Genetic Overlap between Apparently Sporadic Motor Neuron Diseases

Marka van Blitterswijk¹*, Lotte Vlam¹, Michael A. van Es¹, W-Ludo van der Pol¹, Eric A. M. Hennekam², Dennis Dooijes², Helenius J. Schelhaas³, Anneke J. van der Kooi⁴, Marianne de Visser⁴, Jan H. Veldink¹*, Leonard H. van den Berg¹*

¹ Department of Neurology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Utrecht, The Netherlands, ² Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands, ³ Department of Neurology, Donders Institute for Brain, Cognition and Behavior, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, ⁴ Department of Neurology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Abstract

Progressive muscular atrophy (PMA) and amyotrophic lateral sclerosis (ALS) are devastating motor neuron diseases (MNDs), which result in muscle weakness and/or spasticity. We compared mutation frequencies in genes known to be associated with MNDs between patients with apparently sporadic PMA and ALS. A total of 261 patients with adult-onset sporadic PMA, patients with sporadic ALS, and control subjects of Dutch descent were obtained at national referral centers for neuromuscular diseases in The Netherlands. Sanger sequencing was used to screen these subjects for mutations in the coding regions of superoxide dismutase-1 (SOD1), angioinogen (ANG), fused in sarcoma/translated in liposarcoma (FUS/TLS), TAR DNA-binding protein 43 (TARDBP), and multivesicular body protein 2B (CHMP2B). In our cohort of PMA patients we identified two SOD1 mutations (p.D90A, p.I113T), one ANG mutation (p.K17I), one FUS/TLS mutation (p.R521H), one TARDBP mutation (p.N352S), and one novel CHMP2B mutation (p.R69Q). The mutation frequency of these genes was similar in sporadic PMA (2.7%) and ALS (2.0%) patients, and therefore, our findings demonstrate a genetic overlap between apparently sporadic PMA and ALS.


Introduction

Motor neuron diseases (MNDs) are a heterogeneous group of disorders characterized by muscle weakness and/or spasticity due to degeneration of motor neurons. Progressive muscular atrophy (PMA) refers to a subgroup of the MND patients with rapidly or gradually developing muscle weakness. PMA accounts for 5–10% of adult-onset MNDs, and is caused by a progressive loss of lower motor neurons (LMNs) [1,2]. Differentiation of PMA from amyotrophic lateral sclerosis (ALS) is important, since the median survival of patients with PMA is significantly longer than that of patients with ALS [3].

The etiology of MNDs is complex. Most of the ALS cases, for instance, are sporadic in nature and thought to be caused by an interaction of genetic and environmental factors [4]. Currently, many genes appear to be involved in the pathogenesis of ALS, including chromosome 9 open reading frame 72 (C9orf72), superoxide dismutase-1 (SOD1), angioinogen (ANG), fused in sarcoma/translated in liposarcoma (FUS/TLS), TAR DNA-binding protein 43 (TARDBP/TDP-43), vesicle-associated membrane protein B (VAPB), optineurin (OPTN), valosin-containing protein (VCP), ubiquilin-2 (UBQLN2), sequestosome-1 (SQSTM1), and profilin-1 (PFN1) [5,6,7,8,9,10,11]. We have recently shown that C9orf72 repeat expansions can also be detected in apparently sporadic PMA, but at a lower frequency (1.6%) than in apparently sporadic ALS (6.1%) [12]. Moreover, we have demonstrated that mutations in four major MND-associated genes, SOD1, ANG, FUS/TLS and TARDBP, account for less than two percent of the sporadic ALS cases [13]. The combined mutation frequency of these four MND-associated genes is unknown for sporadic PMA patients. Mutations in charged multivesicular body protein 2B (CHMP2B) have, however, been reported in sporadic PMA patients [14,15].

The objective of this study is to determine the mutation frequency of major MND-associated genes in patients with apparently sporadic PMA. We compared their mutation frequencies to those in a large cohort of patients with apparently sporadic ALS, revealing a genetic overlap.

Materials and Methods

Patient Selection

We included 261 patients with apparently sporadic PMA and screened their DNA for mutations in SOD1, ANG, FUS/TLS,
TARDBP, and CHMP2B. PMA patients had already been screened for mutations in transient receptor potential vanilloid 4 (TRPV4) and repeat expansions in C9orf72 [12,16]. Their diagnosis was based on LMN involvement on clinical and electrophysiological examination at time of referral. We excluded patients with a family history of PMA, a history of acute polynuromyelitis, spinal radiculopathy, diabetic amyotrophy, thyrotoxicosis, or hyperparathyroidism, clinical signs of upper motor neuron (UMN) involvement, sensory signs on neurological examination, structural lesions on magnetic resonance imaging or computed tomography of head and spine, and motor conduction block on extensive standardized nerve conduction studies [2].

Cohorts of sporadic ALS patients had already been screened for mutations in SOD1 (N = 431), ANG (N = 941), FUS/TLS (N = 1,192), TARDBP (N = 1,192), and C9orf72 (N = 1,422) [12,13,17,18,19]. We screened 1,002 sporadic ALS patients for mutations in CHMP2B. ALS patients were recruited through the Dutch Prospective Population-based ALS registry; they were diagnosed according to the El Escorial Criteria at national referral centers for neuro muscular diseases (University Medical Center Utrecht, Academic Medical Center Amsterdam, or Radboud University Nijmegen Medical Center) [20,21].

Mutations in SOD1 (N = 1,894), ANG (N = 1,582), FUS/TLS (N = 970), and TARDBP (N = 1,415) had previously been reported in Dutch control subjects [13,18,19]. We screened a total of 750 control subjects of Dutch descent for mutations in CHMP2B.

Ethics Statement
All material was obtained with approval of the medical ethics committee for research in humans of the University Medical Center Utrecht, The Netherlands, and all participants gave written informed consent.

Genetic Analysis
Coding regions of SOD1 (NM_000454.4), ANG (NM_001145.4), FUS/TLS (NM_004960.3, exon 5, 6, 14, 15), TARDBP (NM_007375.3, exon 6), and CHMP2B (NM_014043.3) were screened for mutations using touchdown PCR, as described previously [13,22]. Sanger sequencing and data analysis were performed with BigDye Terminator 3.1 sequencing kit (Applied Biosystems, Foster City, California), DNA Analyzer 3730XL (Applied Biosystems) and PolyPhred [23]. Each mutation was confirmed on genomic DNA and its impact on the structure, and function of the protein was predicted with PolyPhen-2 (PolyPhen-2 version 2.1.0; http://genetics.bwh.harvard.edu/pph2/) and PMut (http://mmb.pcb.ub.es/PMut/PMut.jsp).

Genealogical Analysis
Lists of descendants were compiled for index patients. Based on these lists, civil records/registers, and church records of the Dutch population, pedigrees were generated (containing two parents, four grandparents, eight great-grandparents, etc.). This information was then used to determine whether index patients were related, and detailed family trees were constructed.

Haplotype Analysis
Extended haplotype analysis, using six extragenic polymorphic markers flanking TARDBP (D1S1612, D1S503, D1S244 proximal of TARDBP, and D1S2667, D1S2740 and D1S1397 distal of TARDBP), was performed to construct a haplotype segregating with the identified p.N352S mutation in TARDBP. Validity of the constructed haplotype was determined by segregation analysis in families and patients whose DNA was available for testing.

Statistical Analysis
A Fisher’s exact test or Chi-square test was used to compare mutation frequencies, gender, site of onset, and current status (alive/deceased) between PMA and ALS patients; a Mann-Whitney test was used to compare age at onset and disease duration (GraphPad Prism version 5; http://www.graphpad.com). P-values below 0.05 were considered significant.

Results

Study Population
Baseline characteristics of the 261 sporadic PMA patients and 1,002 sporadic ALS patients are shown in Table 1. Patients with PMA were more likely to be male (72% versus 59%); furthermore, they lived longer (7.6 year versus 3.8 year), and had a lower age at onset (50.8 year versus 60.6 year) than patients with ALS.

Mutation Frequencies
Table 2 summarizes the mutations found in patients and control subjects. In individual PMA patients we detected heterozygous mutations in SOD1 [p.D90A [c.1078A>G], p.I113T [c.1147T>C], ANG [p.K171 [c.122A>T], FUS/TLS [p.R521H [c.1562G>A] and TARDBP [p.N352S [c.1055A>G]], accounting for 2.3% of the patients. Previously, we showed that missense mutations in SOD1, ANG, FUS/TLS and TARDBP were present in 1.7% of the ALS patients, and 0.4% of the control subjects [13,17,18,19]. In our current study, we also identified four novel CHMP2B mutations, one of which was present in a PMA patient [p.R69Q [c.206G>A], and three in ALS patients [p.R22Q [c.65G>A], p.N54T [c.161A>C], p.T33I [c.248C>T]]. All four CHMP2B mutations are located in a domain that is important for the formation of multivesicular bodies (MVBs), involved in sorting of cargo proteins to intraluminal vesicles [24]. These mutations are located in well conserved areas (Figure S1) and predicted to be pathological (Table 2). They account for 0.38% of the sporadic PMA patients and 0.30% of the sporadic ALS patients. None of these CHMP2B mutations was present in our control subjects; however, in one control subject (0.13%) we did detect a mutation [p.S194L [c.581C>T]] that had previously been reported in a patient with frontotemporal dementia (FTD) [25].

Clinical Characteristics
The average age at onset of PMA patients with missense mutations was 48 years, and five of them were male (71%). Although only one of these patients had died, their average disease duration already exceeded 114 months (range 37–316). These clinical characteristics of sporadic PMA patients with missense mutations were consistent with the characteristics of our entire PMA cohort. More detailed signs and symptoms are provided in Table 3.

Genealogical- and Haplotype Analyses
Previously, we have shown that the p.N352S mutation in TARDBP is a founder mutation in the Dutch ALS population [13]. Hence, we performed a thorough genealogical analysis and demonstrated that our PMA patients with p.N352S mutations had common ancestors, dating back to the 17th century in the north of France (Figure S2). Haplotype analysis revealed that these patients also shared the haplotype that was reported in Dutch ALS patients [13].
Patients with isolated LMN signs represent a subgroup of the patients with MND. To assess the mutation frequency of MND-associated genes in this subgroup, we compared 261 apparently sporadic PMA patients to apparently sporadic ALS patients. Our PMA patients were more likely to be male and lived significantly longer than ALS patients, as reported previously [3]. We detected two SOD1 mutations (p.D90A, p.I113T), one ANG mutation (p.K17I), one FUS/TLS mutation (p.R521H), one TARDBP mutation (p.N352S), and one novel CHMP2B mutation (p.R69Q) in individual PMA patients. For each of these genes we compared mutation frequencies between our PMA patients and ALS patients, and did not detect significant differences.

Clinical and pathological similarities between PMA and ALS have already been reported: more than twenty percent of the patients with isolated LMN signs will develop UMN signs within six years, especially in the first years after symptom onset [1,3,26]. Nonetheless, it can be difficult to diagnose these UMN signs due to LMN wasting and pathophysiological abnormalities caused by...

**Table 1.** Baseline characteristics of study population.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Number (N)</th>
<th>Male/female (N) (%)</th>
<th>Age at onset (y) (CI)</th>
<th>Alive/deceased (N) (%)</th>
<th>Duration (y) (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>261</td>
<td>187/74 (72/28)</td>
<td>58.0 (56.4–59.7)</td>
<td>137/116 (54/46)</td>
<td>7.6 (6.7–8.5)</td>
</tr>
<tr>
<td>ALS</td>
<td>1,002</td>
<td>593/409 (59/41)</td>
<td>60.6 (59.8–61.3)</td>
<td>135/854 (14/86)</td>
<td>3.8 (3.6–4.1)</td>
</tr>
</tbody>
</table>

Abbreviations: PMA = progressive muscular atrophy, ALS = amyotrophic lateral sclerosis, N = number, y = years, and CI = 95% confidence interval. Disease duration is defined as the interval between age at onset and age at death, or between age at onset and age last known to be alive. Patients with sporadic PMA are more likely to be male (p-value 0.001), to have a lower age at onset (p-value 0.010), to be alive (p-value < 0.001), and to have a longer disease duration than patients with sporadic ALS (p-value < 0.001).

doi:10.1371/journal.pone.0048983.t001

**Table 2.** Missense mutations found in SOD1, ANG, FUS/TLS, TARDBP, and CHMP2B.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Exon</th>
<th>PMA (N)</th>
<th>ALS (N)</th>
<th>CON (N)</th>
<th>PolyPhen-2 Prediction</th>
<th>PMut Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD1</td>
<td>p.D90A</td>
<td>4</td>
<td>1/261*</td>
<td>1/451</td>
<td>3/1,894</td>
<td>Benign</td>
<td>Pathological</td>
</tr>
<tr>
<td></td>
<td>p.I113T</td>
<td>4</td>
<td>1/261</td>
<td>0/451</td>
<td>0/1,894</td>
<td>Probably damaging</td>
<td>Pathological</td>
</tr>
<tr>
<td></td>
<td>p.I99V</td>
<td>4</td>
<td>0/261</td>
<td>1/451</td>
<td>0/1,894</td>
<td>Benign</td>
<td>Neutral</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td></td>
<td>2/261 (0.77)</td>
<td>2/451 (0.44)</td>
<td>3/1,894 (0.16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG</td>
<td>p.G(–10)D</td>
<td>2</td>
<td>0/261*</td>
<td>1/941</td>
<td>0/1,582</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>p.K17I</td>
<td>2</td>
<td>1/261</td>
<td>3/941</td>
<td>2/1,582</td>
<td>Benign</td>
<td>Pathological</td>
</tr>
<tr>
<td></td>
<td>p.T80S</td>
<td>2</td>
<td>0/261</td>
<td>1/941</td>
<td>0/1,582</td>
<td>Possibly damaging</td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td>p.F100I</td>
<td>2</td>
<td>1/261</td>
<td>1/941</td>
<td>0/1,582</td>
<td>Probably damaging</td>
<td>Neutral</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td></td>
<td>1/261 (0.38)</td>
<td>6/941 (0.64)</td>
<td>2/1,582 (0.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUS/TLS</td>
<td>p.S115N</td>
<td>5</td>
<td>0/261*</td>
<td>1/1,192</td>
<td>0/970</td>
<td>Unknown</td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td>p.Q210H</td>
<td>6</td>
<td>0/261</td>
<td>0/1,192</td>
<td>1/970</td>
<td>Unknown</td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td>p.R487C</td>
<td>14</td>
<td>0/261</td>
<td>1/1,192</td>
<td>0/970</td>
<td>Probably damaging</td>
<td>Pathological</td>
</tr>
<tr>
<td></td>
<td>p.R495X</td>
<td>14</td>
<td>0/261</td>
<td>1/1,192</td>
<td>0/970</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>p.R521H</td>
<td>15</td>
<td>1/261</td>
<td>0/1,192</td>
<td>0/970</td>
<td>Probably damaging</td>
<td>Pathological</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td></td>
<td>1/261 (0.38)</td>
<td>3/1,192 (0.17)</td>
<td>1/970 (0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TARDBP</td>
<td>p.N352S</td>
<td>6</td>
<td>2/261*</td>
<td>3/1,192</td>
<td>0/1,415</td>
<td>Benign</td>
<td>Pathological</td>
</tr>
<tr>
<td></td>
<td>p.I383V</td>
<td>6</td>
<td>0/261</td>
<td>1/1,192</td>
<td>0/1,415</td>
<td>Benign</td>
<td>Neutral</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td></td>
<td>2/261 (0.77)</td>
<td>4/1,192 (0.34)</td>
<td>0/1,415 (0.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHMP2B</td>
<td>p.R22Q</td>
<td>2</td>
<td>0/261*</td>
<td>1/1,002</td>
<td>0/750*</td>
<td>Possibly damaging</td>
<td>Pathological</td>
</tr>
<tr>
<td></td>
<td>p.N54T</td>
<td>3</td>
<td>0/261</td>
<td>1/1,002</td>
<td>0/750</td>
<td>Probably damaging</td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td>p.R69Q</td>
<td>3</td>
<td>1/261</td>
<td>0/1,002</td>
<td>0/750</td>
<td>Probably damaging</td>
<td>Pathological</td>
</tr>
<tr>
<td></td>
<td>p.T83I</td>
<td>3</td>
<td>0/261</td>
<td>1/1,002</td>
<td>0/750</td>
<td>Probably damaging</td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td>p.S194L</td>
<td>6</td>
<td>0/261</td>
<td>0/1,002</td>
<td>1/750</td>
<td>Benign</td>
<td>Neutral</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td></td>
<td>1/261 (0.38)</td>
<td>3/1,002 (0.30)</td>
<td>1/750 (0.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td></td>
<td>7 (2.7)</td>
<td>18 (2.0)</td>
<td>7 (0.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CON = control subjects, and N/A = not applicable. Mutations in SOD1, ANG, FUS/TLS, TARDBP, and CHMP2B were present in 2.7% of the PMA patients, 2.0% of the ALS patients, and 0.5% of the control subjects. No PMA patients were detected with mutations in multiple MND-associated genes. A Fisher’s exact test or Chi-square test was used to compare mutation frequencies between patients with PMA and ALS for each gene; no significant differences were detected (data not shown for simplicity).

Cohort described in the present study.

doi:10.1371/journal.pone.0048983.t002
damaged motor pathways, motor neurons and interneurons [27]. Pathological studies have also revealed ubiquitin inclusions and involvement of the corticospinal tract in PMA patients, which are typical for ALS patients and emphasize similarities between these diseases [28,29].

Previously, mutations in \textit{SOD1}, \textit{ANG}, \textit{FUS/TLS}, \textit{TARDBP} and \textit{CHMP2B} have been identified in patients with a range of clinical phenotypes, including combinations of FTD, Parkinson’s disease, and ALS [14,18,22,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45]. \textit{CHMP2B} mutations have also been described in sporadic PMA patients, while mutations in MND-associated genes have been detected in familial ALS patients with predominantly LMN signs [15,19,28,46,47,48,49,50]. In addition, we have recently shown that \textit{C9orf72} repeat expansions are present in sporadic PMA patients, but at a lower frequency than in sporadic ALS patients (1.6% versus 6.1%) [12]; other studies have shown that \textit{C9orf72} repeat expansions were present in approximately 7% of white sporadic ALS patients from the USA, Europe and Australia, and that bulbar onset ALS is frequently encountered in patients with these expansions [51,52,53,54]. Our current findings demonstrate that mutations in \textit{SOD1}, \textit{ANG}, \textit{FUS/TLS}, \textit{TARDBP} and \textit{CHMP2B} are also associated with apparently sporadic PMA, thus expanding the wide range of clinical phenotypes reported to date. Furthermore, the comparable mutation frequencies between PMA and ALS patients show that, apart from clinical and pathological similarities, these diseases demonstrate a genetic overlap as well, suggesting that PMA is a subtype of ALS.

We detected a \textit{SOD1} mutation (p.D90A) in a patient with sporadic PMA, a patient with classical sporadic ALS, and control subjects. This is the most common \textit{SOD1} mutation, and causes both autosomal dominant and recessive ALS [55,56]. Although it behaves dominantly in many families, it is a polymorphism in the Swedish population, primarily causing ALS when in the homozygous state [57]. Another \textit{SOD1} mutation (p.I113T) was also present in a PMA patient; it is known for its clinical heterogeneity, including asymptomatic subjects, patients with mild fasciculations, patients with typical ALS, and patients with ALS-FTD and chorea [41,38]. Both these \textit{SOD1} mutations appear to result in ALS through aggregation of mutant \textit{SOD1} protein [59].

In addition, we identified an \textit{ANG} mutation (p.K17I) in one PMA patient, and in two out of 1,582 Dutch control subjects. The p.K17I mutation has already been reported in ALS patients and in control subjects [19]. Despite its presence in control subjects, it does affect the neuroprotective-, angiogenic- and ribonucleolytic activity of \textit{ANG} [50,60,61]. It seems likely that this mutation raises ALS susceptibility and/or acts as a genetic modifier, a hypothesis supported by recent reports of families that harbor a p.K17I mutation in combination with \textit{TARDBP} or \textit{FUS/TLS} mutations, and a large international collaborative study, which demonstrates that \textit{ANG} mutations confer a substantial risk for ALS [13,18,62].

In one PMA patient, we identified a \textit{FUS/TLS} mutation (p.R521H); one of the most common \textit{FUS/TLS} mutations with a disease duration of approximately four years [63,64]. In two other PMA patients we detected a \textit{TARDBP} mutation (p.N352S), which has been described in German and Japanese ALS patients [65,66,67]. We have recently reported that p.N352S is a founder mutation in the Dutch ALS population [13]. In the present study, we revealed that our PMA patients had common ancestors and shared a haplotype also detected in Dutch ALS patients.

The four \textit{CHMP2B} mutations we detected (p.R69Q, p.R22Q, p.N54T, p.T83I) are novel, absent in control subjects, located in well conserved areas, and predicted to be pathogenic. One of these mutations was identified in a patient with sporadic PMA (0.38%), three in patients with sporadic ALS (0.30%). These mutation frequencies demonstrate that \textit{CHMP2B} mutations are not specific for PMA, but are present in patients with ALS, FTD, ALS-FTD, and ALS. We also detected one previously reported mutation (p.S194L) in a control subject [25]. Since this variant is located within an area of low complexity and predicted to have neutral effects, it probably represents a rare benign polymorphism.

Recently, we have provided evidence for an oligogenic etiology of familial ALS [13]. We reported five families with mutations in multiple MND-associated genes: \textit{ANG} mutations were detected in combination with \textit{FUS/TLS} and \textit{TARDBP} mutations, and \textit{C9orf72} repeat expansions in combination with \textit{TARDBP}, \textit{SOD1}, and \textit{FUS/TLS} mutations (p-value $1.57\times10^{-7}$). In our present study, we did not identify mutations in multiple MND-associated genes in a single patient; however, the high phenotypic variability that is seen amongst patients with mutations in these genes (ranging from FTD to Parkinson’s disease and MNDs) does suggest that other genetic and/or environmental factors influence disease characteristics of sporadic MNDs.
To summarize, we have detected comparable mutation frequencies in patients with apparently sporadic PMA and ALS, indicating a genetic overlap between these two diseases. Thus, our findings favor the hypothesis that PMA is a subtype of ALS and not a distinct entity, broadening the disease spectrum of ALS.

Supporting Information

Figure S1 CHMP2B mutations and conservation. Conservation of amino-acid residues across species was generated using ClustalW2 online tool, http://www.ebi.ac.uk/Tools/msa/clustalw2/.

(DOC)

References


Figure S2 Pedigree of two PMA patients with p.N352S mutations in TARDBP.

(DOC)

Author Contributions

Conceived and designed the experiments: MB LV MAE WLdP JHV LHvdB. Performed the experiments: MB LV EAMH DD HJS Ajvdk MVJ HVH LHvdB. Analyzed the data: MB LV MAE WLdP EAMH DD JHV LHvdB. Contributed reagents/materials/analysis tools: MB LV MAE WLdP EAMH DD HJS Ajvdk MVJ HVH LHvdB. Wrote the paper: MB. Revising manuscript for content: MB LV MAE WLdP EAMH DD HJS Ajvdk MVJ HVH LHvdB. Statistical analysis: MB LV JHV. Study supervision or coordination: JHV LHvdB.