

Neurofunctional and neuroimaging readouts for designing a preclinical stem-cell therapy trial in experimental stroke

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1 **Neurofunctional and neuroimaging readouts for designing a preclinical**
2 **stem-cell therapy trial in experimental stroke**

3 14 words (< 20 words)

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28 **Key words:** ischemic stroke; tMCAO; diffusion tensor imaging; sensorimotor deficits; internal
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30

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33

34 **Abstract**

35 With the aim of designing a preclinical study evaluating an intracerebral cell-based therapy for
36 stroke, an observational study was performed in the rat suture model of ischemic stroke.
37 Objectives were threefold: (i) to characterize neurofunctional and imaging readouts in the first
38 weeks following transient ischemic stroke, according to lesion subtype (hypothalamic, striatal,
39 corticostriatal); (ii) to confirm that intracerebral administration does not negatively impact these
40 readouts; and (iii) to calculate sample sizes for a future therapeutic trial using these readouts as
41 endpoints. Our results suggested that the most relevant endpoints were side bias (staircase test)
42 and axial diffusivity (AD) (diffusion tensor imaging). Hypothalamic-only lesions did not affect
43 those parameters, which were close to normal. Side bias in striatal lesions reached near-normal
44 levels within 2 weeks, while rats with corticostriatal lesions remained impaired until week 14.
45 AD values were decreased at 4 days and increased at 5 weeks post-surgery, with a subtype
46 gradient: hypothalamic < striatal < corticostriatal. Intracerebral administration did not impact
47 these readouts. After sample size calculation (18-147 rats per group according to the endpoint
48 considered), we conclude that a therapeutic trial based on both readouts would be feasible only
49 in the framework of a multicenter trial.

50

51 197 words (< 200 words)

52 **Introduction**

53 Ischemic stroke is a leading cause of mortality and disability worldwide (1). To date, the only
54 therapeutic option is to reopen the occluded artery mechanically and/or pharmacologically. This
55 option is applicable only in the acute phase for selected patients. In case of persisting disability,
56 there is no treatment in the chronic phase to restore function, except rehabilitation.

57 Stem-cell therapy is a promising therapeutic option to restore function in the chronic phase of
58 ischemic stroke (2, 3). Mesenchymal stem cells are of major interest due to their low
59 immunogenicity profile, good availability and absence of ethical concerns. These pluripotent
60 cells have the capacity to differentiate into different cell types but their use is mainly based on
61 their immunoregulatory properties. Adipose mesenchymal stem cells (ASCs) are the more
62 accessible source compared to bone marrow mesenchymal stem cell (4, 5). Human adipose-
63 derived mesenchymal stem cells (hASCs) are already used in stroke clinical trials
64 ([NCT03570450](#))(6). Intracerebral injection has been shown to be the most efficient route in
65 terms of preclinical treatment efficacy, due to the direct delivery of stem cells (7). Recent phase
66 0/1 clinical trials have also reported the safety of this administration route in patients in the
67 chronic stage of stroke (6, 8, 9). In this context, our global aim was to design a preclinical study
68 to evaluate the effects of intracerebral administration of clinical-grade hASCs in ischemic
69 stroke, with a study design that aligns with clinical functional evaluation methods for long-term
70 recovery studies (2, 10).

71 As is well-known, there are several obstacles to the translation of stem-cell research in ischemic
72 stroke from the preclinical to the clinical arena. The rigor of study design, the inclusion of
73 different stroke subtypes, the choice of appropriate primary readout parameters and well-
74 defined sample sizes have been identified as key factors to overcome the translational roadblock
75 (10-14). The assessment of neurofunctional outcome in chronic stroke patients relies on clinical
76 scores such as the National Institutes of Health Stroke Scale (NIHSS) (6) and the upper limb

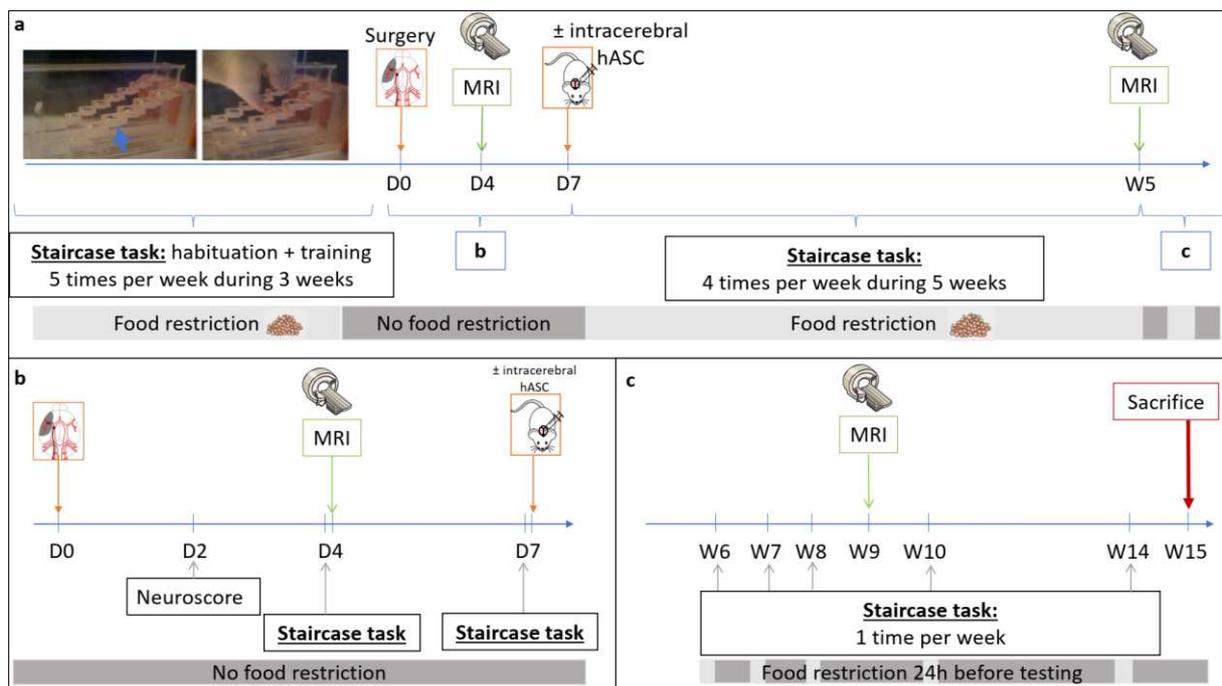
77 movement section of the Fugl-Meyer (FM) scale (15, 16). Combining clinical scores with the
78 assessment of ipsilesional corticospinal tract (CST) remodeling with diffusion tensor imaging
79 (DTI) can improve prediction of motor outcome (16-19). Accordingly, our preclinical stem-cell
80 trial aimed at combining neuroscores and the staircase test, a skilled reaching task that assesses
81 forelimb function in rodent models (11), with the DTI evaluation of CST structural integrity
82 (internal capsule).

83 Ideally, a rigorous study design implies to thoroughly investigate these endpoints according to
84 stroke subtype, in order to determine the optimal frequency of measurements, the post-stroke
85 period during which data should be monitored, the quantitative modifications of readouts in
86 time, and the within-laboratory variances. The specific aims of the present observational study
87 with a limited number of subjects were threefold: (i) to characterize neurofunctional readouts
88 (neuroscores and staircase test) and DTI metrics in the first weeks following transient middle
89 cerebral artery occlusion (tMCAO) according to stroke subtype; (ii) to confirm that
90 intracerebral administration of hASCs does not negatively impact these readouts (because of
91 the invasiveness of the procedure); and (iii) to determine the most appropriate functional and
92 imaging endpoints, at which time-points they should be evaluated, and to calculate the sample
93 size required to achieve statistically significant differences with these endpoints for a preclinical
94 exploratory therapeutic trial.

95 **Results**

96 Figure 1 shows the experimental design of the study. Briefly, after a 3-week period of training
97 at the staircase task, transient (60 minutes) middle cerebral artery occlusion was performed at
98 day 0 (D0) (Fig. 1A). Neuroscores were obtained at D2 post-surgery (Fig. 1B). The staircase
99 test was then performed at D4 (before baseline MRI) and D7 (before treatment administration).
100 Baseline MRI, including T2-weighted imaging (T2WI) and diffusion tensor imaging (DTI)
101 sequences, was performed at D4 post-surgery (Fig. 1B). Cerebral lesions were stratified

102 into 3 subtypes according to their location on baseline MRI: corticostriatal, striatal or
 103 hypothalamic-only (20). Half of the rats received clinical-grade hASCs intracerebrally at D7.
 104 All rats were then monitored for 5 weeks with longitudinal neurofunctional tests and MRI. By
 105 this time, most rats had completely recovered according to neurofunctional testing, except those
 106 with corticostriatal lesions; for these rats, follow-up was extended to week 14 (W14) (Figure
 107 1C).



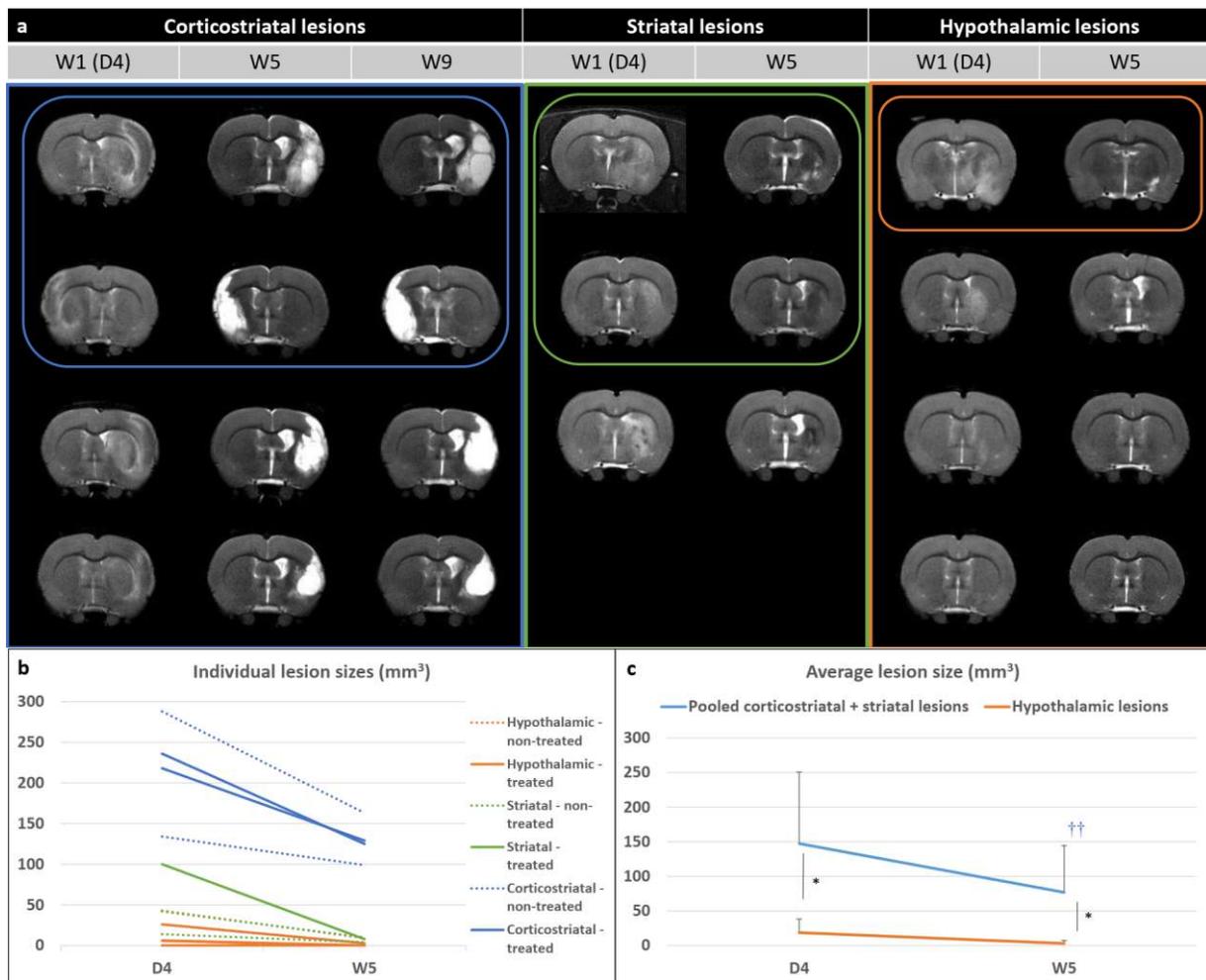
108 **Figure 1- Study design. a.** Experimental design; **b.** Focus on the first week of the experiment;
 109 **c.** Focus on weeks 6 to 15: extended follow-up only for rats with corticostriatal lesions. D:
 110 Days, MRI: magnetic resonance imaging, hASC: Human adipose mesenchymal stem cells, W:
 111 Weeks.
 112

113 **Stroke subtypes**

114 Supplementary Fig. 1 presents the CONSORT-like chart of the study. Of the 25 rats trained at
 115 the staircase test, 18 matched the inclusion criteria and were thus selected to undergo surgery.
 116 Seven rats died before the end of the experiment: 2 during the surgical procedure, 4 in the first
 117 24h and 1 in the first 48h post-surgery, the 5 latter probably due to malignant edema. Of the 11
 118 rats included in the study, 4 had a corticostriatal lesion, 3 a striatal lesion and 4 a hypothalamic

119 lesion (Fig. 2, Supplementary Fig. 1). Five animals received intracerebral hASC treatment (Fig.
 120 2, Supplementary Fig. 1).

121



122

123 **Figure 2- Evaluation of lesions on T2-weighted imaging. a.** Longitudinal T2-weighted
 124 imaging of all included rats according to lesion subtype (only one central slice is shown).
 125 Treated rats (that received intracerebral administration of hASCs) are presented in top rows and
 126 circled. **b.** Individual lesion sizes are presented according to lesion subtype (striatal,
 127 corticostriatal and hypothalamic lesions) and treatment group (plain line: treated; dashed line:
 128 non-treated) at day 4 (D4) and week 5 (W5) post-surgery. **c.** Average lesion sizes are presented
 129 according to lesion subtype: pooled (corticostriatal+striatal) vs hypothalamic lesions. Data are
 130 displayed as mean \pm SD. W: weeks, D: days; * $p < 0.05$, (corticostriatal + striatal) vs
 131 hypothalamic, Wilcoxon-Mann-Whitney test; † $p < 0.05$, †† $p < 0.01$, W5 vs D4, Friedman test.

132 **Neurofunctional readouts**

133 *Staircase test*

134 Figure 3A presents individual results for side bias according to lesion subtype and treatment
 135 group. Rats were slightly lateralized before surgery (side bias: 66% [56 ; 66%]). Side bias in
 136 the hypothalamic-only lesion group was maintained around 50% (i.e., no difference between

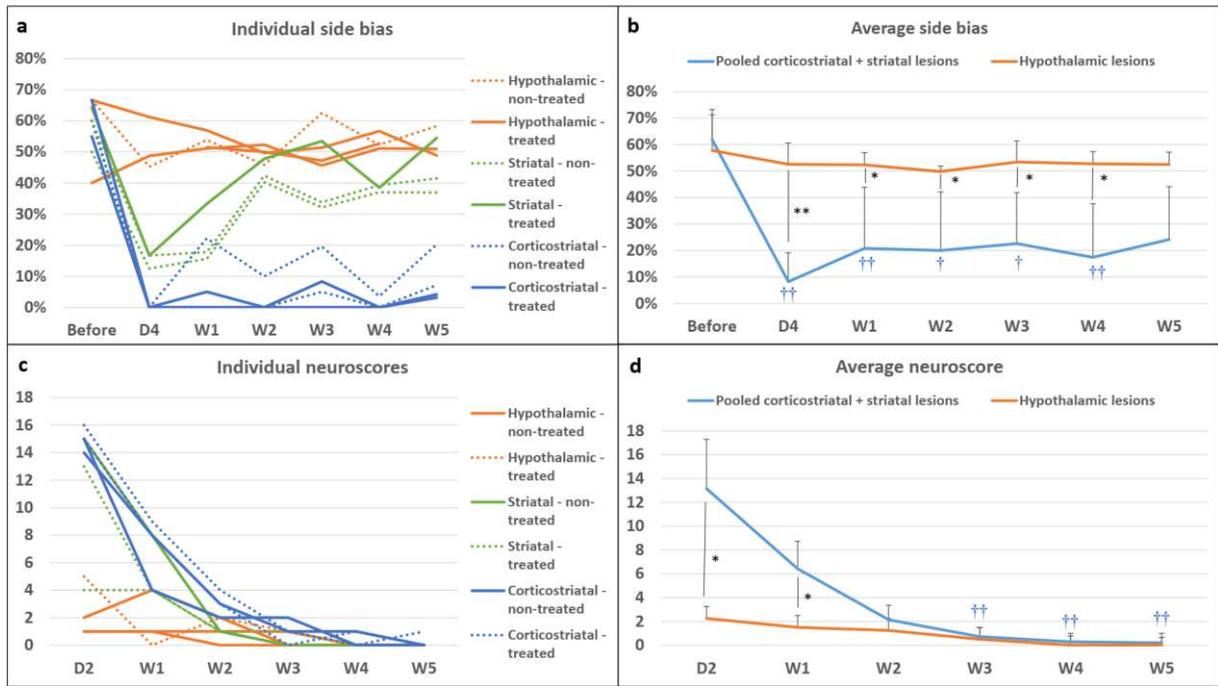
137 right and left paw performances) right after surgery and until the end of testing. In rats with
138 striatal and corticostriatal lesions, side bias was severely increased in the first days after stroke
139 (i.e., marked difference in favor of the ipsilateral paw).

140 In rats with striatal and corticostriatal lesions, side bias was severely decreased in the first days
141 after stroke (*i.e.* marked difference between the contralateral and ipsilateral paw, in favor of the
142 ipsilateral one), with a progressive recovery afterwards, until reaching a plateau at W2. Rats
143 with striatal lesions recovered nearly to the level of rats with hypothalamic lesions at W2, while
144 rats with corticostriatal lesions remained severely impaired until W5 W5 (Fig. 3A; W5: 6%
145 [4% ; 10%] for corticostriatal lesions vs 41% [39% ; 48%] for striatal lesions and 51% [50% ;
146 55%] for hypothalamic-only lesions). Staircase performance remained low until W14 in rats
147 with corticostriatal lesions (Supplementary Fig. 2). Intracerebral administration of hASCs did
148 not negatively impact staircase test performance (Table 1).

149 Because hypothalamic lesion did not have a neurofunctional impact on the staircase test, rats
150 with hypothalamic-only lesions were assimilated to sham-like animals, while rats with striatal
151 and corticostriatal lesions were considered as tMCAO rats and pooled for performing statistical
152 analysis between 2 groups (N=7 (corticostriatal + striatal) lesions vs N=4 hypothalamic-only
153 lesions). There was no difference in side bias across time in the hypothalamic-only lesion group
154 (Friedman test – $p=0.8291$), contrary to the pooled (corticostriatal + striatal) lesion group
155 (Friedman test – $p=7.266e-05$). Post-surgery side biases were statistically lower from pre-
156 surgery ones, except at W5 (post-hoc Conover test – D4: $p=0.0002$; W1: 0.0090; W2: 0.0249;
157 W3: 0.0357; W4: 0.0080; W5: 0.1154). Side bias was statistically lower in pooled
158 (corticostriatal + striatal) lesion group than in hypothalamic-only lesion group at all time-points,
159 except before surgery (Fig. 3B; Wilcoxon-Mann-Whitney test – Before surgery: $p=1$; D4:
160 $p=0.0088$; W1: $p=0.0105$; W2: $p=0.0171$; W3: $p=0.0424$; W4: $p=0.0100$; W5: 0.0424).

161 *Neuroscores*

162 Figures 3C and 3D present individual and averaged neuroscores according to lesion subtype
163 and treatment group. Intracerebral administration of hASCs did not aggravate neuroscores
164 (Table 1). At D2, neuroscores were in the same order of magnitude in rats with striatal lesion
165 (13 [9 ; 14]) and rats with corticostriatal lesion (15 [15 ; 15]), while rats with hypothalamic-
166 only lesion had much lower neuroscores (2 [1 ; 3]) (Fig. 3C), thus confirming our previous
167 observations with regard to rats with hypothalamic-only lesions behaving as sham-like animals.
168 There was a significant difference in neuroscores across time in the hypothalamic-only lesion
169 group (Friedman test – $p=0.02727$); however, none of the Conover post-hoc test were
170 significant. In the pooled (corticostriatal + striatal) lesion group, the difference in neuroscores
171 across time was statistically different (Friedman test – $p=4.897e-06$), with W3, W4 and W5
172 neuroscores that were statistically lower than D2 neuroscores (Conover post-hoc test – W1:
173 $p=0.3998$; W2: 0.0955; W3: 0.0055; W4: 0.0015; W5: 0.0011). There was a statistically
174 significant difference between the 2 groups at D2 that was attenuated but maintained at W1
175 (Fig. 3D; Wilcoxon-Mann-Whitney test – D2: $p=0.0168$; W1: $p=0.0188$). Starting at W2,
176 neuroscores were no longer significantly different between groups, as all neuroscores had
177 reached sham-like levels (Fig. 3D; Wilcoxon-Mann-Whitney test – W2: $p=0.3276$; W3:
178 $p=0.754$; W4: $p=0.3241$; W5: $p=0.5708$).



179

180 **Figure 3- Neurofunctional readouts. a.** Individual side bias according to lesion subtype and
 181 treatment group (plain line: treated; dashed line: non-treated) in the first 5 weeks post-surgery.
 182 **b.** Average side biases according to lesion subtype: pooled (corticostratial+striatal) vs.
 183 hypothalamic lesions. **c.** Individual neuroscores according to lesion subtype and treatment
 184 group (plain line: non-treated; dashed line: treated) in the first 5 weeks post-surgery. **d.** Average
 185 neuroscores according to lesion subtype: pooled (corticostratial+striatal) vs. hypothalamic
 186 lesions. Data are displayed as mean \pm SD. W: weeks, D: days. * $p < 0.05$, ** $p < 0.01$,
 187 (corticostratial + striatal) vs. hypothalamic, Wilcoxon-Mann-Whitney test; † $p < 0.05$, †† $p < 0.01$,
 188 D4 to W5 vs. before, Friedman test.
 189

<i>Biomarkers</i>		Time points		Treated (N=5)	Non-treated (N=6)	p
<i>Neuroscores</i>		Before treatment D2		11 ± 6	8 ± 7	0.4059
		Before treatment W1		5 ± 4	4 ± 3	0.7766
		After treatment W2		2 ± 1	2 ± 1	0.4493
		After treatment W3		1 ± 1	1 ± 1	1
		After treatment W4		0 ± 0	0 ± 0	1
		After treatment W5		1 ± 1	0 ± 0	1
<i>Side bias (%)</i>		Before stroke		58% ± 5%	58% ± 11%	0.576
		Before treatment D4		17% ± 20%	28% ± 27%	0.7787
		Before treatment W1		26% ± 18%	30% ± 27%	1
		After treatment W2		30% ± 23%	31% ± 25%	1
		After treatment W3		32% ± 19%	33% ± 25%	0.9307
		After treatment W4		27% ± 24%	33% ± 26%	0.9266
		After treatment W5		35% ± 22%	34% ± 24%	0.9307
<i>Lesion size (mm³)</i>		Before treatment D4		129 ± 120	77 ± 92	0.5368
		After treatment W5		61 ± 78	41 ± 56	0.6473
<i>DTI</i>	FA	Contra	Before tt D4	0.28 ± 0.03	0.27 ± 0.03	0.5368
			After tt W5	0.26 ± 0.05	0.26 ± 0.02	0.9307
		Ipsi	Before tt D4	0.19 ± 0.03	0.22 ± 0.05	0.2468
			After tt W5	0.38 ± 0.02	0.36 ± 0.02	0.2468
	MD	Contra	Before tt D4	0.76 ± 0.01	0.78 ± 0.04	0.5704
			After tt W5	0.77 ± 0.01	0.75 ± 0.01*	0.0365
		Ipsi	Before tt D4	0.71 ± 0.06	0.75 ± 0.07	0.358
			After tt W5	0.86 ± 0.17	0.86 ± 0.09	0.9307
	AD	Contra	Before tt D4	0.97 ± 0.10	1.02 ± 0.06	0.407
			After tt W5	0.98 ± 0.04	0.98 ± 0.03	0.8541
		Ipsi	Before tt D4	0.91 ± 0.13	0.92 ± 0.10	0.7144
			After tt W5	1.33 ± 0.19	1.24 ± 0.15	0.4286
	RD	Contra	Before tt D4	0.65 ± 0.01	0.66 ± 0.03	0.5778
			After tt W5	0.64 ± 0.04	0.65 ± 0.02	1
		Ipsi	Before tt D4	0.63 ± 0.05	0.65 ± 0.04	0.583
			After tt W5	0.72 ± 0.12	0.69 ± 0.09	0.9269

190 **Table 1- Neurofunctional and neuroimaging readouts according to treatment group.**
191 Contra: contralateral; D: Days; Ipsi: ipsilateral; tt: treatment; W: Weeks; FA: fractional
192 anisotropy, MD: mean diffusivity, AD: axial diffusivity, RD: radial diffusivity. P-values are
193 given for Wilcoxon-Mann-Whitney test, *p<0.05.
194

195 **Imaging readouts**

196 *Brain lesions*

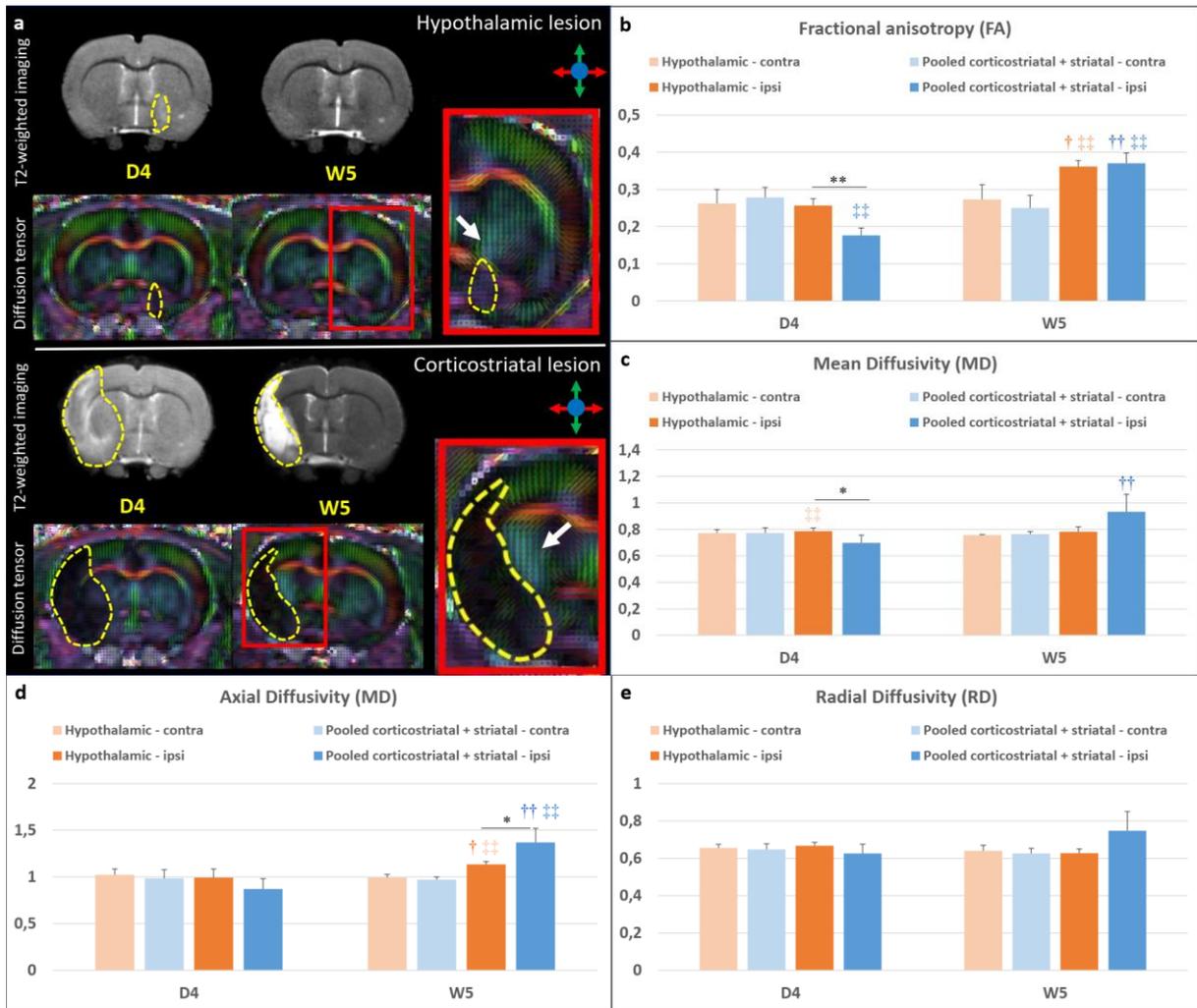
197 Figures 2B and 2C show individual and average lesion sizes according to lesion subtype and
198 treatment group. As expected, at D4, there was a gradient in lesion size according to subtype
199 (D4: hypothalamic-only: 16 [5; 30] < striatal: 42 [28 ; 71] < corticostriatal: 227 [197 ; 249]
200 mm³ and W5: hypothalamic-only: 2 [0; 5] < striatal: 8 [6 ; 9] < corticostriatal: 127 [119 ; 138]
201 mm³). Corticostriatal lesion volumes remained stable after W5 (Supplementary Fig. 2; W9: 124
202 [111 ; 141] mm³). Intracerebral administration of hASCs did impact lesions sizes (Table 1).
203 Lesion shrinkage between D4 and W5 was statistically significant in pooled (corticostriatal +
204 striatal) lesion group (Friedman test – p=0.0081) but not in hypothalamic-only lesion group
205 (Friedman test – p= 0.0832). Lesion size was statistically larger in pooled (corticostriatal +
206 striatal) lesion group than in hypothalamic-only lesion group at both time-points (Fig. 2C;
207 Wilcoxon-Mann-Whitney test – D4: p= 0.0424 and W5: 0.0293).

208 *Microstructural alterations*

209 The internal capsule appeared as a region characterized by a high fractional anisotropy (FA)
210 value localized between the lateral ventricle and the caudate putamen (Fig. 4A, white arrows).
211 Supplementary Fig. 3 and Fig. 4B-E present individual and average DTI metrics (MD: mean
212 diffusivity, AD: axial diffusivity and RD: radial diffusivity) according to lesion subtype and
213 treatment group. DTI metrics were not impacted by intracerebral administration of hASCs
214 (Table 1). DTI metrics did not change over time in the contralateral internal capsule in
215 hypothalamic-only lesion group (Friedman test – FA: p=1; MD: p=0.3173; AD: 0.3173; RD:
216 0.5637) and in pooled (corticostriatal + striatal) lesion group (Friedman test – FA: p=0.2568;
217 MD: 0.4142; AD: 0.2568; RD: 1). Also, there was no significant difference between these 2
218 groups in the contralateral internal capsule at D4 (Wilcoxon-Mann-Whitney test – FA: p=
219 0.5273; MD: p= 0.769; AD: 0.5044; RD: 0.6311) and W5 (Wilcoxon-Mann-Whitney test – FA:

220 $p= 0.3152$; MD: $p= 0.3028$; AD: 0.1829 ; RD: 0.7035) (Supplementary Fig. 3, Fig. 4B-E). In
221 the ipsilesional internal capsule, FA and MD were significantly decreased at D4 in the pooled
222 (corticostriatal+striatal) lesion group compared to the hypothalamic-only lesion group (Fig. 4B-
223 C; Wilcoxon-Mann-Whitney test – FA: $p=0.0060$ and MD: $p=0.0363$, represented by *). FA
224 and AD were significantly increased in the ipsilesional internal capsule at W5 vs. D4
225 (represented by †) in both hypothalamic-only lesion group (Friedman test – FA: $p= 0.0455$ and
226 AD: $p=0.0455$) and (corticostriatal+striatal) lesion group (Friedman test – FA: $p= 0.0081$ and
227 AD: $p=0.0081$),. MD was significantly increased at W5 vs. D4 in the (corticostriatal+striatal)
228 lesion group only (Friedman test – $p=0.0081$). FA was significantly decreased in the ipsilesional
229 vs. contralesional internal capsule (represented by ‡‡) at D4 in (corticostriatal+striatal) lesion
230 group (Friedman test – $p= 0.0081$). At W5, it was significantly increased in the ipsilesional vs.
231 contralesional internal capsule both in hypothalamic-only lesion group (Friedman test – $p=$
232 0.0455) and in (corticostriatal+striatal) lesion group (Friedman test – $p= 0.0081$). AD was also
233 significantly increased in the ipsilesional vs. contralesional internal capsule both in
234 hypothalamic-only lesion group (Friedman test – $p= 0.0455$) and (corticostriatal+striatal) lesion
235 group (Friedman test – $p=0.0081$) at W5. In rats with a corticostriatal lesion, FA remained
236 relatively stable in the ipsilesional internal capsule while MD, AD and radial diffusivity (RD)
237 continued to increase at W9 (Supplementary Fig. 2).

238



239

240 **Figure 4- DTI readouts. a.** Example of T2-weighted imaging and color-coded fractional
 241 anisotropy for two individual rats: one with a hypothalamic lesion and one with a corticostriatal
 242 lesion (dotted yellow line). Only the central slice is presented. The internal capsule is pointed
 243 out by the white arrow. **b-e.** Average DTI metrics (respectively FA, MD, AD and RD) are
 244 presented according to lesion subtype: pooled (corticostriatal+striatal) vs. hypothalamic lesions
 245 at day 4 (D4) and week 5 (W5) post-surgery. FA: fractional anisotropy, MD: mean diffusivity,
 246 AD: axial diffusivity, RD: radial diffusivity. * $p < 0.05$, ** $p < 0.01$, (corticostriatal+striatal) vs.
 247 hypothalamic, Wilcoxon-Mann-Whitney test; † $p < 0.05$, †† $p < 0.01$, W5 vs. D4, Friedman test;
 248 ‡ $p < 0.05$, ‡‡ $p < 0.01$, ipsilateral (ipsi) vs. contralateral (contra) side, Friedman test.

249 **Therapeutic trial design**

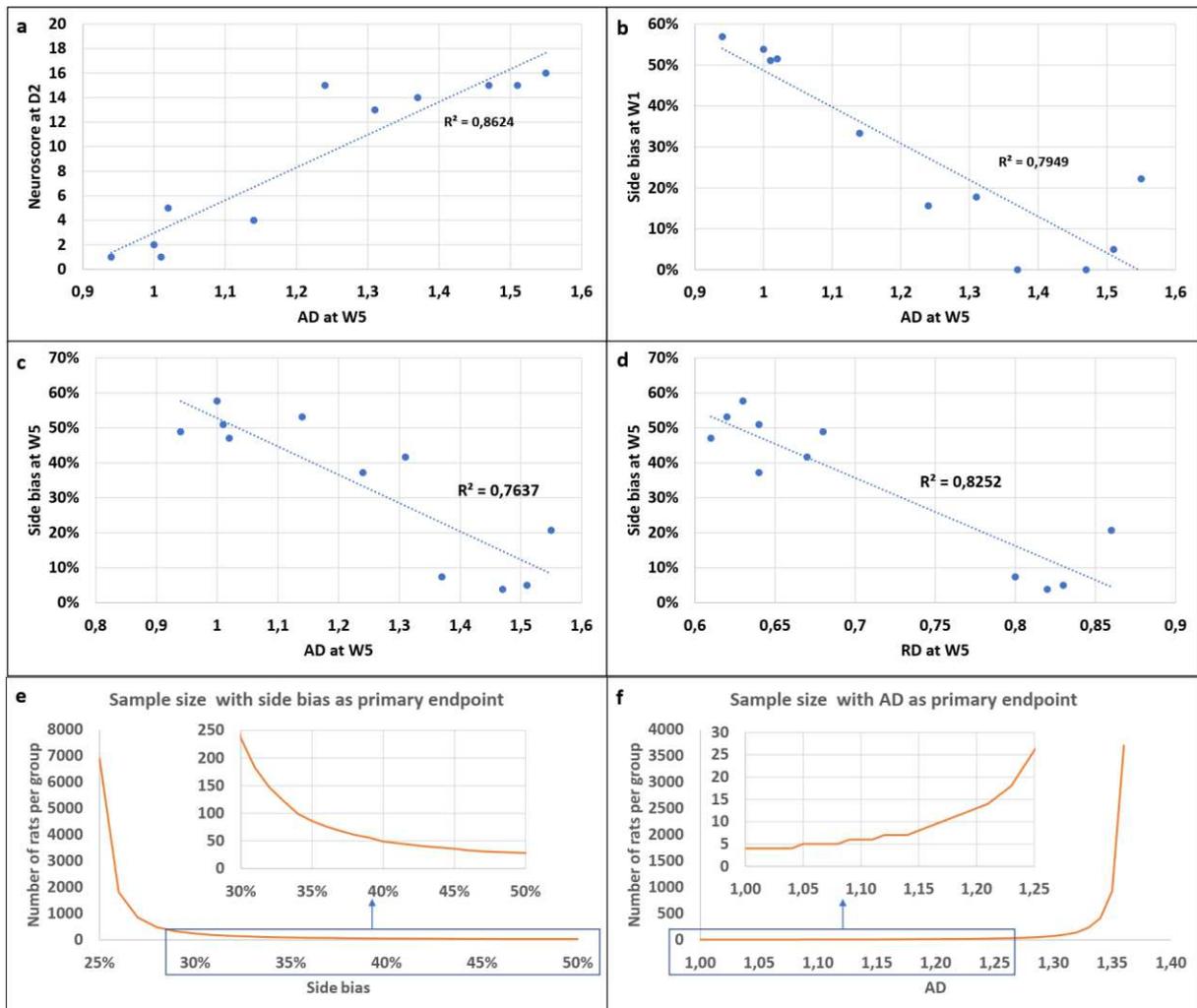
250 *Correlation between imaging and neurofunctional readouts*

251 Several correlations were observed between imaging and neurofunctional readouts.
 252 Neuroscores at D1 and side bias at W5 were linearly related to lesion size at D4 (Supplementary
 253 Fig. 4). Figure 5 shows the most significant correlations between side bias and DTI metrics.
 254 AD at W5 correlated with neuroscore at day 2 (Fig. 5A; Pearson's correlation – $p = 3.65e-05$)

255 and with side bias at W1 (Fig. 5B; Pearson's correlation – $p=0.0002$) and W5 (Fig. 5C;
256 Pearson's correlation – $p=0.0003$). Hence this parameter seemed the most relevant for detecting
257 a treatment effect.

258 *Sample size calculation*

259 To assist in the design of future therapeutic trials, we performed calculations using our data to
260 determine sample size in order to detect a deficit in treated compared to non-treated tMCAO
261 rats at W5 after stroke. We assumed that the tMCAO group included corticostriatal and striatal
262 lesions and excluded hypothalamic-only lesions. The primary endpoints were side bias (Fig.
263 5E) and the DTI metric AD (Fig. 5F). For the staircase test, we assumed a side bias value of
264 $24\% \pm 20\%$ in the non-treated tMCAO group (i.e., mean \pm SD from the pooled
265 (corticostriatal+striatal) lesion group of the current study at W5). The side bias in the treated
266 group was then varied from 25% to 50% (corresponding to hypothalamic-only lesion levels at
267 W5) by steps of 1% and sample size was calculated for each side bias (Fig. 5E). For side bias
268 increasing from 24% to 32% (30% improvement in side bias), sample size needs to be 147 rats
269 per group in order to detect a significant difference between groups. For side bias increasing
270 from 24% to 36% (50% improvement in side bias, *i.e.* to reach the level of rats with striatal
271 lesions), sample size needs to be 76 rats per group. For DTI metrics, we assumed an AD value
272 of 1.37 ± 0.15 in the non-treated group (i.e., mean \pm SD from the pooled (corticostriatal+striatal)
273 lesion group of the current study at W5). AD in the treated group was then varied from 1.36 to
274 0.99 (corresponding to contralateral hypothalamic-only lesion levels at W5) by steps of 0.01
275 and sample size was calculated for each AD value (Figure 5F). For an AD value decreasing
276 from 1.37 to 1.23 (10% improvement in AD value, reaching the level of rats with striatal
277 lesions), sample size needs to be 18 rats per group. For an AD value decreasing from 1.37 to
278 1.16 (15% improvement in AD value, *i.e.* to reach an intermediate value between rats with
279 striatal and hypothalamic lesions), sample size needs to be 9 rats per group only.



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Figure 5- Correlations between neurofunctional and imaging outcomes and sample size calculation. **a-d.** Main correlations between neurofunctional and imaging readouts. **a.** Correlation between neuroscores at D2 and AD at D5 (Pearson correlation test, $p=3.65e-05$). **b.** Correlation between side bias at W1 and AD at W5 (Pearson correlation test, $p=0.00022$). **c.** Correlation between side bias at W5 and AD at W5 (Pearson correlation test, $p=0.00034$). **d.** Correlation between side bias at W1 and RD at W5 (Pearson correlation test, $p=6.543e-05$). **e-f.** Sample size calculation for future pre-clinical therapeutic trial. **e.** Side bias as primary endpoint. The x-axis represents the hypothesized value of side bias in the treatment group and the x-axis represents the corresponding number of rats per group. **f.** AD as primary endpoint. The x-axis represents the hypothesized value of AD in the treatment group and the x-axis represents the corresponding number of rats per group. AD: axial diffusivity, RD: radial diffusivity.

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Discussion

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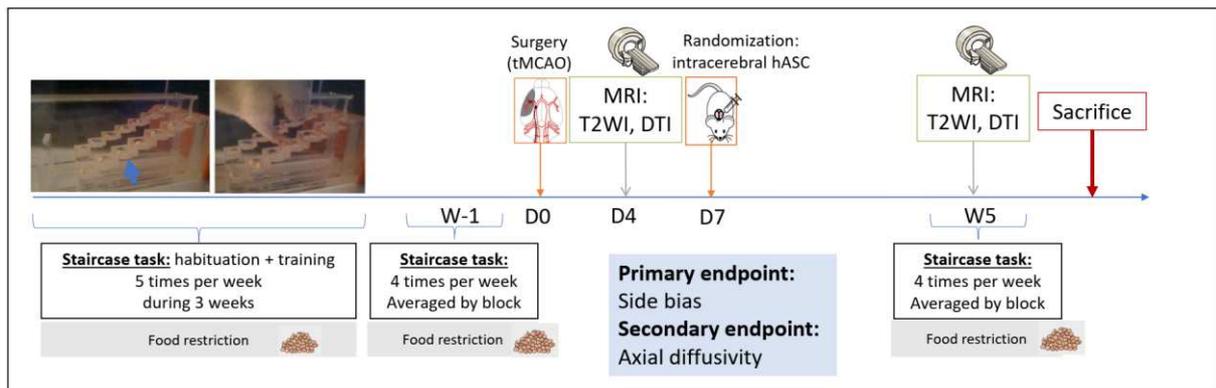
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We here report an observational, descriptive study aiming to design a larger-scale therapeutic trial to evaluate intracerebral administration of hASCs in the chronic stage of ischemic stroke. Figure 6 summarizes the final protocol design.

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 300 **Figure 6- Design of preclinical therapeutic trial.** The study design involves 2 staircase tasks
 301 (one before tMCAO surgery and one 5 weeks after, *i.e.* 4 weeks post-treatment) and 2 MRIs
 302 with T2-weighted imaging and DTI sequences (one at D4 post-surgery and one at 5 weeks after
 303 tMCAO surgery, *i.e.* 4 weeks post-treatment).

304 Although such preclinical trials have already been published in the literature (21-23), several
 305 methodological aspects, including choice of biomarkers, need to be considered to produce
 306 robust data that may be translated to the clinical realm. We chose to model ischemic stroke in
 307 rats using transient occlusion of the middle cerebral artery with an intraluminal thread. This
 308 model produces cerebral damage with a variety of lesion sizes and anatomical locations
 309 (hypothalamus, striatum and cortex) (20), as is common in ischemic stroke patients (12).
 310 Because lesion location, in addition to lesion size, is a main determinant of functional outcome,
 311 we reported our results according to these 3 lesion subtypes.

312 Long-term neurofunctional tests remain a challenge due to the quick compensation of rodents
 313 and the difficulty to differentiate adaptive strategy from motor improvement (10, 24, 25). The
 314 test must be quantitative, allow repetition and it must reveal long term and stable deficits with
 315 enough sensitivity to show an improvement in treated *vs* non-treated subjects (26, 27). The
 316 staircase test, a skilled reaching task that assesses forelimb function, fulfills these criteria for
 317 the long-term evaluation of motor recovery in stroke-induced rats (28, 29). However, the
 318 optimal frequency of testing, the timescale and side bias modifications in time still remained to
 319 be determined (30-33). Our results confirm that the staircase test is an appropriate
 320 neurofunctional biomarker for the long-term evaluation of rats with corticostriatal and striatal
 321 lesions. To the best of our knowledge, this is the first study to report detailed changes in side

322 bias over time according to lesion subtype using the tMCAO intraluminal thread model.
323 Because of the heterogeneity between striatal and corticostriatal lesions, the number of subjects
324 per group to detect a side bias difference in a stem-cell trial is relatively high (76-147 rats per
325 group). This is not unfeasible but necessitates a multicenter design, probably including at least
326 5 centers, as in (34). Alternatively, pre-treatment T2WI may be used to include rats with
327 corticostriatal lesions only, in order to reduce variability and hence sample size. This might be
328 relevant for translational research, as half of stroke patients experience persistent loss of upper-
329 limb function in the chronic stage (13).

330 One limitation of the staircase test is that it requires intensive training and is prone to large
331 exclusion rates of low-learner rats (29). In addition, implementation and analysis are quite
332 strenuous and time-consuming. Hence we aimed at simplifying the neurofunctional follow-up.
333 As side bias remained stable in all groups 2 weeks after tMCAO, a single week of testing (with
334 block averaging) may be sufficient to assess stem-cell treatment effects. We suggest choosing
335 W5, because we found no further spontaneous improvement after this stage, while a follow-up
336 of at least one month after treatment is usually recommended for stem-cell studies (26, 35).

337 Advanced neuroimaging modalities such as DTI are commonly used in clinical stroke research
338 as complementary outcome measures to neurofunctional evaluation (16). Axial diffusivity
339 appeared to be the most relevant parameter. We observed a decrease (although not significant)
340 in AD in the ipsilesional internal capsule in the acute stage of ischemic stroke and a significant
341 increase in the chronic stage. There was also a trend toward an increase in RD in the chronic
342 stage in the ipsilesional internal capsule. This is consistent with acute axonal damage followed
343 by chronic axon and myelin damage (36). The hypothesis to test in a stem cell trial would be
344 that CST microstructural remodeling by treatment ‘normalizes’ the AD value. Because DTI
345 metrics are quite consistent over time and across animals, the number of subjects per group if
346 this metric is used as a primary endpoint (N=9-18 according to the expected size effect) would

347 be compatible with a single-center exploratory study. However, such a study would be
348 underpowered to evaluate side bias at the same time, and should therefore be considered
349 preliminary.

350 We did not observe any impact of intracerebral administration on neurofunctional and imaging
351 readouts. This is important to report as the main drawback of this route of administration is its
352 invasiveness. We thus confirm the safety of the procedure. On the other hand, there was no
353 trend toward an improvement of any of the endpoints evaluated in the group of rats that were
354 injected with hASC compared to those who were not. Although the study was not powered to
355 detect such an effect, this suggests that cell therapy regimen may benefit from being optimized
356 before proceeding to the larger-scale preclinical trial.

357 The main limitation of the present study was the small number of animals that were included.
358 This was due in part to a mortality rate that was higher than expected, probably due to
359 complications such as hemorrhagic transformation and malignant edema that are difficult to
360 anticipate. Exclusion rates are rarely reported in stroke research, which actually represents one
361 of the methodological flaw of preclinical studies. Basalay et al recently reported a 25%
362 exclusion rate at 24h post-surgery due to the combination of mortality rate and hemorrhagic
363 transformations in a bicentre international study using the same tMCAO model in rats (37). For
364 long-term studies, the mortality rate is increased as seen in the current study, where the
365 mortality post-surgery exceeded our a priori hypothesis: this is a further element to be taken
366 into account when designing a therapeutic trial to reach the adequate statistical power.
367 Nevertheless, the study was designed as an observational study, results are shown for individual
368 animals and statistical analysis was performed between pooled (corticostriatal and striatal
369 lesions) lesions and sham-like (hypothalamic lesions) groups only. We therefore believe these
370 results are robust and that they are of interest to the stroke community by addressing the need
371 to standardize preclinical stem-cell trials and design high-quality studies.

372 **Conclusion**

373 This study determined the optimal neurofunctional and imaging readouts for the follow-up of
374 rats in the chronic stage of ischemic stroke, the relevant timescale, and adequate sample size to
375 evaluate the therapeutic effects of intracerebral administration of hASCs, in line with
376 international recommendations (26). We conclude that an exploratory preclinical trial based on
377 both readouts would be feasible only in the framework of a multicenter trial, which in turn
378 would necessitate appropriate funding and/or an industrial partnership. Such rigorous
379 approaches are paramount for the successful translation of preclinical stem-cell research for the
380 benefit of stroke patients.

381 **Methods**

382 **Animals and ethics statement**

383 All experimental procedures involving animals and their care conformed to European
384 regulations for animal use (APAFIS agreement number: APAFIS#4688-2016032514131943).
385 This study was approved by our institutional ethic committee “Comité d’éthique pour
386 l’Expérimentation Animale Neurosciences Lyon” (CELYNE-CNREEA number: C2EA-42).
387 The rats were housed three to four per cage (except in the first 2 days post-surgery where they
388 were housed one per cage) in a temperature and humidity-controlled environment ($21.2 \pm 3^\circ\text{C}$),
389 on 12:12h light-dark cycle, having free access to tap water and standard diet except during
390 neurofunctional testing when they were put under food restriction for motivation (see details
391 below). Rats were housed, regardless of type of lesion or treatment, in a standard Plexiglas box
392 covered with mulch and enriched with red-colored cylindrical plastic tubes. Male Sprague
393 Dawley OFA rats (CrI:OFA(SD), Charles River, France) aged 6-8 weeks were used, with a
394 mean weight of 199 ± 13 g at the start of the experiment.

395 **Sample size, inclusion criteria and blinding**

396 The RIGOR guidelines were used to design the study (38). Data were reported according to
397 ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (39). Inclusion
398 criteria were: lesion on D4 T2-weighted imaging, regardless of size and location, and complete
399 neurofunctional and imaging follow-up. No formal sample size was calculated for this
400 observational, descriptive study. We aimed at including 15 rats within 1 year. We thus planned
401 to enroll 25 animals, assuming a 20% exclusion rate for low-learners on the staircase test (29)
402 and 25% exclusion for the tMCAO model due either to mortality or to absence of lesion on T2-
403 weighted imaging at D4 (37). All data were anonymized and analyses were performed blindly.
404 For the staircase test analysis, treatment group allocation was concealed by filming the rats
405 from the side so that rats that had undergone intracerebral administration could not be identified.

406 **Ischemic stroke model**

407 The animal model of ischemic stroke was the previously described tMCAO model (40). Rats
408 were anesthetized with a mixture of isoflurane and ambient air (4% during induction and
409 between 1% and 2% during surgery) (ISO-VET, Piramal Healthcare, Morpeth, UK). Analgesia
410 was obtained with subcutaneous administration of buprenorphine (Buprecare, Axience) at 0.05
411 mg/kg, injected after anesthesia induction. Briefly, the model was performed by introducing the
412 thread (Doccol corporation, USA) through the external carotid artery. The thread was kept in
413 place for 60 minutes. Because we aimed at inducing variability in a limited number of subjects
414 (in order to investigate lesion subtypes), the thread size was the same for all rats (0.39 mm) and
415 not adapted to the rat weight as we usually do. Definitive occlusion of the internal carotid artery
416 and external carotid artery was performed. Temperature was controlled with a rectal probe
417 throughout the surgical procedure with a heating pad set at 37°C. The effectiveness of occlusion
418 was checked by the presence of a lesion on D4 T2-weighted imaging.

419 **Stem-cells**

420 Surgical residue was harvested according to French regulations and declared to the Research
421 Ministry (DC n° 2008162) following written informed consent from the patients. Human
422 stromal vascular fraction (SVF) was isolated from lipoaspirate obtained from healthy volunteers
423 undergoing liposuction. Adipose tissue was digested with collagenase (0.120 U/ml, Roche,
424 Indianapolis, IN, USA) at 37°C for 30 min and under constant shaking. Digestion was stopped
425 by adding Dulbecco's Modified Eagle's Medium (DMEM with Glutamax®, Gibco®,
426 Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS, HyClone, Logan, UT,
427 USA). Floating adipocytes were discarded and cells from the SVF were pelleted, rinsed with
428 medium, centrifuged (300g for 5 min at 20°C) and incubated in an erythrocyte lysis buffer for
429 20 min at 37°C. The cell suspension was centrifuged (300g for 5 min, 20 °C) and cells were
430 counted using the Trypan blue exclusion method. A total of 40,000 SVF cells/cm² were plated
431 and grown in proliferation medium containing DMEM (Gibco®, Life technologies), HAM-F12
432 L-Glutamine (Gibco®, Life Technologies, St Aubin, France) (v/v), 10% FCS (HyClone), 10
433 ng/ml basic fibroblast growth factor (FGF2, Miltenyi Biotec, Paris, France), 10 µg/ml
434 Gentamicin and 100 IU/ml Penicillin (Panpharma, Fougères, France). The medium was
435 changed three times a week until 80% confluence was reached. At subconfluency, cells were
436 detached with Trypsin-0.01%-EDTA (Gibco® (Invitrogen, Carlsbad, CA, USA) and
437 centrifuged for 10 min at 300g and amplified in subculture at 4,000 cells/cm² density during
438 two passages.

439 Stem-cell administration

440 A subgroup of animals received clinical-grade human mesenchymal stem cells derived from
441 human adipose tissue (hASCs, codecoh number AC 2019-3476). Coordinates for stereotaxic
442 injection were calculated from D4 MRI to inject cells in the striatal part of the lesion. Rats were
443 anesthetized with the same protocol as for stroke induction and placed in a stereotaxic frame
444 (Stoelting, Chicago, IL, USA) with a mask delivering isoflurane during the procedure. After

445 opening the skin and careful drilling of the chosen entry point, 500,000 hASCs were prepared
446 in 10- μ l medium solution and injected in 1 minute through a 26-gauge needle (RN-type, NH-
447 BIO) with a UltraMicropump III with Micro4Controller (World Precision Instruments,
448 Friedberg, Germany). The needle was kept in place for 2 minutes before careful progressive
449 removal. The control subgroup did not undergo intracerebral surgery, as we aimed at assessing
450 the impact of intracerebral administration on neurofunctional and imaging readouts.

451 **Neurofunctional testing**

452 Staircase test:

453 Staircase testing was performed under restricted feeding to maintain body weight at 90% of
454 normal (0.05-g/g of weight) (28, 30). Weight was checked every day during the restriction
455 period and the quantity of diet given daily after the staircase experiment was adapted in case of
456 weight loss. No diet restriction was imposed for 2 days before and 7 days after stroke to allow
457 good recovery from surgery. After a period of habituation to the experimenter (1 week) and to
458 the pellets (Dustless precision pellets, purified, 45mg, Bio-Serv, Flemington), rats were placed
459 for 10 minutes 5 days a week in the home-made staircase box (30, 31). Rats with sufficient
460 training (pellet retrieval \geq 6 pellets in 10 minutes) were selected before stroke induction.
461 Unilateral stroke was performed to impair the dominant side when rats were lateralized (i.e.,
462 taking more pellets on one side than the other: side bias $>60\%$), otherwise they were operated
463 on the right side. Rats were filmed during their task (Sony Xperia ZD compact) and analyses
464 were made by a blinded observer after anonymization of the movies. The number of pellets
465 retrieved per side using the paw only was evaluated for each test session. With this apparatus,
466 only the ipsilateral paw can take the pellet on the ipsilateral side and vice-versa. Data were
467 averaged by blocks of 4 tests to obtain 1 side-bias value per week. Side bias was used to evaluate
468 neurofunctional deficit and was calculated as $\text{contra}/(\text{ipsi}+\text{contra})$ performance, with 'ipsi'

469 corresponding to the brain-spared side and ‘contra’ to the brain ischemic side (28). Staircase
470 tests were carried out in the morning or in the afternoon in alternation.

471 Neuroscore:

472 The neuroscore was a scale from 0 to 20 that included sensorimotor tasks: gait, limb placing,
473 parachute reflex, lateral resistance, beam walk (41). A higher score indicates a more severe
474 deficit. The test was performed in the morning.

475 **MRI**

476 For *in vivo* MRI, anesthesia was induced and maintained in the same way as during the surgery.
477 The animals were placed in an MRI-compatible rats cradle. The respiratory rhythm was
478 monitored by a pressure sensor linked to a monitoring system (ECG Trigger Unit HR V2.0,
479 RAPID Biomedical, Rimpfing, Germany). MRI acquisitions were performed on a horizontal 7T
480 BRUKER Biospec MRI system (Bruker Biospin MRI GmbH Bruker, Germany) equipped with
481 a set of gradients of 440 mT/m and controlled via Bruker ParaVision 5.1 workstation. A Bruker
482 birdcage volume coil (inner diameter = 72 mm and outer diameter = 112 mm) was used for the
483 signal transmission, and a Bruker single loop surface coil (25 mm diameter) was used for signal
484 reception. Two sequences were used: anatomical T2-weighted imaging (T2WI) and diffusion
485 tensor imaging (DTI). Supplementary Table 1 presents the acquisition parameters.

486 Images analyses:

487 Bruker raw data were converted in Nifti format using the open source medical image converter
488 Dicomifier (<https://github.com/lamyj/dicomifier>). For assessment of lesion size, T2WI data
489 were analyzed blindly using ImageJ software (National Institute of Health, USA
490 imagej.nih.gov/ij/) by manually contouring the lesion, the ipsilateral and the contralateral
491 hemispheres and applying a correction for edema/atrophy (42). The DTI parametric maps
492 (fractional anisotropy FA, mean diffusivity MD, axial diffusivity AD and radial diffusivity RD)
493 thus directional color-coded fractional anisotropy maps were obtained, after motion correction

494 between volumes based on a rigid registration, using FSL (FMRIB Software Library, The
495 University of Oxford). Then, affine registration according to the FA map at W5 was applied to
496 individual maps using FSL. The internal capsule was analyzed to evaluate the ipsilesional
497 corticospinal tract disruption and remodeling in analogy to patient studies (16, 43). This was
498 automatically obtained by thresholding an individual region of interest (ROI) manually defined
499 close to the lesion, in the ipsilesional striatum, according to a FA value superior to 0.3. The
500 contralateral ROI was obtained by mirroring the ipsilateral ROI.

501 **Statistical analysis**

502 Statistical analysis was performed with R for Mac (The R foundation for statistical Computing).
503 Data are given as median [25%;75%] interquartile unless specified otherwise. Because the
504 residual normality hypothesis was not verified, for longitudinal data, differences between time-
505 points were evaluated with Friedman test followed by Conover post hoc test with p-value
506 adjustment according to Holm method. Differences between two groups at a given time point
507 were evaluated with a two-sided Wilcoxon-Mann-Whitney tests. The Pearson correlation test
508 was used for correlation analysis. A p-value inferior to 0.05 was considered significant. Sample
509 size calculation were made with G*Power 3.9.11.2 for a power of 0.8, an alpha error of 0.05
510 and 2-sided Wilcoxon-Mann-Whitney tests for two groups using the data obtained in the study
511 at week 5 post-stroke (as further specified in the Result section).

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648 Conceptualization: MW, CP, CR, FC

649 Data curation: CD, MW, CP

650 Formal analysis: CD, CP, LC, MC, CA

651 Funding acquisition: MW, FC, CR, ECS

652 Investigation: CD, LC, EO, VH, RB, MC, CA, FC, MW

653 Methodology: CA, MW, CP, FC, CD

654 Project administration: MW

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662

663 **Conflict of interest statement**

664 Declarations of interest: none.

665 **Data availability statement**

666 The processed data required to reproduce these findings and perform the statistical analyses
667 are available to download at the figshare repository— <https://figshare.com>
668 (<https://figshare.com/s/15af2a099076389d2a5e>).

669 **Figure legends**

670 **Figure 1- Study design**

671 **a.** Experimental design; **b.** Focus on the first week of the experiment; **c.** Focus on weeks 6 to
672 15: extended follow-up only for rats with corticostriatal lesions. D: Days, MRI: magnetic
673 resonance imaging, hASC: Human adipose mesenchymal stem cells, W: Weeks.

674 **Figure 2- Evaluation of lesions on T2-weighted imaging.**

675 **a.** Longitudinal T2-weighted imaging of all included rats according to lesion subtype (only one
676 central slice is shown). Treated rats (that received intracerebral administration of hASCs) are
677 presented in top rows and circled. **b.** Individual lesion sizes are presented according to lesion
678 subtype (striatal, corticostriatal and hypothalamic lesions) and treatment group (plain line:
679 treated; dashed line: non-treated) at day 4 (D4) and week 5 (W5) post-surgery. **c.** Average lesion
680 sizes are presented according to lesion subtype: pooled (corticostriatal+striatal) vs
681 hypothalamic lesions. Data are displayed as mean \pm SD. W: weeks, D: days; * $p < 0.05$,
682 (corticostriatal + striatal) vs hypothalamic, Wilcoxon-Mann-Whitney test; † $p < 0.05$, †† $p < 0.01$,
683 W5 vs D4, Friedman test.

684 **Figure 3- Neurofunctional readouts**

685 **a.** Individual side bias according to lesion subtype and treatment group (plain line: treated;
686 dashed line: non-treated) in the first 5 weeks post-surgery. **b.** Average side biases according to
687 lesion subtype: pooled (corticostriatal+striatal) vs. hypothalamic lesions. **c.** Individual
688 neuroscores according to lesion subtype and treatment group (plain line: non-treated; dashed
689 line: treated) in the first 5 weeks post-surgery. **d.** Average neuroscores according to lesion
690 subtype: pooled (corticostriatal+striatal) vs. hypothalamic lesions. Data are displayed as mean
691 \pm SD. W: weeks, D: days. * $p < 0.05$, ** $p < 0.01$, (corticostriatal + striatal) vs. hypothalamic,
692 Wilcoxon-Mann-Whitney test; † $p < 0.05$, †† $p < 0.01$, D4 to W5 vs. before, Friedman test.

693 **Figure 4- DTI readouts**

694 **a.** Example of T2-weighted imaging and color-coded fractional anisotropy for two individual
695 rats: one with a hypothalamic lesion and one with a corticostriatal lesion (dotted yellow line).
696 Only the central slice is presented. The internal capsule is pointed out by the white arrow. **b-e.**
697 Average DTI metrics (respectively FA, MD, AD and RD) are presented according to lesion
698 subtype: pooled (corticostriatal+striatal) vs. hypothalamic lesions at day 4 (D4) and week 5
699 (W5) post-surgery. FA: fractional anisotropy, MD: mean diffusivity, AD: axial diffusivity, RD:
700 radial diffusivity. * $p < 0.05$, ** $p < 0.01$, (corticostriatal+striatal) vs. hypothalamic, Wilcoxon-
701 Mann-Whitney test; † $p < 0.05$, †† $p < 0.01$, W5 vs. D4, Friedman test; ‡ $p < 0.05$, ‡‡ $p < 0.01$,
702 ipsilateral (ipsi) vs. contralateral (contra) side, Friedman test.

703 **Figure 5- Correlations between neurofunctional and imaging outcomes and sample size**
704 **calculation.**

705 **a-d.** Main correlations between neurofunctional and imaging readouts. **a.** Correlation between
706 neuroscores at D2 and AD at D5 (Pearson correlation test, $p = 3.65e-05$). **b.** Correlation between
707 side bias at W1 and AD at W5 (Pearson correlation test, $p = 0.00022$). **c.** Correlation between
708 side bias at W5 and AD at W5 (Pearson correlation test, $p = 0.00034$). **d.** Correlation between
709 side bias at W1 and RD at W5 (Pearson correlation test, $p = 6.543e-05$). **e-f.** Sample size
710 calculation for future pre-clinical therapeutic trial. **e.** Side bias as primary endpoint. The x-axis
711 represents the hypothesized value of side bias in the treatment group and the x-axis represents
712 the corresponding number of rats per group. **f.** AD as primary endpoint. The x-axis represents
713 the hypothesized value of AD in the treatment group and the x-axis represents the
714 corresponding number of rats per group. AD: axial diffusivity, RD: radial diffusivity.

715 **Figure 6- Design of preclinical therapeutic trial**

716 The study design involves 2 staircase tasks (one before tMCAO surgery and one 5 weeks after,
717 *i.e.* 4 weeks post-treatment) and 2 MRIs with T2-weighted imaging and DTI sequences (one at
718 D4 post-surgery and one at 5 weeks after tMCAO surgery, *i.e.* 4 weeks post-treatment).

Supplementary Files

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