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

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33

34 Abstract

35 With the aim of designing a preclinical study evaluating an intracerebral cell-based therapy for stroke, an observational study was performed in the rat suture model of ischemic stroke. 36 37 Objectives were threefold: (i) to characterize neurofunctional and imaging readouts in the first weeks following transient ischemic stroke, according to lesion subtype (hypothalamic, striatal, 38 39 corticostriatal); (ii) to confirm that intracerebral administration does not negatively impact these readouts; and (iii) to calculate sample sizes for a future therapeutic trial using these readouts as 40 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 levels within 2 weeks, while rats with corticostriatal lesions remained impaired until week 14. 44 45 AD values were decreased at 4 days and increased at 5 weeks post-surgery, with a subtype gradient: hypothalamic < striatal < corticostriatal. Intracerebral administration did not impact 46 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

Ischemic stroke is a leading cause of mortality and disability worldwide (1). To date, the only
therapeutic option is to reopen the occluded artery mechanically and/or pharmacologically. This
option is applicable only in the acute phase for selected patients. In case of persisting disability,
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 ischemic stroke (2, 3). Mesenchymal stem cells are of major interest due to their low 58 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 adiposederived mesenchymal stem cells (hASCs) are already used in stroke clinical trials 63 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 to evaluate the effects of intracerebral administration of clinical-grade hASCs in ischemic 68 69 stroke, with a study design that aligns with clinical functional evaluation methods for long-term 70 recovery studies (2, 10).

As is well-known, there are several obstacles to the translation of stem-cell research in ischemic stroke from the preclinical to the clinical arena. The rigor of study design, the inclusion of different stroke subtypes, the choice of appropriate primary readout parameters and welldefined sample sizes have been identified as key factors to overcome the translational roadblock (10-14). The assessment of neurofunctional outcome in chronic stroke patients relies on clinical scores such as the National Institutes of Health Stroke Scale (NIHSS) (6) and the upper limb movement section of the Fugl-Meyer (FM) scale (15, 16). Combining clinical scores with the
assessment of ipsilesional corticospinal tract (CST) remodeling with diffusion tensor imaging
(DTI) can improve prediction of motor outcome (16-19). Accordingly, our preclinical stem-cell
trial aimed at combining neuroscores and the staircase test, a skilled reaching task that assesses
forelimb function in rodent models (11), with the DTI evaluation of CST structural integrity
(internal capsule).

83 Ideally, a rigorous study design implies to thoroughly investigate these endpoints according to stroke subtype, in order to determine the optimal frequency of measurements, the post-stroke 84 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 with a limited number of subjects were threefold: (i) to characterize neurofunctional readouts 87 (neuroscores and staircase test) and DTI metrics in the first weeks following transient middle 88 89 cerebral artery occlusion (tMCAO) according to stroke subtype; (ii) to confirm that intracerebral administration of hASCs does not negatively impact these readouts (because of 90 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**

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

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



108



113 <u>Stroke subtypes</u>

- 114 Supplementary Fig. 1 presents the CONSORT-like chart of the study. Of the 25 rats trained at
- 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
- rats included in the study, 4 had a corticostriatal lesion, 3 a striatal lesion and 4 a hypothalamic

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





- 132 <u>Neurofunctional readouts</u>
- 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
- the hypothalamic-only lesion group was maintained around 50% (i.e., no difference between

right and left paw performances) right after surgery and until the end of testing. In rats with
striatal and corticostriatal lesions, side bias was severely increased in the first days after stroke
(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 after stroke (*i.e.* marked difference between the contralateral and ipsilateral paw, in favor of the 141 142 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 presurgery ones, except at W5 (post-hoc Conover test – D4: p=0.0002; W1: 0.0090; W2: 0.0249; 156 157 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, except before surgery (Fig. 3B; Wilcoxon-Mann-Whitney test – Before surgery: p=1; D4: 159 160 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 (Table 1). At D2, neuroscores were in the same order of magnitude in rats with striatal lesion 164 165 (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 treatment group (plain line: treated; dashed line: non-treated) in the first 5 weeks post-surgery. 181 182 **b.** Average side biases according to lesion subtype: pooled (corticostriatal+striatal) vs. 183 hypothalamic lesions. c. Individual neuroscores according to lesion subtype and treatment group (plain line: non-treated; dashed line: treated) in the first 5 weeks post-surgery. d. Average 184 neuroscores according to lesion subtype: pooled (corticostriatal+striatal) vs. hypothalamic 185 186 lesions. Data are displayed as mean ± SD. W: weeks, D: days. *p<0.05, **p<0.01, (corticostritatal + striatal) vs. hypothalamic, Wilcoxon-Mann-Whitney test; $\frac{1}{p} < 0.05$, $\frac{1}{p} < 0.01$, 187 188 D4 to W5 vs. before, Friedman test.

Biomarkers		Time points		Treated (N=5)	Non-treated (N=6)	р
Neuroscores		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
Side bias (%)		Before stroke		58% ± 5%	58% ± 11%	0.576
		Before treatment D4		17% ± 20%	$28\% \pm 27\%$	0.7787
		Before treatment W1		26% ± 18%	$30\% \pm 27\%$	1
		After treatment W2		30% ± 23%	31% ± 25%	1
		After treatment W3		32% ± 19%	33% ± 25%	0.9307
		After treatment W4		$27\% \pm 24\%$	$33\% \pm 26\%$	0.9266
		After treatment W5		35% ± 22%	$34\% \pm 24\%$	0.9307
Lesion size		Before treatment D4		129 ± 120	77 ± 92	0.5368
(mm^{3})		After treatment W5		61 ± 78	41 ± 56	0.6473
DTI	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	$0.75 \pm 0.01*$	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

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

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 (D4: hypothalamic-only: 16 [5; 30] < striatal: 42 [28 ; 71] < corticostriatal: 227 [197 ; 249] 199 200 mm^3 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= 0.5273; MD: p=0.769; AD: 0.5044; RD: 0.6311) and W5 (Wilcoxon-Mann-Whitney test – FA: 219

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 \dagger) 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= 0.0081and 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 significantly increased in the ipsilesional vs. contralesional internal capsule both in 233 hypothalamic-only lesion group (Friedman test -p=0.0455) and (corticostriatal+striatal) lesion 234 235 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) continued to increase at W9 (Supplementary Fig. 2). 237



240 Figure 4- DTI readouts. a. Example of T2-weighted imaging and color-coded fractional anisotropy for two individual rats: one with a hypothalamic lesion and one with a corticostriatal 241 242 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 presented according to lesion subtype: pooled (corticostriatal+striatal) vs. hypothalamic lesions 244 at day 4 (D4) and week 5 (W5) post-surgery. FA: fractional anisotropy, MD: mean diffusivity, 245 AD: axial diffusivity, RD: radial diffusivity. *p<0.05, **p<0.01, (corticostriatal+striatal) vs. 246 hypothalamic, Wilcoxon-Mann-Whitney test; †p<0.05, ††p<0.01, W5 vs. D4, Friedman test; 247 [‡]p<0.05, [‡][‡]p<0.01, ipsilateral (ipsi) vs. contralateral (contra) side, Friedman test. 248

Therapeutic trial design 249

250 Correlation between imaging and neurofunctional readouts

251 Several correlations were observed between imaging and neurofunctional readouts.

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

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

258 Sample size calculation

To assist in the design of future therapeutic trials, we performed calculations using our data to 259 260 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. 5E) and the DTI metric AD (Fig. 5F). For the staircase test, we assumed a side bias value of 263 264 $24\% \pm 20\%$ 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 increasing from 24% to 32% (30% improvement in side bias), sample size needs to be 147 rats 268 269 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 striatal and hypothalamic lesions), sample size needs to be 9 rats per group only. 279



280

281 282 Figure 5- Correlations between neurofunctional and imaging outcomes and sample size 283 calculation. a-d. Main correlations between neurofunctional and imaging readouts. a. 284 Correlation between neuroscores at D2 and AD at D5 (Pearson correlation test, p=3.65e-05). b. 285 Correlation between side bias at W1 and AD at W5 (Pearson correlation test, p=0.00022). c. 286 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-287 f. Sample size calculation for future pre-clinical therapeutic trial. e. Side bias as primary 288 289 endpoint. The x-axis represents the hypothesized value of side bias in the treatment group and 290 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 291 represents the corresponding number of rats per group. AD: axial diffusivity, RD: radial 292 293 diffusivity.

294 Discussion

295 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.

297 Figure 6 summarizes the final protocol design.







Although such preclinical trials have already been published in the literature (21-23), several methodological aspects, including choice of biomarkers, need to be considered to produce robust data that may be translated to the clinical realm. We chose to model ischemic stroke in rats using transient occlusion of the middle cerebral artery with an intraluminal thread. This model produces cerebral damage with a variety of lesion sizes and anatomical locations (hypothalamus, striatum and cortex) (20), as is common in ischemic stroke patients (12). 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.

Long-term neurofunctional tests remain a challenge due to the quick compensation of rodents 312 313 and the difficulty to differentiate adaptive strategy from motor improvement (10, 24, 25). The test must be quantitative, allow repetition and it must reveal long term and stable deficits with 314 315 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 5 centers, as in (34). Alternatively, pre-treatment T2WI may be used to include rats with 326 327 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).

One limitation of the staircase test is that it requires intensive training and is prone to large exclusion rates of low-learner rats (29). In addition, implementation and analysis are quite strenuous and time-consuming. Hence we aimed at simplifying the neurofunctional follow-up. As side bias remained stable in all groups 2 weeks after tMCAO, a single week of testing (with block averaging) may be sufficient to assess stem-cell treatment effects. We suggest choosing W5, because we found no further spontaneous improvement after this stage, while a follow-up 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 this metric is used as a primary endpoint (N=9-18 according to the expected size effect) would 346

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

We did not observe any impact of intracerebral administration on neurofunctional and imaging readouts. This is important to report as the main drawback of this route of administration is its invasiveness. We thus confirm the safety of the procedure. On the other hand, there was no trend toward an improvement of any of the endpoints evaluated in the group of rats that were injected with hASC compared to those who were not. Although the study was not powered to detect such an effect, this suggests that cell therapy regimen may benefit from being optimized 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 anticipate. Exclusion rates are rarely reported in stroke research, which actually represents one 360 361 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 rats in the chronic stage of ischemic stroke, the relevant timescale, and adequate sample size to 374 375 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 approaches are paramount for the successful translation of preclinical stem-cell research for the 379 380 benefit of stroke patients.

381 Methods

382 Animals and ethics statement

All experimental procedures involving animals and their care conformed to European 383 384 regulations for animal use (APAFIS agreement number: APAFIS#4688-2016032514131943). 385 This study was approved by our institutional ethic committee "Comité d'éthique pour l'Expérimentation Animale Neurosciences Lyon" (CELYNE-CNREEA number: C2EA-42). 386 387 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 $(21.2 \pm 3^{\circ}C)$, 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 (Crl: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 criteria were: lesion on D4 T2-weighted imaging, regardless of size and location, and complete 398 399 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 stromal vascular fraction (SVF) was isolated from lipoaspirate obtained from healthy volunteers 422 423 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 medium, centrifuged (300g for 5 min at 20°C) and incubated in an erythrocyte lysis buffer for 428 429 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 ng/ml basic fibroblast growth factor (FGF2, Miltenyi Biotec, Paris, France), 10 µg/ml 433 434 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 centrifuged for 10 min at 300g and amplified in subculture at 4,000 cells/cm² density during 437 two passages. 438

439 <u>Stem-cell administration</u>

A subgroup of animals received clinical-grade human mesenchymal stem cells derived from human adipose tissue (hASCs, codecoh number AC 2019-3476). Coordinates for stereotaxic injection were calculated from D4 MRI to inject cells in the striatal part of the lesion. Rats were anesthetized with the same protocol as for stroke induction and placed in a stereotaxic frame (Stoelting, Chicago, IL, USA) with a mask delivering isoflurane during the procedure. After opening the skin and careful drilling of the chosen entry point, 500,000 hASCs were prepared
in 10-µl medium solution and injected in 1 minute through a 26-gauge needle (RN-type, NHBIO) with a UltraMicropump III with Micro4Controller (World Precision Instruments,
Friedberg, Germany). The needle was kept in place for 2 minutes before careful progressive
removal. The control subgroup did not undergo intracerebral surgery, as we aimed at assessing
the impact of intracerebral administration on neurofunctional and imaging readouts.

451 Neurofunctional testing

452 <u>Staircase test:</u>

Staircase testing was performed under restricted feeding to maintain body weight at 90% of 453 454 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 weight loss. No diet restriction was imposed for 2 days before and 7 days after stroke to allow 456 457 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 training (pellet retrieval ≥ 6 pellets in 10 minutes) were selected before stroke induction. 460 461 Unilateral stroke was performed to impair the dominant side when rats were lateralized (i.e., taking more pellets on one side than the other: side bias >60%), otherwise they were operated 462 463 on the right side. Rats were filmed during their task (Sony Xperia ZD compact) and analyses were made by a blinded observer after anonymization of the movies. The number of pellets 464 465 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 neurofunctional deficit and was calculated as contra/(ipsi+contra) performance, with 'ipsi' 468

469 corresponding to the brain-spared side and 'contra' to the brain ischemic side (28). Staircase470 tests were carried out in the morning or in the afternoon in alternation.

471 <u>Neuroscore</u>:

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

475 MRI

For *in vivo* MRI, anesthesia was induced and maintained in the same way as during the surgery. 476 477 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, RAPID Biomedical, Rimpar, Germany). MRI acquisitions were performed on a horizontal 7T 479 480 BRUKER Biospec MRI system (Bruker Biospin MRI GbmH 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 <u>Images analyses</u>:

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

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

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

size calculation were made with G*Power 3.9.11.2 for a power of 0.8, an alpha error of 0.05

- 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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- 636

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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
- 655 Resources: CD, MC, VH, EO, CA
- 656 Software: MC
- 657 Supervision: MW, CP, CR, FC, CA
- 658 Validation: MW, CP, CR, FC, ECS
- 659 Visualization: CD, CP, MC
- 660 Writing original draft: MW
- 661 Writing review & editing: All authors
- 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—<u>https://figshare.com</u>
- 668 (<u>https://figshare.com/s/15af2a099076389d2a5e</u>).

669 Figure legends

670 Figure 1- Study design

a. Experimental design; b. Focus on the first week of the experiment; c. Focus on weeks 6 to
15: extended follow-up only for rats with corticostriatal lesions. D: Days, MRI: magnetic
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 ± SD. W: weeks, D: days; *p<0.05, (corticostritatal + striatal) vs hypothalamic, Wilcoxon-Mann-Whitney test; †p<0.05, ††p<0.01, 682 683 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 ± SD. W: weeks, D: days. *p<0.05, **p<0.01, (corticostritatal + 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 subtype: pooled (corticostriatal+striatal) vs. hypothalamic lesions at day 4 (D4) and week 5 698 699 (W5) post-surgery. FA: fractional anisotropy, MD: mean diffusivity, AD: axial diffusivity, RD: radial diffusivity. *p<0.05, **p<0.01, (corticostriatal+striatal) vs. hypothalamic, Wilcoxon-700 701 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.

Figure 5- Correlations between neurofunctional and imaging outcomes and sample sizecalculation.

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 the hypothesized value of AD in the treatment group and the x-axis represents the 713 714 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,

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).

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