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# METHYLATION OF THE CPG SITES IN THE MYOTONIC DYSTROPHY LOCUS DOES NOT CORRELATE WITH CTG EXPANSION SIZE OR WITH THE CONGENITAL FORM OF THE DISEASE

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1 METHYLATION OF THE CPG SITES IN THE MYOTONIC  
2 DYSTROPHY LOCUS DOES NOT CORRELATE WITH CTG  
3 EXPANSION SIZE OR WITH THE CONGENITAL FORM OF THE  
4 DISEASE.

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16  
17 **ABSTRACT**

18 We have studied the methylation status of the sequence 152 nucleotides upstream of  
19 the CTG repeat of the DM1 locus in patients' peripheral blood. We used the methylation-  
20 sensitive endonucleases SacII, HpaII and HhaI, followed by PCR. This allowed to  
21 correlate the methylation status of each CTG allele with its size. Contrary to previous  
22 findings (Steinbach P et al., 1988, Am.J.Hum.Genet. 62:278-285), only the SacII site is  
23 often but not always differentially methylated amongst expanded CTG alleles.  
24 Importantly, this methylation was not restricted to congenital DM1, nor to large  
25 expansions, as it was also present in DM1 patients with a classical phenotype and various  
26 expansion sizes. On the other hand, we did not find any methylated alleles on the HhaI

27 and HpaII sites, as was reported by Steinbach et al, which is in line with the results of  
28 Shaw and collaborators (1993, J.Med.Genet. 30:189-192). The size range of the repeat  
29 expansions with methylation was from as small as 300 to as large as 2800 repeats.

30

## 31 REPORT

32 Myotonic dystrophy type 1 (DM1, [MIM 160900]) is a dominantly inherited  
33 multisystemic disorder, with an incidence of approximately 1 in 8000 individuals. DM1  
34 has a complex phenotype, with symptoms including progressive skeletal muscle wasting,  
35 impaired muscle relaxation (myotonia), cardiac conduction defects resulting in  
36 arrhythmias, early-onset iridescent cataracts, insulin resistance and hyperinsulinemia  
37 (Harper, 2001). These symptoms are caused by several mechanisms, which include the  
38 aberrant processing of the *DMPK* mRNA, leading to decreased levels of DMPK (Krahe et  
39 al., 1995), toxic gain of function of the mRNA (Mankodi et al., 2000), decreased  
40 expression of the neighbouring *SIX5* gene (Klesert et al., 1997; Thornton et al., 1997)  
41 and disrupted alternative splicing of numerous other genes (Ranum and Cooper, 2006).

42 DM1 is caused by a CTG expansion in the 3' untranslated region of the *DMPK* gene  
43 located on chromosome 19q13.3. Expanded CTG tracts show both somatic and germinal  
44 instability; unaffected individuals have from 5 to 40 CTG repeats, whereas individuals  
45 with the disease carry more than 100 repeats. Disease severity and age of onset  
46 correlate with repeat size, and expansions of >1500 repeats often result in a severe  
47 congenital form of DM1.

48 CpG methylation has been suggested to play a role in the stability of repetitive  
49 sequences in general (Gourdon et al., 1997, Nichol and Pearson, 2002), and more  
50 particularly in the behaviour of the CTG repeat in the DM1 locus (Steinbach et al., 1998,  
51 Brock et al., 1999, Gorbunova et al., 2004) as well as in the pathogenesis of the disease  
52 (Shaw et al., 1993, Steinbach et al., 1998, Filippova et al., 2001 ).

53 The relationship between the semiology of the disease and the methylation of the CpG  
54 sites in the DM1 locus was first investigated by Shaw and collaborators (1993), who

55 could not find any clear correlation between the methylation and the age of onset or the  
56 severity of the disease. On the other hand, Steinbach and coworkers found that several  
57 CpG sites close to the CTG repeat were fully methylated in blood samples containing  
58 expansions larger than 1000 repeats. They also reported that, since these expansions  
59 appeared as single well-defined signals on Southern blot, the methylation of these CpG  
60 sites would correlate with triplet repeat stability. This idea was later supported by *in vitro*  
61 and *in silico* work (Brock et al., 1999, Gorbunova et al., 2004, Gourdon et al., 1997,  
62 Nichol and Pearson, 2002). The work of Filippova built further on the idea that  
63 methylation would be related to the pathogenesis of congenital DM1 (CMD). The authors  
64 discovered that there are two binding sites for the zinc-finger protein CTCF flanking the  
65 CTG repeat, and that the hypermethylation of these sequences, which coincide with the  
66 region described by Steinbach and collaborators (1998), would be incompatible with the  
67 binding of the CTCF and its proper function mediating the inhibition of promoter-  
68 enhancer interactions by insulator elements (Bell et al., 1999). Conversely, Libby and  
69 collaborators (2008) found that CpG methylation of CTCF binding sites may rather lead to  
70 repeat instability, instead of stability as previously suggested.

71 In this work we studied 8 of the 18 CpG sites in 152 nucleotides upstream of the CTG  
72 repeat of the DM1 locus (see figure 1). These sites are part of the CpG sites investigated  
73 by Steinbach and collaborators and did not include the sites affecting the CTCF binding  
74 (Filippova et al., 2001 ). We did not investigate the distal SacII site, located over 1 Kb  
75 upstream of the CTG repeat, which Steinbach and collaborators reported as constitutively  
76 methylated, nor the HpaII and HhaI sites located in this same region. We analysed our  
77 samples in duplicate by methylation sensitive endonuclease digestion, followed by PCR.  
78 The PCRs were designed so that they included both the restriction site and the CTG  
79 repeat and were performed on small quantities of DNA. Consequently, smears could be  
80 resolved into individual bands, and sizing was much more accurate than by using  
81 Southern blot on total genomic DNA. Furthermore, it enabled us to correlate the  
82 methylation status with the exact length of each allele, which would not have been  
83 possible with Southern blot on total genomic DNA or bisulphite sequencing. It is

84 important to notice that in the case of the HpaII restriction enzyme, the studied region  
85 contained 6 sites. This means that we could not distinguish the methylation state of each  
86 independent site, but could only asses whether at least one of the sites was  
87 unmethylated.

88 The aim of this study was to establish the threshold for the expansion size necessary for  
89 the hypermethylation of the CpG sites close to the CTG repeat and this within one DNA  
90 sample. We also aimed to investigate the possibility that hypermethylation is patient- or  
91 allele specific.

92 DNA samples from peripheral blood from 22 DM1 patients were digested with the  
93 methylation-sensitive restriction enzymes SacII, HhaI and HpaII (Biolabs, Westburg,  
94 Leusden, The Netherlands). Both digested and undigested samples were analysed by  
95 PCR. In the case of the SacII digested samples, we used the primer set indicated in  
96 figure 1 as SacII primer and reverse primer. The DNA input was of 200 pg. For the HhaI  
97 and HpaII samples, we used the HhaI-HpaII and the reverse primers. Initially, the DNA  
98 input was of 200 pg, but after the first results, DNA input was increased to 2 ng to  
99 ensure the detection of possible uncleaved alleles present at a very low quantity. The  
100 PCR protocol was further performed as previously described (De Temmerman et al.,  
101 2008). The expansions were visualized by denaturing Southern blot using a GAC probe,  
102 and the size calculated by comparison to two molecular weight markers (markers VI and  
103 VII, Roche diagnostics, Vilvoorde, Belgium).

104 Table 1 shows the results for the 22 DM1 patients included in this study. The table  
105 outlines the results of the PCR before and after endonuclease cleavage by SacII, along  
106 with the disease status and inheritance of the mutation in all those patients for which  
107 these data were known. The cohort of patients includes cases of congenital DM1, infantile  
108 and classic DM1, both paternally and maternally inherited. The age at sampling ranges  
109 from birth to 43 years, and expansions were between 180 to 2800 repeats. Figure 2  
110 shows an example of the Southern blot results after SacII restriction enzyme cleavage  
111 and PCR. The results for HhaII and HpaII cleavage, and the complete data for the SacII  
112 digestion can be found in the supplementary figures 1, 2 and 3.

113 After HhaI endonuclease cleavage, none of the DNA samples showed amplification,  
114 whereas the undigested samples worked as a positive control for the PCR. This meant  
115 that all alleles were unmethylated, both the wild type alleles as well as all of the  
116 expansions. We verified that the lack of amplification in the digested samples was not  
117 due to DNA degradation by amplifying the material with a PCR for 4 short tandem  
118 repeats located on chromosome 19q13.41, 19q13.33, 19p13.3 and 19p13.2 (tandem  
119 repeats located using the database [www.microsatellites.org](http://www.microsatellites.org)). The primer sequences and  
120 PCR conditions are available upon request. The results showed that the DNA was intact in  
121 all samples, and proved that the restriction by HhaI had been specific for its target.  
122 These results suggest that, contrary to the findings of Steinbach and collaborators, not  
123 only is this CpG site constitutively unmethylated, but also that it is not necessarily  
124 methylated in expansions larger than 1000 repeats or in congenital DM1 patients.

125 Identical results were obtained after cleavage with HpaII. Both the wild type and the  
126 expanded alleles were unmethylated (for at least one of the six HpaII sites in the  
127 sequence) in all the samples studied. These results are in concordance with the findings  
128 of Shaw and collaborators (1993) and contradict those of Steinbach and co-workers  
129 (1998).

130 From our results it seems that of the CpG sites we studied the SacII site immediately  
131 upstream of the repeat is the only one in the region that can be differentially methylated  
132 between wild type alleles, which are never methylated, and expanded alleles, which may  
133 be methylated. Our data are difficult to harmonize with the previously published study  
134 (Steinbach et al., 1998). We did not find a correlation between the methylation status of  
135 this site on one hand and the allele size or on the other hand with the presence of a  
136 smear on the Southern blot -indicative of triplet repeat instability- , as was suggested by  
137 Steinbach et al. Alleles as small as 400 repeats could be methylated (patient 20), and  
138 alleles of 1600 repeats unmethylated (patient 8), indicating that there is no correlation  
139 between the allele size and the methylation status. We found several patients showing  
140 intermediate methylation patterns (some alleles were methylated while others were not),  
141 and even patients without any methylation at this site. A patient could present a smear

142 in which all alleles appeared to be methylated (i.e. patient 7), indicating that the  
143 methylation status apparently does not correlate with the stability of the repeat.  
144 However, it is clear that the presence of CTG length mosaicism either with or without  
145 methylation does not necessarily make one the cause or effect of the other. The time  
146 during which one occurred may or may not have coincided with the other. Furthermore,  
147 the link between methylation of this site and congenital DM1 does not seem to be as  
148 strict as previously assumed. It is true that both congenital DM1 cases analysed here  
149 showed methylation of the SacII site in all the expanded CTG alleles (patients 4 and 6),  
150 but non-congenital DM1 patients also presented it (patients 5 and 7). Furthermore, we  
151 did not find the correlation previously described (Steinbach *et al.*, 1998) between the  
152 methylation of the SacII site and of the HhaI and HpaII sites, since we never found  
153 methylation in the latter two, not even in the congenital DM1 patients. The fact that we  
154 did not find a methylation pattern specific to the CMD patients contradicts the hypothesis  
155 that differential methylation may be responsible for the distinct features of CMD by  
156 modulating the binding of CTCF in the DM1 locus (Flippova et al., 2001). It is, however,  
157 important to bear in mind that we did not study the methylation of all the CpG sites  
158 present in the CTCF binding sites, and that methylation may still play an important role in  
159 the pathogenesis of DM1.

160 These discordances do not seem attributable to incomplete restriction or technical  
161 differences. In the works of Shaw et al. (1993) and Steinbach et al. (1998), as in ours,  
162 incomplete restriction would have been detected by the incomplete cleavage of the wild  
163 type allele. To ensure that the results for HpaII and HhaI were not due to the  
164 degradation of the samples after restriction, we confirmed the presence of intact DNA by  
165 PCR for microsatellite markers. The fact that in our work we analysed PCR products by  
166 Southern blot, whereas Shaw et al. (1993) and Steinbach et al. (1998) performed direct  
167 Southern blotting of the restricted DNA only adds to the resolution of the expanded  
168 alleles. Furthermore, it is possible that our method is more sensitive in picking up a few  
169 unrestricted alleles. Nevertheless, for the HhaI and HpaII digests, we never found any  
170 uncleaved allele.

171 In the work of Shaw et al (1993) it is suggested that the patient's age might play a role  
172 on the methylation levels in CMD. The authors suggest that, since their CMD patients  
173 were rather of mature age, the methylation could have been lost. It is important to bear  
174 in mind that these authors were investigating a possible relationship between imprinting  
175 of the DM1 locus and CMD, and hypothesized that this imprinting would only be of  
176 importance *in utero*, and would be subsequently lost after birth. In Steinbach's paper,  
177 the oldest CMD patient is 24 years, and shows full methylation of the expanded allele for  
178 all the SacII, HhaI and HpaII sites. Shaw et al. (1993) did not mention the exact age of  
179 the patients, but if they were older than 24 years, their hypothesis would harmonize the  
180 difference in results between Shaw and Steinbach's papers, but not with ours, where the  
181 two CMD patients were very young, and showed no methylation for the HhaI and HpaII  
182 sites. An age effect on the classical DM1 patients can be ruled out, as both Steinbach's  
183 work as ours has patients of all ages (Steinbach's range was 19-62, ours 4-45 years).  
184 Furthermore, there does not seem to be a parent of origin effect.

185 In conclusion, we have studied the methylation status of the proximal sequence  
186 upstream of the CTG repeat of the DM1 locus. From our results, we establish that,  
187 contrary to previous findings, only one of the studied sites is differentially methylated in  
188 wild type and expanded CTG alleles, and that this methylation does not strictly correlate  
189 with the length of the allele, nor with the disease status of the patient. Further research  
190 on this topic is necessary to elucidate the reasons for the discrepancy with some of the  
191 previously published results.

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198



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206 Competing interests

207 None declared

208

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 267

## 268 **FIGURE LEGENDS**

269 **Figure 1.** Schematic overview of the sequence analyzed in this study and its  
 270 relationship to the genomic regions studied by Shaw et al (1993) and Steinbach et al  
 271 (1998). The figures are modified versions of the original figures found in the respective  
 272 publications. A: Genomic region studied by Shaw et al.(1993). Three fragments were  
 273 created using EcoRI and EcoRV, and then restricted by HpaII. The results showed that at  
 274 least some of the HpaII sites contained unmethylated cytosines, independently of the  
 275 form of the disease or expansion size. B: Genomic region studied by Steinbach et al.  
 276 (1998). The samples were restricted using combinations of SacI and HindIII, and SacII,  
 277 HpaII or HhaI. The results showed that the upstream SacII site was constitutively  
 278 methylated, and the downstream SacII site only methylated on very largely expanded  
 279 alleles of patients with congenital DM1. The HpaII and HhaI sites were also all

280 methyated in these patients. C: Sequence studied in this paper. Genomic DNA was  
281 restricted using SacII, HpaII or HhaI and amplified by PCR using the primers indicated on  
282 the sequence as arrows. The highlighted sequence corresponds to the CTCF binding site  
283 1 (Fillippova et al., 2001). The results showed no correlation between the methylation of  
284 the SacII site and the disease status of the patient or the expansion size. No methylation  
285 of the HhaI site was detected, and the results for HpaII suggested that there was at least  
286 always one unmethylated site present in the sequence.

287 **Figure 2.** Example of the Southern blot results after SacII restriction enzyme cleavage  
288 and PCR. Lanes 1, 2, 3, 4 and 5 show restricted samples, lanes 1', 2', 3', 4' and 5' are  
289 the same DNA samples, but unrestricted. These numbers correspond also to the patient  
290 identification numbers found in table 1. The lane marked with MWM contains the  
291 molecular weight marker VII (Roche Diagnostics, Vilvoorde, Belgium). The lane numbers  
292 correspond to the patient numbers. Unrestricted samples still contain the wild type allele  
293 (wt), whereas in the restricted samples, the wt is completely cleaved. This can be used  
294 as an internal restriction control.

295 **Supplementary figures 1, 2 and 3.** Raw data of the Southern blot analysis of the  
296 PCRs after restriction enzyme digestion of the patient's DNA samples. Figure 1 shows the  
297 results for the SacII cleavage, figure 2 for the HpaII cleavage, and figure 3 for the HhaI  
298 cleavage. All Southern blots contain the PCR products of restricted and unrestricted DNA  
299 samples, for comparison and as PCR control. The lanes are labeled with the patient's  
300 identification numbers. When the number is followed by a 'd', it is a digested sample. All  
301 blots show one or two molecular weight markers. On the left side, molecular weight  
302 marker VI is shown, on the right, marker VII of Roche Diagnostics (Vilvoorde, Belgium).  
303 Some samples have been analyzed in duplicate on the same blot, and in some cases a  
304 replicate can be found on another blot. In the case of the HpaII and HhaI cleavage, the  
305 blots show smears in the control lanes because the PCRs were performed with a higher  
306 input to ensure the detection of possible uncleaved alleles present in a low quantity.  
307 Nevertheless, none of the digested samples showed any amplification, revealing that  
308 they all contained unmethylated sites. The signal at the lower end of the blot corresponds

309 to the wild type allele. Its full cleavage serves as an internal control for the digestion, as  
310 it is assumed to be always unmethylated. In some lanes, heteroduplexes can be seen as  
311 a signal between the wild type and the expanded allele.  
312

## 313 TABLE

**Table 1.** Details of the studied patients and results after SacII cleavage

Patient	Disease status and origin of mutation	Age at sampling	Expansion size (repeats)	Description of the expansion	Alleles after SacII digestion	Description of the alleles after SacII digestion
1	DM, paternal	33	900-2800	Numerous discrete bands	900-2800	Numerous discrete bands: full methylation
2	Unknown	30	400-1200	Smear	Fully cleaved	Fully unmethylated
3	DM, paternal	33	200-400	Two groups of very close bands	Fully cleaved	Fully unmethylated
4	CMD, probably maternal	3 months	1400	One band	1400	One band: full methylation
5	DM, paternal	41	300-900	Two groups of very close bands	300-900	Two groups of very close bands: full methylation
6	CMD, maternal	Newborn	700-1300	Smear with predominant band at 1300	700-1300	Smear with predominant band at 1300: full methylation
7	Unknown	4	700-1300	Smear with predominant band at 1300	700-1300	Smear with predominant band at 1300: full methylation
8	DM, maternal	20	700-1600	Smear with predominant allele at 1600	860	One band: partial methylation
9	Unknown	Unknown	200-500	Smear	Fully cleaved	Fully unmethylated
10	DM, paternal	33	700-1600	Smear with predominant allele at 1600	700-1160	Bands of 700, 990, 840 and 1160: partial methylation
11	DM, maternal	30	600-1200	Smear	680-1170	Bands of 680 and 1170: partial methylation
12	DM, paternal	24	200-480	Smear	Fully cleaved	Fully unmethylated
13	DM, probably maternal	33	200-480	Smear	Fully cleaved	Fully unmethylated
14	DM, maternal	27	680-900	Smear	680-860	Bands of 680 and 860: partial methylation
15	DM, paternal	45	500-1300	Smear	840-990	Bands of 840 and 990: partial methylation
16	DM, paternal	38	500-1300	Smear	Fully cleaved	Fully unmethylated
17	Unknown	43	500-1650	Smear	1300	One band: partial methylation
18	Unknown	30	220-370	Smear	Fully cleaved	Fully unmethylated
19	DM, maternal	29	250-1400	Smear	850-1000	Bands of 850 and 1000: partial methylation
20	DM	Unknown	180-2200	Smear	400-1700	discrete bands of 400, 900 and 1700: partial methylation
21	Unknown	Unknown	300-2600	Smear	600-2600	discrete bands of 600, 950, 1200, 1500, 1600 and 2600: partial methylation
22	Infantile form, maternal inheritance	14	1000-2600	Smear	1300	One band: partial methylation

DM: disease onset is not congenital, CMD: disease onset is congenital.

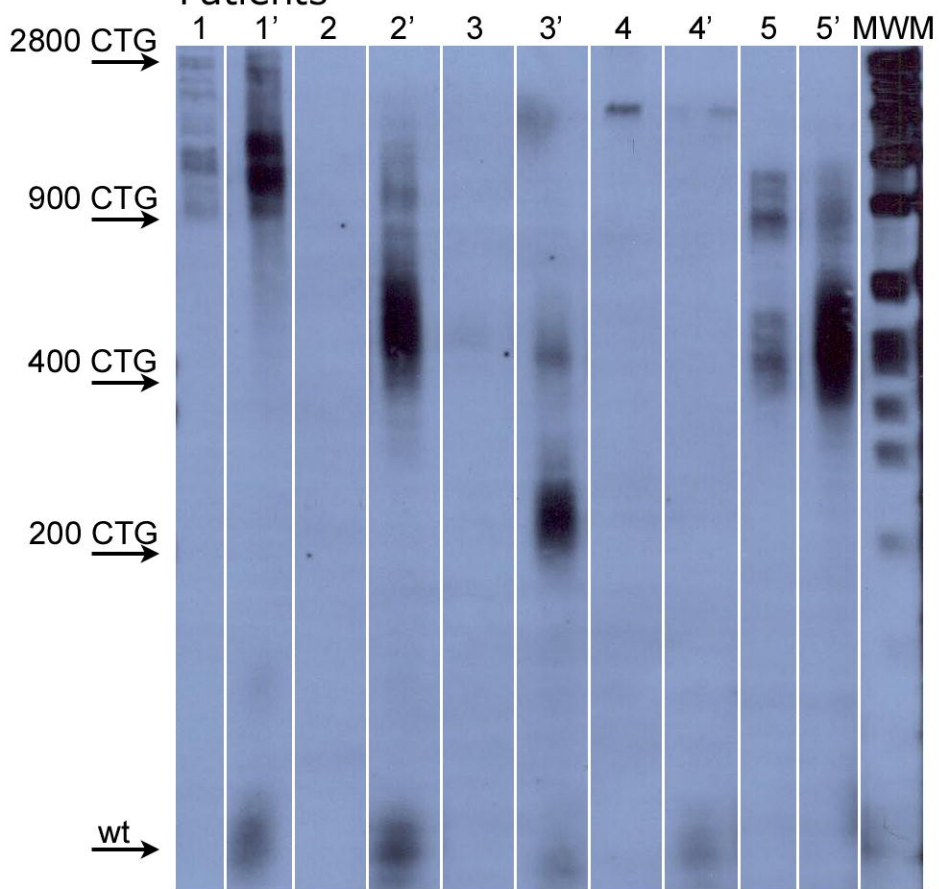
Full methylation indicates that all alleles appeared to carry a methylated SacII site.

314

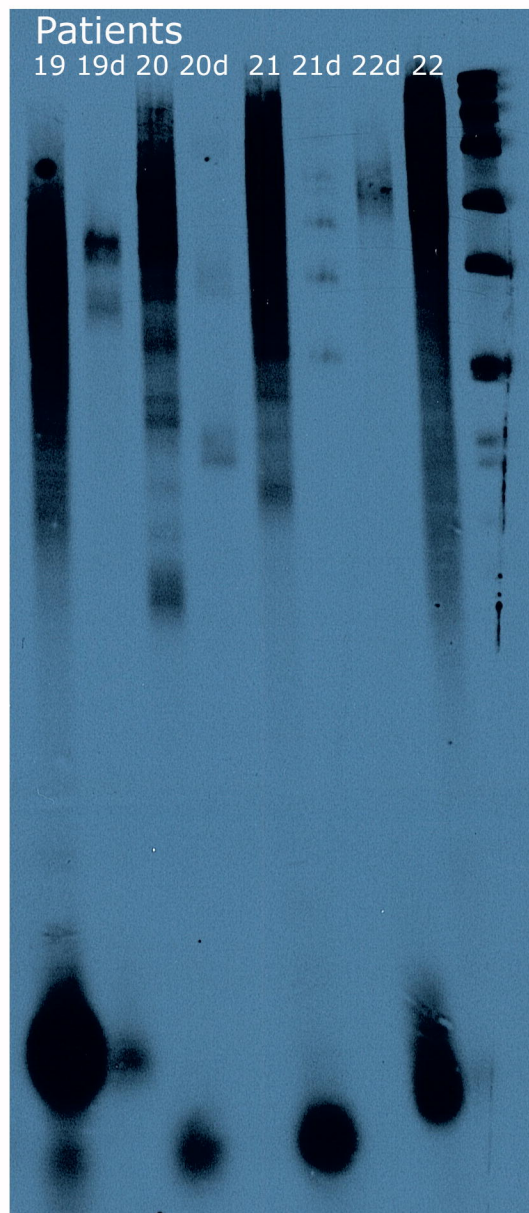
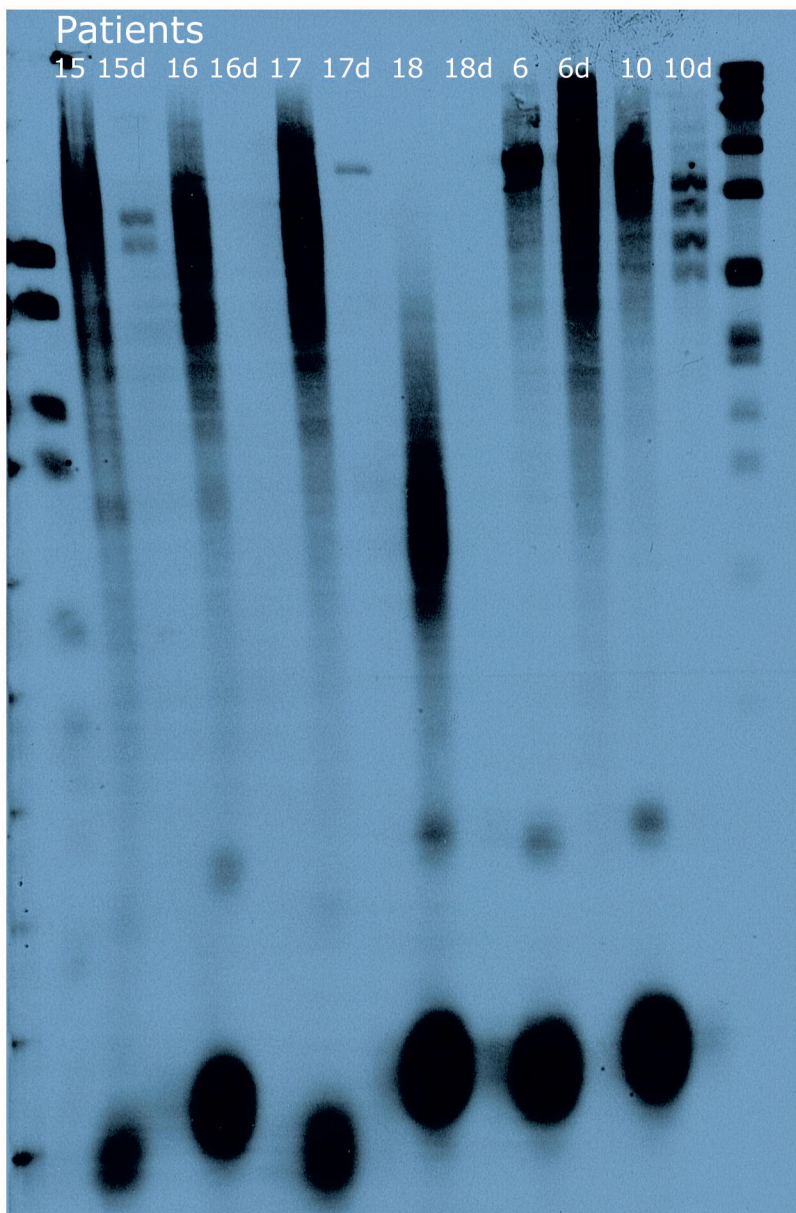
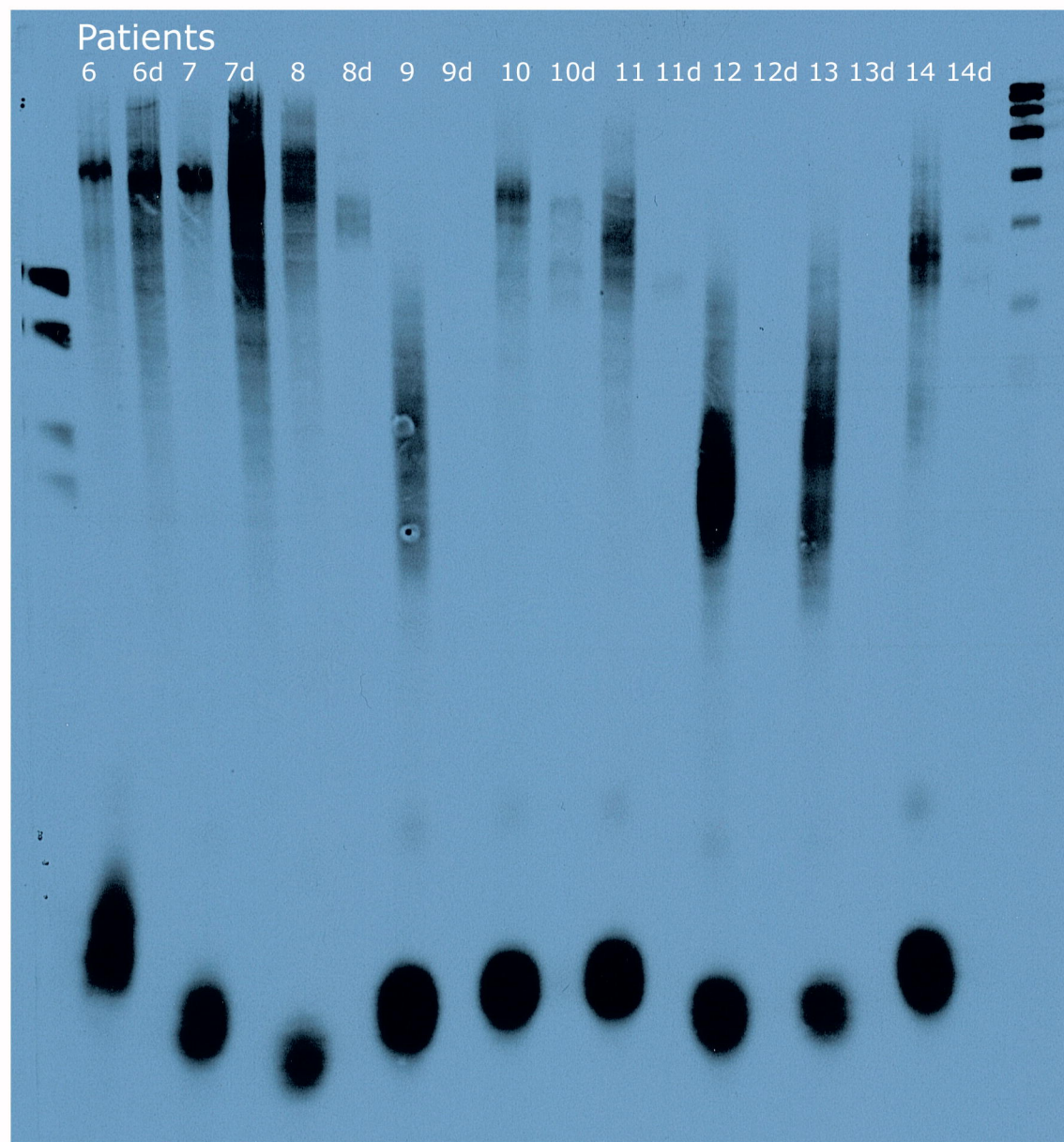
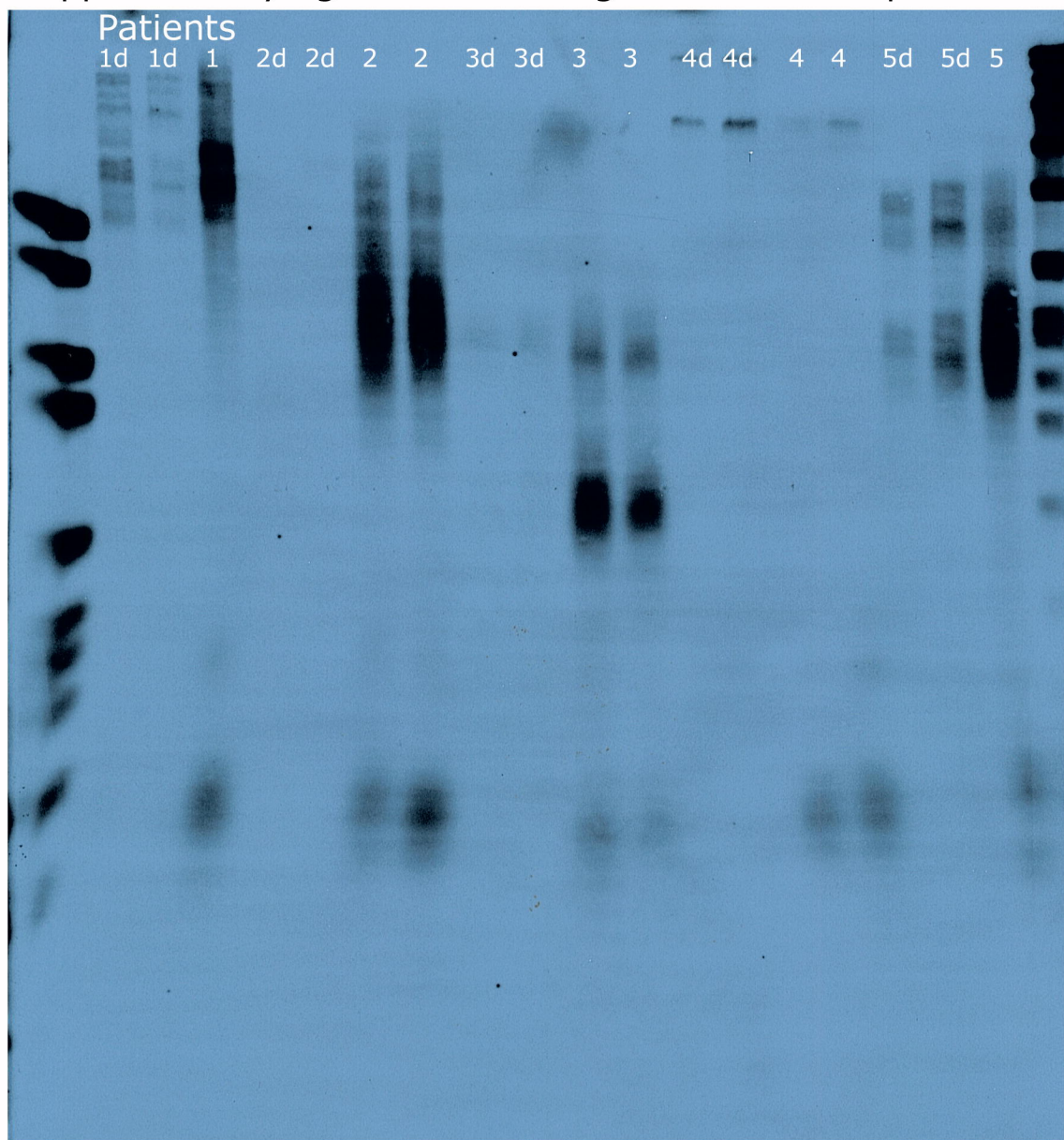
315



Patients

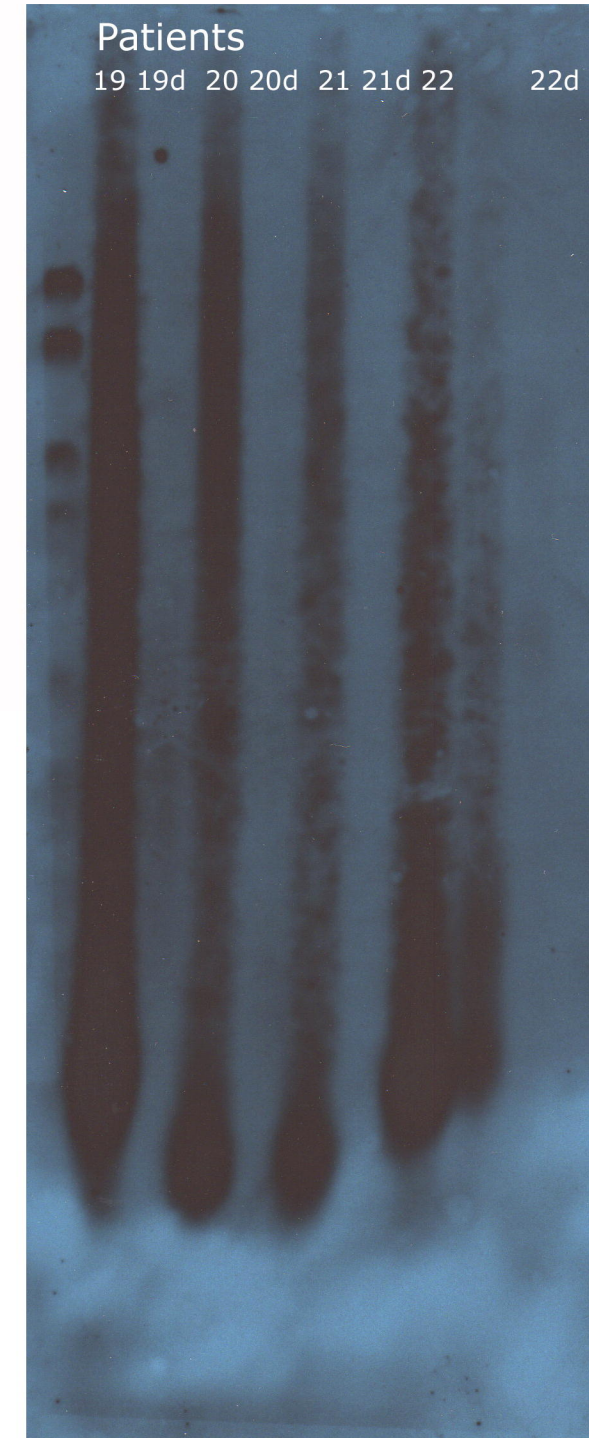
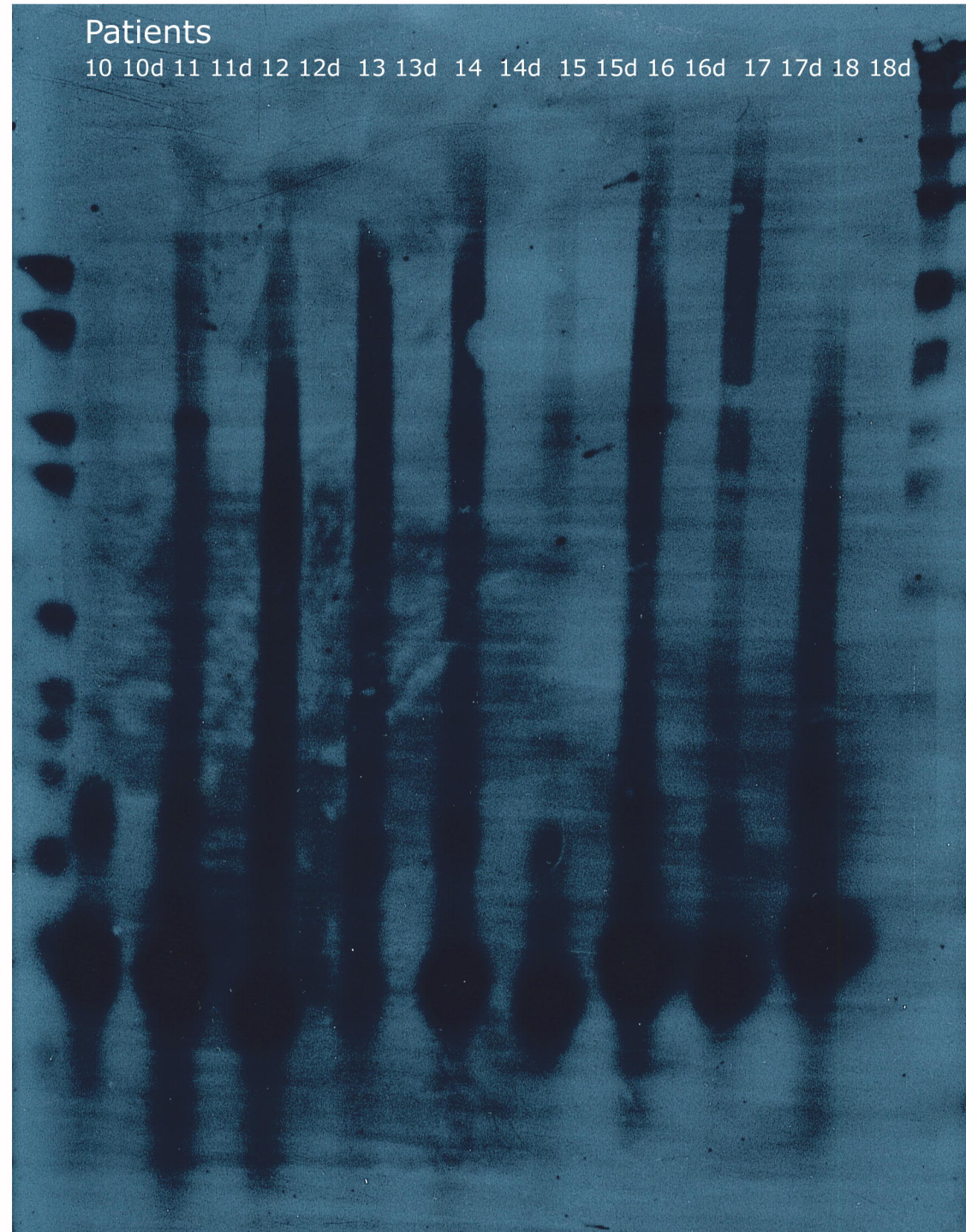
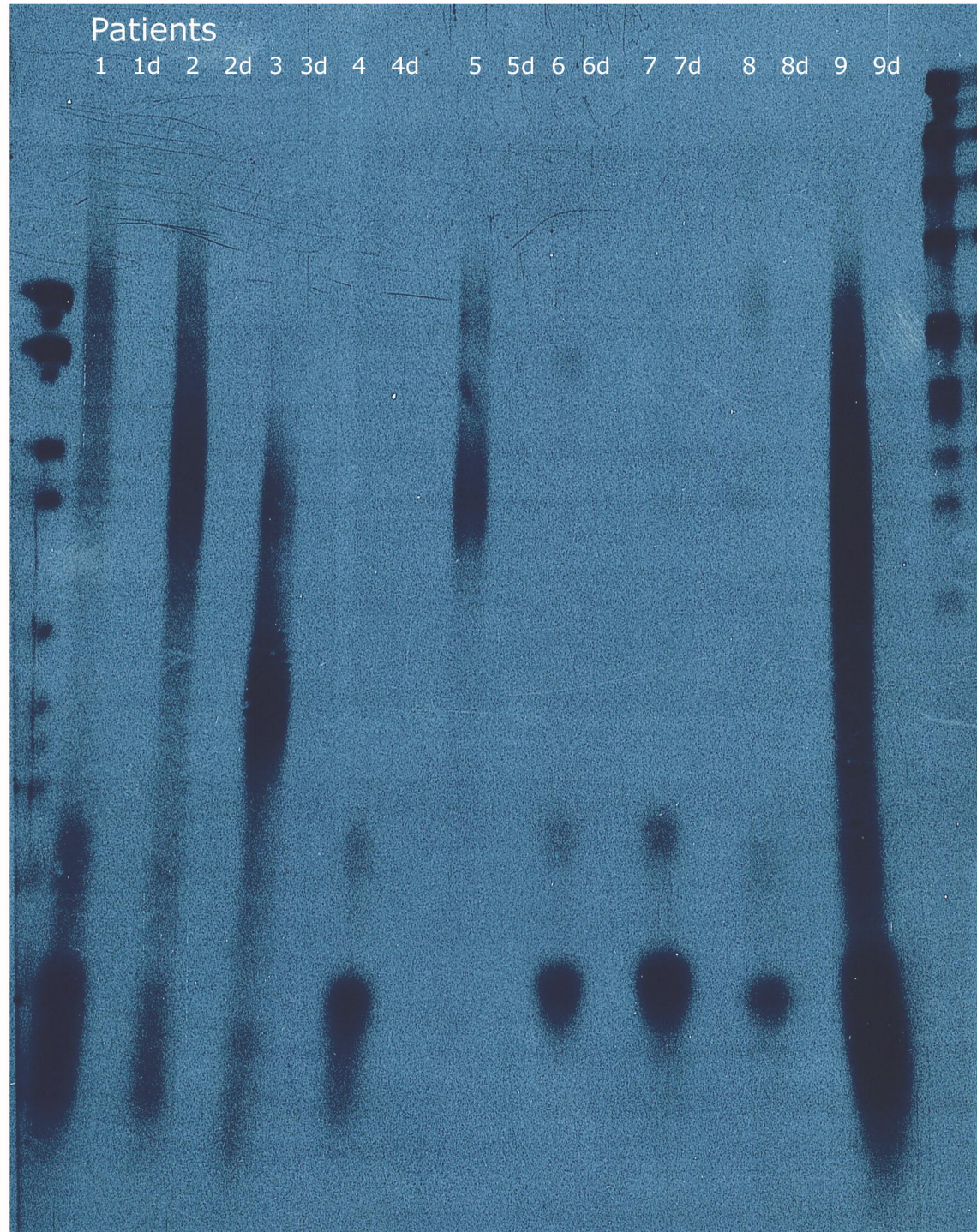


Supplementary figure 1: SacII digested DNA samples.





Supplementary figure 2: HpaII digested DNA samples.



Supplementary figure 3: HhaI digested DNA samples.

