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***In silico* analysis of the effects of disease-associated mutations of β -Hexosaminidase A in Tay-Sachs disease**

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Abstract

Tay-Sachs disease (TSD), a deficiency of β -Hexosaminidase A (Hex A), is a rare but debilitating hereditary metabolic disorder. Symptoms include extensive neurodegeneration and often result in death in infancy. We report an *in silico* study of 42 Hex A variants associated with the disease. Variants were separated into three groups according to age of onset: infantile (n=28), juvenile (n=9) and adult (n=5). Protein stability, aggregation potential and the degree of conservation of residues were predicted using a range of *in silico* tools. We explored the relationship between these properties and the age of onset of TSD. There was no significant relationship between protein stability and disease severity or between protein aggregation and disease severity. Infantile TSD had a significantly higher mean conservation score than non-disease associated variants. This was not seen in either juvenile or adult TSD. This study has established that the degree of residue conservation may be predictive of infantile TSD. It is possible these more highly conserved residues are involved in trafficking of the protein to the lysosome. In addition, we developed and validated software tools to automate the process of *in silico* analysis of proteins involved in inherited metabolic diseases. Further work is required to identify the function of well-conserved residues to establish an *in silico* predictive model of TSD severity.

Keywords: GM2 gangliosidosis; Sphingolipidosis; inherited metabolic disease; HexA; protein stability

Introduction

Tay-Sachs disease (TSD; Type I GM₂-gangliosidosis; OMIM#272800) is a rare, progressive autosomal recessive disease with an incidence of one in 320,000 births in the general populations and a carrier frequency of 1 in 250 (Lew *et al.*, 2015). Commonly fatal, it is caused by the abnormal metabolism of GM1 ganglioside, a molecule that accounts for 10-12% of the total lipid content of neuronal cellular membranes and contributes to approximately 1.5% of dry brain weight (Svennerholm & Fredman, 1980; Tettamanti, 2004). In healthy individuals, degradation of GM1 ganglioside occurs in the acidic confines of the lysosome (Mark *et al.*, 2003). Subunits are removed sequentially from the ganglioside with each step being catalysed by specific enzymes (Figure 1a). The conversion of GM2 to GM3 is catalysed by β -Hexosaminidase A (HexA/EC 3.2.1.52), a heterodimeric enzyme composed of two subunits, α and β , encoded by the genes *HexA* and *HexB* respectively (Henrissat & Davies, 1997). The α -subunit is uniquely able to bind the negatively charged GM2 ganglioside/activator protein complex and hydrolyse the terminal *N*-acetylgalactosamine (GalNAc) (Mark *et al.*, 2003; Sharma *et al.*, 2003a). If a mutation in *HexA* causes HexA to dysfunction, GM2 accumulates within the neuron resulting in TSD.

The human enzyme is a heterodimer of one α - and one β -subunit encoded by the *HEXA* and *HEXB* genes respectively, β -Hexosaminidase A (Korneluk *et al.*, 1986; Neote *et al.*, 1988). Two other isoforms of the β -hexosaminidase protein are present in humans – an $\alpha\alpha$ homodimer (β -hexosaminidase S) and a $\beta\beta$ homodimer (β -hexosaminidase B) (Aruna & Basu, 1976; Hayase & Kritchevsky, 1973; Hepbldikler *et al.*, 2002; Ikonne *et al.*, 1975; Robinson & Stirling, 1968). Structurally the α - and β -subunits have a similar overall fold (root mean square deviation of approximately 0.7 Å over approximately 460 equivalent C $_{\alpha}$ atoms) (Lemieux *et al.*, 2006b). Both subunits contain an active site which catalyses the hydrolysis of terminal *N*-acetylgalactosamine and *N*-acetylglucosamine residues from complex polysaccharide moieties. The catalytic mechanism is postulated to involve two residues – Glu-323 which acts as a base and protonates the glycosidic oxygen atom and Asp-322 which acts as a base, polarising the carbonyl in the *N*-acetyl group and stabilising the transition state (Lemieux *et al.*, 2006b;

Passos *et al.*, 2011). However, only the α -subunit can catalyse the hydrolysis of polysaccharides containing sialic acid groups (Kytzia & Sandhoff, 1985). This specificity arises from different active site structures: the α -subunit has a positively charged arginine residue which is able to bind to the negatively charged sialic acid (Lemieux *et al.*, 2006b; Mark *et al.*, 2001; Sharma *et al.*, 2003b).

TSD occurs in four forms: infantile, juvenile, adult and the B1 variant. In infantile TSD, the most severe form, patients appear normal until, within the first 6 months of life, there is developmental retardation, muscular weakness and an increased startle response. As the disease progresses, there is a loss of hearing and vision, cognitive impairment, seizures and paralysis resulting in death between 3 and 5 years of age (Fernandes Filho & Shapiro, 2004; Maegawa *et al.*, 2006b; Masingue *et al.*, 2020). In juvenile TSD, onset occurs in childhood with one cohort study of 21 patients stating a mean onset time of 5.3 ± 4.1 years with symptoms such as gait disturbances, ataxia, dysarthria and developmental delay with a median survival time of 14.5 years (Maegawa *et al.*, 2006b). As the disease progresses cognitive and motor skills deteriorate and anarthria, muscle wasting and incontinence occurs (Maegawa *et al.*, 2006a; Specola *et al.*, 1990). Adult TSD has the most variable time of onset and is the least severe of the three as it is not **always** fatal (Masingue *et al.*, 2020). One case series of 21 patients reported a mean onset age of 18.1 years (Neudorfer *et al.*, 2005), whereas another study reported onsets of 15 and 20 years (Hurowitz *et al.*, 1993). A separate case report reported onset age of 47 years (Steiner *et al.*, 2016). Symptoms involve psychiatric disturbance, dysarthria, gait abnormalities, and leg weakness. As the disease progresses the patient may require a wheelchair and experience dysphagia and worsening of psychiatric state (Neudorfer *et al.*, 2005). The B1 variant can present as any of the other variants. However, it exhibits a unique biochemical profile. HexA in B1 TSD is able to catalyse the conversion of the neutral synthetic substrate 4-methylumbelliferyl-*N*-acetyl-fl-D-glucosamine, which is sometimes used in diagnostic tests, but is unable to hydrolyse GM2 ganglioside *in vivo* or the acidic synthetic substrate 4-methylumbelliferyl- β -D-*N*-acetylglucosamine-6-sulphate (Gordon *et al.*, 1988). Although the neurological symptoms can be partially mitigated with drugs and other therapies, there are no effective treatments available for Tay-Sachs disease. Potential treatments under development include inhibition of substrate synthesis (thus reducing the accumulation of non-degraded G_{M2}

ganglioside), gene therapy, enzyme replacement therapy, neuronal stem cell transplants, pharmacological chaperones and gene editing (Akeboshi *et al.*, 2007; Cachon-Gonzalez *et al.*, 2006; Desnick & Kaback, 2001; Gray-Edwards *et al.*, 2018; Karumuthil-Meethil *et al.*, 2016; Kato *et al.*, 2017; Maegawa *et al.*, 2007; Ornaghi *et al.*, 2020; Ou *et al.*, 2020; Platt *et al.*, 2001; Rountree *et al.*, 2009; Tropak *et al.*, 2010; Tropak *et al.*, 2004; Tsuji *et al.*, 2011).

Here, we report an *in silico* investigation to explore how molecular changes in the HexA protein relate to the severity of TSD. The aim was to build the foundation for a predictive framework. Similar work has been done in the past to successfully develop predictive frameworks for several other diseases such as mevalonate kinase deficiency (Browne & Timson, 2015), type III galactosemia (McCorvie & Timson, 2013) and triose phosphate isomerase deficiency (Oliver & Timson, 2017). Developing a predictive framework that is reliable, accurate and effective could assist in the genetic counselling of a TSD carrier or in guiding prognoses for a TSD sufferer. As all studies were to be performed *in silico*, the opportunity arose to build software tools that could aid the accuracy and speed and reduce the cost of future work in this field.

Materials and Methods

Datasets

The identification of mutations known to be pathogenically associated with TSD was performed by a literature search using the NCBI PubMed database (<http://www.ncbi.nlm.nih.gov/PubMed>). Only mutations reported and categorised as pathogenic in the NCBI clinvar database were included (<http://www.ncbi.nlm.nih.gov/clinvar>). The classification of the disease was noted along with molecular consequence. Mutants were omitted if they resulted in early termination of the peptide, frameshift or were compound heterozygous. The crystal structure of wildtype human HexA (PDB: 2GJX) (Lemieux *et al.* 2006) was obtained from the Protein Data bank (<https://www.rcsb.org/>) (Berman *et*

1 *al.*, 2000). The amino acid sequence of wildtype HexA was obtained from the UniProt database
2 (<http://www.uniprot.org/uniprot/P06865>) (Pundir *et al.*, 2017; UniProt Consortium, 2018).
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8 *Structural analysis* 9

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11 YASARA (<http://www.yasara.org>) (Krieger *et al.*, 2009) is based on the AMBER force-field. It
12 computationally solvates and energy minimises protein structures. All PDB files were processed
13 through YASARA. Where possible, these energy-minimised structures were used for any analysis
14 involving protein structure. Superpose (<http://wishart.biology.ualberta.ca/superpose/>) (Maiti *et al.*,
15 2004) calculates protein structure superpositions of two or more PDB files based on a modified
16 quaternion eigenvalue approach. Comparisons were made between the wildtype PDB and each
17 mutant variant PDB in turn, the root-mean-square deviation (RMSD) was recorded.
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33 *Prediction of biochemical effects of each missense mutation on HexA aggregation* 34 35

36 Predicting the effect of a mutation on the aggregation propensity of the protein was done by using
37 freely available software. Fluorescence probe measurement of live macrophages has shown
38 intralysosomal pH to be 4.7 to 4.8 with other studies placing estimates between 4.5 and 5 (Coen *et al.*,
39 2012; Lange *et al.*, 2006; Ohkuma & Poole, 1978; Tabeta *et al.*, 2006). With this in mind, a pH of 5 was
40 selected as the webserver only accepted pH as an integer. Additionally, a temperature of 310 K and
41 an ionic strength of 0.15 M were used to replicate the human lysosomal conditions as best as the
42 software allowed. All HexA sequences had the first 22 residues removed to simulate the initial
43 proteolytic cleavage that occurs in the endoplasmic reticulum before HexA is trafficked to the lysosome
44 (Little *et al.*, 1988).
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66 TANGO (<http://tango.crg.es/>) uses a statistical mechanics algorithm to estimate the propensity for a
67 protein to aggregate (Fernandez-Escamilla *et al.*, 2004). The C- and N-terminus status was set to free.

Zygggregator (<http://www-mvsoftware.ch.cam.ac.uk/index.php/zygggregator>) is a web server that uses the Zygggregator algorithm to make predictions of the propensity of polypeptide chains to form protofibrillar assemblies (Tartaglia & Vendruscolo, 2008). CamSol (<http://www-mvsoftware.ch.cam.ac.uk/index.php/camsolintrinsic>) is a web server that predicts the solubility and the generic aggregation propensity of a protein from a given sequence (Sormanni *et al.*, 2015).

Prediction of biochemical effects of each missense mutation on HexA stability and structure

Prediction of the effect of mutations on the stability of the protein was done by using several freely available web servers. I-Mutant 3.0 (<http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi>) predicts the effect of a mutation on the stability of a protein (Capriotti *et al.*, 2008). It performs a requested point mutation on a provided PDB file or a single letter code amino acid sequence and returns the change in the Gibbs free energy change of unfolding compared to the wildtype protein ($\Delta\Delta G$). The wildtype HexA PDB file was used as an input and a pH of 5 and a temperature of 37 °C were chosen to replicate human lysosomal conditions. The Cologne University protein stability analysis tool (CUPSAT) available from (<http://cupsat.tu-bs.de/>) predicts the effects of point mutations on the overall protein stability. It returns the $\Delta\Delta G$ for every possible point mutation at a requested location using a PDB file (Parthiban *et al.*, 2006). The SDM server (<http://131.111.43.103/>) uses a knowledge-based system to predict the effect of a given list of mutations on a wildtype PDB file and returns the $\Delta\Delta G$ (Pandurangan *et al.*, 2017). The mutation Cutoff Scanning Matrix (mCSM) (<http://biosig.unimelb.edu.au/mcsm/stability>) (Pires *et al.*, 2014) uses predictive models trained with graph-based signatures to predict the effects of a given list of point mutations on the stability of a wildtype PDB file and returns the $\Delta\Delta G$.

Multiple sequence alignment and comparison of residue conservation of HexA variants

Wildtype HexA amino acid sequences were obtained from the NCBI protein database (<http://www.ncbi.nlm.nih.gov/protein>). Only complete sequences of HexA found in the refSeq database were included. A total of 101 sequences were identified including 79 mammals, 14 fish, five birds, one lizard, one nematode and one plant. After elimination of duplicates, 73 homologous sequences remained (for a full list, see Supplementary Table S1). These were input to a multiple sequence alignment server Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) (Sievers *et al.*, 2011) with human HexA added to the first position. The resultant multiple aligned sequence was entered into the Scorecons server to output Valdar conservation scores, an estimate of a residue's conservation throughout multiple sequences (Valdar, 2002).

Development of software tools to simplify and increase accuracy of data acquisition

Python scripts were created using PyCharm (JetBrains, 2017) with manipulation of protein structures (PDB files) performed by communicating with PyMol (Schrödinger, 2018) via its Application Programming Interface (API). The openpyxl module (Gazoni, 2018) was used to read/write Microsoft Excel files (ISO/IEC 29500-1:2016) in python. A python script loaded the energy minimised wildtype HexA structure and a Microsoft Excel File containing a list of mutations stored as comma-separated values (e.g. Arg,252,His). The comma-separated values were split and input sequentially to the mutagenesis function within PyMol to produce PDB files for all mutants.

A command line based program was written in Pascal using Lazarus (Team, 2018) to facilitate the production of mutant sequences and to output them as text files formatted for further analysis. The program held a copy of the wildtype HexA sequence in memory. The user selects a residue and mutates it to an amino acid single letter code of choice and saves it to a text file – the sequence is reset to wildtype after every save. The program also allows for deletions, early terminations and can output the length of the current sequence. A batch file was created in Notepad++ (Ho, 2018) (Ho, 2018) on

which the user can drag and drop text files to input it directly into TANGO thereby bypassing any command line interaction.

R scripts were created using RStudio (Team, 2016). The RSelenium package (Harrison, 2017) was used to automate interaction with and to extract data from web servers. The xlsx package (Dragulescu, 2014) was used to read/write Microsoft Excel files in R. An R script was created to automate user interaction with the Zygggregator web server. The script holds a copy of the wildtype HexA sequence in memory and loads a Microsoft Excel file containing a list of mutations stored as comma-separated values. The comma-separated values were split to yield the position of the mutation and the amino acid to mutate to. The amino acids were converted from three letter code to single letter code (i.e. Arg to R) and the wildtype HexA sequence was modified accordingly before being input to Zygggregator along with the experimental conditions. The script is able to identify when the Zygggregator calculation is complete and navigate to the results page. The relevant data is captured and stored in memory before being saved to a Microsoft excel file. An identical approach was used to create an R script for CamSoli.

An R script was created to automate the user interaction of iMutant 3.0. The script contains the file path to the energy minimised wildtype HexA structure and loads a comma separated values file (CSV) containing a list of mutations. The comma-separated values are split to yield the location of mutation and desired amino acid. The wildtype HexA structure is uploaded to iMutant 3.0 and experimental conditions are entered. The script waits for iMutant 3.0 to finish its calculations before extracting the $\Delta\Delta G$ values. An identical approach was used to create an R script for CUPSAT. An R script was also created to automate the user interaction of Superpose. An approach similar to the iMutant 3.0 script was taken. However, key differences were the need to upload both the wildtype and mutant PDB rather than entering mutant locations and amino acids.

An important step in the automation of web servers was the identification of the desired result. Superpose was the only web server that consistently presented the result in the same cell of an html table. In this case, it was straightforward to use RSelenium's getElementText request. The other web servers presented the result in either plain text (Zygggregator, CamSoli, iMutant 3.0) or as a table

where the result could be in any row (CUPSAT). In this case a pattern recognition method (R-Project, 2018) was used to identify text containing the desired result. (For the patterns used, see supplements E-H under the code comment “#Define patterns”.) All code was run with test data and samples of the final outputs were manually examined to assess the level of function of the software tools.

Data analysis

All data analysis and graph production was carried out using Graphpad Prism.(GraphPad Software Inc., 2017) Where data normalisation was performed, the equation $x_{new} = x_n/x_{wildtype}$ was used to normalise data with respect to the wildtype. Where no wildtype value was available, the equation $x_{new} = (x_n - x_{min})/(x_{max}-x_{min})$ was used to normalise the data with respect to the maximum and minimum value. One-way ANOVA was used to assess whether there was any statistically significant difference between the means of groups. Tukey post-hoc tests were done to identify which groups were significantly different to each other. All graphs were plotted as the mean $\pm$ standard deviation. The significance threshold was set at $p<0.05$.

Results

Identification of point mutations pathogenic for Tay-Sachs Disease

A total of 42 pathogenic variants were identified and included in this study. Of these, five exhibited an adult TSD phenotype, 27 exhibited an infantile TSD phenotype and six exhibited a juvenile phenotype. Additionally, two B1 variants exhibited an infantile phenotype and were included in the infantile group and two B1 variants exhibited a juvenile phenotype and were included with this group. Table 1 outlines the details of the variants identified. Infantile mutants were regarded as the most severe form, adult the least severe and juvenile as intermediate. The positions of the residues affected were mapped

onto the HexA crystal structure (Figure 1b). These positions were located throughout the structure and did not appear to cluster in any specific region.

Effect of point mutations on aggregation potential

The results from three aggregation calculators (Zygggregator, Camsoli and TANGO) were normalised with respect to the wildtype. A mean aggregation score for each variant was then calculated. The mean for each phenotype was then calculated and the results plotted in Figure 2a along with the wildtype. There was no significant difference between any of the groups.

Effect of point mutations on protein stability and structure

The $\Delta\Delta G$ for all variants were obtained from four webserver (iMutant 3.0, CUPSAT, SDM, mCSM) and normalised with respect to the minimum and maximum values. The mean $\Delta\Delta G$ for each variant was calculated and an overall mean for each phenotype was plotted in Figure 2b. There was no significant difference in mean $\Delta\Delta G$ between groups. The root mean square deviation (RMSD) for the energy minimised model of each variant compared to wildtype was calculated using the Superpose webserver. Mean RMSD was calculated for each phenotype and plotted in Figure 2c. There was no significant difference in mean RMSD between the groups.

Degree of conservation of residues affected by mutations

The mean conservation scores of non-disease associated residues were compared to the mean conservation scores of each phenotype and plotted in Figure 2d. There was a highly significant difference ($p=0.001$) in mean conservation scores between the non-disease associated residues and the infantile phenotype residues. There were no significant differences seen with the adult or juvenile groups.

Software tools to simplify and increase accuracy of data acquisition

A number of tools were created to help automate these analyses (and similar analyses for other diseases). These tools have been made publicly available at the Open Science Framework (www.osf.io/a5gsb) and examples of their implementation are shown in Figure 3. A python script was created to automate the production of variant PDB files. This enabled the automated generation of (unminimised) variant models of the protein in pdb file format from an Excel file containing details of the variants (Figure 3a). To automate analysis using the TANGO webserver, a pascal command line program was created along with a system to enable operation via a batch file (Figure 3b, c). R-scripts were generated to automate the use of Zyggregator (Figure 3d), Camsoli (Supplementary Figure S1a), iMutant 3.0 (Supplementary Figure S1b), CUPSAT (Supplementary Figure S1c) and Superpose (Supplementary Figure S1d).

Discussion

The 45 variants that were included in this study represent all point mutations known to be pathogenic for TSD. The separation of the variants into each phenotype and the placement of the phenotypes on a spectrum from severe to least severe is appropriate when considering the description of each clinical case (see Table 1). The exclusion criteria omitted those mutations where the cause of reduced HexA activity is obvious and where the approach adopted in this paper would not be appropriate. For example, the most common infantile TSD mutation is a 4bp insertion causing a frame-shift and early termination resulting in a protein considerably different to wildtype (Boles & Proia, 1995).

The webservers and software used in this project, are manageable when used individually or for small numbers of variants. However, when used in combination the manual entry of data becomes a time-consuming and error prone process. A study using 195 computer literate participants, found that manually entering data and visually checking it against the source resulted in an error rate nearly three

times as high as when using computer assisted double entry data validation (Barchard & Pace, 2011).

Given the multiple entry of data was not an option here, the automation of data entry proved an attractive alternative. The scripts and software we have developed have been shown to work correctly.

Some HexA mutants have been reported to have a propensity to aggregate (Dersh *et al.*, 2016; Proia & Neufeld, 1982) and it has been shown that aggregation plays a role in other metabolic disorders.(Bang *et al.*, 2009; McCorvie & Timson, 2013) Considering this, we explored whether aggregation plays a role in determining TSD severity. After comparing the mean aggregation scores, there were no significant differences in predicted aggregation between the TSD phenotypes or wildtype, suggesting that in any form of TSD, HexA is no more likely to aggregate than wildtype and so the loss of HexA function lies elsewhere.

$\Delta\Delta G$ is a useful indicator of protein stability, the higher it is the less stable the mutant protein compared to the wildtype (Funahashi *et al.*, 2003). Some webserver predicted much larger $\Delta\Delta G$ values than others did, for example, Lys197Thr was predicted to have a $\Delta\Delta G$ of -0.94 kcal/mol by iMutant 3.0 but -11.01 kcal/mol by CUPSAT. Considering this, it was necessary to normalise the data with respect to the minimum and maximum values to make it easier to draw comparisons from each server. It is important to note that in doing this we assumed that the servers are equally accurate. From calculating the mean $\Delta\Delta G$ from all four servers and comparing the mean $\Delta\Delta G$ of each phenotype, there was no significant difference between any groups. This suggests that changes in enzyme stability are not responsible for the increased loss of HexA function seen in more severe TSD.

Conservation analysis relies on the principle that in homologous proteins, residues that are more conserved throughout different organisms play a more important role in the function of the enzyme. It has been used to identify functionally important residues in protein interaction interfaces (Mintseris & Weng, 2005), ligand-binding sites (Liang *et al.*, 2006) and in maintaining overall protein structure (Valdar & Thornton, 2001). In this study, we carried out a multiple protein sequence alignment of human HexA with the enzyme from 73 other species. By comparing the mean Valdar conservation score of disease-associated residues, there was no significant difference between TSD phenotypes.

However, when residues associated with TSD phenotypes were compared with non-disease associated residues there was a highly significant difference in conservation ($p=0.001$) between infantile TSD and the non-disease associated residues. Infantile TSD had a greater mean conservation score than wildtype (0.8425 ± 0.1564 vs 0.6635 ± 0.2517). This suggests that the location of the mutation plays an important role in determining the severe loss of HexA function in infantile TSD but not in the other phenotypes.

Considering this in the context of the predictions of protein stability it is unlikely these conserved residues play a role in maintaining stability. Mutations of the active site of HexA are regarded as rare, only seen in the B1 variant (Lemieux *et al.*, 2006a; Ohno *et al.*, 2008). It is more likely that the affected residues are involved in the trafficking of the protein into the lysosome or the interaction between the α and β subunits. In fact, Dersh *et al* observed that an infantile TSD variant, Glu482Lys, fails to associate with the β subunit while an adult TSD variant, Gly269Ser, does so correctly (Dersh *et al.*, 2016). Lemieux *et al*, reiterate this and additionally state the variant is unable to exit the endoplasmic reticulum (Lemieux *et al.*, 2006a). When the HEXB crystal structure was compared with HexA molecular models, the interaction of the α and β subunit creates the docking site for the substrate/GM2 activator complex so that a correct protein-protein interface here is critical for enzyme function (Mark *et al.*, 2003). Immunoblotting using an anti- α subunit antibody in whole-cell lysates of HEK293T cells expressing either wildtype, Glu482Lys (infantile TSD) and Gly269Ser (adult TSD) HexA showed that only the wildtype was proteolytically cleaved to the final mature form suggesting either the variants were unable to be cleaved or that they never reached the lysosome. Although these experiments focussed on only two variants, they demonstrate that several factors influence the severity of disease. HexA leaving the endoplasmic reticulum, being trafficked to the lysosome, being cleaved correctly, correct interaction between the α and β subunits and having an intact active site capable of interacting with the substrate are all important considerations.

This *in silico* study has established that protein stability and aggregation potential of HexA variants do not appear play significant roles in determining the severity of TSD. Residue conservation is

1 implicated in infantile TSD. It is possible these more highly conserved residues are involved in
2 trafficking of the protein to the lysosome but experimental studies are required to confirm this. It
3 appears that TSD has a different underlying molecular pathology compared to a number of other
4 inherited metabolic diseases. A recent study demonstrated that lysosomal proteins, like HexA, are
5 unusually thermally stable compared to those in other cellular compartments (Collier *et al.*, 2020).
6 This suggests that small changes in thermal stability are unlikely to have a significant effect on *in vivo*
7 function.

8 In several other cases, *in silico* studies have predicted that reduced protein stability is a key factor in
9 the loss of enzymatic activity (Timson, 2015). The majority of these studies concerned enzymes which
10 are synthesised and which function in the cellular cytoplasm. In contrast HexA is trafficked to the
11 lysosome, normally a much more acidic environment. This trafficking involves the recognition and
12 cleavage of a signal sequence. These events provide additional steps which are vulnerable to point
13 mutations and structural changes. This study suggests that these steps are the ones critically affected
14 by the majority of mutations associated with Tay-Sachs Disease.

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Declarations of interest: The authors have none to declare.

Data Availability: Data will be available from the authors on request and will be placed in the
University of Brighton online repository: research.brighton.ac.uk

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Figure legends

Figure 1: The pathway of GM1 ganglioside degradation and structure of β -Hexosaminidase A α subunit (HexA). (a) Tay-Sachs Disease occurs when a mutated α subunit causes β -Hexosaminidase A to dysfunction resulting in the accumulation of GM2 and eventual neuronal cell death. (b) The structure of one monomer of the HexA protein with the location of residues known to be affected by disease-mutations mapped. The backbone of the protein is shown in cartoon format in grey. Affected residues are shown in stick format coloured red for those associated with the infantile onset form of the disease, blue for those associated with the juvenile form and yellow for the adult form. A small number of residues were associated with both the infantile and juvenile forms (purple), the infantile and adult forms (orange) and the juvenile and adult forms (green). The images represent a single subunit from the crystal structure of HexA (PDB: 2GJX) which had been subjected to energy minimisation. The lower image related to the upper one by a rotation of 180° around the y-axis. The images were generated using PyMol (www.pymol.org).

Figure 2: *In silico* analysis of HexA variants associated with Tay-Sachs Disease. (a) Mean and SD of normalised aggregation potential of the three TSD phenotype groups and wildtype calculated from three aggregation web servers (Zygggregator, Camsoli and TANGO). P-values obtained from Tukey post hoc test. There was no significant difference in aggregation potential between the groups. (b) Mean and SD of normalised $\Delta\Delta G$ of the three TSD phenotype groups calculated from four protein stability web servers (iMutant 3.0, CUPSAT, SDM, mCSM). P-values obtained from Tukey post hoc test. There was no significant difference in protein stability between the groups. (c) Mean and SD of RMSD of the three TSD phenotype groups calculated from a RMSD web server (Superpose). P-values obtained from Tukey post hoc test. There was no significant difference in protein RMSD between the groups. (d) Figure 2 Mean and SD for conservation score for disease associated and non-disease associated HexA residues calculated by the Scorecons server using a human HexA sequence aligned with HexA sequences from 74 other species. There is a highly significant difference between no disease and the severe infantile form.

Figure 3: Examples of the implementation of software tools developed in this study. (a) PDB file produced by python script for the mutant Arg252His visualised in PyMol. A command to select residue 252 has been executed (red box) to highlight position 252 in the single code sequence (red arrow). Residue 252 is Histamine rather than the wildtype Arginine demonstrating the mutation has taken place and the python script functions correctly. (b) Input (left) and output (right) of the pascal program for automating analysis by TANGO. A mutation at position 25 (correlating to position 3 after post-translational cleavage) has been carried out. Position 3 of the output is Histidine as expected and the sequence is preceded with the correct parameters for TANGO. (c) Dragging Test.txt onto the batch file (upper left) results in execution of TANGO analysis of the correct file (upper right) and as expected outputs an aggregation score in Test_Aggregation.txt (bottom) matching the output seen when running TANGO manually (not shown). (d) A test input of Arg,252,His (left) is correctly read and split by the R script. This displays variables in memory during execution of the code, note 'res' and 'mut' correctly correspond to the input (upper middle) and the file output from the script (right). The aggregation score is the same as that obtained from the input being entered manually (lower middle).

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Table

Table 1: The variants included in this study, their phenotype and references to the studies describing them.

Variant	TSD Variant	Reference	Provean Score	SIFT Score
Leu39Arg	Infantile	(Akli <i>et al.</i> , 1993b)	-4.49 (Deleterious)	0.001 (Damaging)
Glu114Lys	Infantile	(Mistri <i>et al.</i> , 2012) (Sheth <i>et al.</i> , 2014)	-3.68 (Deleterious)	0.001 (Damaging)
Leu127Arg	Infantile	(Akli <i>et al.</i> , 1993b; Montalvo <i>et al.</i> , 2005)	-5.48 (Deleterious)	0.000 (Damaging)
Arg170Gln	Infantile	(Drucker <i>et al.</i> , 1992; Kaufman <i>et al.</i> , 1997; Nakano <i>et al.</i> , 1990)	-3.85 (Deleterious)	0.000 (Damaging)
Arg170Trp	Infantile	(Mistri <i>et al.</i> , 2012; Sheth <i>et al.</i> , 2014; Triggs-Raine <i>et al.</i> , 1991)	-7.71 (Deleterious)	0.000 (Damaging)
Arg178Cys	B1 (Infantile)	(Navon <i>et al.</i> , 1995; Tanaka <i>et al.</i> , 1990a)	-7.71 (Deleterious)	0.000 (Damaging)
Arg178Leu	B1 (Infantile)	(Triggs-Raine <i>et al.</i> , 1991)	-6.74 (Deleterious)	0.000 (Damaging)
Val192Leu	Infantile	(Ainsworth & Coulter-Mackie, 1992; Hou <i>et al.</i> , 1996)	-1.26 (Neutral)	0.002 (Damaging)
His204Arg	Infantile	(Akli <i>et al.</i> , 1993b)	-7.8 (Deleterious)	0.000 (Damaging)
Ser210Phe	Infantile	(Akli <i>et al.</i> , 1991)	-5.82 (Deleterious)	0.000 (Damaging)

Phe211Ser	Infantile	(Akli <i>et al.</i> , 1993b; Brown <i>et al.</i> , 1989; Montalvo <i>et al.</i> , 2005)	-7.73 (Deleterious)	0.000 (Damaging)
Arg252Leu	Infantile	(Tanaka <i>et al.</i> , 2003a)	-6.83 (Deleterious)	0.002 (Damaging)
Asn295Ser	Infantile	(Tanaka <i>et al.</i> , 2003a)	-4.19 (Deleterious)	0.05 (Damaging)
Met301Arg	Infantile	(Akli <i>et al.</i> , 1993b)	-4.87 (Deleterious)	0.001 (Damaging)
Asp322Asn	Infantile	(Mistri <i>et al.</i> , 2012) (Sheth <i>et al.</i> , 2014)	-4.88 (Deleterious)	0.000 (Damaging)
Asp322Tyr	Infantile	(Mistri <i>et al.</i> , 2012; Sheth <i>et al.</i> , 2014)	-8.73 (Deleterious)	0.000 (Damaging)
Gln374Arg	Infantile	(Montalvo <i>et al.</i> , 2005)	-3.92 (Deleterious)	0.001 (Damaging)
Gln374Pro	Infantile	(Sheth <i>et al.</i> , 2014)	-5.88 (Deleterious)	0.001 (Damaging)
Arg393Pro	Infantile	(Mistri <i>et al.</i> , 2012; Sheth <i>et al.</i> , 2014)	-2.57 (Deleterious)	0.005 (Damaging)
Trp420Cys	Infantile	(Matsuzawa <i>et al.</i> , 2003; Tanaka <i>et al.</i> , 2003a; Tanaka <i>et al.</i> , 1990b)	-12.44 (Deleterious)	0.000 (Damaging)
Cys458Tyr	Infantile	(Matsuzawa <i>et al.</i> , 2003; Tanaka <i>et al.</i> , 1994)	-9.5 (Deleterious)	0.000 (Damaging)
Glu462Val	Infantile	(Mistri <i>et al.</i> , 2012; Sheth <i>et al.</i> , 2014)	-6.62 (Deleterious)	0.000 (Damaging)
Gly478Arg	Infantile	(Mistri <i>et al.</i> , 2012; Sheth <i>et al.</i> , 2014)	-2.16 (Neutral)	0.012 (Damaging)

Glu482Lys	Infantile	(Dersh <i>et al.</i> , 2016; Nakano <i>et al.</i> , 1988; Proia & Neufeld, 1982)	-3.71 (Deleterious)	0.000 (Damaging)
Leu484Gln	Infantile	(Tanaka <i>et al.</i> , 1994)	-5.57 (Deleterious)	0.000 (Damaging)
Leu484Pro	Infantile	(Tanaka <i>et al.</i> , 1994)	-6.5 (Deleterious)	0.000 (Damaging)
Arg504Cys	Infantile	(Akli <i>et al.</i> , 1991; Boonyawat, 2016; Montalvo <i>et al.</i> , 2005; Neudorfer <i>et al.</i> , 2005; Paw <i>et al.</i> , 1990; Raghavan <i>et al.</i> , 1985; Shapiro & Natowicz, 2009)	-7.43 (Deleterious)	0.003 (Damaging)
Trp485Arg	Infantile	(Akalin <i>et al.</i> , 1992)	-13 (Deleterious)	0.000 (Damaging)
Cys58Tyr	Juvenile	(Najmabadi <i>et al.</i> , 2011)	-8.58 (Deleterious)	0.001 (Damaging)
Arg178His	B1 (Juvenile)	(Brown <i>et al.</i> , 1989; Giraud <i>et al.</i> , 2010; Maegawa <i>et al.</i> , 2006a; Montalvo <i>et al.</i> , 2005; Ou <i>et al.</i> , 2019; Tanaka <i>et al.</i> , 1990a)	-4.82 (Deleterious)	0.000 (Damaging)
Gly250Asp	Juvenile	(Trop <i>et al.</i> , 1992)	-6.83 (Deleterious)	0.001 (Damaging)
Asp258His	B1 (Juvenile)	(Fernandes <i>et al.</i> , 1992; Fernandes <i>et al.</i> , 1997)	-6.83 (Deleterious)	0.000 (Damaging)
Ser279Pro	Juvenile	(Drucker <i>et al.</i> , 1997)	-2.89 (Deleterious)	0.06 (Tolerated)
Trp474Cys	Juvenile	(Maegawa <i>et al.</i> , 2006a; Neudorfer <i>et al.</i> , 2005; Petroulakis <i>et al.</i> , 1998)	-12.21 (Deleterious)	0.000 (Damaging)

Arg499His	Juvenile	(Maegawa <i>et al.</i> , 2006a; Ou <i>et al.</i> , 2019; Paw <i>et al.</i> , 1990; Shapiro & Natowicz, 2009; Tanaka <i>et al.</i> , 2003a; Zampieri <i>et al.</i> , 2012)	-4.64 (Deleterious)	0.000 (Damaging)
Arg499Cys	Juvenile	(Akli <i>et al.</i> , 1993a; Mules <i>et al.</i> , 1992; Tanaka <i>et al.</i> , 2003b)	-7.43 (Deleterious)	0.000 (Damaging)
Arg504His	Juvenile	(Giraud <i>et al.</i> , 2010; Paw <i>et al.</i> , 1990)	-4.64 (Deleterious)	0.001 (Damaging)
Tyr180His	Adult	(De Gasperi <i>et al.</i> , 1996)	-4.45 (Deleterious)	0.000 (Damaging)
Lys197Thr	Adult	(Akli <i>et al.</i> , 1993b)	-5.85 (Deleterious)	0.000 (Damaging)
Arg252His	Adult	(Ribeiro <i>et al.</i> , 1996)	-4.88 (Deleterious)	0.001 (Damaging)
Gly269Ser	Adult	(Maegawa <i>et al.</i> , 2006a; Navon & Proia, 1989; Neudorfer <i>et al.</i> , 2005; Shapiro & Natowicz, 2009)	-5.51 (Deleterious)	0.177 (Tolerated)
Val391Met	Adult	(Navon <i>et al.</i> , 1995)	-2.79 (Deleterious)	0.001 (Damaging)

The screenshot shows a Jupyter Notebook interface. The top cell contains the following code:

```

mutinstabx - Excel
Copyright (C) Schroedinger, LLC

This Executable Build integrates and extends Open-Source PyMOL.

start
hello
pdb: C:\Users\Ihsan\OneDrive\Medicine\IRP\hexmod\Structures\WT.pdb
name: Arg352His
selection: A/352/
mutant: HIS

```

The second cell contains the following code:

```

Cmload: PDB-string loaded into object "Arg352His", state 1.
PyMOL>select res 352
Selector: selection "aale" defined with 35 atoms.

```

The third cell contains the following code:

```

PyMOL>

```

The output of the third cell shows the sequence of the protein structure, with the residue Arg352 highlighted in red:

```

...Arg352His 231 236 241 246 251 256 261 266 271 276 281 286 291 296
...LPPKGVNIVNIVTADQNVIVTAPKGLVLAETDTHGHLGAGPGIPSLITFCYSGLPGLTTFVIVSLNITY

```

A red arrow points to the residue Arg352 in the sequence.

```
enter residue number [auto subtracts 22]:  
25  
Residue 5 is P  
Change P to?  
H  
  
Residue 5 is now H  
  
enter residue number [auto subtracts 22]:  
save  
Filename?  
Test  
##### Now A HILEY #####  
2 Test M N S 310 0.15 LHHWTQNY
```

[illegible]

Supplementary Figure S1: R-scripts for the automated use of webserver. (a) A test input of Arg,252,His (left) is correctly read and split by the CamSoli R script (left). The script displays variables in memory during execution of the code, note 'res' and 'mut' correctly correspond to the input (upper middle) and displays the file output from the script (right). The aggregation score is the same as that obtained from the input being entered manually (lower middle). (b) A test input of Arg,252,His is correctly read and split by the iMutant 3.0 R script (left). This displays variables in memory during execution of the code, note 'wt', 'res' and 'mut' correctly correspond to the input (upper middle) and the file output from the script (right). The aggregation score is the same as that obtained from the input being entered manually (lower middle). (c) A test input of Arg,252,His is correctly read and split by the CUPSAT R script (left). The script displays variables in memory during execution of the code, note 'x', 'wt', 'res' and 'mut' correctly correspond to the input (upper middle) and displays the file output from the script (right). The aggregation score is the same as that obtained from the input being entered manually (lower middle). (d) A test input of Arg,252,His is correctly read and split by the Superpose R script (left). The script displays variables in memory during execution of the code, note 'x', 'wt', 'res' and 'mut' correctly correspond to the input (upper middle) and the file output from the script (right). The RMSD for all atoms is the same as that obtained from the input being entered manually (lower middle).

Supplementary data

Supplementary Table S1 Identifiers of HexA sequences used in alignment

XP_003413972.1 beta-hexosaminidase subunit alpha isoform X2 [*Loxodonta africana*]
 XP_023408658.1 beta-hexosaminidase subunit alpha isoform X1 [*Loxodonta africana*]
 XP_023408659.1 beta-hexosaminidase subunit alpha isoform X3 [*Loxodonta africana*]
 XP_020018929.1 beta-hexosaminidase subunit alpha [*Castor canadensis*]
 NP_001133930.1 beta-hexosaminidase subunit beta [*Salmo salar*]
 XP_011395527.1 Beta-hexosaminidase subunit alpha [*Auxenochlorella protothecoides*]
 XP_022435662.1 beta-hexosaminidase subunit alpha isoform X1 [*Delphinapterus leucas*]
 XP_022435663.1 beta-hexosaminidase subunit alpha isoform X2 [*Delphinapterus leucas*]
 XP_022435664.1 beta-hexosaminidase subunit alpha isoform X3 [*Delphinapterus leucas*]
 XP_021409901.1 beta-hexosaminidase subunit alpha [*Lonchura striata domestica*]
 XP_020659346.1 beta-hexosaminidase subunit alpha [*Pogona vitticeps*]
 XP_010410730.2 beta-hexosaminidase subunit alpha [*Corvus cornix cornix*]
 XP_004628811.1 beta-hexosaminidase subunit alpha isoform X1 [*Octodon degus*]
 XP_023568375.1 beta-hexosaminidase subunit alpha isoform X2 [*Octodon degus*]
 XP_544758.2 beta-hexosaminidase subunit alpha isoform X1 [*Canis lupus familiaris*]
 XP_013965054.1 beta-hexosaminidase subunit alpha isoform X2 [*Canis lupus familiaris*]
 XP_022268255.1 beta-hexosaminidase subunit alpha isoform X3 [*Canis lupus familiaris*]
 XP_019687278.1 beta-hexosaminidase subunit alpha isoform X1 [*Felis catus*]
 XP_012996870.1 beta-hexosaminidase subunit alpha [*Cavia porcellus*]
 XP_022367876.1 beta-hexosaminidase subunit alpha [*Enhydra lutris kenyoni*]
 XP_005069690.1 beta-hexosaminidase subunit alpha isoform X2 [*Mesocricetus auratus*]
 XP_012968476.1 beta-hexosaminidase subunit alpha isoform X1 [*Mesocricetus auratus*]
 XP_012968477.1 beta-hexosaminidase subunit alpha isoform X3 [*Mesocricetus auratus*]
 XP_012623694.1 beta-hexosaminidase subunit alpha isoform X1 [*Microcebus murinus*]
 XP_012623695.1 beta-hexosaminidase subunit alpha isoform X2 [*Microcebus murinus*]
 XP_020784475.1 beta-hexosaminidase subunit alpha [*Boleophthalmus pectinirostris*]
 XP_022610337.1 beta-hexosaminidase subunit alpha [*Seriola dumerili*]
 XP_021549757.1 beta-hexosaminidase subunit alpha isoform X1 [*Neomonachus schauinslandi*]
 XP_021549758.1 beta-hexosaminidase subunit alpha isoform X2 [*Neomonachus schauinslandi*]

XP_021262712.1 beta-hexosaminidase subunit alpha [*Numida meleagris*]
 XP_001494361.1 beta-hexosaminidase subunit alpha [*Equus caballus*]
 XP_004070040.1 beta-hexosaminidase subunit alpha isoform X1 [*Oryzias latipes*]
 XP_020559701.1 beta-hexosaminidase subunit alpha isoform X2 [*Oryzias latipes*]
 XP_020852539.1 beta-hexosaminidase subunit alpha isoform X1 [*Phascolarctos cinereus*]
 XP_020852541.1 beta-hexosaminidase subunit alpha isoform X2 [*Phascolarctos cinereus*]
 XP_011373887.1 beta-hexosaminidase subunit alpha isoform X1 [*Pteropus vampyrus*]
 XP_023377626.1 beta-hexosaminidase subunit alpha isoform X2 [*Pteropus vampyrus*]
 XP_023377628.1 beta-hexosaminidase subunit alpha isoform X3 [*Pteropus vampyrus*]
 XP_006087896.1 beta-hexosaminidase subunit alpha [*Myotis lucifugus*]
 XP_012306688.1 beta-hexosaminidase subunit alpha isoform X1 [*Aotus nancymaae*]
 XP_012306689.1 beta-hexosaminidase subunit alpha isoform X2 [*Aotus nancymaae*]
 XP_012306690.1 beta-hexosaminidase subunit alpha isoform X3 [*Aotus nancymaae*]
 XP_012306697.1 beta-hexosaminidase subunit alpha isoform X6 [*Aotus nancymaae*]
 XP_021494470.1 beta-hexosaminidase subunit alpha [*Meriones unguiculatus*]
 XP_021165578.1 beta-hexosaminidase subunit alpha [*Fundulus heteroclitus*]
 XP_012931717.1 beta-hexosaminidase subunit alpha isoform X2 [*Heterocephalus glaber*]
 XP_021092627.1 beta-hexosaminidase subunit alpha isoform X1 [*Heterocephalus glaber*]
 XP_021092628.1 beta-hexosaminidase subunit alpha isoform X3 [*Heterocephalus glaber*]
 XP_004463787.1 beta-hexosaminidase subunit alpha isoform X1 [*Dasypus novemcinctus*]
 XP_004463788.1 beta-hexosaminidase subunit alpha isoform X2 [*Dasypus novemcinctus*]
 XP_020749943.1 beta-hexosaminidase subunit alpha isoform X1 [*Odocoileus virginianus texanus*]
 XP_020749946.1 beta-hexosaminidase subunit alpha isoform X2 [*Odocoileus virginianus texanus*]
 XP_003901203.1 beta-hexosaminidase subunit alpha [*Papio anubis*]
 XP_008071051.1 beta-hexosaminidase subunit alpha isoform X1 [*Carlito syrichta*]
 XP_021561799.1 beta-hexosaminidase subunit alpha isoform X2 [*Carlito syrichta*]
 XP_020953572.1 beta-hexosaminidase subunit alpha isoform X1 [*Sus scrofa*]
 XP_021156454.1 beta-hexosaminidase subunit alpha [*Columba livia*]
 XP_021027405.1 beta-hexosaminidase subunit alpha [*Mus caroli*]
 XP_023278359.1 beta-hexosaminidase subunit alpha [*Seriola lalandi dorsalis*]
 XP_021062583.1 beta-hexosaminidase subunit alpha [*Mus pahari*]
 XP_003784529.1 beta-hexosaminidase subunit alpha [*Otolemur garnettii*]

XP_023205502.1 beta-hexosaminidase subunit alpha [*Xiphophorus maculatus*]
XP_022045995.1 beta-hexosaminidase subunit alpha [*Acanthochromis polyacanthus*]
XP_003755208.1 beta-hexosaminidase subunit alpha [*Sarcophilus harrisii*]
XP_005316823.1 beta-hexosaminidase subunit alpha isoform X1 [*Ictidomys tridecemlineatus*]
XP_021583780.1 beta-hexosaminidase subunit alpha isoform X2 [*Ictidomys tridecemlineatus*]
XP_004374779.1 beta-hexosaminidase subunit alpha isoform X1 [*Trichechus manatus latirostris*]
XP_023585564.1 beta-hexosaminidase subunit alpha isoform X2 [*Trichechus manatus latirostris*]
XP_003378067.1 beta-hexosaminidase subunit alpha [*Trichinella spiralis*]
XP_023076435.1 beta-hexosaminidase subunit alpha [*Piliocolobus tephrosceles*]
XP_004553154.1 beta-hexosaminidase subunit alpha isoform X1 [*Maylandia zebra*]
XP_023008256.1 beta-hexosaminidase subunit alpha isoform X2 [*Maylandia zebra*]
XP_021326163.1 beta-hexosaminidase subunit alpha isoform X1 [*Danio rerio*]