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Chapter 4
Effect of Post-Mortem Delay on N-terminal Huntingtin Protein Fragments in Human Control and Huntington Disease Brain Lysates

Menno H. Schut1, Stefano Patassini3, Eric H. Kim3, Jocelyn Bullock3, Henry J. Waldvogel3, Richard L.M. Faull3, Barry A. Pepers1, Johan T.den Dunnen1,2, Gert-Jan B. van Ommen1, and Willeke M.C. van Roon-Mom1*

1 Department of Human Genetics, Leiden University Medical Center, 2333ZA Leiden, The Netherlands
2 Leiden Genome Technology Center, Leiden University Medical Center, 2333ZA Leiden, The Netherlands
3 Centre for Brain Research and Department of Anatomy with Radiology, University of Auckland, Auckland 1023, New Zealand

* Corresponding author: Willeke M.C. van Roon-Mom, Department of Human Genetics, Leiden University Medical Center, Albinusdreef 2, 2333ZA Leiden, The Netherlands. Tel: +31 71 5269435; Fax: +31 71 5268285; Email: w.vanroon@lumc.nl

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Abstract
Huntington disease is associated with elongation of a CAG repeat in the HTT gene that results in a mutant huntingtin protein. Several studies have implicated N-terminal huntingtin protein fragments in Huntington disease pathogenesis. Ideally, these fragments are studied in human brain tissue but the use of human brain tissue comes with certain unavoidable variables such as post mortem delay, artefacts from freeze-thaw cycles and subject-to-subject variation. Knowledge on how these variables affect N-terminal huntingtin protein fragments in post mortem human brain is important for a proper interpretation of study results. The effect of post mortem delay on protein in human brain is known to vary depending on the protein of interest. In the present study, we have assessed the effect of post mortem delay on N-terminal huntingtin protein fragments using western blot. We mimicked post mortem delay in one individual control case and one individual Huntington disease case with low initial post mortem delay. The influence of subject-to-subject variation on N-terminal huntingtin fragments was assessed in human cortex and human striatum using two cohorts of control and Huntington disease subjects. Our results show that effects of post mortem delay on N-terminal huntingtin protein fragments are minor. Additionally, we show that one freeze-thaw cycle decreases the huntingtin western blot signal intensity, but does not introduce additional N-terminal huntingtin fragments. We conclude that subject-to-subject variation contributes more to variability in N-terminal huntingtin fragments than post mortem delay.

Introduction
Huntington disease (HD), is an autosomal dominant neurodegenerative disease that primarily affects the striatum and is caused by a CAG repeat expansion in the first exon of the HTT gene [1]. This results in a toxic huntingtin protein (htt) with an N-terminally expanded polyQ-stretch [2, 3]. Subsequent studies using HD mouse models with comparable polyQ-repeats expressing either N-terminal, or full length htt showed that N-terminal htt fragments invoke a more severe phenotype [4-6], with smaller fragments inducing the most severe phenotype [7, 8]. The htt N-terminal domain contains several proteolytic cleavage sites, some of which have been linked to HD pathology [9-12]. Results obtained from different mouse-models are sometimes conflicting. YAC128 mouse models expressing full length mutant htt resistant against caspase-3 or caspase-6 cleavage suggested that caspase-6 cleavage at amino acid 586 contributes more to HD pathogenesis than caspase-3 cleavage at amino acids 513 and 552 [13]. On the other hand, murine expression of the N552-htt fragment was shown to be more lethal compared with other caspase-associated htt fragments including N586-htt [7]. With regards to HD research on N-terminal htt protein fragments, it is important to note that studies in rat, mouse and post mortem human brain tissue have indicated that calpain-mediated proteolysis also occurs during post mortem delay (PMD) which is the time between death and tissue preservation. In rat brain, PMD-related fodrin calpain cleavage fragments were observed [14], and in mouse and human brain tissue PMD-related GSK-3 truncation by calpain was demonstrated [15]. Hence, it is important to distinguish biologically relevant N-terminal htt fragments from those that are formed during PMD. Available data on N-terminal htt protein
fragments in human post mortem brain is limited, but also indicates a role in HD pathology. N-terminal htt fragments in post mortem human HD striatum differ with post mortem human control striatum [16]. Also, the N552 htt fragment that is associated with HD pathology in a HD mouse model was detected in post mortem human HD and control brain tissue lysates [11]. Therapeutic strategies involving prevention of formation of N-terminal htt fragments are currently pursued [17]. However, extrapolation of results obtained in cell and animal models to the human brain is difficult. N-terminal htt fragments may vary between different biological systems [18]. Furthermore, research involving post mortem human brain tissue involves unavoidable variables that are difficult to control such as PMD, post mortem processing, and interpersonal variation. Effects of PMD on biological parameters have been well described [19]. In rat brain, synaptic density and vesicles decline after a PMD of 15hr, with subtle changes on synaptic structure [20]. Binding sites for forskolin, an activator for adenylate cyclase, were already reduced in rat striatum after a PMD of 4 hours [21]. PMD related effects vary between different molecules and proteins. No effect of PMD for up to 72 hours was observed for fatty acid molecules in post mortem human brain tissue [22]. PMD was shown to variously affect different proteins in mouse CNS [23]. A study on different post mortem human brain samples indicated that levels of synaptic proteins PSD-95 and syntaxin, but not synaptophysin, decline with PMD [24]. However, care must be taken when comparing different subjects with comparable PMD because PMD related effects might also vary between subjects [25, 26]. In the current study, we have assessed effects of PMD, freezing and inter-individual variation between human subjects on the profile of N-terminal htt fragments on western blot. Our study shows a small effect of artificially induced PMD on N-terminal htt fragments. Furthermore, one freeze-thaw cycle already adversely affected the western blot signal for huntingtin in human temporal lobe tissue, but did not result in the appearance of novel N-terminal htt fragments. Finally, analysis of nine control versus nine HD subjects matched for age, gender and PMD showed that there was more inter-individual variability in the profile of N-terminal htt fragments than the variability introduced by PMD.
Materials and Methods

Human brain tissue
Post mortem human cortical and striatal brain tissue from control and HD subjects was obtained from the Neurological Foundation of New Zealand Human Brain Bank, Centre for Brain Research, University of Auckland. The temporal lobe tissue was obtained 1 hour after surgery on a patient suffering from severe epilepsy. Tissue was obtained with the approval by the University of Auckland Human Participants Ethics Committee and informed consent from all families. See Table 1 for the complete list of samples with clinical information.

Human brain tissue for analysis of post mortem delay effects.
To mimic post mortem delay, tissue samples were taken from the main tissue specimen at regular time intervals (sampling). During sampling, tissue was left at room-temperature under sterile conditions. 1 ml of chilled homogenization buffer (150 mM Sucrose, 15 mM HEPES pH 7.9, 60 mM KCl, 0.5 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1% Triton X-100) was added. Tissue was homogenized with a bullet blender (Next Advance) for 3 minutes, strength 8 using 0.5 mm stainless steel beads. Homogenized tissue was kept on ice for 1 hour and centrifuged cold at full speed for 10 minutes. Supernatant was aliquoted in 100 µl portions, snap-frozen and stored at -80°C. To mimic post mortem delay with and without freeze-thaw cycle, the temporal lobe tissue was divided into two halves immediately after obtaining the tissue. One half was subsequently sampled, while the other was snap-frozen and stored at -80°C according to [27] for 11 days before sampling.

Post mortem human brain tissue lysates.
For every subject, sensory/motor cortex and caudate nucleus tissue was collected separately. Per subject and region, 15 slides (thickness: 30 µm) of unfixed tissue were collected on glass slides with a cryostat-microtome (LEICA). Grey matter was scraped off, weighed and collected in 0.5 ml eppendorf tubes. 10 µl of chilled homogenization buffer was added per µg of tissue with a minimum of 200 µl. Tissue was homogenized with a bullet blender (Next Advance) for 3 minutes, strength 8 using 0.5 mm stainless steel beads. Homogenized tissue was kept on ice for 1 hour, centrifuged cold at full speed for 10 minutes. Supernatant was aliquoted in 100 µl portions, snap-frozen and stored at -80°C.
Table 1 – Clinical information

| Type   | Number | Name  | Age | Gender | PMD | CAG 1 | CAG 2 | Grade |
|--------|--------|-------|-----|--------|-----|-------|-------|-------|
| HD     | n.a.   | HC107 | 75  | M      | 3   | 43    | 19    | 3     |
| Control| C1     | H110  | 83  | F      | 14  | N.A.  | N.A.  | n.a.  |
| Control| C2     | H202  | 83  | M      | 14  | N.A.  | N.A.  | n.a.  |
| Control| C3     | H157  | 66  | M      | 15  | N.A.  | N.A.  | n.a.  |
| Control| C4     | H155  | 61  | M      | 7   | N.A.  | N.A.  | n.a.  |
| Control| C5     | H146  | 61  | M      | 15  | N.A.  | N.A.  | n.a.  |
| Control| C6     | H159  | 53  | M      | 16.5| N.A.  | N.A.  | n.a.  |
| Control| C7     | H174  | 59  | M      | 24.5| N.A.  | N.A.  | n.a.  |
| Control| C8     | H130  | 32  | M      | 13  | N.A.  | N.A.  | n.a.  |
| Control| C9     | H200  | 56  | M      | 23  | N.A.  | N.A.  | n.a.  |
| HD     | HD1    | HC111 | 91  | F      | 18  | 40    | 15    | 2     |
| HD     | HD2    | HC137 | 83  | M      | 13  | 41    | 17    | 1     |
| HD     | HD3    | HC133 | 65  | M      | 14  | 43    | 17    | 3     |
| HD     | HD4    | HC134 | 62  | M      | 9   | 43    | 18    | 2     |
| HD     | HD5    | HC113 | 58  | M      | 14  | 44    | 28    | 2     |
| HD     | HD6    | HC120 | 51  | M      | 15  | 46    | 10    | 2     |
| HD     | HD7    | HC115 | 56  | M      | 16  | 46    | 16    | 2     |
| HD     | HD8    | HC132 | 32  | M      | 14  | 47    | 17    | 1     |
| HD     | HD9    | HC119 | 51  | M      | 15.5| 48    | 17    | 3     |

Age: Age in years at death. F: female, M: male. PMD: post mortem delay in hours. CAG1 = CAG repeat length of mutant allele, CAG2 = CAG repeat length of normal allele. N.A. Not assessed. n.a. Not applicable. HD grade according to [28].

Average age for control subjects = 61.6 ± 15.5 years, HD subjects = 61.0 ± 17.6 years. Average PMD for control subjects = 15.8 ± 5.3 hours, HD subjects = 14.3 ± 2.5 hours. Average CAG repeat length in HD subjects = 44.2 ± 2.7 CAGs (mutant allele), 17.2 ± 4.7 CAGs (normal allele).

Western Blotting.

100 µg of human brain lysate was used per sample. To detect full length htt, proteins were separated by SDS-PAGE according to the “shorter CAG repeats” protocol [29]. Proteins were transferred to a nitrocellulose membrane (Trans-Blot Turbo Transfer Pack Midi #170-4158, Bio-Rad, Hercules CA, USA) using the Trans-blot Turbo Transfer system (BioRad) with settings 2.5 A for 10 minutes. Blots were blocked with TBS containing 5% (w/v) non-fat milk (Nutricia, Schiphol, The Netherlands), and incubated with primary antibody 3702-1 (Epitomics, Burlingame CA, USA) that binds the htt N17 terminus [30]. Secondary antibody goat anti rabbit IRDye800 (LI-COR, Lincoln, USA) diluted 1:5000 in TBS containing 5% non-fat milk (w/v). Blots were analyzed with the Odyssey infrared imaging system and Odyssey software version 3.0 (LI-COR).

To detect htt fragments, proteins were separated by SDS-PAGE by running at 40 mA constant through the stacking gel and at 50 mA constant through the 10% separating gel alongside a protein size marker (PageRuler, Thermo Fisher, St Leon-Rot, Germany). Proteins were blotted onto either a nitrocellulose membrane for Control versus HD subjects (Trans-Blot Turbo Transfer Pack Midi #170-4158, Bio-Rad, Hercules CA, USA), or a PVDF-membrane for post mortem delay samples (Trans-Blot Turbo Transfer Pack Midi #170-4157), using the Trans-Blot Turbo Transfer System (Bio-Rad) with settings 1.0 A for 30 minutes. Blots were blocked with TBS containing 5% non-fat milk (Nutricia). For nitrocellulose-blots, primary incubation was performed with antibodies 3702-1 (Epitomics) and mouse anti β-actin. Secondary incubation
was performed with goat anti rabbit IRDye800 (LI-COR) and goat anti mouse IRDye680 (LI-COR). All antibodies were diluted 1:5000 in TBS containing 5% non-fat milk (Nutricia). Blots were analysed with the Odyssey reader and viewed using the Odyssey software version 3.0 (LI-COR) with the linear manual sensitivity set at 5, or 6 if blot was viewed at “high sensitivity”. For PVDF-blots, primary incubation was performed with antibody 3702-1 (Epitomics) diluted 1:1000. Secondary incubation was performed with a Goat anti Rabbit antibody conjugated with Horse Radish Peroxidase (Santa Cruz) diluted 1:10.000. Blots were visualized using ECL+ substrate (#32132, ThermoFisher Scientific), and Hyperfilm ECL (#28906837, GE healthcare). For the β-actin loading control, blots were stripped, re-blocked and incubated with mouse β-actin diluted 1:1000, followed by a Goat anti Mouse antibody conjugated with Horse Radish Peroxidase (Santa Cruz) diluted 1:10.000. Densitometric analysis was performed with image J. Intensities of relevant bands were reported as percentages of the total htt signal within the associated lane. Statistical significance (n=4) between timepoints was calculated in Excel using a paired T-test (two sided).
**Results**

Two wild-type N-terminal htt fragments increase with artificial PMD in control temporal lobe tissue.

To assess the effect of PMD on N-terminal htt fragments, we obtained human control temporal lobe tissue one hour post-surgery. One half was stored at -80°C and the other half was sampled at regular time-intervals to mimic PMD (artificial PMD). Western blot analysis with an antibody that binds huntingtin at the N-terminal end showed no change in the full length htt signal, and a subtle gradual increase in band intensity compared with T=0 was observed for N-terminal htt fragments of 50 kDa (1.49% ± 1.12% on average), and 65 kDa (4.01% ± 1.53 on average) over an eight-hour time period (**Figs 1a and 1c**). The increase became significant for the 65 kDa band at T=4. Next, we performed the same experiment using the frozen half of the temporal lobe. Here, we detected N-terminal fragments of the same molecular weight compared with the non-frozen tissue, but western blot bands corresponding to full-length and N-terminal htt fragments of more than 100 kDa became weaker with increasing artificial PMD (**Fig 1b**). The relative intensities of the 50kDa and 65kDa bands at T=0hr from the frozen sample were higher compared to the non-frozen tissue at T=0hr. This difference was 2.06% ±1.38% (p=0.06) for the 50kDa band, and for the 65 kDa band the difference was 4.63% ±1.88% (p <0.05). Furthermore, we observed an increase in band intensity in the frozen sample after an artificial PMD of 8 hours for N-terminal htt fragments of 50 kDa (10.53% ±5.16%) and 65 kDa (13.33% ±2.04%) (**Fig 1d**) compared with T=0, that was already significant after 2 hours. This is in contrast to the subtle gradual increase observed in the non-frozen tissue. We did not detect additional N-terminal htt fragments that were introduced by the freeze-thaw cycle. This indicates that freezing of brain tissue affects htt signal strength, but not the profile of htt protein fragments in control brain tissue samples.
Fig 1. **N-terminal huntingtin fragments in control temporal lobe tissue.** Western blot analysis of temporal lobe tissue samples from tissue with no (a) and one freeze-thaw cycle (b) before sampling. Upper blot: full length htt. Middle and lower blot: N-terminal htt fragments with β-actin loading control. Squares: position of bands that increase with increasing artificial PMD (black = 65kDa, white = 50kDa). kDa = Molecular weight in kilodaltons. Artificial post mortem delay in hours (hr). (c) Image J quantification of PMD-related htt bands at indicated timepoints relative to total htt (0 freeze-thaw cycles). (d) Image J quantification of PMD-related htt bands at indicated timepoints relative to total htt (1 freeze-thaw cycle). Columns represent the average of four independent western blots. Black columns: 65 kDa, White columns: 50 kDa. Error bars indicate standard deviation. * = P<0.05, *** = P<0.001, **** = P<0.0001.
Artificial PMD introduces N-terminal htt fragments in HD striatal tissue.

Using tissue from an HD subject with an initial PMD of 3 hours, we analyzed the effect of an increasing artificial PMD in human HD striatal tissue. Western blot analysis of full length htt showed two bands corresponding to wild-type and mutant huntingtin which is in agreement with findings in human HD fibroblast lysates [29]. Artificial PMD decreased band intensities of full-length htt and most N-terminal htt fragments in HD striatal tissue. However, we observed a band at 43 kDa that appeared after an artificial PMD of 24hr (Fig 2a). Additionally, we were able to detect a band corresponding to a small N-terminal htt fragment (<26 kDa) that increased after 24hr, but this low molecular weight band could not reliably be quantified. ImageJ analysis of the 43 kDa band (Fig 2b) revealed a significant increase of 11.61% ±3.88% at T=30hr compared with T=24hr.

Fig 2. Effect of artificial post mortem delay on N-terminal huntingtin fragments in post mortem HD striatal tissue. (a) Western blot analysis of HD striatal tissue (1 freeze-thaw cyclus). Upper blot: full length htt. Middle and lower blot: N-terminal htt fragments with β-actin loading control. Gray square: Band increased with increasing artificial PMD. kDa = Molecular weight in kilodaltons, Artificial post mortem delay in hours (hr). (b) Image J quantification of the 43kDa band at indicated timepoints relative to total htt. Gray columns represent the average of four independent western blots. Error bars indicate standard deviation. **=P<0.01.
Huntington disease subjects show greater variation in full-length and N-terminal huntingtin protein fragment profiles.

Having identified N-terminal htt fragments that can be attributed to longer PMDs, we next analyzed N-terminal htt fragments in post mortem tissue in nine control subjects (mean age = 61.6 ± 15.5 years, mean PMD = 15.8 ± 5.3 hours) and nine HD subjects (mean age = 61.0 ± 17.6 years, mean age = 14.3 ± 2.5 hours). For further clinical information see Table 1. We observed that the western blot signal for full-length htt and N-terminal htt fragments was stronger in control subjects compared to HD subjects, and overall stronger in cortical tissue compared to striatal tissue, while no differences in the β-actin loading controls were observed (S1 Fig). Therefore, for optimal comparison, western blot results for the HD subjects in Figures 3 and 4 are shown with the odyssey software configured at a higher viewing-sensitivity. In control cortical tissue, results were similar for subjects C2-C9 showing a consistent banding pattern with subtle interpersonal differences. The band-pattern for subject C1 was very different, showing relatively more N-terminal htt fragments below 55 kDa (Fig 3a). In HD cortical tissue (Fig 3b), interpersonal variation was more prominent and subjects HD1, HD4 and HD5 showed a weaker N-terminal htt profile. Smaller N-terminal htt fragments below 55 kDa appeared more pronounced with respect to the total htt signal in HD cortical tissue compared with control cortical tissue (S1 Fig). For both control and HD cortical subjects, western blot bands for N-terminal htt fragments were most prominent between 43 kDa and 95 kDa. This was in concordance with the N-terminal htt fragment profile induced by the artificial PMD of > 2 hours in temporal lobe tissue stored at -80°C (Fig 1b). N-terminal htt fragments of 65kDa and 50kDa that were associated with PMD in temporal lobe tissue were detected in both control and HD cortical tissue. However, we did not observe a correlation with the PMD’s of the different subjects.
Next, we performed western blot on post mortem striatal tissue from the same control and HD subjects (Fig 4). Between control subjects (Fig 4a) the N-terminal htt fragment profile was similar, but we observed some interpersonal variation in signal intensity. The overall htt western blot signal in striatum was weak for all HD subjects (Figs 4b and S1). Between HD subjects the N-terminal htt fragment profile was similar with small interpersonal variation. Comparison between control and HD subjects for striatum revealed that the most prominent N-terminal htt fragment was detected at 80kDa in most control subjects, while in most HD subjects (HD1-HD6) the most prominent N-terminal htt fragment was detected at 55kDa. In subjects HD8-HD9, both 80kDa and 55kDa N-terminal htt fragments were detected equally. For control subjects, N-terminal htt fragments of similar sizes can be observed in both cortical and striatal tissue (Figs 3a and 4a). On the other hand, in HD subjects the 55kDa N-terminal fragment is less prominent in cortical tissue compared with striatum (Figs 3b and 4b).
Fig 4. Comparison of N-terminal huntingtin fragments in post mortem control and HD striatum Western blot analysis of (a) Control subjects, (b) HD subjects. Control and HD subjects were age, sex and post mortem delay matched. Upper blot: full length huntingtin (fl htt). Middle and lower blot: N-terminal htt fragments with β-actin loading control. Arrow: position of bands associated with a post mortem delay related increase in intensity in HD striatal tissue. kDa = Molecular weight in kilodaltons, Post mortem delay in hours (hr). High sensitivity: Blot analyzed at a higher viewing sensitivity.
**Discussion**

In the current study we have examined PMD effects on N-terminal htt protein fragments in human brain tissue using western blot analysis on temporal lobe tissue obtained directly post-surgery from a control human subject. Our results show that increasing PMD resulted in a gradual increase of N-terminal htt fragment bands between 50 kDa and 65 kDa on western blot. Other studies detected a similar N-terminal htt fragment of 50 kDa in post mortem human cortical brain tissue obtained from control subjects with a PMD of 9 to 16 hour [16, 18]. Within the context of our study, this 50kDa N-terminal htt fragment could be associated with PMD. However, other N-terminal htt fragments detected in [16, 18], and the caspase-3 related N-terminal htt fragment [11] detected in the cortex of human control subjects were not associated with PMD in our study. Our results further indicate that the effect of one freeze-thaw cycle on N-terminal htt fragments is quantitative, but not qualitative. We observed that one freeze-thaw cycle reduced the htt signal, especially for larger (>95 kDa) htt fragments and the full-length protein. Furthermore, PMD-related N-terminal htt fragments at 50kDa and 65kDa appeared at earlier time points for tissue that underwent one freeze-thaw cycle, suggesting their formation is enhanced due to freeze-thawing. However, no additional N-terminal htt fragments were observed due to freeze-thaw effects. Hence, the common practice of tissue storage at -80°C and subsequent re-thawing for use is not expected to greatly influence study results on N-terminal htt fragments. This is an important finding because there is a well-established link between HD-pathology and small N-terminal htt fragments [7, 13]. Our experiments showed that artificial PMD gave rise to different N-terminal htt fragments in post mortem control temporal lobe tissue when compared with post mortem HD striatal tissue. This could suggest that PMD differently affects HD and control brains or different brain regions, but this difference might also be due to interpersonal differences between both subjects. The results on striatal tissue suggest that the 43 kDa band that is associated with PMD in HD striatum, appears between 8 and 24 hours. A striatal 43 kDa N-terminal fragment was reported before in HD striatum with PMD’s of 9 to 16 hours [16]. In our cortical control and HD subjects, we observed inter individual differences in N-terminal htt fragments, especially for HD subjects. However, we did not observe a correlation between N-terminal htt bands and the PMD’s of individual subjects. It is likely that the observed inter individual differences between subjects are mainly caused by other factors such as genetic or environmental factors. We did not observe different N-terminal htt fragment profiles between control and HD cortical tissue, which is in accordance with previous studies [16, 18]. However compared with these studies, we detected more larger N-terminal htt fragments. Possibly, this is due to the use of different subject-cohorts or western blotting protocols. In our striatal samples, the molecular weight of the most prominent N-terminal htt fragment differed between HD and controls. This suggests a difference in htt proteolytic cleavage in striatum between controls and HD. This is in agreement with [16] although Mende-Mueller et al reported N-terminal htt fragments of smaller sizes. We were probably not able to show these smaller N-terminal htt fragments due to the weak htt western blot signal obtained for HD striatal tissue. El-Daher et al, whom utilized the 4C8 antibody also observed weaker htt signals for HD striatum compared with controls [31]. On the other hand, studies using an anti N17 antibody did not observe a
difference in htt signal between control and HD striatum. [16, 18]. Hence, the observed difference in htt western blot signal between control and HD and cortex versus striatum could be due to variability between different human post mortem brain tissue specimens.

**Conclusion**

By mimicking PMD and a freeze-thaw cycle separately, we were able to provide an overview of expected effects on N-terminal htt fragments in post mortem human brain tissue. According to our results, PMD has a mild qualitative influence on N-terminal htt fragments because it introduces new fragments. A freeze-thaw cycle has a quantitative effect as it affects huntingtin signal strength. Hence, our study contributes to the correct interpretation of data on N-terminal htt fragments obtained from human post mortem brain tissue. Furthermore, our results show that subject to subject variation has a larger, both qualitative and quantitative effect on N-terminal htt fragments. Hence, our study underscores the need for larger subject cohorts in studies involving post mortem human brain tissue.

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S1 Fig– Levels of N-terminal huntingtin fragments in cortical and striatal tissue.
All western blots shown at the same sensitivity. (a) Control subjects, Cortical region. (b) HD subjects, Cortical region. (c) Control subjects, Striatal region. (d) HD subjects, Striatal region. Control and HD subjects are age, sex and PMD matched. Upper blot: full length htt (fl htt). Middle blot: N-terminal htt fragments. Lower blot: β-actin. Post mortem delay in hours (hr).