Regional Brain and Spinal Cord Volume Loss in Spinocerebellar Ataxia Type 3

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ABSTRACT: Background: Given that new therapeutic options for spinocerebellar ataxias are on the horizon, there is a need for markers that reflect disease-related alterations, in particular, in the preataxic stage, in which clinical scales are lacking sensitivity.

Objective: The objective of this study was to quantify regional brain volumes and upper cervical spinal cord areas in spinocerebellar ataxia type 3 in vivo across the entire time course of the disease.

Methods: We applied a brain segmentation approach that included a lobular subsegmentation of the cerebellum to magnetic resonance images of 210 ataxic and 48 preataxic spinocerebellar ataxia type 3 mutation carriers and 63 healthy controls. In addition, cervical cord cross-sectional areas were determined at 2 levels.

Results: The metrics of cervical spinal cord segments C3 and C2, medulla oblongata, pons, and pallidum, and the cerebellar anterior lobe were reduced in preataxic mutation carriers compared with controls. Those of cervical spinal cord segments C2 and C3, medulla oblongata, pons, midbrain, cerebellar lobules crus II and X, cerebellar white matter, and pallidum were reduced in ataxic compared with nonataxic carriers. Of all metrics studied, pontine volume showed the steepest decline across the disease course. It covaried with ataxia severity, CAG repeat length, and age. The multivariate model derived from this analysis explained 46.33% of the variance of pontine volume.

Conclusion: Regional brain and spinal cord tissue loss in spinocerebellar ataxia type 3 starts before ataxia onset. Pontine volume appears to be the most promising imaging biomarker candidate for interventional trials that aim at slowing the progression of spinocerebellar ataxia type 3.© 2021 The Authors. Movement Disorders published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: spinocerebellar ataxia; MRI; volumetry; biomarker

Spinocerebellar ataxia type 3/Machado–Joseph disease (SCA3) is worldwide the most common autosomal dominantly inherited ataxia disorder. It is caused by unstable expansions of polyglutamine encoding CAG repeats in the ATXN3 gene, resulting in the formation of abnormally elongated disease proteins. Although partial loss of the physiological role of ataxin-3 contributes to the development of SCA3, its pathogenesis is mainly because of newly acquired deleterious actions of elongated ataxin-3.

SCA3 is a multisystem disorder characterized by degeneration of spinocerebellar tracts, dentate nucleus, cerebellar cortex, brain stem nuclei, and basal ganglia. The clinical syndrome is characterized by prominent cerebellar ataxia in combination with supranuclear gaze palsy and peripheral neuropathy. SCA3 takes a progressive course and leads to severe disability and premature death, with a median survival after ataxia onset of 25 years.

Currently, there is no causal treatment for SCA3. However, as the understanding of the molecular mechanisms is rapidly advancing, there are several new treatment approaches. Among the most promising ones are approaches for downregulating or silencing the ATXN3 gene. Like in other neurodegenerative diseases, in particular those with a clinically presymptomatic phase, there is a need for markers with known evolution throughout the time course of the disease that have the potential to map disease activity.

Magnetic resonance imaging (MRI) allows the study of structural abnormalities of the brain and spinal cord in vivo. Previous studies in patients with SCA3 showed a pattern of regional brain tissue loss that faithfully reflected the distribution of neurodegenerative changes described in autopsy studies and revealed that regional volume loss already starts in the preataxia stage. Three longitudinal volumetric studies in small numbers of patients with SCA3 suggested that MRI volume is more sensitive to change than clinical scales, which makes MRI volumes promising candidates for biomarkers in clinical trials. This applies in particular to preventive trials in preataxic mutation carriers, as clinical measures lack sensitivity before ataxia onset. However, further steps toward the validation of MRI regional volumes as biomarkers for SCA3 are hampered by the small numbers of studied MRIs and the
time-consuming procedures for segmentation and volumetry.

In this study, we applied an automated method for MRI volumetry, which includes subsegmentation of the cerebellum into its lobules to a large number of existing T1-weighted MRIs of preataxic and ataxic SCA3 mutation carriers that were acquired at 14 centers worldwide. The advantage of the applied method is that it provides individual single-point values that can be used in future longitudinal studies to map individual trajectories.

Materials and Methods

MRI Scans

We collected T1-weighted (T1W) MRIs of 295 SCA3 mutation carriers and 72 healthy controls from 14 sites in 8 countries. There were no restrictions regarding manufacturer, software version, or field strength. Scans with a resolution of greater than 2 mm on at least 1 axis, based on the limited ability to accurately measure small regional brain volumes in scans with large voxel sizes, and scans that were acquired after application of contrast agents were excluded (for details, see Table S2 and Fig. S1). Age, sex, and total Scale for Assessment and Rating of Ataxia (SARA) score of all SCA3 participants and healthy controls were available. Information about CAG repeat length was available for SCA3 mutation carriers. Using a SARA cutoff value of 3, SCA3 mutation carriers were divided into preataxic (SARA < 3) and ataxic (SARA ≥ 3) individuals. This SARA threshold for ataxia was defined as the mean SARA +2 SDs of the healthy control group in the original SARA validation study. For complementary analyses, which can be found in the Supplementary data, groups were defined on the basis of subject report, whether and since when gait disturbances were present, here using the terms presymptomatic and symptomatic. Scans with incomplete clinical information were excluded (for details, see Fig. S1). All participants gave their written informed consent.

Image Analysis

The T1W images of each subject were processed with a fully automated image-processing pipeline to obtain volumes of 122 distinct anatomical regions covering the entire brain and 7 compartments containing cerebrospinal fluid. For the cerebellar subsegmentation, a reference database was developed to provide a parcellation scheme including fine-granularity cerebellar subsegments on the level of cerebellar lobules into 30 disjoint volumes. These reference data set segmentations were generated from a gold standard segmentation set of 17 T1W images with manual segmentations of the cerebellar lobules provided by J. Diedrichsen (http://www.diedrichsenlab.org). The image-processing and segmentation methods are described in detail in the supplementary data. To enable comparison of subject volume, data adjustment to account for head size was undertaken, using a normalization factor that was estimated from a template registration approach based on Buckner et al. In brief, the affine (9-parameter) transformation was computed for the subject T1W to the MNI152 linear T1 template. The magnitude of the scaling factors of the estimated affine matrix was then applied as a normalization factor to estimated volumes. For the final analysis, each 2 hemispheric volumes of hemispheric bilateral volumes were combined, and for the cerebellum the 8 subdivisions of the vermis were combined. In addition, the following compound volumes were analyzed: anterior lobe (cerebellar lobules I–V), superior posterior lobe (cerebellar lobules VI, VIIA [crus I, crus II], and VIIIB), inferior posterior lobe (cerebellar lobules VIIA, VIIIB, and IX), cerebellar gray matter (lobules I–X and vermis) and basal ganglia (palidum, caudate, putamen). A full list of all volumes is given in Table S1, and an example segmentation is shown in Figure S2.

The upper portion of the cervical spinal cord was depicted on all available MRIs. Analysis of the C3 level was applicable in 256 cases and analysis of the C2 level in 297 of 321 cases. In a semiautomated approach, we used the Spinal Cord Toolbox in combination with manual corrections of the automated delineation to compute the cross-sectional area under consideration of an angle correction along the center line. We calculated the mean of the angle-corrected cross-sectional areas for all slices of cervical spinal cord segments C2 and C3 separately. The image processing and segmentation methods are described in detail in the supplementary data. To improve readability, we do not state the term “mean cross-sectional area” each time, but instead we will subsume the values of the mean cross-sectional areas of C2 and C3 and brain regional volumes under the term “metric.”

Statistical Analysis

All analyses were performed using R Software for Statistical Computing, version 3.5.1. R Foundation for Statistical Computing, Vienna.

To investigate group differences between ataxic SCA3 mutation carriers, preataxic mutation carriers, and healthy controls, we used separate linear mixed-effects models (R package lme4) to analyze the relationship of each metric — each brain volume and the mean cross-sectional area of spinal cord levels C2 and C3 — with the covariables age, sex, and group (preataxic, ataxic, healthy control). The latter variables were represented by fixed effects, whereas scanner type was represented by a random effect. Group differences were
evaluated using the R package multcomp (function glht). In all metrics that showed a significant group effect, a post hoc multiple comparison via Benjamini–Hochberg correction was applied afterward.21 Given the heterogeneous sample, we chose a strict significance level to reduce the probability of false-positive effects. \( P < 0.001 \) after post-hoc Benjamini–Hochberg correction21 was considered significant. In a second analysis, analysis groups were defined by self-report as presymptomatic or symptomatic SCA3 mutation carriers (Supplementary Data).

Only those metrics that showed a significant difference between preataxic SCA3 mutation carriers, either compared with healthy controls or ataxic SCA3 mutation carriers, were considered for the further analyses to meaningfully cover the entire time course of the disease (regional volume loss in relation to disease duration, influencing factors, sample sizes).

To describe the regional changes in relation to disease duration, we \( z \)-transformed each metric in relation to healthy controls of the same age and plotted \( z \) values against the time scale of estimated disease duration, as described in detail in the supplementary data. We used a uniform time scale for all SCA3 mutation carriers, defined by the predicted time of ataxia onset calculated on the basis of CAG repeat length.22 On this scale, negative values for disease duration indicate the predicted time to ataxia onset and positive values the time from the predicted onset. In a second analysis (Supplementary Data), we used a compound time scale. For presymptomatic carriers, we calculated the time to ataxia onset based on CAG repeat length and present age,22 and in symptomatic carriers, we used the reported time from ataxia onset. The \( x \) axis was restricted to \((-20 \text{ years}; 20 \text{ years})\). We applied locally weighted scatterplot smoothing for interpolation to avoid any preassumptions about the curve course, for example, assuming a linear or parabolic curve course.

To identify factors that covary with regional volume loss, we applied linear regression analysis with SARA score, CAG repeat of the longer allele, age, and sex as independent variables. We calculated \( R^2 \), which indicates the overall proportion of the variance of each metric that is explained by the independent variables and the \( P \) value for each independent variable. Here, the \( P \) value indicates whether the respective variable contributes to the model in a statistically significant way.

As a measure of effect size, we calculated Cohen’s \( d \) values for a presumed 50% reduction of the decrease of SARA and each MRI metric. Calculations were based on the estimated metric slopes of linear models with calculated disease duration as covariate. To allow comparison of the effect sizes of the MRI metrics, we calculated relative values by dividing Cohen’s \( d \) of each MRI metric by Cohen’s \( d \) of SARA.

### Results

#### Demographic and Clinical Data

Of the 367 collected data sets, 46 had to be excluded (Fig. S1). We analyzed the remaining 321 data sets that comprised data from 210 ataxic SCA3 mutation carriers, 48 preataxic SCA3 mutation carriers, and 63 healthy controls. Demographic data are given in Table 1. Age differed among the 3 subgroups. Ataxic mutation carriers had the highest age (mean ± SD, 46.84 ± 11.24 years) and preataxic mutation carrier the lowest age (mean ± SD, 37.75 ± 9.47 years). Age of the control group was between the 2 groups of mutation carriers (mean ± SD, 42.81 ± 13.65 years). SARA score of the ataxic mutation carriers was on average ± SD, 12.41 ± 5.50. The mean SARA scores of preataxic mutation carriers (mean ± SD, 1.31 ± 0.94) and healthy controls (mean ± SD, 0.22 ± 0.46) was below the cutoff of 3. The number of CAG repeats of the longer allele was higher in ataxic (mean ± SD, 71.10 ± 4.23) than in the preataxic (mean ± SD, 68.29 ± 3.55) mutation carriers.

#### Group Comparisons

To identify regions subject to volume loss before ataxia onset in SCA3, we compared SCA3 preataxic mutation carriers with healthy controls. Volumes of the following brain regions and mean cross-sectional areas of the spinal cord levels were reduced in preataxic mutation carriers compared with healthy controls: cervical spinal cord segments C2 and C3 (\( P < 0.0001 \)), medulla oblongata

### Table 1: Demographic and characterizing cohort data

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age</th>
<th>Male/female</th>
<th>Age of onset*</th>
<th>Disease duration in years*</th>
<th>CAG repeats, longer allele</th>
<th>SARA sum score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>63</td>
<td>42.81 (13.65)</td>
<td>35/28</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>0.22 (0.46)</td>
</tr>
<tr>
<td>Preataxic</td>
<td>48</td>
<td>37.75 (9.47)</td>
<td>18/30</td>
<td>39.73 (7.87)</td>
<td>-1.98 (9.83)</td>
<td>68.29 (3.55)</td>
<td>1.31 (0.94)</td>
</tr>
<tr>
<td>Ataxic</td>
<td>210</td>
<td>46.84 (11.24)</td>
<td>118/92</td>
<td>34.12 (9.45)</td>
<td>12.67 (9.45)</td>
<td>71.10 (4.23)</td>
<td>12.41 (5.50)</td>
</tr>
</tbody>
</table>

Data are expressed as mean and standard deviation for age, age at onset, disease duration, CAG repeat length of the longer allele, and SARA sum score and as number for the group size and the male/female distribution.

*Estimated age at onset on the basis of CAG repeat length following the model provided by Tezenas et al (Tezenas du Montcel et al, 2014) and disease duration in years, defined as the actual age minus the estimated age at onset, resulting in negative values for the expected time to onset in preataxic SCA3 mutation carriers and positive values for ataxic SCA3 mutation carriers.
TABLE 2 Group differences between preataxic and ataxic SCA3 mutation carriers and healthy controls (HC)

<table>
<thead>
<tr>
<th>Metric</th>
<th>Preataxic SCA3 (&lt;) HC</th>
<th>Ataxic SCA3 (&lt;) preataxic SCA3</th>
<th>Ataxic SCA3 (&lt;) HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical spinal cord</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Cervical spinal cord, level C3, CSAa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical spinal cord, level C2, CSAa</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Brain stem</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Pons</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Midbrain</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum white matter</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum I–IV</td>
<td>*</td>
<td>*</td>
<td></td>
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<tr>
<td>Cerebellum V</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum VI</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum crus I</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum crusII</td>
<td>*</td>
<td>*</td>
<td></td>
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<tr>
<td>Cerebellum VIIb</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum IX</td>
<td>*</td>
<td>*</td>
<td></td>
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<tr>
<td>Cerebellum X</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum (b) vermis</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate</td>
<td></td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Pallidum</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound volumes</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum, anterior lobe</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Cerebellum, superior posterior lobe (c)</td>
<td>*</td>
<td>*</td>
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</tr>
</tbody>
</table>
Among the regions that showed smaller metrics in ataxic SCA3 mutation carriers compared with healthy controls, a number of regions also showed reduced metrics compared with preataxic mutations carriers, indicating that these regions undergo progressive volume loss in the SCA3 disease course. These regions included cervical spinal cord segments C2 and C3 ($P < 0.0001$), medulla oblongata ($P < 0.0001$), pons ($P < 0.0001$), midbrain ($P < 0.0001$), cerebellar lobules crus II and X ($P < 0.0001$), cerebellar white matter ($P < 0.0001$), and pallidum ($P < 0.0001$); see Table 2. In addition, the volume of the third ventricle was larger than in controls ($P < 0.0001$). Estimates, standard errors, and 95% confidence intervals (CIs) for each group comparison as well as the ANOVA statistics are given in Tables S3 and S4.

When we performed the same analysis in groups defined by self-report as presymptomatic or symptomatic SCA3 mutation carriers, results were generally similar. However, the pallidum and anterior lobe of the cerebellum were not smaller in presymptomatic mutation carriers compared with healthy controls, whereas the superior posterior lobe and total cerebellar gray matter was smaller in presymptomatic compared with symptomatic SCA3 mutation carriers (Table S5).

### Regional Volume Loss in Relation to Disease Duration

To study regional volume loss in SCA3 in relation to disease duration, we applied local regression on a time scale defined by the predicted time of ataxia onset, calculated on the basis of CAG repeat length. On this scale, negative values indicate the predicted time to ataxia onset and positive values the time from the predicted onset. For this analysis, we selected those metrics that showed significant alterations in any comparison of preataxic mutation carriers, with either healthy controls or ataxic mutation carriers. Already around 10 to 15 years before ataxia onset, the metrics of cervical spinal cord segments C2 and C3 and the pallidum were reduced by about 1 standard deviation (SD) compared with healthy controls of the same age. Metrics of all regions steadily decreased until a period of 5 to 15 years after ataxia onset. Thereafter, volume loss decelerated, and metrics remained stable or even increased relative to controls, except for the pons and cerebellar lobule X. In the time interval lasting from 5 years before until 5 years after ataxia onset, the metrics of all regions decreased almost linearly. The decline was steepest in the pons and midbrain. At the time of ataxia onset, the metrics of cervical spinal cord segments C2 and C3, the pons, midbrain, and pallidum ranged between about 1 and 2 SD below the control group, whereas the other volumes, in particular the cerebellar volumes, were reduced by less than 1 SD (Fig. 1).

When we performed the same analysis on a compound time scale that used the calculated time to expected ataxia onset in presymptomatic mutation carriers and the reported time since ataxia onset in symptomatic mutation carriers, the results were generally similar (Fig. S4).
Factors Determining Regional Volume Loss

To identify factors that covary with regional tissue loss in SCA3, we performed a linear regression analysis with SARA sum score, CAG repeat length of the longer allele, age, and sex as potential determining factors. Again, we selected those metrics for analyses that showed significant alterations in any comparison of preataxic mutation carriers, either with healthy controls or with ataxic mutation carriers. SARA sum score contributed highly significantly to the models of cervical spinal cord segments C2 and C3, the medulla oblongata, pons, and midbrain. Furthermore, CAG repeat length influenced pons, midbrain, and pallidum, age on pons, midbrain anterior cerebellum, and cerebellar crus II, and sex on medulla oblongata and midbrain (each P < 0.001). The multivariate models derived from this analysis explained between 5.82% and 46.33% of the variance of the respective metrics. The proportion of the explained variance was highest for the pons (46.33%) and midbrain (33.49%) and lowest for the cerebellar regions (5.82%–16.60%); see Table 3. Estimates, standardized beta, standard error, and 95% confidence intervals are given in Table S6.

Calculation of Effect Sizes

Based on the estimated slopes of linear models of regional volume changes in relation to disease duration, we calculated Cohen’s d values as a measure of effect size for each metric. We report the effects sizes of each metric relative to the effect size of SARA to allow a...
comparison. Cohen's $d$ of pontine volume was larger than that of SARA (1.03 times the Cohen's $d$ of SARA). Cohen's $d$ values of cervical spinal cord segment C3 (0.81 times the Cohen's $d$ of SARA) and C2 (0.68 times the Cohen's $d$ of SARA), medulla oblongata (0.61 times the Cohen's $d$ of SARA), midbrain (0.97 times the Cohen's $d$ of SARA), and pallidum (0.97 times the Cohen's $d$ of SARA) were in the same magnitude, but smaller than that of SARA (Table 4). For all cerebellar regions, Cohen's $d$ values were less than 0.5 times the Cohen's $d$ of SARA (Table 4).

Discussion

In this cross-sectional MRI study, we assessed regional tissue loss in a large cohort of preataxia and ataxic SCA3 mutation carriers. We applied a refined and optimized brain segmentation approach that allowed reliable sub-segmentation of the cerebellum into the cerebellar lobules. For the spinal cord, we relied on the assessment of the mean cross-sectional area of the upper cervical levels. The advantage of the applied methodology is that the availability of individual single-point metrics allows consideration of brain regional volumes as outcome measures in future interventional trials. To study the change of metrics in relation to disease duration, we used a uniform time scale defined by the predicted age of ataxia onset for all mutation carriers according to a previously published model. To check whether this methodological approach treating all mutation carriers consistently distorts the disease course, we repeated the analysis using a compound time scale in which ataxia length, age, and sex could only explain a small proportion of the variance in cerebellar volume, whereas the pontine volume showed the highest proportion, with almost 50% of its variance explained by a model including these factors. This is remarkable, in particular, given the very heterogeneous sample of MRIs included in this study.

Calculation of effect size has 2 major limitations. First, our calculations were based on cross-sectional data, which do not allow an accurate estimation of between-subject variability in the longitudinal rate of change. However, as we calculated effect size for all metrics and SARA in the same way, we believe that the data are useful for comparison between metrics. Consequently, we provided effect sizes of the MRI metrics in relation to the effect size of SARA. Second, we assumed a linear progression throughout the whole time course of the disease, although the evolution of almost all metrics was nonlinear. The effect size of pontine volume marginally exceeded that of SARA. The effect size of all other metrics was smaller than that of SARA, although those of cervical spinal cord segment C2 and C3, pons, midbrain, and pallidum were of the same magnitude as that of SARA. The current values are not comparable to effect sizes calculated on the basis of longitudinal data.

We suggest that extracerebellar regions represent the core sites of the disease process in SCA3. This suggestion
is based on the observed time course of volume changes and the close relation of the metrics of extracerebellar regions with clinical disease severity and CAG repeat length. Our observation that the effect size of the pontine volume was marginally higher than that of SARA and that a relevant proportion of pontine volume variance was explained with a model including ataxia severity, CAG repeat length, and age suggests that pontine volume is a promising biomarker candidate for interventional studies that aim to slow the progression of SCA3. However, longitudinal studies including of preataxic SCA3 mutation carriers are inevitable to adequately test the validity of such imaging biomarker candidates.

References


Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.
Authors’ Roles

1) Research project: 1A. Conception, 1B. Organization, 1C. Execution; 1D. Patient recruitment.
2) Statistical Analysis: 2A. Design, 2B. Execution, 2C. Review and Critique;
3) Manuscript: 3A. Writing of the first draft, 3B. Review and Critique.

J.F.: 1A, 1B, 1C, 1D, 2A, 2B, 2C, 3A.
T.S.: 2A, 2B.
K.B.: 1C.
K.R.: 1D, 2C, 3B.
M.C.F. Jr: 1D, 2C, 3B.
T.J.R.R.: 1D, 2C, 3B.
J.H.: 1D, 2C, 3B.
W.L.: 1D, 2C, 3B.
B.v.W.: 1D, 2C, 3B.
J.v.G.: 1D, 2C, 3B.
A.D.: 1D, 2C, 3B.
F.M.: 1D, 2C, 3B.
P.G.: 1D, 2C, 3B.
H.G.-M.: 1D, 2C, 3B.
L.S.: EU: 1D, 2C, 3B.
H.H.: 1D, 2C, 3B.
M.S.: 1D, 2C, 3B.
B.B.: 1D, 2C, 3B.
G.O.: 1D, 2C, 3B.
J.J.: 1D, 2C, 3B.
J.d.V.: 1D, 2C, 3B.
J.-S.K.: 1D,
D.T.-B.: 1D, 2C, 3B.
H.J.: 1D, 2C, 3B.
I.I.: 1D, 2C, 3B.
R.J.: 1C, 2C, 3B.
S.R.: 1D, 2C, 3B.
J.D.: 1D, 2C, 3B.
M.S.: 2A 2C, 3B.
R.W.: 1C 2C, 3B.
T.K.: 1A, 1D, 2A, 2C, 3B.

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