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Characterization of novel SLC6A8 variants with the use of splice-site analysis tools and implementation of a newly developed LOVD database.

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1 Running Title:

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26 **Characterization of novel *SLC6A8* variants with the use of splice-site analysis tools**
27 **and implementation of a newly developed LOVD database.**

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57 **Abstract**

58 The X-linked creatine transporter defect is caused by mutations in the *SLC6A8* gene.
59 Until now, 66 synonymous and intronic variants in *SLC6A8* were detected in our
60 laboratory. To gain more insight in the effect of the detected variants, we applied five
61 free web based splice-site analysis tools to 25 published variants that were stratified as
62 (non-)disease causing. All were correctly predicted to have no effect (n=18) or to cause
63 erroneous splicing (n=7), with the exception of a pathogenic *de novo* 24 bp intronic
64 deletion. Secondly, 41 unclassified variants, including 28 novel, were subjected to
65 analysis by these tools. At least four splice-site analysis tools predicted that 3 of the
66 variants would affect splicing as the mutations disrupted the canonical splice site.
67 Urinary creatine/creatinine and brain MRS confirmed creatine transporter deficiency in
68 five patients (four families), including one female. Another variant was predicted to
69 moderately affect splicing by all five tools. However, transient transfection of a
70 minigene containing the variant in a partial *SLC6A8* segment showed no splicing errors,
71 and thus was finally classified as non disease causing. This study shows that splice tools
72 are useful for the characterization of the majority of variants, but also illustrates that the
73 actual effect can be misclassified in rare occasions. Therefore, further laboratory studies
74 should be considered before final conclusions on the disease causing nature are drawn.
75 To provide an accessible database, the 109 currently known *SLC6A8* variants, including
76 35 novel ones, are included in a newly developed LOVD DNA variation database
77 (<http://www.LOVD.nl/SLC6A8>).

78

79 **Introduction**

80 In 2001, the X-linked mental retardation (XLMR) syndrome, SLC6A8 deficiency, was
81 identified due to a creatine deficiency in the brain caused by mutations in the creatine
82 transporter (*SLC6A8*) gene (MIM 300036). *SLC6A8* has been mapped to Xq28¹ and is a
83 member of the solute-carrier family 6 (neurotransmitter transporters). The clinical
84 presentation of males affected with SLC6A8 deficiency is mental retardation (MR),
85 expressive speech and language delay, epilepsy, developmental delay and autistic
86 behavior. Laboratory hallmarks include a reduction of the creatine signal in the proton
87 magnetic resonance spectroscopy (H-MRS) of the brain, an increased urinary
88 creatine/creatinine ratio and impaired creatine uptake in cultured fibroblasts. In female
89 carriers, learning disabilities of varying degrees have been noted. Two studies of males
90 with X-linked MR estimated the prevalence of SLC6A8 deficiency to be 2.1% (CI: 0.44
91 - 3.76)² and 1.5% (CI: 0 – 4.46).³ A third study of males with MR revealed a prevalence
92 of 0.8% (CI: 0.02 – 1.7%).⁴ Within the last decade many novel variants in the *SLC6A8*
93 gene have been detected. These variants are difficult to classify as pathogenic or non
94 disease causing since the variants are either located in the coding region, but are
95 synonymous, or are found in the intronic regions. However, such variants may affect
96 proper splicing and are potentially pathogenic.⁵ We therefore not only tested if such
97 variants could be properly analyzed by studying previously classified variants, but also
98 subjected 28 novel variants to these tools. Moreover, to facilitate worldwide diagnostic
99 and research laboratories interested in the *SLC6A8* gene, we developed a novel LOVD
100 database which includes clinically and genetically relevant data.

101

102 **Materials and Methods**

103

104 ***Subjects***

105 In our diagnostic unit, a total of 1900 patients with a differential diagnosis of SLC6A8
106 deficiency were analyzed by DNA sequence analysis. This resulted in the detection of
107 66 individuals with intronic or synonymous variants (including 28 novel). These
108 variants are addressed in the current study. Also, 5 novel patients affected with SLC6A8
109 deficiency are reported.

110

111 **Methods**

112 ***PCR of exon 1 to 13 of SLC6A8 (NM_005629.1)***

113 Exon 2 to 13 and flanking intronic sequences of *SLC6A8* were amplified using
114 HotStarTaq Polymerase (Qiagen, USA). Amplification consisted of an initial
115 denaturation step at 95°C for 15 minutes, followed by 38 cycles of 94°C for 45 seconds,
116 66°C for 45 seconds and 72°C for 80 seconds. For the amplification of exon 1 and its
117 flanking sequences, Takara LA Taq (Takara Bio Inc, Japan) Polymerase was used. After
118 an initial denaturing step for one minute at 94°C, amplification was allowed in 35 cycles
119 composed of 95°C for 30 seconds, 66°C for 30 seconds and 72°C for 80 seconds.

120

121 ***RT-PCR analysis of SLC6A8 cDNAs and minigenes***

122 RNA was isolated from lymphoblasts or fibroblasts using the SV RNA kit (Promega) or
123 from PAX blood tubes (Qiagen). cDNA was synthesized from isolated RNA using
124 Omniscript Reverse Transcriptase (Qiagen, USA) according to the manufacturer's
125 instructions. RT-PCR was performed using cDNA primers designed for the exons of

126 interest of the *SLC6A8* gene. To rule out possible amplification of genomic DNA, RT-
127 PCR for each RNA was also performed without reverse transcriptase.

128

129 ***Construction and transfection of the minigenes***

130 Due to the large size of the *SLC6A8* gene and its highly GC-rich 5' region, we only
131 cloned the region of interest of the *SLC6A8* gene. As templates for the PCR, the
132 patients' and a wild type genomic DNAs were used. The fragment, covering exons 3 to
133 7 including 53 and 55 nucleotides of the flanking 5' and 3' intronic regions respectively,
134 was amplified with forward primer 5'-

135 CCGGAATTCGTAAAACGACGGCCAGCAGGGGGAGGTGGCCAGGG -3'

136 containing an EcoR1 site and reverse primer 5'-

137 GCGTCGACCAGGAAACAGCTATGACATGCATCTGGGTAGCACTC -3' with a

138 Sall restriction site for cloning into the pBABE-puro plasmid. The fragment was

139 amplified using Takara LA Taq (Takara Bio Inc, Japan), cloned into the TOPO-TA

140 (Invitrogen, Breda, The Netherlands) vector and either wild type or mutant sequence

141 was confirmed by sequencing. For the construction of the positive control (i.e.

142 c.777+2T>A), site directed mutagenesis was performed on the TOPO vector containing

143 the wild type fragment with forward primer 5'-

144 AAATCCACGGGAAAGGAACCACTAGAGGCATGC-3' and reverse 5'-

145 GCATGCCTCTAGTGGTTCCTTTCCCGTGGATTT-3'. Presence of the desired

146 mutation and absence of PCR-artifacts were confirmed by sequencing of the complete

147 fragments. After digestion with EcoR1 and Sall, the inserts were sub cloned into the

148 pBABE-puro plasmid.

149 Minigene constructs were transiently transfected into *SLC6A8* deficient primary
150 fibroblasts. With the use of polyethylenimine (PEI), 25 µg of construct, in a ratio of 3:1
151 (PEI:DNA), was transfected in fibroblasts grown to 70% confluence in a 75 cm² culture
152 disc. After 48 hours, cells were harvested.

153

154 ***DNA sequence analysis***

155 Sequence analysis was performed using BigDye v3.1 terminator and an ABI 3110XL
156 (Applied Biosystems, The Netherlands). The obtained sequences were analyzed using
157 the Mutation Surveyor software package (Softgenetics, State College, PA, USA).

158

159 ***Analysis of amplicons with DHPLC***

160 For DHPLC analysis, optimal heteroduplex formation was realized by denaturing PCR
161 products at 95°C for 5 min and gradual cooling to 25°C over a period of 60 min.

162 Subsequently, 5 µl of the heteroduplex/homoduplex mixture was loaded on a WAVE
163 3500HT DNA fragment analysis system (Transgenomic, Omaha, NE). Elution of the
164 PS-DVB DNAsep column (Transgenomic, Omaha, NE) was performed in high-
165 throughput mode with a linear gradient of increasing acetonitril (ACN) concentration
166 with a runtime of 3 min and a constant flow rate of 1.5 ml/min. Gradient was realized
167 with the use of buffer A (0.1 M triethylammonium acetate [TEAA] and 0.025% ACN)
168 and buffer B (0.1 M TEAA and 25% ACN). Navigator 2.0 software (Transgenomic,
169 Omaha, NE) was used to calculate the ratio of buffer A and B during the gradient, as
170 well as the optimal partially denaturing temperature (T_m) for the amplicon. The T_m of
171 this amplicon was determined as 64°C.

172

173 ***In silico analysis***

174 The analysis of splicing efficiencies in the normal and mutant sequences was carried out
175 using the following five splice-site analysis tools which are commonly used in
176 diagnostic laboratories: the Berkeley Drosophila Genome Project⁶ (BDGP,
177 http://www.fruitfly.org/seq_tools/splice.html), Netgene2⁷
178 (<http://www.cbs.dtu.dk/services/NetGene2/>), Splice Predictor⁸
179 (<http://deepc2.psi.iastate.edu/cgi-bin/sp.cgi>), GenscanW⁹
180 (<http://genes.mit.edu/GENSCAN.html>) and FSplice
181 (<http://linux1.softberry.com/berry.phtml?topic=fssplice&group=programs&subgroup=gfi>
182 nd). The parameters used for analysis were the default settings of all tools.

183

184 ***LOVD Database***

185 The target of the LOVD database is to include all published and unpublished mutations
186 and variants. It is localized at a server in Leiden, The Netherlands, which can be reached
187 by the following URL <http://www.LOVD.nl/SLC6A8> or through the Variation
188 Databases page of the Human Genome Variation Society (HGVS, www.HGVS.org/).
189 The *SLC6A8*-specific database has been developed with the recently described LOVD
190 software,¹⁰ which is in agreement with the HGVS guidelines.¹¹ All variants are checked
191 and approved by Mutalyzer.¹² So far 38 pathogenic mutations have been reported in 44
192 male patients affected with *SLC6A8* deficiency.

193 **Results**

194 ***Validation of splice-site analysis using previously reported variants***

195 We analyzed 7 pathogenic mutations (Table 1) detected in patients affected with
196 SLC6A8 deficiency by applying five splice-site analysis tools (see methods). In 6 out of
197 7 pathogenic mutations at least four of the five tools predicted a reduced recognition of
198 the splice sites, varying with a reduction of the probability score between 9% and 100%.
199 Three mutations had significant reduction scores of more than 99% in four of the five
200 tools, whereas 3 other variants had reduction of their scores in a much lower range. One
201 mutation, c.1392+24_1393-30del, reducing the intron from 76 to 52 bp, was not
202 recognized by all the tools. Even though analysis of this mutation with Splice Predictor
203 resulted in the loss of both rho and gamma values, this was not ascribed to the mutation
204 itself but to Splice Predictor's precondition of an intron size of at least 60 bp.
205 In addition, 18 previously reported variants that are not disease causing were correctly
206 predicted not to cause erroneous splicing by all five splice-site analysis tools, with the
207 exception of one synonymous variant that showed in four tools minor reduction scores
208 of equal or less than 7%. These variants are all confirmed to be non disease causing,
209 since we now either established at the cDNA level that no erroneous splicing occurred
210 or that the variants were detected in healthy control males or were published in dbSNP
211 (See table 1; See www.lovd.nl/slc6a8).

212

213 ***Detection and classification of novel variants***

214 Within the last decade we have analyzed about 1900 individuals (referred to our
215 department because of e.g. mental retardation, increased urinary creatine/creatinine) by
216 DNA sequence analysis of the *SLC6A8* gene to exclude/confirm creatine transporter

217 defect. In this endeavor we identified 41 variants including 28 novel synonymous and
218 intronic variants that were not included in the dbSNP database and of which their (non-)
219 disease causing nature needed to be established. All were subjected to the five splice-
220 site analysis tools. In total, 3 novel pathogenic mutations (c.263-1G>C, c.778-2A>G,
221 c.1596+1G>A) were detected that were predicted to cause aberrant splicing with
222 reduction scores of 100% by at least three splice-site analysis tools (Table 2). Twenty-
223 seven variants showed no effect by all five tools. By only one or two tools 8 variants
224 were predicted to decrease the possibility of correct splicing by 10% or less. One
225 synonymous unclassified variant (c.780C>T) showed in three splice-site analysis tools
226 reduction scores that appear significant in terms of percentage, but if the normal range is
227 taken into account these differences are considered minimal (e.g. the score by the
228 FruitFly tool shows a decrease of 32% while the actual score drops from 0.22 to 0.15
229 with the range of low probability to high probability being 0 – 1.00). All these variants
230 were considered non disease causing. Only 1 intronic unclassified variant (c.777+4C>T)
231 was predicted by all five splice-site analysis tools to reduce the probability of the
232 canonical donor site of exon 4 (Table 2) by 10%, 2%, 23%, 10% and 15% compared to
233 the canonical donor site score. cDNA was not available from this patient and thus this
234 variant was studied by overexpression of a minigene (i.e. a genomic *SLC6A8* segment
235 containing the c.777+4C>T variant). Both overexpression of the wild type minigene as
236 well as the mutant minigene in *SLC6A8* deficient primary fibroblasts showed normal
237 splicing as detected by RT-PCR and sequence analysis. This is in contrast to the
238 positive control minigene that contained the c.777+2T>A mutation and resulted in
239 skipping of exon 4 (Figure 1).

240

241 ***Five novel SLC6A8 deficient patients.***

242 The first case (Patient I) was a young female (DOB 01/93) born at term following a
243 normal pregnancy and delivery with normal weight, length and head circumference.
244 From the age of 2 she developed a behavior disturbance and was assessed by a
245 pedopsychiatric institution that also diagnosed mild mental retardation. At age 14 she
246 was affected with auditory hallucinations and the diagnosis of chronic hallucinatory
247 psychosis was made. Her cognitive impairment was stable with conserved language
248 capacities and social contact, no motor impairment and no specific neurological signs.
249 Her total IQ was tested at 45 (verbal: 61, reason: 45, memory: 53; working velocity:
250 66). A brain H-MRS was performed which showed a reduced cerebral creatine level
251 with a normal appearance to choline and *N*-acetylaspartic acid (Figure 2). Urinary
252 creatine levels were within the upper normal range. Plasma creatine was determined
253 twice resulting in values of 39.9 $\mu\text{mol/L}$ and 75.4 $\mu\text{mol/L}$ (normal 30 – 124 $\mu\text{mol/L}$).
254 Genomic DNA sequencing revealed a c.263-1G>C mutation. This mutation arose *de*
255 *novo*. Somatic mosaicism was not detected in her parents' DNA by both DNA analysis
256 and the use of DHPLC (Figure 3). mRNA isolated from PAX tubes, followed by cDNA
257 synthesis showed approximately 90% r.263_325del (p.Gly88_Leu108del) and 10%
258 wildtype product. The X-inactivation was studied in blood with the analysis of *Hha*I
259 digested and undigested DNA followed by PCR of the highly polymorphic CAG repeat
260 of the androgen receptor gene and showed a 90:10 pattern. Also, fibroblasts incubated
261 with a physiological concentration of creatine (25 μM) showed no uptake. All these
262 findings combined confirmed creatine transporter deficiency.

263 The second case (Patient II) was a 7 year old male with epilepsy, expressive language
264 difficulties and a movement disorder. He was tested for mutations in *SLC6A8* after H-

265 MRS of the brain revealed a diminished creatine peak with a normal appearance to
266 NAA and choline and urinary analysis showed an increased Cr/Crn value. This resulted
267 in the detection of a hemizygous c.778-2A>G variant. His brother (Patient III), currently
268 10 years old, showed a similar clinical phenotype with an increased urinary Cr/Crn
269 value and absence of cerebral creatine. He was also found to be hemizygous for this
270 variant while his mother was proven to be heterozygous.

271 The fourth patient (Patient IV) was a young male with a reduced cerebral creatine level
272 measured by H-MRS. Further investigation resulted in the detection of a c.1596+1G>A
273 variant, which was also found to be heterozygous in DNA from his mother.

274 The fifth patient (Patient V) was unmistakably diagnosed with SLC6A8 deficiency at
275 the age of 6 years. The patient suffered from moderate mental retardation and severe
276 language delay. Quantitative localized single voxel magnetic resonance spectroscopy
277 was performed over the basal ganglia and revealed a markedly diminished creatine level
278 with a normal appearance to choline. Urinary analysis revealed a creatine/creatinine
279 value of 1.7 (normal 0.017 – 0.72).¹³ The creatine uptake ability of the patients'
280 fibroblasts was significantly decreased in comparison with control cells when incubated
281 at a physiological creatine concentration of 25µM. Moreover, the mutation could not be
282 detected in the DNA of the mother and is therefore considered *de novo*. At the cDNA
283 level, two erroneous transcripts were revealed (r.[1392_1393ins1392+1_1393-
284 1;1392+24_1393-30del,1393_1495del])(Figure 4). This conclusively classified the
285 c.1392+24_1393-30del mutation as the pathogenic event. Also, the same *de novo*
286 mutation was detected in an unrelated patient.¹⁴

287

288 ***LOVD database***

289 In total, the LOVD database of *SLC6A8* lists 44 *SLC6A8* deficient families, with a total
290 of 43 mutations. Of these, 38 are proven pathogenic (+/+), whereas 5 others are
291 presumed to be pathogenic (+?/+?). The latter are all missense variants, which have not
292 been investigated by overexpression studies yet.¹⁵

293 The database (Figure 5) provides detailed information on the nature of the variants, but
294 also exon/intron location of the DNA change (both according to current nomenclature
295 and as published), RNA change and protein change. To all variants a unique database
296 number is assigned. The column “variant remarks” provides a detailed description on
297 the nature of the variant. The final conclusion is presented in the first column “path.”
298 where the first sign provides the conclusion of the report and the second one that of the
299 curator (e.g. ++ meaning confirmed pathogenic). Other columns include detailed
300 information on the variant origin, reference, template, technique, frequency, disease
301 status, patient remarks, times reported, gender, geographic origin and ethnic origin.
302 Moreover, clinical symptoms are continuously archived (non-public). The database is
303 an excellent tool to get an up-to-date overview of the type of mutations, the frequency
304 of specific variants and more details. This data can be found in the different tabs by any
305 user, but an advanced search function is also available. Researchers or clinicians who
306 are interested in the nature of a specific variant can use the database to search if the
307 variant has been detected in other patients. This information may have been published
308 in reports or on the website only, but also may include unpublished variants which have
309 not yet been made public. The latter allows contact between clinicians even if the data is
310 not publicly released. All scientists/clinicians are invited to submit their data to the
311 database. The identity of the submitter is included in the database.

312

313 **Discussion**

314 In this study, the nature of 67 intronic and synonymous variants have been studied by
315 free web based splice-site analysis tools. These have been characterized and are all
316 being included in a newly developed LOVD database. In addition, all previously
317 published mutations are included in this database as well. This is of high relevance for
318 researchers and clinicians who detect difficult to interpret variants in the *SLC6A8* gene.
319 In an ideal diagnostic setup, an unclassified variant is further investigated at the mRNA,
320 protein and/or functional level to confirm/exclude its pathogenic nature. However, in
321 diagnostic laboratories time is usually limited and proper materials are often not
322 available. Therefore, alternative methods are warranted for variant classification such as
323 frequently used splice-site analysis tools, which predict the possible effect on RNA
324 splicing of a variant based on known and computed conserved splicing sequences. Here
325 we demonstrate that the use of five splice-site analysis tools, indeed is very helpful for
326 proper classification.

327 Twenty four out of 25 (96%) variants were properly classified by the combined use of
328 the five tools. The importance of using more than one splice-site analysis tool is
329 illustrated by the fact that 4 out of 7 proven pathogenic mutations were not predicted to
330 have a strong effect (i.e. 90% or higher reduction) by one or more of the individual
331 analysis tools, whereas the combined data of all the tools clearly predicted erroneous
332 splicing for 6 of these variants (Table 1). In one case, only one tool predicted a
333 reduction in the probability of correct splicing. The fact that this “missed” mutation
334 (c.1392+24_1393-30del) comprises an intronic deletion of 24 base pairs out of a 76
335 base pairs intron, easily explains this omission as none of the five splice-site analysis
336 tools have intron size as a parameter. This caveat is an important finding for diagnostic

337 laboratories that encounter this specific type of mutations (e.g. deletions, insertions in
338 small introns). We demonstrated that this deletion indeed results in erroneous spliced
339 transcripts (r.[1392_1393ins1392+1_1393-1;1392+24_1393-30del,1393_1495del]),
340 (Abdul-Rahman et al, in press, present study).

341 In 17 out of 18 proven variants, no change in prediction scores was detected by any of
342 the five tools. Only 1 variant, that was proven not to interfere with proper splicing, had
343 a reduced score of 7% or less compared to the canonical site by four of the five tools.
344 Due to this variant, we arbitrarily decided to only consider variants for further molecular
345 workup if at least three tools predicted a mild effect (10 < > 80%), or if one or two tools
346 predicted a significant effect (>80%). In addition, we took into account the prediction
347 score of the wild type splice site. Thus a 50% reduction of a canonical site with a 0.99
348 score is more likely to have an effect on splicing than a 50% reduction in a splice site
349 with a much weaker score of for instance 0.22.

350 Utilizing these criteria we analyzed the potential effect of 41 unclassified variants on the
351 *SLC6A8* mRNA with the use of these five splice-site analysis tools. This resulted in the
352 identification of 3 pathogenic mutations (c.263-1G>C, c.778-2A>G, c.1596+1G>A),
353 and 38 variants that have no apparent relation with creatine transporter deficiency. The
354 effect on splicing for 2 of the 3 novel pathogenic mutations could not be confirmed at
355 the cDNA level. However, given the fact that all 3 mutations affect the donor or
356 acceptor splice-site, all were classified as pathogenic. Pathogenicity was in excellent
357 agreement with the fact that the mutations were not detected in 280 control
358 chromosomes, the biochemical findings (increased urinary Cr/Crn) and/or reduced
359 cerebral creatine detection by H-MRS.

360 Interestingly, one novel mutation (c.263-1G>C) was found in a girl, who according to
361 our knowledge is the first reported index girl affected with SLC6A8 deficiency. The girl
362 was not correctly diagnosed until the age of 14 when a brain H-MRS revealed a reduced
363 cerebral creatine level. This led to DNA analysis which resulted in the discovery of the
364 pathogenic c.263-1G>C mutation. Additional investigations revealed a urinary creatine
365 to creatinine ratio within the normal range as well as normal values for plasma creatine.
366 The discrepancy between the urinary, plasma and cerebral creatine levels is possibly
367 caused by variation of the X-inactivation pattern in different tissues, which was shown
368 to be skewed in blood of this patient.

369 In a recently described cohort of female relatives who were heterozygous for a
370 pathogenic mutation in *SLC6A8*, it was shown that symptoms of SLC6A8 deficiency
371 (e.g. mental retardation, learning difficulties and constipation) can occur in female
372 heterozygotes. However, it should be noted that the level of biochemical markers
373 (urinary creatine to creatinine ratio and/or cerebral creatine) usually overlap with that of
374 normal controls. This is in concordance with the clinical and biochemical findings of
375 the above mentioned female index patient and confirms the recommendation of
376 screening for mutations in *SLC6A8* in females with (mild) mental
377 retardation/psychiatric disorders as this method appears to be the most sensitive and
378 specific test.¹⁶

379 In only 1 of the 38 UVs, mild reduction values of the probability scores were detected
380 by all five splice-site analysis tools. Unfortunately it was not possible to obtain
381 additional material of the patient to isolate mRNA, and clinically it was also not
382 possible to exclude/confirm SLC6A8 deficiency. We therefore selected this variant
383 (c.777+4C>T) for overexpression studies of a minigene containing the variant. These

384 studies revealed no aberrant splicing and therefore this variant is also considered non-
385 pathogenic.

386 After evaluating the results of the splice-site analysis tools reported in this study, we
387 defined our criteria for the analysis of *SLC6A8*. If at least three out of five splice-site
388 analysis tools predict a reduction of more than 10% or one out of five predicts a
389 reduction of 80% of the site score compared to the canonical site, further research is
390 warranted. However a critical view needs to be kept which is illustrated by the fact that
391 in our study 1 out of the 10 proven pathogenic mutations would have escaped proper
392 identification. The c.1392+24_1393-30del mutation was not recognized by the overall
393 prediction score of all five splice-site analysis tools. It is of note that these scores do not
394 include composition, size and branch site of the intron as parameters. One exception is
395 Splice predictor, which does have additional intron dependent scores, namely rho and
396 gamma. Unfortunately, these scores are not calculated and set at zero when an intron is
397 smaller than 60 base pairs, therefore making it difficult to deduce the actual reduction
398 with this variant as the deletion leads to a 52 base pair intron. This of course reduces the
399 reliability of these scores since naturally occurring introns can have sizes of 60 base
400 pairs and less.

401 In conclusion, splice-site analysis tools are very important in the process of classifying
402 novel variants. For definite classification of novel variants outside the canonical donor
403 and acceptor sites, *in vitro* experimentation, either with mRNA analysis or with the use
404 of a minigene (if no additional material is available), is an essential procedure.

405

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414

415

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482

483 **Titles and Legends to Tables and Figures**

484 ***Table 1***

485 **Proven pathogenic mutations and non disease associated variants in *SLC6A8*.**

486 **Validation of the splice-site analysis tools.**

487 ^a This mutation arose *de novo*.

488 ^b r.[1392_1393ins1392+1_1393-1;1392+24_1393-30del, 1393_1495del]

489 Location refers to the intron (i) or exon (2 digits) number in which the variant was
490 found. In the upper panel the proven pathogenic variants are denoted. The lower panel
491 includes both intronic and synonymous variants.

492 NC: no change. Variant did not influence the predicted score compared to the canonical
493 site.

494 Path.: Reported and concluded pathogenicity. +: Pathogenic; -: No known
495 pathogenicity.

496 r.spl?: the variant is expected to affect splicing; however, no further mRNA analysis is
497 performed.

498 Ref: Reference. Ref 2: ²; Ref 4: ⁴; Ref 17: ¹⁷; Ref 18: ¹⁸; Ref 19: ¹⁹; Ref 20: ²⁰; Ref 21:
499 ²¹.

500 cDNA denotation is based on the reference sequence NM_005629.3, where +1
501 corresponds to the first nucleotide of the initiation codon. All variants are annotated
502 according to the guidelines of Den Dunnen and Antonarakis
503 (<http://www.genomic.unimelb.edu.au/mdi/mutnomen/>).

504 **Table 2**

505 **Twenty eight novel intronic variants and 13 previously reported unclassified**
506 **intronic variants in *SLC6A8*.**

507 None of these variants, including 3 pathogenic mutations and 13 previously reported
508 variants were detected in 280 control alleles.

509 Location refers to the intron (i) or exon (2 digits) number in which the variant was
510 found. r.spl?: the variant is expected to affect splicing; however, no further mRNA
511 analysis is performed.

512 ^a This variant was cloned into a minigene. Overexpression studies indicated that this
513 variant does not affect splicing.

514 NC: no change (i.e. the variant did not influence the predicted score compared to the
515 canonical site)

516 SND: site not detected. The canonical donor or acceptor site is not recognized as a
517 naturally occurring site by this splice-site analysis tool.

518 Path.: Reported / concluded pathogenicity. -: No known pathogenicity; -?: Probably no
519 pathogenicity; ?: Unknown; +?: Probably pathogenic; +: Pathogenic.

520 Deduced effect: Indicates that the variant has not been tested at the mRNA level;
521 r.(spl?): the variant is expected to affect splicing; p.(=), no effect predicted, but not
522 tested at the mRNA level.

523 Ref: Reference. Ref 2: ²; Ref 4: ⁴.

524 cDNA denotation is based on the reference sequence NM_005629.3, where +1
525 corresponds to the first nucleotide of the initiation codon. All variants are annotated
526 according to the guidelines of Den Dunnen and Antonarakis

527 (<http://www.genomic.unimelb.edu.au/mdi/mutnomen/>).

528 **Figure 1**

529 RT-PCR results of spliced products after transfection of the minigenes in SLC6A8
530 deficient fibroblasts.

531 SLC6A8 deficient fibroblast were transfected with a minigene, containing an exon 3 to
532 7 fragment of *SLC6A8* with either a wild type sequence, the c.777+2T>A transversion
533 or the c.777+4C>T variant. Cells were harvested and RNA was isolated using the
534 Promega RNA Isolation Kit. Subsequent RT-PCR of the wild type and the c.777+4C>T
535 minigene transfected fibroblasts resulted in an amplicon of 384 base pairs, indicating
536 the variant not to cause aberrant splicing. The positive control (c.777+2T>A
537 transfectants) resulted in a 251 base pair amplicon. Sequencing analysis showed that
538 exon 4 was skipped in the 251 base pair amplicon.

539

540 **Figure 2**

541 Proton MRS of the brain of an index girl with SLC6A8 deficiency (Patient I).

542 A proton MRS was performed on a 14 year old girl with mild mental retardation.

543 Creatine (Cr) was found to be reduced with a normal appearance to choline (Cho) and

544 *N*-acetylaspartic acid (NAA). Subsequent genomic DNA sequencing revealed a

545 pathogenic c.263-1G>C mutation, which confirmed the diagnosis of SLC6A8

546 deficiency.

547

548 **Figure 3**

549 Patient I with a c.263-1G>C mutation in the *SLC6A8* gene.

550 Somatic mosaicism for the c.263-1G>C mutation was not detected in both parents of

551 Patient I. The DHPLC elution profile of the exon 2 amplicon of EDTA blood DNA of

552 patient I with a c.263-1G>C variant in *SLC6A8* including his parents and a male control.

553 At the retention time of the heteroduplex peak clearly visible with the patient, no peak is

554 visible with the parents, indicating both are not somatic mosaic for the mutation.

555

556 **Figure 4**

557 Schematic representation of the c.1392+24_1393-30del variant in *SLC6A8*.

558 The c.1392+24_1393-30del variant was found in two independent patients and occurred

559 *de novo* in both cases. In the patients' DNA a hemizygous variant, c.1392+24_1393-

560 30del, was found (upper pane). mRNA analysis showed that this variant affects splicing

561 and results in different splice products, r.[1392_1393ins1392+1_1393-

562 1;1392+24_1393-30del, 1393_1495del](lower pane), resulting in *SLC6A8* deficiency.

563 For the second patient see Hathaway *et al.*¹⁴ This variant is annotated according to the

564 guidelines of Den Dunnen and Antonarakis

565 (<http://www.genomic.unimelb.edu.au/mdi/mutnomen/>).

566

567 **Figure 5**

568 Screenshot of the newly developed LOVD/SLC6A8 database.

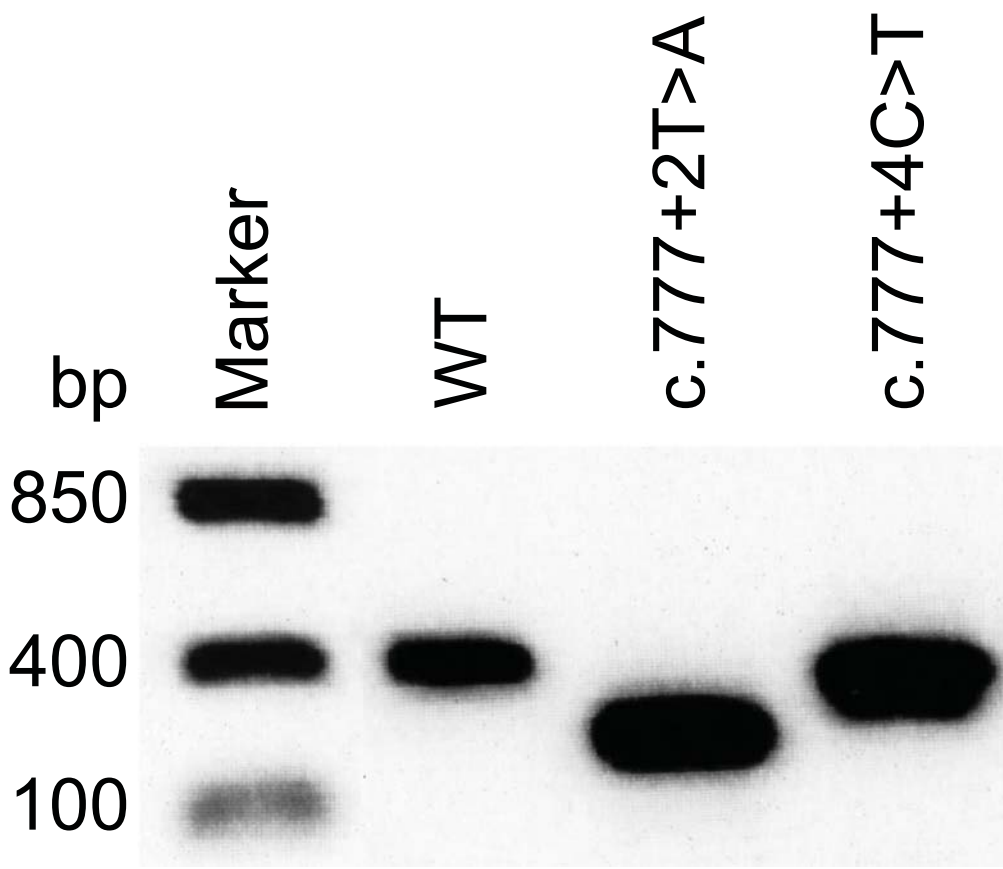
569 The currently known variants and pathogenic mutations of *SLC6A8* are described in the
570 newly developed LOVD database including the corresponding clinical data. Search
571 queries can be performed for many parameters (i.e. specific variant, exon number,
572 biochemical findings). In order to submit variants it is required to log in, after which the
573 variants will be checked for correctness and completeness by the curator.

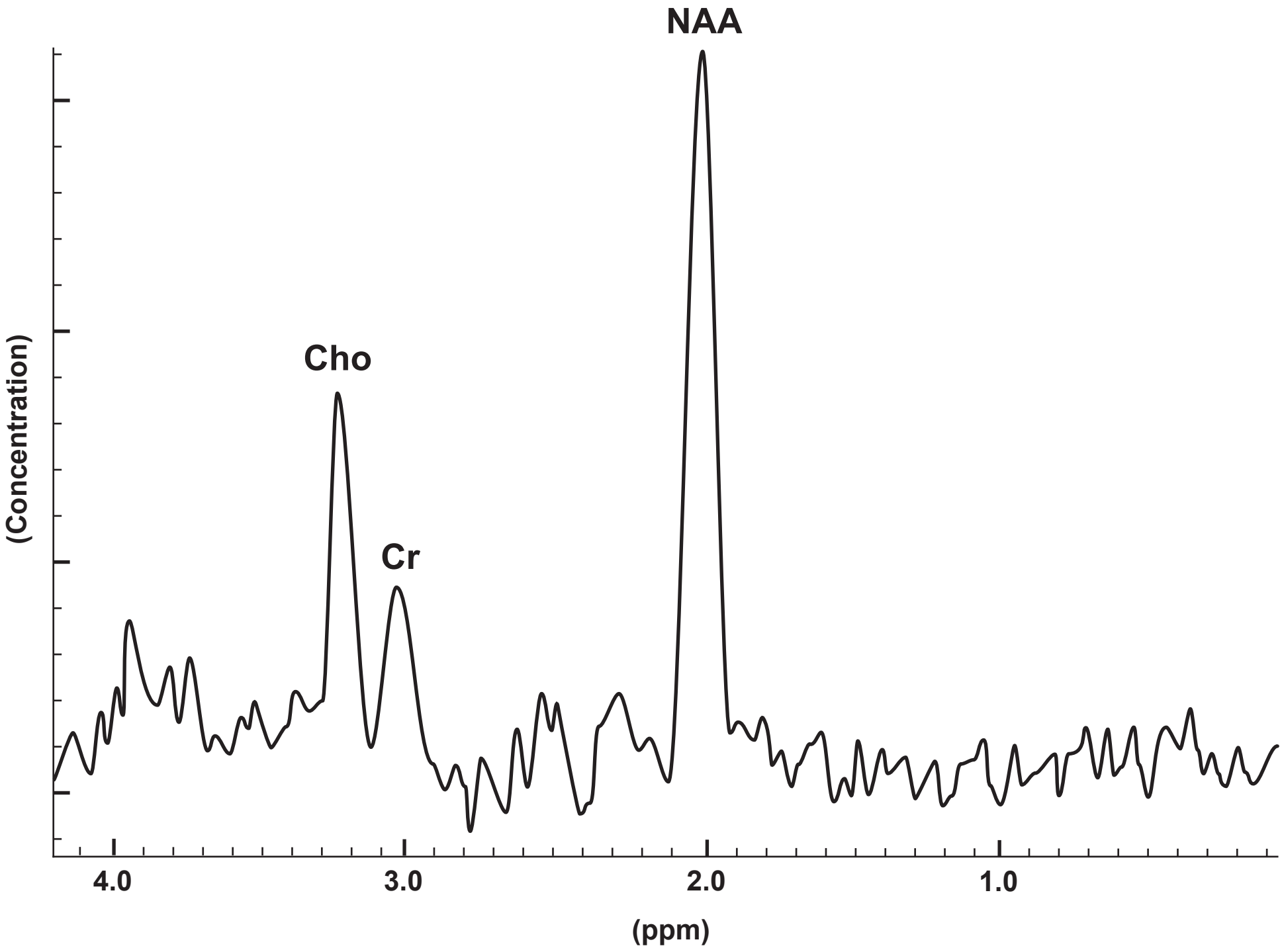
Table 1.
 Proven pathogenic mutations and non disease associated variants in *SLC6A8*.
 Validation of the splice-site analysis tools.

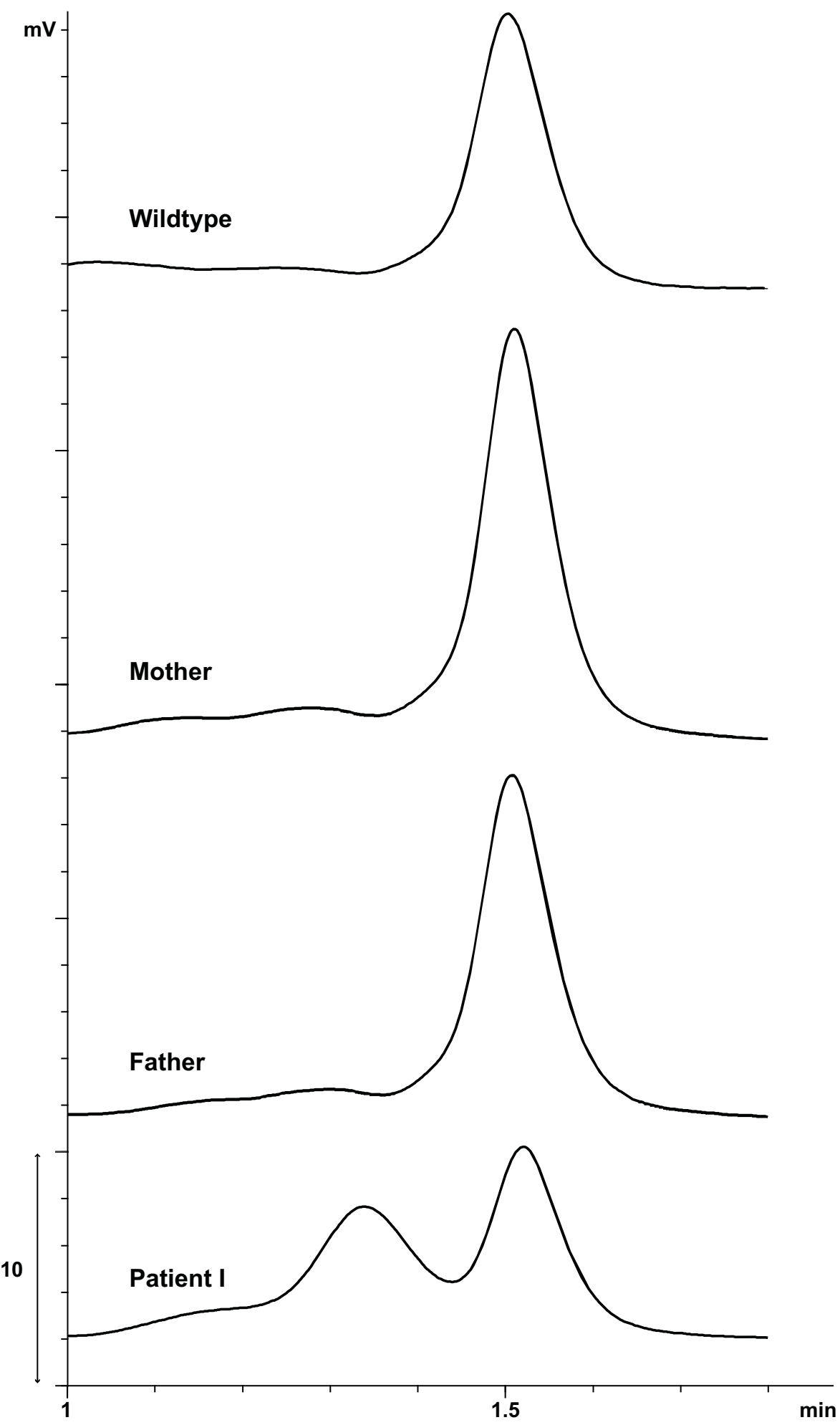
| Location | cDNA | RNA | Protein | Controls | Netgene 2 | Fruitfly | Splice Predictor | Genscan W | FSplice | Path. | Reference |
|----------|----------------------------------|---------------------------|-------------------------------|-------------------|--------------------|--------------------|--------------------|--------------------|-------------------|-------|-----------|
| 01i | c.262_262+1delinsTT ^a | r.(spl?) | p.(?) | 0/280 | 1.00 > 0 (-100%) | 0.99 > 0.01 (-99%) | 0.99 > 0 (-100%) | 57.0 > 39.1 (-31%) | 12.4 > 0 (-100%) | +/+ | 21 |
| 01i | c.263-2A>G | r.263_328del | p.Gly88_Glu109del | 0/280 | 0.95 > 0 (-100%) | 0.61 > 0 (-100%) | 0.97 > 0 (-100%) | 21.9 > 5.9 (-73%) | 12.5 > 0 (-100%) | +/+ | 20 |
| 05 | c.912G>A | r.772_912del | p.Ile260_Gln304del | 0/280 | 0.99 > 0.80 (-19%) | 0.95 > 0.67 (-30%) | 0.99 > 0.88 (-11%) | 12.4 > 9.1 (-27%) | 11.6 > 6.0 (-48%) | +/+ | 18 |
| 06i | c.1016+2T>C | r.(spl?) | p.(?) | 0/280 | 0.99 > 0 (-100%) | 0.99 > 0 (-100%) | 0.97 > 0 (-100%) | 18.2 > 10.9 (-40%) | 10.16 > 0 (-100%) | +/+ | 2 |
| 07 | c.1141G>C | r.[1141G>C, 1129_1141del] | p.[Gly381Arg, Val377GlyfsX15] | 0/280 | 0.99 > 0.90 (-9%) | 0.92 > 0.17 (-82%) | 0.99 > 0.90 (-10%) | 24.3 > 18.7 (-23%) | 11.0 > 1.5 (-86%) | +/+ | 17 |
| 09i | c.1392+24_1393-30del | r.[1392...] ^b | p.(?) | 0/280 | NC | NC | NC | NC | NC | +/+ | |
| 10i | c.1495+5G>C | r.1393_1495del | p.Gly465GlufsX12 | 0/280 | 0.95 > 0.70 (-26%) | 0.96 > 0 (-100%) | 0.99 > 0.86 (-13%) | 17.9 > 15.7 (-12%) | 11.0 > 1.5 (-86%) | +/+ | 19 |
| 01i | c.262+26T>C | ND | p.(=) | 6/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 02i | c.394+88G>C | ND | p.(=) | rs6643763, 7/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 05 | c.813C>T | ND | p.= | 4/280 | NC | NC | NC | NC | NC | -/- | 4 |
| 06i | c.1016+9C>T | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 07i | c.1141+37G>A | r.= | p.= | rs2071028, 9/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 07i | c.1141+87A>G | r.= | p.= | rs41302172, 6/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 07i | c.1142-152_151del | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 07i | c.1142-35G>A | r.= | p.= | 2/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 08i | c.1254+28C>T | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 08i | c.1255-35G>A | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 09i | c.1393-36G>A | r.= | p.= | 1/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 10 | c.1494C>T | r.= | p.= | 1/280 | 0.95 > 0.91 (-4%) | 0.96 > 0.89 (-7%) | NC | 17.9 > 16.7 (-7%) | 11.0 > 10.3 (-6%) | -/- | 2 |
| 10i | c.1496-18C>T | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 11i | c.1596+21G>A | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 12 | c.1678A>G | ND | p.= | 1/280 | NC | NC | NC | NC | NC | -/- | 2.4 |
| 12i | c.1767+15C>T | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 12i | c.1767+32C>A | r.= | p.= | 0/280 | NC | NC | NC | NC | NC | -/- | 2 |
| 12i | c.1768-82G>C | ND | p.(=) | 1/280 | NC | NC | NC | NC | NC | -/- | 2 |

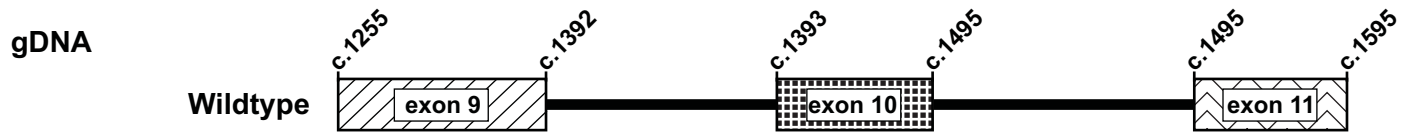
Table 2. Twenty eight novel intronic variants and 13 previously reported unclassified intronic variants in *SLC6A8*.

| Location | cDNA | Deduced effect | Netgene 2 | Fruitfly | Splice Predictor | Genscan W | FSplice | Path. | Ref |
|----------|-------------------------|----------------|--------------------|--------------------|-------------------|-------------------|-------------------|-------|-----|
| 01i | c.263-1G>C \$ | r.263_325del | 0.95 > 0 (-100%) | 0.93 > 0.32 (-66%) | 0.97 > 0 (-100%) | 21.9 > 5.9 (-73%) | 12.5 > 0 (-100%) | +/+ | |
| 01i | c.262+65G>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 02 | c.306A>G | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 02i | c.394+52C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 02i | c.394+66C>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 02i | c.394+72C>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 02i | c.394+88G>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 03 | c.495C>T | p.(=)? | NC | NC | NC | 33.3 > 32.8 (-2%) | NC | -?/-? | |
| 03 | c.603C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 4 |
| 03i | c.644+9G>A | p.(=)? | NC | NC | 0.85 > 0.79 (-7%) | NC | NC | -?/-? | |
| 04i | c.778-2A>G | r.(spl)? | 1.00 > 0 (-100%) | 0.22 > 0 (-100%) | SND | 12.4 > 7.7 (-38%) | 3.3 > 0 (-100%) | +/+ | |
| 04i | c.777+4C>T ^a | p.(=)? | 0.91 > 0.82 (-10%) | 0.61 > 0.47 (-23%) | 0.94 > 0.92 (-2%) | 7.8 > 6.6 (-15%) | 9.6 > 8.7 (-10%) | -/- | |
| 05 | c.780C>T | p.(=)? | 1.00 > 0.96 (-4%) | 0.22 > 0.15 (-32%) | NC | NC | 3.3 > 2.67 (-20%) | -?/-? | 4 |
| 05 | c.856C>T | p.(=)? | NC | NC | NC | NC | NC | -/- | |
| 05i | c.913-40T>C | p.(=)? | NC | NC | NC | NC | 10.0 > 9.2 (-9%) | -?/-? | |
| 06i | c.1016+41_46del | p.(=)? | 0.99 > 0.96 (-3%) | NC | NC | NC | NC | -?/-? | |
| 06i | c.1016+41_45dup | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 4 |
| 06i | c.1017-38C>G | p.(=)? | NC | NC | NC | NC | 10.2 > 10.0 (-2%) | -?/-? | 4 |
| 07i | c.1141+18G>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 07i | c.1142-130C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 4 |
| 07i | c.1142-122C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 07i | c.1142-100G>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 07i | c.1142-98C>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 2 |
| 07i | c.1142-19G>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 08 | c.1162G>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 08i | c.1254+39G>T | p.(=)? | NC | NC | 0.92 > 0.89 (-3%) | NC | NC | -?/-? | 4 |
| 08i | c.1254+54C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 4 |
| 09i | c.1392+12G>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 09i | c.1392+31T>C | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 4 |
| 10 | c.1416G>A | p.(=)? | NC | NC | NC | NC | NC | -/- | 2 |
| 10 | c.1437C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 4 |
| 10i | c.1496-8C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 10i | c.1496-7G>A | p.(=)? | 1.00 > 0.96 (-4%) | 0.72 > 0.67 (-7%) | NC | NC | NC | -?/-? | |
| 10i | c.1496-5C>T | p.(=)? | NC | NC | 0.86 > 0.81 (-6%) | NC | NC | -?/-? | 4 |
| 11i | c.1596+1G>A | r.(spl)? | 0.95 > 0 (-100%) | 1.00 > 0 (-100%) | 0.99 > 0 (-100%) | 14.19 > 0 (-100%) | 11.98 > 0 (-100%) | +/+ | |
| 11i | c.1596+24_1597-41dup | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 11i | c.1596+28C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 11i | c.1597-20C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |
| 12 | c.1629G>A | p.(=)? | NC | NC | 0.57 > 0.55 (-4%) | 27.9 > 25.3 (-9%) | NC | -?/-? | |
| 12 | c.1662G>A | p.(=)? | NC | NC | NC | NC | NC | -?/-? | 4 |
| 12i | c.1767+20C>T | p.(=)? | NC | NC | NC | NC | NC | -?/-? | |

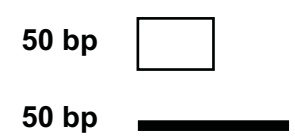
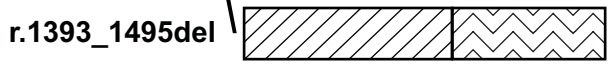
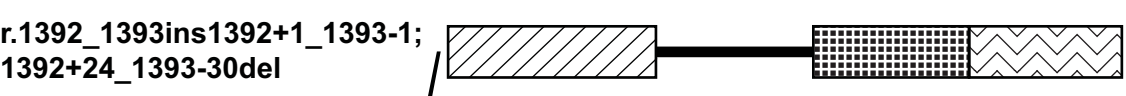
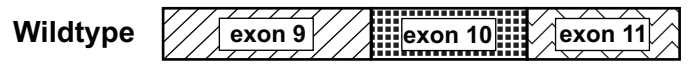








mRNA



LOVD - Variant listings

| Path. | Exon | DNA change | RNA change | Protein change | Variant remarks | Reference | Frequency | Disease | Geographic origin |
|-------|------|--------------------------|--------------|--------------------|---|---|----------------|--|-------------------|
| +/+ | 01 | c.219delC + c.0 | - | p.(Asn74ThrfsX23) | - | Dezortova 2008 | - | SLC6A8 defect | Czech Republic |
| +/+ | 01 | c.259G>A + c.0 | - | p.(Gly87Arg) | This variant is a proven pathogenic mutation since overexpression of this variant in SLC6A8 deficient fibroblasts did not restore creatine uptake. (Rosenberg et al. 2007) | Rosenberg 2004 , Rosenberg 2007 | - | SLC6A8 defect | France |
| +/+ | 01i | c.263-2A>G + c.0 | r.263_325del | p.Gly88_Leu108del | cDNA analysis revealed splice error. 5 out of 5 splice predictors predict the complete loss of the splice site. | Schiaffino 2005 | - | SLC6A8 defect | Italy |
| +/+ | 02 | c.321_323delICTT + c.0 | - | p.(Phe107del) | This variant is a proven pathogenic mutation since overexpression of this variant in SLC6A8 deficient fibroblasts did not restore creatine uptake. (Rosenberg et al. 2007) | degrauw 2002 , Rosenberg 2007 | - | SLC6A8 defect | OH,USA |
| +/+ | 03 | c.462G>A + c.0 | - | p.(Trp154X) | - | Fons 2009 | - | SLC6A8 defect | Spain |
| +/+ | 03 | c.570_571delITG + c.0 | - | p.(Ala191GlnfsX10) | - | de Kort, Nederlands Tijdschrift voor Geneeskunde, 2006 | - | SLC6A8 defect | - |
| +/+ | 04i | c.778-2A>G + c.= | r.(spl?) | - | 4 out of 4 splice predictors predict the complete loss of the splice site. | - | - | SLC6A8 heterozygote | Germany |
| +/+ | 05 | c.786 C>G + c.0 | - | p.(Tyr262X) | This mutation has been detected de novo in at least one patient with SLC6A8 deficiency. | degrauw 2002 | - | SLC6A8 defect | OH,USA |
| +/+ | 05 | c.878_879delITC + c.0 | - | p.(Lys293fsX3) | - | Poo-Arquelles 2006 | - | SLC6A8 defect | Spain |
| +/+ | 05 | c.912G>A + c.0 | r.772_912del | p.Ile260_Gln304del | cDNA analysis revealed splice error. 5 out of 5 splice predictors predict the complete loss of the splice site. | Lion-Francois 2006 | - | SLC6A8 defect | France |
| +/+ | 06 | c.942_944delICTT + c.0 | - | p.(Phe315del) | - | Fons 2009 | - | SLC6A8 defect | - |
| +/+ | 06 | c.950dupA + c.0 | - | p.(Tyr317X) | This variant is a proven pathogenic mutation since overexpression of this variant in SLC6A8 deficient fibroblasts did not restore creatine uptake. (Rosenberg et al. 2007) | Rosenberg 2004 | - | SLC6A8 defect | - |
| +/+ | 06 | c.1006_1008delAAC + c.0 | - | p.(Asn336del) | This mutation has been detected de novo in at least one patient with SLC6A8 deficiency. Overexpression of this variant in SLC6A8 deficient fibroblasts did not restore creatine uptake. (Rosenberg et al. 2007) | Clark 2006 , Rosenberg 2007 | 0/280 controls | SLC6A8 defect | SC,USA |
| +/+ | 06 | c.1011C>G + c.0 | - | p.(Cys337Trp) | This variant is a proven pathogenic mutation since overexpression of this variant in SLC6A8 deficient fibroblasts did not restore creatine uptake. (Rosenberg et al. 2007) | Rosenberg 2004 , Rosenberg 2007 | - | SLC6A8 defect | - |
| +/+ | 06i | c.1016+2T>C + c.0 | r.(=) | p.(=) | 5 out of 5 splice predictors predict the complete loss of the splice site. | Clark 2006 | 0/280 controls | Mental retardation, most likely no SLC6A8 defect | SC,USA |
| +/+ | 07 | c.1040_1042delITCA + c.0 | - | p.(Ile347del) | This variant is a proven pathogenic mutation since overexpression of this variant in SLC6A8 deficient fibroblasts did not restore creatine uptake. (Rosenberg et al. 2007) | Clark 2006 , Rosenberg 2007 | 0/280 controls | SLC6A8 defect | SC,USA |
| +/+ | 07 | c.1059_1061delICTT + c.0 | - | p.(Phe354del) | - | Betsalel 2008 | - | SLC6A8 defect | - |