A NEW GENERATION OF BRAIN-TARGETED NANOPARTICLES FOR CHOLESTEROL DELIVERY IN HD: KINETICS, DRUG RELEASE AND BEHAVIORAL EFFECTS IN MOUSE MODELS

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Background
Cholesterol (chol), an essential molecule for brain function, is produced locally in the brain as the blood-brain barrier (BBB) prevents its uptake from blood. Huntington disease (HD) is associated with reduced synthesis within the brain, especially in the striatum (Shankaran 2017).
Previous studies showed that the delivery of a low dose of chol (20µg) to the brain of R6/2 mice via systemic injection of brain-permeable polymeric nanoparticles (NPs-Chol_1.0) improved synaptic and cognitive but not motor defects (Valenza 2015). Recently, by infusing three doses of chol (20-250-500µg over 4 weeks) into the striatum of R6/2 mice through osmotic minipumps, we identified the optimal dose of chol (500µg) to ameliorate both motor and cognitive defects, rescue morphological and functional abnormalities of striatal neurons, and reduce mutant huntingtin aggregates (Birolini 2020).

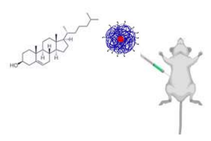
Aims and Methods
To develop a cholesterol-based strategy for HD, a new generation of brain-permeable NPs (NPs-Chol_2.0), with enhanced drug loading capacity, has been developed. Distribution, kinetics, drug release, efficacy and safety have been explored in two HD mouse models.

Results
NPs-Chol_2.0 rapidly target neural cells. Chol is released in a controlled manner and accumulates over time in the brain, while being rapidly removed from peripheral tissues and plasma. Systemic and repeated injections of NPs-Chol_2.0 prevent cognitive decline and ameliorate motor defects in R6/2 mice, without any inflammatory reaction (Birolini 2021). Different therapeutic regimens are ongoing in Q175 mice to explore the long-term effects of Chol on behavioural, molecular, and functional parameters. Preliminary data show that NPs-Chol_2.0 prevents cognitive decline and muscular strength loss, and rescues paw clasping behaviour and late-onset motor defects in these HD mice.

Conclusions
This study provides insights about the benefits of chol delivery through advanced brain-permeable NPs for HD treatment.

1. Background: the rational of cholesterol based therapies for HD

- Circulating cholesterol is not able to cross the BBB and is produced locally in the brain *(Dietschy and Turley 2001)*
- Cholesterol is critical for brain development and function *(Mauch et al., 2001; Funfschilling et al., 2012; Ferris et al., 2017)*

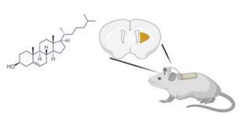


NPs-Chol_1.0

- **Proof-of-concept**
- Amelioration of synaptic defects
- Prevention of cognitive decline
- No motor rescue
- Low drug loading

(Valenza et al., Embo Mol Med 2015)

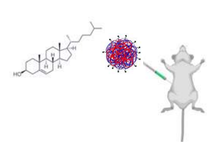
- Brain cholesterol biosynthesis is early reduced in HD animal models *(Valenza et al., 2005; 2007; 2010; Shankaran et al., 2017)*
- The striatum is the most affected area *(Shankaran et al., 2017)*
- The molecular mechanism is known *(Valenza et al., 2005; 2015; Di Pardo et al. 2020; Birolini et al., 2021)*
- The cerebral dysfunction is measurable in blood of HD patients *(Leoni et al., 2008; 2013)*



Osmotic mini-pumps

- Dose escalation study
- **Optimal dose identified**
- Prevention of cognitive decline and synaptic defects
- Dose-dependent motor rescue
- Rescue of other HD phenotypes
- **Mechanisms of action**

(Birolini et al., Embo Mol Med 2020)

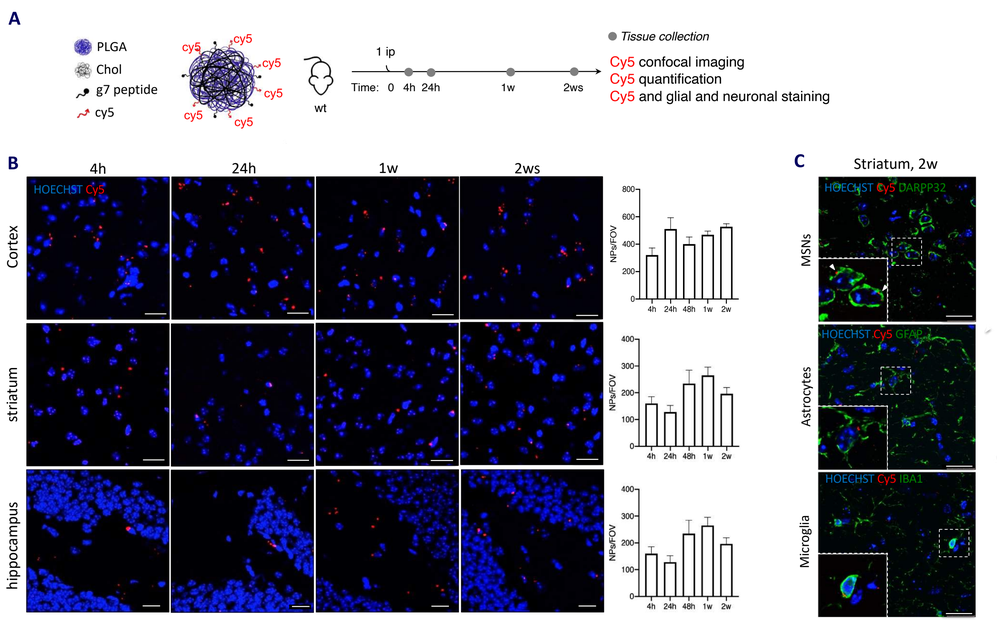


NPs-Chol_2.0

- **Increased drug loading**

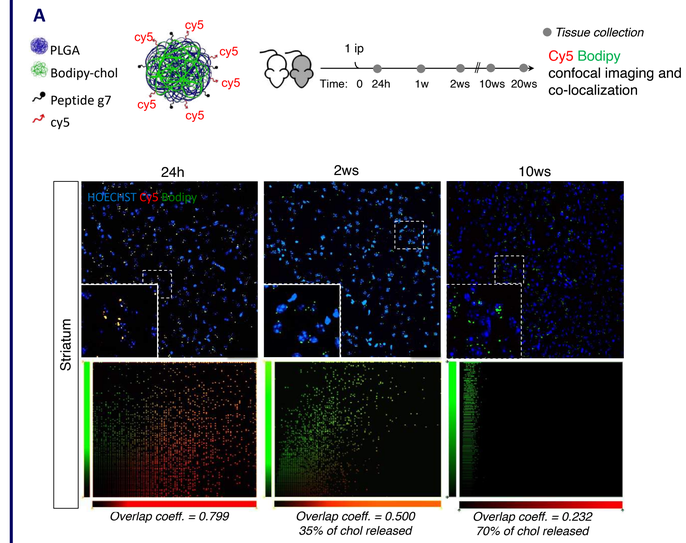
(Belletti et al., Int J Pharm 2018)

2. NPs-Chol_2.0 cross the BBB and reach the brain

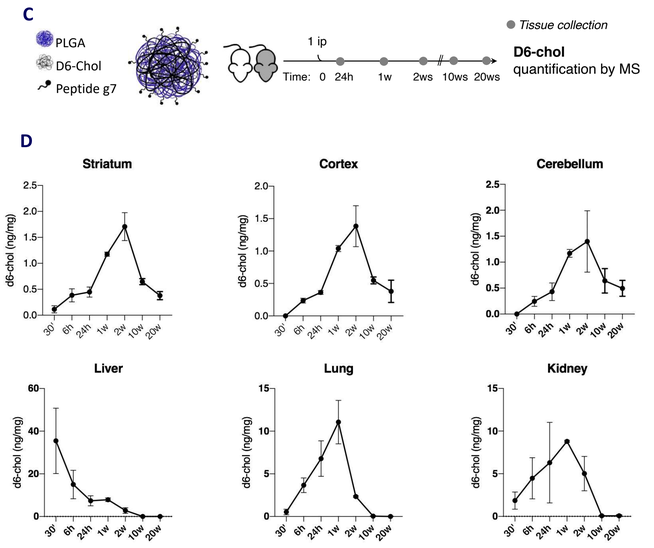


A. 7 weeks-old wt mice were treated with a single I.P. injection of NP-Chol_2.0 (1,18 mg) labelled with cy5. Mice were sacrificed at different time points (n=2/time point) to analyse distribution of NPs (Cy5). **B.** Distribution of NP-Chol_2.0 in different brain regions after a single I.P. injection and relative quantification (right). Graphs indicate the numbers of NPs in a field of view (FOV) (n=5 images/time point). **C.** Cellular distribution of NP-Chol_2.0 in medium spiny neurons (MSNs), astrocytes and microglia in the brain of wt mice following a single I.P. injection. Scale bars: 15 μm (B) and 25 μm (C).

3. Chol release and quantification from NPs-Chol_2.0 *in vivo*

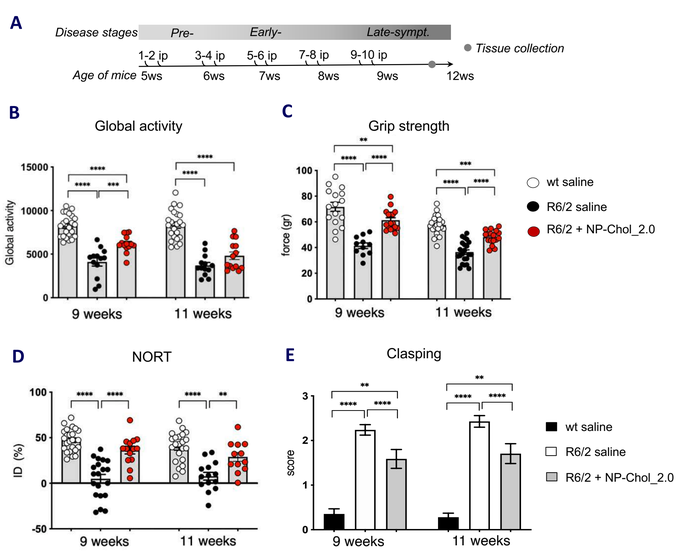


A. To assess whether cholesterol is released from NPs, wt and HD mice were treated with a single I.P. injection of NP-Chol_2.0 labelled with cy5 and loaded with bodipy-chol, a fluorescent cholesterol derivative. Mice were sacrificed at different time point (24h, 1 week, 2 weeks, 10 weeks and 20 weeks after the I.P. injection. **B.** Distribution of NP-Chol_2.0 (red dots) and of bodipy-chol (green signal) was analyzed by IF analysis in the striatum (B) and in cortex (not shown). 24h after a single I.P., NPs and bodipy-chol co-localized. However, starting from 2 week after the injection, red and green signals were no longer co-localizing, meaning that cholesterol was released and NPs were degraded over time. Co-localization analysis was performed by Volocity software.



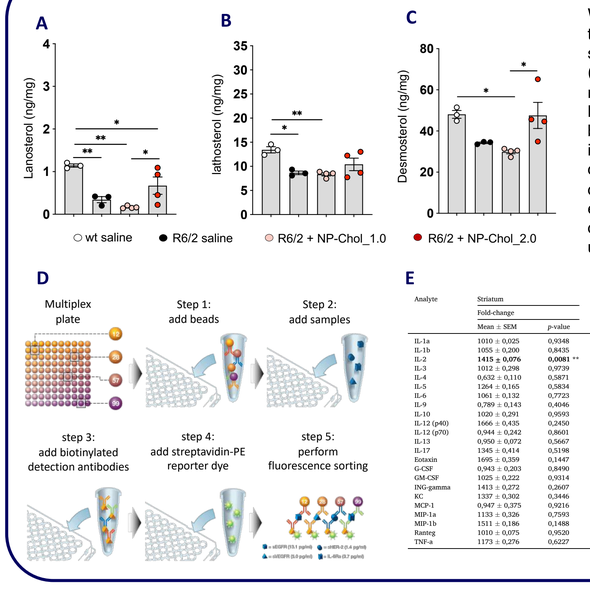
C. Mice were treated with a single I.P. injection of NP-Chol_2.0 and sacrificed at different time point after the I.P. injection). **D.** Striatum, cortex, liver, lung, kidney and plasma were collected to measure the amount of d6-chol in different tissues by means of mass spectrometry. The amount of D6-chol increases over the time in brain tissues, meaning that cholesterol is released from the NPs and accumulates over time. While, D6-chol levels decreases with time in peripheral tissues and in blood, indicating that it is rapidly metabolized in periphery.

4. NPs-Chol_2.0 ameliorates behavioural deficits in R6/2 mice



A. Experimental groups and paradigm: mice were treated twice a week for 5 weeks with saline or with NP-Chol_2.0 (1,8 mg of NPs loaded with 660μg of chol) and sacrificed 2 weeks after the last I.P. injection (at 11-12 weeks of age).
B. Global Activity was reduced in R6/2 mice at both 9 and 11 weeks of age. Of note, at 9 weeks of age there was a partial rescue of this parameter in cholesterol treated mice. **C.** Grip strength allows to study neuromuscular functions and strength by determining the force developed by mouse when the operator tries to pull it out of a grid. HD mice muscular strength was reduced from 9 weeks of age and it was completely rescued by cholesterol treatment. **D.** Novel Object Recognition Test (NORT) is a cognitive test based on the spontaneous tendency of rodents to spend more time exploring a new object than a familiar one, reflected by a positive discrimination index (DI). As expected, the DI of R6/2 mice was decreased at 9 weeks of age compared to wt mice. The delivery of exogenous cholesterol to the brain completely rescued the cognitive defects. **E.** Paw clasping measures hindlimb clasping, a marker of disease progression in several mouse models of neurodegeneration. Saline-treated HD mice retracted one or both the hindlimbs starting from 9 weeks of age. Of note, cholesterol-treated mice ameliorated this phenotype. Statistics: Data represent mean ± SEM. P<0.05 was determined by one-way ANOVA followed by Newmann–Keuls multiple comparison tests (*P<0.05, **P<0.01, ***P<0.001, ***P<0.0001).

5. NPs-Chol_2.0 enhances endogenous cholesterol biosynthesis in R6/2 mice



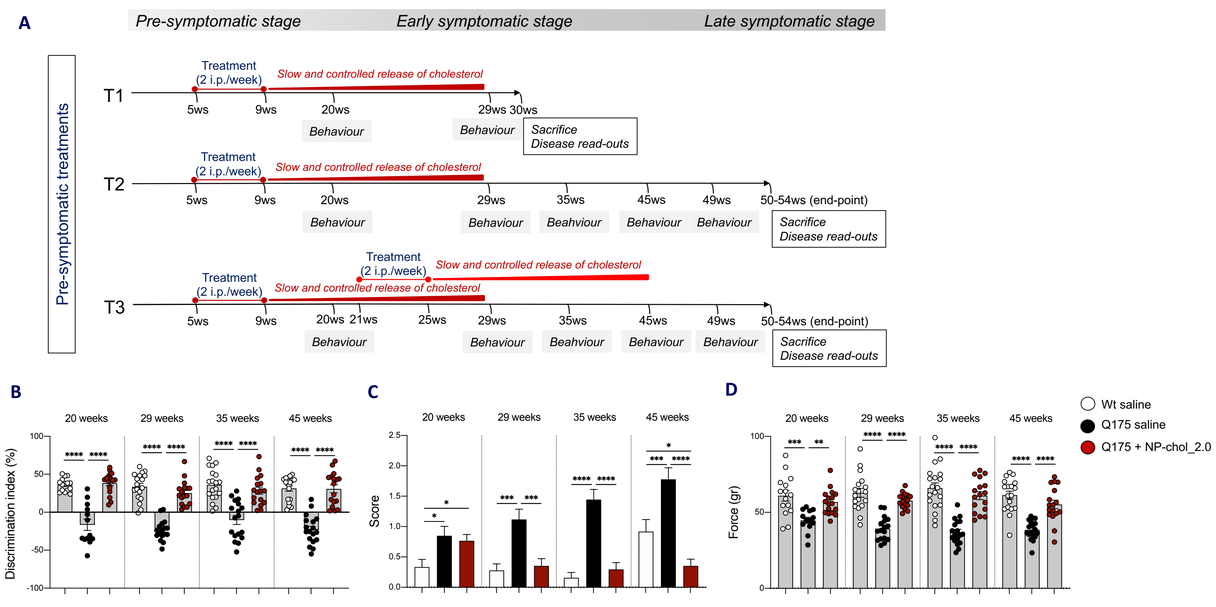
We have recently demonstrated that a therapeutically relevant dose of Chol delivered to the striatum through osmotic mini-pumps indirectly stimulated endogenous cholesterol synthesis, leading to the reversal of both motor and cognitive abnormalities in HD mice (Birolini et al., 2020). In contrast, lower doses delivered to the striatum through osmotic mini-pumps (Birolini et al., 2020) or to the brain *via* NP-Chol_1.0 (Valenza et al., 2015) led to a complete rescue of cognitive decline without significant change in endogenous brain cholesterol biosynthesis or in motor performance. **A-C.** To test whether the increased amount of chol in NP-Chol_2.0 was sufficient to stimulate endogenous cholesterol synthesis in the diseased brain, lanosterol (A), lathosterol (B), and desmosterol (C) in the striatum were measured by mass spectrometry following the experimental paradigm described in 4A. Significant increase in lanosterol and desmosterol levels was found in striatal tissues of R6/2 mice treated with NP-Chol_2.0 unlike NP-Chol_1.0 which did not show any effects.

E

Analyte	Striatum		Cortex		Liver		Plasma	
	Fold-change		Fold-change		Fold-change		Fold-change	
	Mean ± SEM	p-value	Mean ± SEM	p-value	Mean ± SEM	p-value	Mean ± SEM	p-value
IL-1a	1010 ± 0,025	0,9348	0,927 ± 0,033	0,3884	0,976 ± 0,111	0,8622	**0,212 ± 0,032**	**0,0078** **
IL-1b	1055 ± 0,200	0,8435	0,747 ± 0,134	0,3254	1088 ± 0,184	0,7466	0,920 ± 0,381	0,8608
IL-2	**1415 ± 0,076**	**0,0081** **	0,708 ± 0,013	0,1058	1159 ± 0,179	0,4831	**0,398 ± 0,081**	**0,0467** *
IL-3	1012 ± 0,298	0,9739	0,771 ± 0,097	0,2840	1031 ± 0,038	0,7151	N/D	
IL-4	0,632 ± 0,110	0,5871	1472 ± 0,499	0,4103	0,744 ± 0,182	0,4333	0,429 ± 0,184	0,3575
IL-5	1264 ± 0,165	0,5834	1276 ± 0,561	0,6831	0,924 ± 0,158	0,7211	0,330 ± 0,085	0,1610
IL-6	1061 ± 0,132	0,7723	1019 ± 0,102	0,9132	0,889 ± 0,133	0,5446	N/D	
IL-9	0,789 ± 0,143	0,4046	0,836 ± 0,145	0,5136	0,900 ± 0,112	0,6095	N/D	
IL-10	1020 ± 0,291	0,9593	0,677 ± 0,123	0,1301	1109 ± 0,354	0,8100	1175 ± 0,002	0,1610
IL-12 (p40)	1666 ± 0,435	0,2450	0,667 ± 0,176	0,3861	1843 ± 0,113	0,1025	0,923 ± 0,236	0,8124
IL-12 (p70)	0,944 ± 0,242	0,8601	0,798 ± 0,138	0,3859	0,803 ± 0,116	0,4620	N/D	
IL-13	0,950 ± 0,072	0,5667	0,835 ± 0,069	0,2902	0,977 ± 0,097	0,8460	1099 ± 0,152	0,5886
IL-17	1345 ± 0,414	0,5198	0,892 ± 0,125	0,2902	1194 ± 0,104	0,3108	1345 ± 0,414	0,5198
Eotaxin	1695 ± 0,359	0,1447	**0,447 ± 0,078**	**0,0228** *	0,958 ± 0,215	0,8927	0,809 ± 0,195	0,5637
G-CSF	0,943 ± 0,203	0,8490	0,914 ± 0,153	0,6571	1001 ± 0,120	0,9970	0,987 ± 0,148	0,9529
GM-CSF	1025 ± 0,222	0,9314	1060 ± 0,220	0,8302	1090 ± 0,080	0,4218	0,724 ± 0,188	0,2956
ING-gamma	1413 ± 0,272	0,2607	0,899 ± 0,102	0,5404	1155 ± 0,184	0,4968	0,749 ± 0,322	0,7225
KC	1337 ± 0,302	0,3446	1340 ± 0,169	0,1438	0,988 ± 0,313	0,9742	0,489 ± 0,086	0,1494
MCP-1	0,947 ± 0,375	0,9216	0,907 ± 0,196	0,8058	1657 ± 0,414	0,2082	1365 ± 0,689	0,7636
MIP-1a	1133 ± 0,326	0,7593	0,816 ± 0,138	0,4708	1065 ± 0,126	0,6874	0,525 ± 0,222	0,5987
MIP-1b	1511 ± 0,186	0,1488	0,731 ± 0,137	0,3533	0,920 ± 0,192	0,7806	0,614 ± 0,452	0,4762
Rantes	1010 ± 0,075	0,9520	1020 ± 0,134	0,9271	0,882 ± 0,087	0,5915	0,998 ± 0,231	0,9954
TNF-a	1173 ± 0,276	0,6227	0,854 ± 0,249	0,6447	1100 ± 0,352	0,8135	0,851 ± 0,371	0,8106

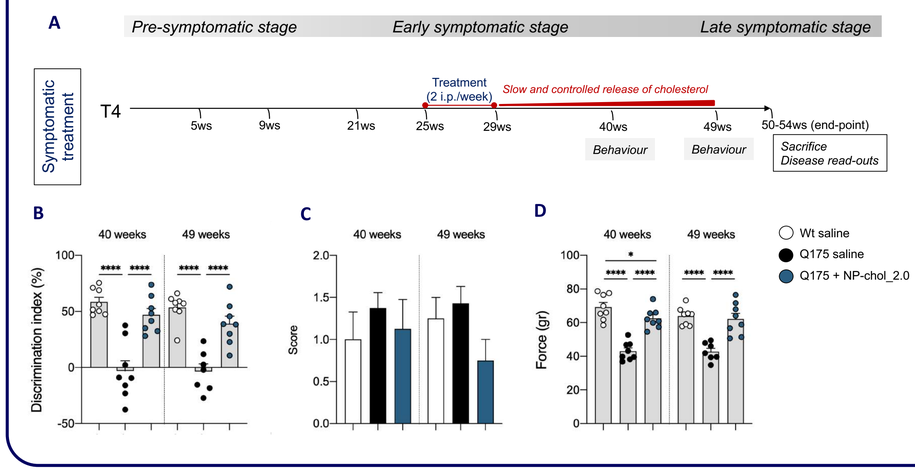
D. To explore any side effects of chronic administration of NPs-chol_2.0, the inflammation status of treated mice was analyzed through the simultaneous detection of 23 analytes in a single well of a 96-well microplate using the Bioplex assay in different brain regions, peripheral tissues and plasma.
E. No substantial changes were observed between groups. Data expressed as fold-change (HD mice treated with NP-Chol_2.0 vs HD mice treated with saline).

6. NP-Chol_2.0 prevent behavioural deficits in Q175 mice (pre-symptomatic treatment)



A. To explore the long-term potential of the new NPs, we used the Q175 mouse model and we tested different therapeutic regimens starting the treatment before the onset of motor and cognitive defects. Trial 1 (T1) and trial 2 (T2) are still ongoing. **B.** At 20 weeks of age Q175 mice exhibited a pronounced cognitive deficit. The amount of cholesterol released from NPs at this time point, following two cycles of treatments (T3) was sufficient to totally rescue cognitive defect in Q175 mice. Importantly, the rescue persisted also at 29, 35 and 45 weeks of age. **C.** Q175 mice manifested low clasping activity at 20 weeks of age which was not rescued in cholesterol-treated mice. At 29 weeks of age, a significant rescue was observed that persisted over time. **D.** The neuromuscular deficit was also prevented in Q175 mice treated with NPs-Chol_2.0 at all time points analysed. Data are shown as mean ± SEM. Statistic: One-way anova followed by Newman Keuls

7. NP-Chol_2.0 rescue behavioural deficits in Q175 mice (symptomatic treatment)



A. To explore whether NP-Chol_2.0 are able also to rescue motor and cognitive deficits in symptomatic Q175 mice, we also tested a therapeutic regimen started when symptoms were already present.
B. At 40 and 49 weeks of age Q175 mice treated with NPs did not show cognitive defect and the behaviour was similar to that observed in wt mice.
C. Clasping deficit at 40 weeks of age was not rescued in cholesterol-treated mice. At 49 weeks of age we observed a trend of amelioration but with no statistical significance.
D. The neuromuscular deficit was rescued in Q175 mice treated with NPs-Chol_2.0 at 40 and 49 weeks of age.
Data are shown as mean ± SEM. Statistic: One-way anova followed by Newman Keuls

Conclusions

- A new generation of NPs (NPs-Chol_2.0) was optimized with enhanced cholesterol loading capacity (*Belletti et al., Int J Pharm 2018*); NPs kinetics and cholesterol release and quantification was also explored (*Birolini, Valenza et al., Journal of Controlled Release, 2021*)
- Delivery of cholesterol to the brain via NPs-Chol_2.0 ameliorates disease phenotypes in R6/2 mice (*Birolini, Valenza et al., Journal of Controlled Release, 2021*)
- Beneficial effects persist long-term in Q175 mice (*unpublished data*)

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