directed testing and actively discouraging testing that is not consistent with the clinical picture or with other laboratory results. Additionally, quality control within the laboratory can be enhanced by monitoring laboratory-based data to look for important correlations that will provide insight for clinicians and laboratory workers with regard to future testing requests.

During the 2001, 2002, and 2003 EV seasons, we collected all available CSF cell count data and CSF protein data (available for 2001 and 2002 only) for patients for whom EV RT-PCR of CSF samples had been requested. Our suspicion that CSF with a nucleated cell count of ≤ 5 per mm^3 from patients more than 2 months old was not likely to be RT-PCR positive proved to be true, although a large number of CSF samples with high cell counts did not test positive for EV.

MATERIALS AND METHODS

CSF specimens (n = 1,706) were submitted to the St. Louis Children's Hospital Virology Laboratory during the 2001, 2002, and 2003 EV seasons (roughly May to November of each year) for EV RT-PCR testing. Relevant CSF cell count data was available from the laboratory information system for 728 of the patients who were seen at Washington University Medical Center and tested by EV RT-PCR. Of the patients included in the study, 549 were pediatric (≤18 years of age) and 179 were adults. The data analyzed included patient age, EV RT-PCR result (243 positive and 485 negative), CSF white blood cell count, and CSF protein level. Pleocytosis was defined as a CSF cell count of >22 cells/mm^3 if the patient's age was <4 weeks, >15 cells/mm^3 if the patient's age was 4 to 8 weeks, and >5 cells/mm^3 if the patient's age was >8 weeks (1). The cutoffs to identify elevated levels of CSF protein were those determined by a study at our institution in which EV RT-PCR was performed on all specimens to eliminate any samples from patients with unsuspected EV infections (19). Those cutoffs were as follows: ≥111 mg/dl for patients aged ≤2 months, ≥79 mg/dl for patients aged >2 months to 4 months, ≥34 mg/dl for patients aged >4 months to 14 years, ≥37 mg/dl for patients aged >14 years to 18 years, and ≥46 mg/dl for patients aged >18 years. When there was CSF pleocytosis (as defined above for each age group) in a patient with a negative EV RT-PCR result during 2002, the results of other bacterial and viral tests performed on the patient were reviewed.

RNA was extracted manually from 140 μl of CSF by using QIAamp Viral RNA Mini Spin columns. This procedure allows purification of viral nucleic acids from cell-free fluid samples, and the columns were used in accordance with the manufacturer's instructions.

The Chemicon Light Diagnostics Pan-Enterovirus ASR kit was used for the qualitative detection of EV RNA through RT-PCR amplification of the 5′ untranslated region of the EV genome. Extracted RNA was combined with reaction mix (QIAGEN RT-PCR mix and Chemicon Pan-Entero Primer mix) and amplified with a Perkin-Elmer 2400 thermocycler. Detection of the amplified product was performed by using a Chemicon Light Diagnostics Pan-Enterovirus hybridization capture assay. Using a serially diluted stock of ECHO 11 grown in cell culture, we determined the analytic sensitivity of the Chemicon assay to be 1.9 times the 50% tissue culture infective dose. The primers and probe for the Chemicon assay have been shown to detect all but 6 of the 64 known EVs that

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TABLE 1. Results of EV RT-PCR by age group and year

Age group	No. positive/no. tested (%) for yr indicated			
	2001	2002	2003	Total
All ages	117/243 (48.1)	44/219 (20.1)	83/266 (31.2)	277/728 (38.1)
≤2 mo	43/68 (63.2)	24/47 (51.1)	16/64 (27.3)	83/179 (46.4)
>2 mo and ≤18 yr	61/127 (48.0)	18/100 (18)	61/143 (42.7)	140/370 (37.8)
>18 yr	13/48 (27.1)	2/72 (2.8)	5/59 (8.5)	20/179 (11.2)

infect humans (13). The six EVs that were not detected are epidemiologically rare.

A z-test was used to test the hypothesis that there were no differences in sensitivities and specificities between the data from 2001 and 2002, the first two years of the study.

RESULTS

Of the 1,706 specimens submitted for EV RT-PCR during the three study seasons, 570 (33.4%) tested positive and 1,136 (67%) tested negative. As shown in Table 1, a higher percentage of submitted specimens (48%) was positive in 2001 than in 2002 (20%) or 2003 (31%). In two of the three seasons (2001 and 2002), the percentage of specimens that was positive was highest for infants ≤2 months of age. In all three seasons, the percentage of specimens that was positive was lowest among those >18 years of age.

We used the 728 samples for which CSF parameters were available to examine the relationship between CSF pleocytosis and results of EV RT-PCR. The percentage of the 728 specimens that was EV RT-PCR positive (33.5%) was very similar to the percentage of the entire group of specimens that was positive (33.4% [570 of 1,706]). Over all three seasons, pleocytosis was present in 410 of 728 patients (56%) for whom CSF cell count data was available, including 216 (88.5%) of those with EV RT-PCR-positive results and 194 (40.1%) of those with EV RT-PCR-negative results. As shown in Table 2, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value of CSF pleocytosis in relation to the EV RT-PCR result were 89, 60, 53, and 91%, respectively.

Interestingly, of the 28 patients who tested EV RT-PCR positive without CSF pleocytosis, 25 (89%) were in the ≤2 months age group (Table 2). These patients accounted for 30% of the 83 patients in this age group who were EV RT-PCR positive. In comparison, pleocytosis was absent in only 3 of 160 patients (1.9%) aged >2 months who were EV RT-PCR positive ($P < 0.001$). This difference translates into large age-related differences in the sensitivities and negative predictive values for CSF pleocytosis in relation to EV RT-PCR (70% sensitivity and 74% negative predictive value for samples from patients ≤2 months of age, 98% sensitivity and 98% negative predictive value for samples from patients aged 2 months to 18 years, and 100% sensitivity and 100% negative predictive value for patients aged >18 years) (Table 2).

In contrast to the finding that lack of pleocytosis in CSF specimens that were EV RT-PCR positive was most common in samples from patients in the ≤2 months age group, the presence of pleocytosis in specimens that were EV RT-PCR negative was more common for older patients. Overall, pleocytosis was present in 194 (40.1%) of the 484 specimens that were EV RT-PCR negative, and it was present in 27% of specimens from patients in the ≤2 months age group, in 39% of specimens from patients in aged 2 months to 18 years, and in 50% of specimens from the >18 years age group. These findings correspond to lower specificities and PPVs for pleocytosis with increasing age (73% specificity and 69% PPV for those patients ≤2 months of age, 61% specificity and 61% PPV for those patients aged 2 months to 18 years, and 50% specificity and 20% PPV for those patients aged >18 years). The PPVs for patients >2 months of age were lowest in 2002, the year that the St. Louis area experienced a large outbreak of West Nile virus (WNV) disease. During this year, the PPV of pleocytosis for a positive RT-PCR was only 36% in the 2 months to 18 years age group and 5% in those over 18 years of age.

We next investigated the possibility that elevated CSF protein level could be used as a marker for EV infection. This analysis was restricted to specimens obtained during the 2001 and 2002 seasons. Of the 465 patients for whom CSF protein values were available, 161 were RT-PCR positive, and 192 had elevated levels of CSF protein. For all ages, the sensitivity, specificity, PPV, and negative predictive value of elevated protein levels in relation to the results of RT-PCR were 38.5%, 61.1%, 47.8%, and 51.7%, respectively, in 2001 and 43.2%, 54.9%, 19.4% and 79.3%, respectively, in 2002. Sensitivities and specificities were low in all age groups and in all years (data not shown). We also examined two other combinations of parameters in relation to the RT-PCR result: the presence

TABLE 2. Presence of pleocytosis and EV RT-PCR results for 2001, 2002, and 2003[a]

Year and age group	No. of samples with indicated EV RT-PCR result when pleocytosis is:				Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Present		Absent					
	PCR+	PCR−	PCR+	PCR−				
2001								
All ages	100	43	17	83	85	66	70	83
≤2 mo	28	5	18	20	65	80	85	57
>2 mo and ≤18 yr	59	19	2	47	97	71	76	96
>18 yr	13	19	0	16	100	46	41	100
2002								
All ages	39	77	5	98	89	56	34	95
≤2 mo	19	10	5	13	79	57	66	72
>2 mo and ≤18 yr	18	32	0	50	100	61	36	100
>18 yr	2	35	0	35	100	50	5	100
2003								
All ages	77	74	6	109	93	60	51	95
≤2 mo	11	11	5	37	69	77	50	88
>2 mo and ≤18 yr	60	38	1	44	98	54	61	98
>18 yr	5	26	0	28	100	52	16	100
All years								
All ages	216	194	28	290	89	60	53	91
≤2 mo	58	26	25	70	70	73	69	74
>2 mo and ≤18 yr	137	89	3	141	98	61	61	98
>18 yr	20	80	0	79	100	50	20	100

[a] PCR+, positive EV RT-PCR results; PCR−, negative EV RT-PCR results; NPV, negative predictive value.

TABLE 3. Z-tests for comparison of two sets of statistical data

Age group and marker	Sensitivity				Specificity				PPV				NPV[a]			
	2001	2002	z-test	*P*	2001	2002	z-test	*P*	2001	2002	z-test	*P*	2001	2002	z-test	*P*
All ages																
Pleocytosis	0.86	0.89	−0.669	0.5031	0.66	0.56	2.138	0.0325								
Protein elevated	0.39	0.43	−1.024	0.3058	0.61	0.55	1.351	0.1767								
Pleocytosis and protein	0.36	0.43	−1.703	0.0886	0.79	0.70	1.516	0.129								
Pleocytosis and/or protein	0.88	0.89	−0.13	0.8966	0.48	0.41	2.209	0.027								
0 to ≤2 mo	0.65	0.79	−1.49	0.137	0.80	0.57	2.443	0.0145	0.85	0.66	2.01	0.044	0.57	0.72	−1.593	0.111
>2 mo to ≤18 yr	0.97	1.0	−0.452	0.651	0.71	0.61	1.506	0.131	0.76	0.36	6.026	<0.001	0.96	1.0	−0.602	0.547
>18 yr	1.0	1.0			0.46	0.50	−0.44	0.66	0.41	0.05	3.96	<0.001	1.0	1.0	0	

[a] NPV, negative predictive value.

of both pleocytosis and elevated protein levels, and the presence of either pleocytosis or elevated protein levels. Neither of these combinations appeared useful: using pleocytosis plus elevated protein levels resulted in decreased sensitivity in relation to RT-PCR, whereas results with either pleocytosis or elevated protein level were very similar to those with pleocytosis alone (data not shown).

In an effort to improve the sensitivity of elevated protein levels for the prediction of EV RT-PCR positive specimens, we evaluated the effect of lower protein cutoff levels on the correlation. The receiver operating characteristic curves for the three different protein cutoff levels (i.e., the normal levels as given in Materials and Methods, 80% of the normal levels, and 60% of the normal levels) and for the three age groups of patients showed that there was not a particularly good CSF protein cutoff level for use as an indicator of EV RT-PCR positivity, since none of the points on any of the curves indicated greater than 80% sensitivity and also greater than 80% specificity (i.e., less than 0.2 on the [1-specificity] axis) (data not shown).

Since WNV was first present in our area during the 2002 EV season but had not been present during the 2001 season, we sought to determine whether the presence of WNV made a noticeable difference in the relationship between the CSF profile and the results of EV RT-PCR. We used the z-test to compare the sensitivities and specificities of CSF pleocytosis and elevated CSF protein levels in relation to the EV RT-PCR results for the two years for all age groups. The results are shown in Table 3. Of note was the fact that the specificity of pleocytosis as a predictor of the EV RT-PCR result in patients of all ages decreased from 66% in 2001 to 56% in 2002 ($P = 0.03$), suggesting the possibility of the presence of a group of patients with pleocytosis resulting from WNV infection (Table 3). Curiously, the difference in specificities between the two years was only significant for the ≤2 months age group (80% in 2001 versus 57% in 2002) (Table 3). Of particular note was the eightfold decrease in PPV in the >18 years cohort, where the PPV was 41% in 2001 and only 5% in 2002 ($P < 0.0001$) (Table 3); this is not surprising and probably reflects the fact that WNV meningitis and encephalitis are more common in adults than in children.

During 2002, a total of 77 patients for whom CSF parameters were available had pleocytosis and a negative EV RT-PCR. Alternative diagnoses were established virologically (based on positive PCR assays or serology) for 18 patients (15%), including 10 with WNV infection, 4 with herpes simplex virus (HSV), 3 with Epstein-Barr virus (EBV), and 1 with both HSV and EBV. None of the 77 patients had a positive culture for a bacterial pathogen associated with meningitis.

DISCUSSION

Nucleic acid amplification using RT-PCR or other techniques provides a more accurate diagnosis of EV meningitis than previously available techniques such as viral culture. With the availability of these sensitive diagnostic techniques, many CSF specimens are now submitted for EV nucleic acid testing. We have monitored the CSF profile, including leukocyte count and protein level, to help assess the performance of the EV RT-PCR used in our laboratory. We have now analyzed the data based on specimens submitted for EV RT-PCR during the 3-year period of 2001 to 2003 to reassess the relationship between CSF parameters and the presence of EV infection of the central nervous system (CNS). During one of those years, a large outbreak of WNV occurred in our area, allowing us to evaluate the impact of the presence of another neurotropic pathogen on the relationship between abnormalities in the CSF profile and the possible presence of EV RT-PCR. Our findings may be useful for clinicians and laboratory workers seeking to optimize their use of EV nucleic acid testing.

The main focus of our study was the relationship between CSF leukocyte counts and the results of EV RT-PCR. We were interested in determining the proportion of patients with both EV CNS infection and an elevated CSF leukocyte count. We were also interested in determining the proportion of patients with elevated CSF leukocyte counts that were accounted for by EV infection. Our data show that both of these proportions are strongly influenced by age. The finding of a lack of CSF pleocytosis in CSF samples that were positive for EV RNA was largely limited to infants under 2 months of age and was quite common (28%) in this age group, probably reflecting the immunologic immaturity of these patients. It is tempting to speculate that a chemokine response required for the recruitment of leukocytes to the site of infection had not yet developed in these patients. Among older patients, fewer than 2% of those with EV infection of the CNS lacked pleocytosis. Conversely,

the finding of CSF pleocytosis with a negative EV RT-PCR was more common among samples from older patients. Not surprisingly, this finding was also more common during the season when a sizable outbreak of WNV disease occurred in the area served by our laboratory.

Previous smaller studies have also focused on a comparison of EV RT-PCR results and CSF parameters. Henquell et al. (5) reported on 61 patients with symptoms of meningitis and compared EV culture and PCR results with CSF findings. Fifty-six of the 61 patients (92%) were PCR positive, but 9 patients who were >1.5 years of age had CSF cell counts of <10 cells/mm^3 and were PCR or culture positive. Thus, in their study, the correlation between pleocytosis and EV RT-PCR positivity was not good. Böttner et al. (2) reported on a cohort of 70 patients with symptoms of meningitis during the summer of 2000. In that study, 29 (46%) of 61 EV RT-PCR assays on CSF were positive. CSF cell counts ranged from 2 to 1,820 cells/mm^3, with a mean of 151. The authors reported that there were no differences in laboratory findings between the patients in their study who were EV RT-PCR positive and those who were negative, but they did not provide detailed CSF cell counts. The explanation for the differences between results of those studies and the present study is not clear, but it could reflect biological differences in the EVs being detected or different performance characteristics of the EV RT-PCR assays.

One of the interesting aspects of the present study is to focus attention on the causes of CSF pleocytosis in patients whose EV RT-PCR assay is negative. One possible explanation is that the assay may not detect all EV infections. We do not know the actual analytic (molecular) sensitivity of the Chemicon assay in terms of its ability to detect low levels of viral RNA. We have investigated the sensitivity and found it to be on the order of 2.0 times the 50% tissue culture infectious dose, but we do not know how many EV genomes this represents, nor is it known how many noninfectious particles are produced in an acute infection in human CSF. However, this is unlikely to be the only explanation since, in our study, 21 of 148 patients (14%) who were ≤2 months old and 169 of 549 patients (31%) who were >2 months old had pleocytosis and were EV RT-PCR negative. The primers and probe used in our study have been shown to detect all but 6 of the 64 known EVs that infect humans (13). One EV, coxsackievirus A15, was not available for testing, and two others that were not detected with this primer and probe combination, echoviruses 22 and 23, have since been reclassified as parechoviruses (16). The six EVs that were not detected are epidemiologically rare. Other infections, such as HSV, EBV, and WNV, were diagnosed in some of the patients with pleocytosis but accounted for only 18 infections or 23% of all patients in this study with pleocytosis and a negative EV RT-PCR in the year 2002 (n = 77). It is possible that some of the remaining patients had infections with other infectious agents, such as parechoviruses (16). Additionally, some of the observed cases of pleocytosis in this study may have been due to noninfectious causes of pleocytosis, including reactions to medications such as cotrimoxazole, nonsteroidal anti-inflammatory agents, and intravenous immunoglobulin. Bacterial urinary tract infection has also been implicated as a cause of CSF pleocytosis in children (18).

The use of CSF protein levels as an indicator of EV RT-PCR positivity was considered. However, the data showed that the CSF protein level was neither sensitive nor specific when used by itself, nor did it increase the sensitivity or specificity when used in conjunction with pleocytosis. The receiver operating characteristic curves showed that the CSF protein level alone never had greater than 80% specificity at any of the cutoff levels investigated, and in no age group nor at any of the three levels we investigated were both the sensitivity and specificity greater than 80%. The best correlation between CSF protein levels and EV RT-PCR positivity occurred in patients who were >2 months old at 80% of the normal cutoff, where the sensitivity was 68% and the specificity was 78%.

The circulation of WNV in the community during the 2002 EV season was potentially a confounding factor in using CSF markers as correlates of EV RT-PCR positivity. Indeed, the z-test data of Table 3 confirm that there were significant differences between data from the 2001 and 2002 seasons on the specificities of pleocytosis alone or pleocytosis and/or elevated protein level as correlates of EV RT-PCR positivity, perhaps due to the presence of WNV in St. Louis during the 2002 season. In 2002, there were 67 CSF specimens from patients who were >2 months old who had pleocytosis that were negative by EV PCR. Of these, 10 (15%) were positive for WNV and 18 (27%) were positive for either WNV or another virus. If all 18 of these false positives were removed from the 2002 calculations, specificity and PPV increased from 56% and 23%, respectively, to 63% and 29%. The specificity and PPV for all specimens from patients who were >2 months old in 2001 were 62% and 66%, respectively. Removing the false positives that were attributable to the presence of other viruses still does not account for the drop in PPV from 2001 to 2002. Other unknown factors must be at work. Thus, the presence of another cause (infectious or otherwise) of CSF pleocytosis in the community decreases the ability of the laboratory to predict EV positivity based on CSF findings.

In addition to focusing attention on the CSF pleocytosis causes other than EV infection, the results of this study have two practical applications. The first is that laboratories performing EV RT-PCR can use the comparison of the RT-PCR results to CSF cell counts to develop metrics that can be used as part of a quality assurance program for that assay. Based on our experience, one metric is that the EV RT-PCR should be positive in approximately 75% of infants less than 2 months of age with CSF pleocytosis, in approximately 65% of infants and children aged 2 months to 18 years with CSF pleocytosis, and in approximately 25% of patients over 18 years of age with CSF pleocytosis. These figures are derived by using data from the two seasons when WNV was either absent or not very common. The circulation of known or new arboviruses or other viruses that may cause meningitis should be considered for the analysis of data related to this metric, because it will cause a decrease in the proportion of patients with pleocytosis who have a positive EV RT-PCR result. Laboratories that do not meet this standard may benefit from investigating the analytic sensitivity of their assay but should also be alert to the possible presence of other agents that might cause CSF pleocytosis.

A second metric that can be used for quality control is that the EV RT-PCR assay should be negative in approximately 98% of patients over 2 months of age who lack CSF pleocytosis, and in approximately 70% of patients under 2 months of

age without pleocytosis. The detection of EV RNA in a larger percentage of patients who lack pleocytosis should cause laboratories to consider whether PCR contamination may be present. It should be noted, however, that these percentages will likely vary from one region to another, from one patient population to another, from one diagnostic assay to another, and from one season to another, possibly depending on the EV that is circulating.

The second practical application of the findings from the present study is that laboratories may use this data to establish criteria to maximize the efficiency of EV RT-PCR testing. For example, because the absence of pleocytosis has a predictive value of greater than 98% for a negative RT-PCR result in patients over 2 months of age, laboratories may choose to establish a cutoff for EV RT-PCR testing based on the presence or absence of pleocytosis. Using the data from the present study, the application of this cutoff would have resulted in excluding 223 of 549 specimens (based on the 549 specimens from patients who were >2 months of age for which CSF cell counts were available), with a failure to detect only 3 positive specimens (1.3%) which would have been in the excluded group of 223 samples. The three patients with EV infection but no pleocytosis were 5 months, 10 years, and 15 years old. One of these patients (the 10 year old) was suspected of having EV meningitis by virtue of a stiff neck, but the other two had symptoms that were less clearly associated with meningitis. The 10-year-old patient would likely have been tested for EV based on clinical suspicion rather than CSF pleocytosis, but the other two might not have been tested. For all three cases, the positive EV PCR result did not significantly impact the case management. If a measure such as we have suggested is adopted, we recommend applying it to patients over 2 months of age and ensuring that a mechanism exists that would allow testing in individual cases with a strong clinical suspicion of EV infection, regardless of the patient age or presence of CSF pleocytosis. Obviously, a screening program based on CSF pleocytosis is practical only for laboratories whose access to CSF cell count data is sufficiently rapid that the application of the screening criterion would not delay the performance of the assay. Previous studies have clearly demonstrated that EV assays must be performed in a very timely fashion in order to have an impact on clinical decision making (14).

ACKNOWLEDGMENTS

We thank Betsy Grant, Washington University Department of Biostatistics, for help with statistical analyses (z-test) and Barbara Hartman for preparation of the manuscript.

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