Identification of Therapeutic Targets in a Glycogen Storage Disease Type IV Mouse Model

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
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Abstract

Adult polyglucosan body disease (APBD) is a neurological, adult-onset variant of glycogen storage disease type IV caused by mutations in the glycogen branching enzyme gene. APBD is characterized by the accumulation of poorly-branched glycogen molecules which aggregate to form pathogenic inclusions, called polyglucosan bodies. Polyglucosan bodies localized to the nervous system are understood to cause the progressive neurological symptoms. We hypothesized that deficiency of the enzyme responsible for glycogen chain elongation, glycogen synthase, or of a protein involved in its activation, protein phosphatase 1 regulatory subunit 3C (PPP1R3C), could mitigate the glycogen branching enzyme deficiency and prevent polyglucosan body formation. Using the APBD mouse model, we characterized the effect of glycogen synthase and PPP1R3C deficiency on the disease phenotype and observed significant behavioural, histological and biochemical rescue. Identification of glycogen synthase and PPP1R3C as effective therapeutic targets represents a critical step towards the development of a therapy for APBD patients.
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Contributions

The author performed experiments included in this thesis with the exception of the below noted contributions. The author conducted experimental design, breeding and behavioural testing for the $Gbe1^{ys}$ $Ppp1r3c^{-}$ mouse line and all histological and data analysis.

Dr. Bart Pederson and Justin Crowder, Jordan Strober and Cody Bennett: Performed the mouse breeding, behavioural experiments and tissue collection for the $Gbe1^{ys}$ $Gys1^{-}$ mouse line.

Dr. Peixiang Wang: Conducted brain and muscle glycogen quantification.

Dr. Mitchell Sullivan: Conducted liver glycogen quantification.

The Pathology Core at The Centre for Phenogenomics: Conducted tissue embedding, Period acid-Schiff diastase staining and immunohistochemistry.

Xiaochu Zhao: Assistance with tissue collection from the $Gbe1^{ys}$ $Ppp1r3c^{-}$ mouse line. Breeding and tissue collection for mice in Figures 2-3.

Yunlin Xue: Assistance with rotarod testing and grip strength measurement for the $Gbe1^{ys}$ $Ppp1r3c^{-}$ mouse line.

Ami Perri: Assistance with tissue grinding for the $Gbe1^{ys}$ $Gys1^{-}$ and $Gbe1^{ys}$ $Ppp1r3c^{-}$ mouse lines.
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List of Abbreviations

AGL  Amylo-alpha-1,6-glucosidase, 4-alpha-glucanotransferase
AIF1  Allograft inflammatory factor 1
APBD  Adult polyglucosan body disease
ANOVA Analysis of variance
ASO  Antisense oligonucleotide
CBM  Carbohydrate binding module
CNS  Central nervous system
DNA  Deoxyribonucleic acid
EMG  Electromyography
EPM2A EPM2A, laforin glucan phosphatase
G6P  Glucose-6-phosphate
GAA  Glucosidase acid, alpha
GBE1  1,4-alpha-glucan branching enzyme
GFAP  Glial fibrillary acidic protein
GSD  Glycogen storage disease
GYG  Glycogenin
GYS  Glycogen synthase
IHC  Immunohistochemistry
LB  Lafora body
mRNA  Messenger ribonucleic acid
NHLRC1  NHL repeat containing E3 ubiquitin protein ligase 1
PAS  Periodic acid-Schiff
PASD  Periodic acid-Schiff diastase
PB  Polyglucosan body
PHK  Phosphorylase kinase
PNS  Peripheral nervous system
PP1  Protein phosphatase 1
PPP1R3  Protein phosphatase 1 regulatory subunit 3
PTC  Premature termination codon
PYG  Glycogen phosphorylase
RBCK1  RANBP2-type and C3HC4-type zinc finger containing 1
SEM  Standard error of the mean
SLM  Stratum lacunosum moleculare
STBD1  Starch binding domain 1
UDP  Uridine diphosphate
Chapter 1: Introduction

1.1 Glycogen metabolism

Glycogen metabolism is responsible for maintaining adequate levels of the simple sugar, glucose, in the human body. This process involves the rapid synthesis and degradation of glycogen in response to high and low glucose concentrations, respectively. This thesis will primarily focus on glycogen synthesis, rather than degradation, and in particular, the pathogenic consequences of abnormal glycogen accumulation in the nervous system.

1.1.1 Glycogen synthesis

Cellular uptake of glucose can be stimulated by a myriad of signals and is mediated by one or several of the glucose transporter isozymes expressed in humans (Thorens and Mueckler, 2010). Upon cell entry, glucose is phosphorylated by hexokinase (EC 2.7.1.1) to produce negatively charged glucose-6-phosphate (G6P), which is unable to exit the cell. Subsequent reactions catalyzed by phosphoglucomutase (EC 5.4.2.2) and UDP-glucose pyrophosphorylase (EC 2.7.7.9) produce uridine diphosphate (UDP)-glucose, which serves as the glucosyl donor for glycogen synthesis.

Glycogen is a large spherical molecule that is comprised of linear and branched glucan chains of up to 55,000 glucose residues. Glycogen molecules are initiated by the self-glucosylating enzyme glycogenin (GYG, EC 2.4.1.186; Lomako et al., 1988). Linear chains are subsequently synthesized by glycogen synthase (GYS, EC 2.4.1.11; Leloir et al., 1959) and glycogen branching enzyme, also known as 1,4-alpha-glucan branching enzyme (GBE1, EC 2.4.1.18), catalyzes the regular formation of branches (Larner, 1953; Figure 1). The concerted actions of GYS and GBE1 result in the highly-branched spherical glycogen structure, optimized for efficient storage and rapid retrieval of large numbers of glucose units (Meléndez et al., 1999).
The occurrence of branch points at regular intervals in glycogen is an important feature critical to the molecule’s hydrophilicity. Oligosaccharides form helical structures and, in the absence of branching, parallel helices can interact to form water insoluble structures (Gessler et al., 1999; Goldsmith et al., 1982). This phenomenon is evident in glycogen’s plant counterpart, starch, in which tiers of unbranched, parallel glucan chains render the particle insoluble (Buléon et al., 1998). Also of structural relevance, glycogen molecules contain small amounts of phosphate, covalently bound to glucose molecules in carbon positions C2, C3 and C6 (Fontana, 1980; Nitschke et al., 2013; Tagliabracci et al., 2011). While the biochemical significance and mechanistic implications of glycogen phosphorylation remain unclear (Nitschke et al., 2017), phosphate incorporation has been associated with glycogen chain length and solubility (Nitschke et al., 2013; Tagliabracci et al., 2007).

**Figure 1. Schematic of the glycogen synthesis pathway.**
UDP-glucose donates glucose for GYG self-glucosylation and GYS-mediated catalysis of alpha-1,4-glycosidic linkages. GBE1 catalyzes the formation of alpha-1,6-linked branch points. Together these enzymes synthesize highly-branched sphere-shaped glycogen molecules.
1.1.1.1 Glycogenin

GYG initiates glycogen molecule synthesis by auto-catalyzing the covalent linkage of glucose units to an internal GYG tyrosine (Tyr) residue (Rodriguez and Whelan, 1985). The proposed reaction mechanism involves dimeric GYG catalyzing intra- or inter-molecular linkage of an average of 12 UDP-glucose units to Tyr195 (Hurley et al., 2006; Issoglio et al., 2012; Lin et al., 1999). This GYG-linked oligosaccharide serves as a primer for further GYS-mediated polymerization (Pitcher et al., 1988).

There are two GYG isozymes in humans, glycogenin-1 (GYG1) and glycogenin-2 (GYG2), located on chromosome 3 and on the X chromosome, respectively (Lomako et al., 1996; Mu and Roach, 1998; Mu et al., 1997). GYG1 is the primary isozyme expressed in skeletal muscle while GYG2 is primary in liver and brain, with both isozymes expressed in heart and other tissues (Imagawa et al., 2014; Mu et al., 1997). Despite catalyzing the same reaction, GYG1 and GYG2 share only 45% amino acid sequence homology (Mu et al., 1997). Experimental findings and structural analyses suggest that beyond their different tissue expression patterns, GYG1 and GYG2 may contribute differently to glycogen particle size and number determination (Mu and Roach, 1998; Skurat et al., 1997; Zeqiraj and Sicheri, 2015). Among mammals, GYG2 is understood to be unique to primates as no second GYG isozyme has been identified in non-primate mammals (Zhai et al., 2001).

1.1.1.2 Glycogen synthase

GYS is the primary enzyme responsible for elongating linear glycogen chains and does so by catalyzing the addition of UDP-glucose via alpha-1,4-glycosidic linkages (Leloir et al., 1959). In mammals, two GYS isoforms are present: i) glycogen synthase 1 (GYS1), a muscle isozyme expressed in most tissues (including brain), and ii) glycogen synthase 2 (GYS2), a liver-specific isozyme (Bai et al., 1990; Kaslow and Lesikar, 1984; Kaslow et al., 1985; Wang et al., 1986). GYS1 and GYS2 share 69% amino acid sequence homology with high sequence conservation across the catalytic site (Nuttall et al.,
Reduced sequence similarity in the N- and C-termini corresponds with differences in isozyme regulation.

Eukaryotic GYS activity is highly regulated via G6P-mediated allosteric activation and multisite phosphorylation. Analysis of *Saccharomyces cerevisiae* glycogen synthase (Gsy2p) demonstrated that allosteric activation by G6P induces a conformation change that enables the glucan chain substrate to better access the Gsy2p active site, thereby increasing catalytic efficiency (Baskaran et al., 2010; Pederson et al., 2000). Multisite phosphorylation, on the other hand, reduces Gsy2p’s catalytic efficiency with regard to substrates UDP-glucose and glycogen, potentially through the promotion of an inhibited protein conformation (Baskaran et al., 2010; Pederson et al., 2000).

GYS phosphorylation occurs on serine residues at the N- and C-termini, with GYS1 having nine phosphorylation sites demonstrated *in vivo* and GYS2 sharing seven of these sites (Roach, 1990). Site-directed mutagenesis of serine residues has demonstrated that not all phosphorylation sites are equal determinants of GYS activity (Ros et al., 2009; Skurat et al., 1994) and the multisite phosphorylation has also been shown to occur in a hierarchal manner (Roach, 1990). At least six kinases, including glycogen synthase kinase 3 have been shown to phosphorylate GYS and dephosphorylation is catalyzed by protein phosphatase 1 (PP1; Roach et al., 2012). *In vitro* and *in vivo* modeling has demonstrated that GYS phosphoregulation is less important than G6P-mediated allosteric activation in determining glycogen accumulation, particularly in response to insulin (Bouskila et al., 2010; McManus et al., 2005; Pederson et al., 2000; von Wilamowitz-Moellendorff et al., 2013).

GYS binding to GYG is also critical for glycogen synthesis to occur (Skurat et al., 2006; Zeqiraj et al., 2014). Crystal structure determination of the binding surface between GYS and GYG in *Caenorhabditis elegans* demonstrated that this interaction is independent of the GYG-bound oligosaccharide primer (Zeqiraj et al., 2014). Recent success in the overexpression and purification of functional human GYS1-GYG1 complexes in insect cells will enable further structural and biochemical characterization, though not without limitations inherent to heterologous expression (Hunter et al., 2015).
1.1.1.2.1 Protein phosphatase 1

PP1 is a serine/threonine protein phosphatase involved in a range of cellular processes including the reciprocal regulation of glycogen synthesis and degradation enzymes. PP1 is comprised of a catalytic subunit and a regulatory subunit.

The PP1 catalytic subunit is responsible for substrate dephosphorylation and in mammals it is encoded by splice variants of three genes (PPP1CA, PPP1CB, PPP1CC; Berndt et al., 1987; Ohkura et al., 1989; Sasaki et al., 1990). These catalytic subunit isozymes have high sequence conservation, especially across the central catalytic domain, and are ubiquitously expressed, with the exception of splice variant PPP1CC2 which is predominantly expressed in testes (Ceulemans and Bollen, 2004; Kitagawa et al., 1990; Zhang et al., 1993). Interrogation of the enzymatic properties of the PP1 catalytic subunit isoforms has yielded few to no differences; however, the presence of phenotypes in isozyme-deficient experimental models demonstrates the isoforms lack functional redundancy (Cheng et al., 2000; Raghavan et al., 2000; Varmuza et al., 1999; Zhang et al., 1993). Furthermore, unique intracellular localization patterns of the catalytic isoforms have been observed, implying that the isoforms differ in their interactions with PP1 regulatory subunits – potentially mediated by their N- and C-termini sequence differences (Andreassen et al., 1998; Trinkle-Mulcahy et al., 2001).

Of the over 200 genes estimated to encode PP1 regulatory subunits, seven are known to target PP1 to glycogen and glycogen metabolizing enzymes (Bollen et al., 2010; Roach et al., 2012). These glycogen-targeting subunits, encoded by genes protein phosphatase 1 regulatory subunit 3A-G (PPP1R3A-G), serve as scaffold proteins which bind: i) the PP1 catalytic subunit via an RVXF docking motif, ii) glycogen particles via a carbohydrate binding module (CBM21) domain, and iii) glycogen metabolizing enzymes (Heroes et al., 2013; Rubio-Villena et al., 2015). In doing so, glycogen-targeting subunits mediate the dephosphorylation of enzymes such as GYS and/or glycogen phosphorylase (PYG, EC 2.4.1.1), resulting in their respective activation and inhibition and therefore the net promotion of glycogen synthesis (Ceulemans and Bollen, 2004).
Despite classification within the same gene family, none of the seven glycogen-targeting subunits share greater than 40% amino acid sequence homology (Munro et al., 2005), supporting functional evidence of different contributions to the regulation of glycogen metabolism. Subunit PPP1R3A (also referred to as $G_M$) is expressed in striated muscles such as skeletal and cardiac muscle, while subunit PPP1R3B ($G_L$) is predominantly expressed in the liver (Doherty et al., 1995; Tang et al., 1991). Subunits PPP1R3C (protein targeting to glycogen, PTG) and PPP1R3D (R6) are expressed ubiquitously (Armstrong et al., 1997; Printen et al., 1997). Less is known about subunits PPP1R3E, PPP1R3F and PPP1R3G, however, they have each been shown to have expression in multiple tissue types (Kelsall et al., 2011; Luo et al., 2015; Munro et al., 2005).

1.1.1.3 Glycogen branching enzyme

GBE1 is responsible for catalyzing the formation of alpha-1,6-linked glycogen branches (Larner, 1953). The human GBE1 gene is located on chromosome 3 (Thon et al., 1993) and the GBE1 amino acid sequence is highly conserved across animals and plants (Fyfe et al., 2007; Moses and Parvari, 2002). Determination of the human GBE1 crystal structure elucidated four main structural domains, including a CBM48 proximal to the central catalytic core (Froese et al., 2015).

The CBM48 domain likely contributes to binding the glycogen molecule, however, an unexpected deficiency of GBE1 in the analysis of glycogen-associated proteins suggests that this binding is somehow regulated or less stable (Caudwell and Cohen, 1980; Stapleton et al., 2010).

GBE1’s central catalytic core catalyzes two reactions: i) hydrolysis, resulting in the cleavage of a minimum of six glucose units from the non-reducing terminal of a glucan chain (Verhue and Hers, 1966), and ii) transglycosylation, which re-attaches this cleaved glucan segment upstream on the same, or a neighbouring, chain (Gibson et al., 1971). The catalytic domain of GBE1 is structurally similar to that of other glycosyl hydrolases, such as alpha-amylase (Froese et al., 2015). Comparison of these two structures suggests a shared mechanism of glycosidic linkage hydrolysis, while a 39
amino acid sequence unique to GBE1 has been proposed to facilitate interaction with the recipient glucan chain and therefore transglycosylation (Froese et al., 2015). Analysis of mammalian glycogen indicates that chain lengths are on average around 13 glucose units long (Illingworth and Cori, 1952; Meléndez-Hevia et al., 1993), however, the mechanisms responsible for regulating chain lengths and the determinants of chain placement (e.g., transglycosylation of the same or a neighbouring chain) remain unclear.

1.1.2 Glycogen degradation

Stimulation of glycogen degradation occurs in response to a myriad of signaling cascades and varies in a cell- and tissue-type dependent manner. Hepatic glycogen degradation and subsequent glucose export is stimulated by glucagon-signaling of low blood glucose concentrations (Unger et al., 1962) while skeletal, cardiac and brain glycogen stores are of particular importance during sudden increases in energy demands or states of hypoglycemia or ischemia (with intra-tissue variation; Goodwin et al., 1996; Henning et al., 1996; Hermansen et al., 1967; Suh et al., 2007; Swanson et al., 1989).

Glycogen is degraded through the combined action of PYG and glycogen debranching enzyme (amylo-alpha-1,6-glucosidase, 4-alpha-glucanotransferase, AGL, EC 2.4.1.25, EC 3.2.1.33) in the cytosol (Cori and Larner, 1951), with a minority fraction of glycogen degraded in lysosomes by glucosidase acid, alpha (GAA, EC 3.2.1.20; Geddes and Stratton, 1977).

In the cytosol, PYG catalyzes degradation of linear glucan chains via phosphorolysis of alpha-1,4-glycosidic linkages and release of glucose-1-phosphate (Cori et al., 1938). Three isozymes of PYG exist with primary expression in brain, muscle and liver (PYGB, PYGM and PYGL, respectively) and a shared 80% amino acid sequence identity (Newgard et al., 1988). As reviewed by Johnson, PYG’s conformation state and therefore activity is highly regulated by the binding of allosteric effectors and by reversible phosphorylation (Johnson, 1992). Binding of adenosine monophosphate and
phosphorylase kinase-mediated (PHK, EC 2.7.11.19) phosphorylation of serine residue 14 promotes a more active PYG configuration.

While PYG is responsible for degrading linear glucan chains, it cannot lyse alpha-1,6-glycosidic linkages and therefore stalls four glucose units away from glycogen branch points. AGL enables lysis of alpha-1,6-linked glycogen branch points via a two-step process (Brown et al., 1963; Nelson et al., 1969). Firstly, AGL acts as a glucanotransferase, transferring the terminal three of four glucose units remaining after PYG degradation onto the terminus of another glucan chain. Secondly, AGL hydrolyses the remaining alpha-1,6-glycosidic linkage, releasing glucose and effectively removing the glycogen branch (Cori and Larner, 1951). Multiple splice variants with different expression patterns are produced from the AGL gene (Bao et al., 1996a) and little is known about AGL regulation.

A minority percentage of glycogen is degraded via an alternate lysosomal pathway. In this pathway, glycogen is transferred to lysosomes and degraded by the predominantly lysosomal enzyme GAA, which hydrolyses both alpha-1,4- and alpha-1,6-glycosidic linkages (Lejeune et al., 1963). Analysis of the liver glycogen fraction hypothesized to be associated with lysosomes suggests that lysosomal glycogen may account for more than 10% of total hepatic glycogen and contains predominantly high molecular weight glycogen (Geddes and Stratton, 1977). The mechanisms responsible for glycogen trafficking to lysosomes are incompletely understood, however, macroautophagy and the enzyme starch binding domain 1 (STBD1) have been implicated in this process in murine skeletal muscle and liver, respectively (Raben et al., 2010; Sun et al., 2016). The determinants of glycogen particle targeting to lysosomes remain unclear, as does the functional significance of this secondary degradation pathway.

1.2 Glycogen storage diseases

Deficiency of an enzyme involved in glycogen metabolism can result in a glycogen storage disease (GSD). GSDs are classified by the affected gene and cumulatively affect approximately one out of every 20,000-43,000 live births (Applegar...
Ozen, 2007). Over 12 types of GSDs have been identified and numbered sequentially upon identification.

GSDs caused by mutations in enzymes involved in glycogen synthesis lend insight into the roles of these enzymes in health and disease, as well as informing the rational pursuit of therapeutic interventions via pathway manipulation.

1.2.1 GSD XV

GSD XV is caused by inheritance of autosomal recessive mutations in GYG. The clinical manifestation of a GYG1 deficiency (OMIM 613507) includes variable skeletal muscle and cardiac involvement while the implications of a GYG2 deficiency remain unclear. Only a handful of GSD XV patients have been reported, however, it is likely that GSD XV patients are under-diagnosed due to the recent, and still incomplete, link between genotype and phenotype.

1.2.1.1 GYG1-deficient GSD XV

GSD XV patients with mutations in GYG1 are often from consanguineous families and several different mutations have been reported, including missense and nonsense mutations and single nucleotide deletions (Luo et al., 2015; Malfatti et al., 2014; Moslemi et al., 2010). The functional consequences of these mutations range from GYG1 protein ablation to impaired self-glucosylation or interaction with GYS1 (Luo et al., 2015; Malfatti et al., 2014; Moslemi et al., 2010; Nilsson et al., 2012).

Patients typically present with adult-onset muscle weakness, myopathic electromyography (EMG) patterns and fatty replacement of skeletal muscle (Akman et al., 2016; Colombo et al., 2016; Luo et al., 2015; Malfatti et al., 2014; Moslemi et al., 2010). Histological examination of skeletal muscle shows accumulation of periodic acid-Schiff (PAS)-positive material within central or subsarcolemmal vacuoles in type I muscle fibers. This PAS-positive material was confirmed to be glycogen via electron
microscopy analysis, however, its ultrastructure, location within vacuoles and partial sensitivity to amylase digestion renders it unique among GSDs.

Given that GYG1 is broadly considered to be the sole GYG isozyme expressed in human skeletal muscle (Mu et al., 1997; Oldfors, 2017), formation of any skeletal muscle glycogen, albeit abnormal, in the absence of functional GYG1 runs counter-intuitive to the assumption that GYG is critical for glycogen particle synthesis. Possible explanations include residual function of mutant GYG1, compensatory expression of GYG2 or use of alternate primers for glycogen synthesis (Meezan et al., 1997; Testoni et al., 2017; Torija et al., 2005). GYG2 messenger ribonucleic acid (mRNA) analysis has, however, shown low levels of GYG2 expression in skeletal muscle (Imagawa et al., 2014) and to the best of my knowledge has not been investigated in skeletal muscle tissue of GYG1-deficient GSD XV patients. Sensitive analysis of GYG2 expression in unaffected and GYG1-deficient skeletal muscle tissue would lend insight into the above hypotheses and mechanisms of GSD XV pathogenesis.

The clinical spectrum of GSD XV due to GYG1 mutations has broadened to include cardiomyopathy with variable skeletal muscle involvement (Hedberg-Oldfors et al., 2017; Moslemi et al., 2010). Four patients with adolescent- or adult-onset cardiomyopathy and accumulation of abnormal glycogen in cardiomyocytes have been described. Two of the patients progressed to require heart transplantation and three of the patients had normal muscle strength. All four patients were unrelated, however, three of the patients were homozygous for what was likely a founder mutation. The reason for variable cardiac or skeletal muscle involvement remains unknown.

1.2.1.2 GYG2-deficient GSD XV

The role of GYG2 in glycogen metabolism and the clinical implications of a GYG2 deficiency have yet to be clarified since the isozyme’s discovery in 1997 (Mu et al., 1997). A hemizygous missense mutation in GYG2 was identified in two brothers diagnosed with Leigh syndrome (OMIM 256000), a severe juvenile-onset progressive neurological condition (Imagawa et al., 2014). The GYG2 mutation was hypothesized to
be causative due to its inheritance pattern (X-linked), *in vitro* functional studies indicating the GYG2 mutation inhibited self-glucosylation, and a lack of alternate exonic variants of pathogenic likelihood (Imagawa et al., 2014). This case and the clinical and biochemical significance of GYG2 has since been challenged, however, by the identification of several males missing the entire GYG2 gene with no associated phenotype (Irgens et al., 2015). It is also possible that GYG2 functional impairment has different pathogenic consequences than complete GYG2 absence.

Gyg knockout in mice results in abnormal cardiac development (thickened myocardium first evident at embryonic day 15.5) and perinatal lethality (Dickinson et al., 2016). Interestingly, these Gyg knockout mice also displayed embryonic abnormalities in brain and spinal cord development, potentially indicative of neural degeneration (Dickinson et al., 2016). Further glycogen-centric analysis of a different Gyg knockout mouse model demonstrated accumulation of abnormally large glycogen particles that lacked a covalently-bound protein primer (Testoni et al., 2017). Perinatal lethality in this mouse model showed incomplete penetrance and the presence of Gyg mRNA several kilobases after the gene trap cassette suggests the allele may result in some hypomorphic expression.

1.2.2 GSD 0

GSD 0 is caused by autosomal recessive mutations in either the GYS1 (GSD 0B, OMIM 611556) or GYS2 (GSD 0A, OMIM 240600) gene. The clinical manifestations of GSD 0B and 0A differ in accordance with the expression profiles of GYS1 and GYS2 (ubiquitous and liver-specific, respectively). GSD 0B and 0A are extremely rare and also likely under-diagnosed (Cameron et al., 2009; Gitzelmann et al., 1996; Kollberg et al., 2007; Orho et al., 1998).
1.2.2.1 GSD 0B

GSD 0B has only recently been identified as a GSD, with three families reported to have affected children (Cameron et al., 2009; Kollberg et al., 2007; Sukigara et al., 2012). In the first family, the eldest sibling presented with exercise fatigability and died due to cardiac arrest at 10.5 years of age, with the autopsy ruling hypertrophic cardiomyopathy as the cause of death (Kollberg et al., 2007). Two younger siblings, one presenting with muscle fatigability and hypertrophic cardiomyopathy at 11 years of age and another presenting with asymptomatic cardiac involvement at 2 years of age, were placed on cardioprotective medication. All three affected individuals were found to be homozygous for a nonsense mutation in \textit{GYS1}. In the second and third families, an 8-year-old male and 12-year-old female died due to sudden cardiac arrest (Cameron et al., 2009; Sukigara et al., 2012). The male was found to have a 2 bp deletion in \textit{GYS1} exon 2, while the female had been diagnosed with compound heterozygous \textit{GYS1} mutations (Cameron et al., 2009; Sukigara et al., 2012). In all cases, skeletal muscle biopsies demonstrated a lack of \textit{GYS1} protein and glycogen, in addition to mitochondrial proliferation and either a predominance of type I fibers or atrophy of type II fibers (Cameron et al., 2009; Kollberg et al., 2007; Sukigara et al., 2012). In two of the three families, the parents were consanguineous and the mothers had an obstetric history including perinatal death(s) of previous children (Cameron et al., 2009; Kollberg et al., 2007).

Several aspects of the human GSD 0B phenotype are modeled in \textit{Gys1} knockout mice. \textit{Gys1} knockout in mice results in perinatal lethality in 90% of pups and an absence of glycogen in all tested non-hepatic tissues (Pederson et al., 2004). The absence of cardiac glycogen during mouse embryogenesis is understood to be causative of abnormal cardiac development and subsequent perinatal lethality (Pederson et al., 2004). While the majority of cardiac adenosine triphosphate under normal conditions is derived from fatty acids (Bing et al., 1954; Neely and Morgan, 1974), cardiac glycogen is considered to be of particular importance during times of anoxia (e.g., during birth; Dawes et al., 1959) and sudden cardiac energy demands (e.g., during exercise; Goodwin et al., 1996; Henning et al., 1996). In contrast to the human phenotype, \textit{Gys1}
knockout mice do not show reduced exercise tolerance (Pederson et al., 2005a). This phenotypic difference is hypothesized to be a function of reduced murine storage of and reliance on skeletal muscle glycogen compared to humans (Baldwin et al., 1973; Hribal et al., 2002; Pederson et al., 2005a). Of note, the 10% of Gys1 knockout pups that do survive the perinatal period have no obvious phenotype but do show increased heart masses and a predominance of oxidative fiber types in skeletal muscle with age (Pederson et al., 2004, 2005b).

1.2.2.2 GSD 0A

GSD 0A, due to deficiency of liver-specific GYS2, was first reported in 1963 with upwards of 20 cases reported in the literature since (Lewis et al., 1963; Soggia et al., 2010; Spiegel et al., 2007; Weinstein et al., 2006). The clinical presentation of GSD 0A is variable. The more severe presentation onsets in infancy (after weaning off night feeding) or in early childhood with the patient showing signs of reduced wellbeing, from fatigue to seizures, prior to breakfast (Aynsley-Green et al., 1977; Gitzelmann et al., 1996; Lewis et al., 1963; Orho et al., 1998; Soggia et al., 2010; Spiegel et al., 2007). The less severe presentation can be essentially asymptomatic (or with mild or transient symptoms in retrospect) with the diagnosis due to incidental findings of metabolic abnormalities or diagnosis of an affected sibling (Aynsley-Green et al., 1978; Bachrach et al., 2002; Gitzelmann et al., 1996; Laberge et al., 2003; Spiegel et al., 2007; Szymańska et al., 2015).

In contrast to the variability in clinical presentation, metabolic profiles and liver biopsy analyses are generally consistent across patients (Aynsley-Green et al., 1977; Bachrach et al., 2002; Gitzelmann et al., 1996; Laberge et al., 2003; Lewis et al., 1963; Orho et al., 1998; Soggia et al., 2010; Spiegel et al., 2007). Most GSD 0A patients display fasting hypoglycemia and hyperketonaemia, with postprandial inversion of the metabolic profile including hyperglycemia and hyperlactatemia. Common metabolic features also include impaired glucose tolerance and a reduced response to glucagon administration in the fasted, but not fed, state. These metabolic perturbations are in large part due to the reduced ability of the liver to store glucose and consequent
shunting of glucose into glycolysis rather than glycogenesis. Liver biopsy analysis shows reduced glycogen storage, fatty liver, and undetectable to low GYS activity. Disease management for GSD 0A patients includes consumption of frequent protein-rich meals in addition to uncooked cornstarch prior to bedtime to provide gluconeogenic precursors and prevent nighttime fasting hypoglycemia, respectively.

Nineteen unique GYS2 mutations have been reported in GSD 0A patients (Nessa et al., 2012; Soggia et al., 2010; Spiegel et al., 2007; Szymańska et al., 2015; Weinstein et al., 2006). In vitro modeling of mutations confirmed their impairment of GYS2 activity, but failed to correlate genotype (and consequent GYS2 activity) with patient phenotype (Orho et al., 1998). This lack of correlation contributes to the puzzling phenomenon in GSD 0A in which undetectable to low GYS activity in the liver is not in theoretical accordance with the only partially reduced and normally structured liver glycogen levels (Aynsley-Green et al., 1977; Orho et al., 1998). The consensus hypothesis is that in the absence of GYS activity, PYG may contribute to glycogen synthesis as has been shown to be possible in vitro (Cori and Cori, 1943; Lewis et al., 1963; Orho et al., 1998). Curiously, the potential contribution of GYS1 to glycogen synthesis in human liver has not been investigated in the context of GSD 0A (expression demonstrated by Kollberg et al., 2007). While the GYS activity assays conducted on liver homogenate should have detected GYS1 activity if present, it is possible that the in vitro activity assays are not sensitively reflecting in vivo glycogen synthesis.

A GSD 0A mouse model, deficient in liver GYS2, replicated several of the human symptoms including impaired glucose tolerance and quicker onset of fasting hypoglycemia compared to controls (Irimia et al., 2010). Additionally, reduced exercise capacity in mice lacking liver GYS2 further supported the hypothesis of greater reliance on hepatic glycogen for exercise in mice compared to humans (Irimia et al., 2010).

1.2.3 GSD IV

GSD IV (OMIM 232500), also called amylopectinosis or Andersen disease, is caused by autosomal recessive mutations in GBE1 (Bao et al., 1996b; Lossos et al., 1998). The
clinical presentation of GSD IV is heterogeneous with variation in the age of onset and tissues affected (Moses and Parvari, 2002). While most accurately described along a continuum, GSD IV is typically subdivided into six variants: i) classical progressive hepatic, ii) non-progressive hepatic, iii) fatal perinatal, iv) congenital, v) childhood and, vi) adult polyglucosan body disease (APBD).

Over 50 mutations in GBE1 have been reported in the Human Gene Mutation Database, including missense, nonsense and splicing mutations in addition to insertions, deletions and complex rearrangements (Stenson et al., 2017). While the latter mutations are likely to result in protein ablation, missense mutations, which account for the majority of mutations, are predominantly located within the catalytic core and structural insights suggest an effect on either protein stability or catalytic efficiency (Froese et al., 2015). The severity of the mutation’s effect on protein activity can be loosely correlated with disease severity, but lacks prognostic power (Bao et al., 1996b; Bruno et al., 2004). Carriers have been shown to have as low as 50% GBE1 activity and are unaffected (Greene et al., 1988).

Confirmation of GBE1 mutations and reduced GBE1 activity are therefore considered gold standards for the diagnosis of GSD IV. GBE1 activity is often measured in patient leukocytes, cultured skin fibroblasts or nerve, muscle or liver tissue and the assay most commonly used indirectly measures GBE1 activity as a function of radiolabelled glucose incorporation into glycogen (Moses and Parvari, 2002). Given the different tissue types used and the indirect nature of the assay, GBE1 activity measurements should be interpreted as approximations.

Deficiency of GBE1 activity in GSD IV patients results in the accumulation of abnormal glycogen, containing longer linear chains with fewer branch points (Illingworth and Cori, 1952). This increased glycogen chain length initially drew similarities to amylopectin (a comparatively less-branched component of starch), hence the name amylopectinosis, however, further analysis confirmed both GSD IV and normal glycogen to have a unimodal chain length distribution, compared to amylopectin’s bimodal distribution (Mercier and Whelan, 1973). Poorly-branched glycogen in GSD IV, termed polyglucosans, is less soluble and therefore has an increased tendency to precipitate
and aggregate to form polyglucosan bodies (PBs; Illingworth and Cori, 1952; Robitaille et al., 1980).

PBs are intracellular inclusions that when visualized under the electron microscope appear filamentous with irregular branching (Robitaille et al., 1980). PBs vary in size, stain PAS-positive and are more resistant to diastase digestion (PASD-positive) than normal glycogen (Robitaille et al., 1980). The critical mechanism hypothesized to underlie PB formation in GSD IV is an imbalance between GYS-mediated chain elongation and GBE1 branching activity. This theory is supported by observations of accumulated poorly-branched glycogen, structurally and histologically indistinguishable from PBs, in a transgenic mouse model and a naturally occurring horse model in which GYS1 activity is abnormally increased (McCue et al., 2008; Pederson et al., 2003; Raben et al., 2001). Disproportionately high GYS1 activity compared to GBE1 activity is also hypothesized to contribute to the formation of skeletal muscle PBs in GSD VII in which G6P, an allosteric activator of GYS1, accumulates (Agamanolis et al., 1980; Hays et al., 1981).

1.2.3.1 Classical progressive hepatic form

The classical progressive liver form of GSD IV, first described by Andersen, presents in early infancy (less than 18 months of age) with failure to thrive and progressive liver disease (Andersen, 1952, 1956). Hepatosplenomegaly is observed along with hepatic cirrhosis and consequent portal hypertension, ascites and esophageal varices. Histological analysis shows enlarged hepatocytes with accumulation of polyglucosans in patient livers and other tissues (Bannayan et al., 1976). The classical progressive liver form of GSD IV is typically fatal before 5 years of age unless liver transplantation is performed.

Liver transplantation improves liver functioning and intriguingly has also been shown to reduce accumulation of PBs in non-hepatic patient tissues (Selby et al., 1991). While the mechanism of PB reduction remains unclear, microchimerism (migration of cells from the liver allograft) has been observed in patients and may be responsible for some
of the extrahepatic improvements (Starzl et al., 1993). It additionally seems possible that an allograft-enabled capacity to construct and therefore utilize normally structured hepatic glycogen may shift systemic energy sources and adaptively reduce reliance on glycogen storage in extrahepatic tissues.

1.2.3.2 Non-progressive hepatic form

A second hepatic form of GSD IV presents with several similar but milder symptoms, and critically, it is not acutely progressive in nature (Greene et al., 1988; Guerra et al., 1986; McConkie-Rosell et al., 1996). Hepatomegaly with or without splenomegaly is observed but has in most cases been an incidental diagnosis occurring in childhood years. Histological analysis demonstrates the presence of partially diastase-resistant PAS-positive inclusions in hepatocytes and other tissues. Follow-up liver biopsies did not depict progressive accumulation and no liver cirrhosis was observed. Longer-term follow-up of patients (eldest at 13 and 20 years of age) showed no liver disease progression, nor evidence of myopathy, cardiomyopathy or neuropathy (McConkie-Rosell et al., 1996).

Patients with the non-progressive hepatic form typically have higher GBE1 activity levels than patients with the progressive hepatic form, however, this difference is not statistically significant (in large part due to the extremely small patient population sizes; Bao et al., 1996b; McConkie-Rosell et al., 1996).

1.2.3.3 Fatal perinatal and congenital forms

The fatal perinatal form and congenital form are typically differentiated, however, a literature review indicates these two forms have largely overlapping clinical manifestations (Alegria et al., 1999; Bruno et al., 2004; Cox et al., 1999; Janecke et al., 2004; Konstantinidou et al., 2006; Li et al., 2012; Nambu et al., 2003; Nolte et al., 2008; van Noort et al., 1993; Raju et al., 2008; Ravenscroft et al., 2013; Tang et al., 1994; Tay et al., 2004). The fatal perinatal and congenital forms commonly present with fetal
abnormalities including reduced in utero movement, polyhydramnios and hydrops fetalis. Joint contractures are comparatively more common in the perinatal form as is pulmonary hypoplasia, resulting in the diagnosis of some patients with fetal akinesia deformation sequence. In almost all cases, infants born present with severe hypotonia, signs of cardiomyopathy and are dependent on artificial respiration. Hepatomegaly and liver cirrhosis have been described in a minority of the congenital form cases.

Both the perinatal and congenital forms are fatal due to cardiopulmonary failure. The age at death is the primary determinant of case classification, with cases classified as the perinatal form including pregnancy termination, in utero death, death during labor or within the first few days of life (Alegria et al., 1999; Cox et al., 1999; van Noort et al., 1993; Ravenscroft et al., 2013). The congenital form is most commonly fatal between 1-5 months of age (frequently determined based on time of extubation; Bruno et al., 2004; Janecke et al., 2004; Konstantinidou et al., 2006; Li et al., 2012; Nambu et al., 2003; Nolte et al., 2008; Raju et al., 2008; Tang et al., 1994; Tay et al., 2004).

In both forms, histological analysis shows accumulation of PBs throughout patient tissues (including skeletal and cardiac muscles, liver and the central nervous system [CNS]) and GBE1 activity is typically less than 5% that of control samples.

1.2.3.4 Childhood form

A unique subset of GSD IV patients have been described that present with symptoms of cardiomyopathy and myopathy in childhood and adolescent years (Nase et al., 1995; Schröder et al., 1993a; Servidei et al., 1987). Investigation revealed dilated cardiomyopathy and cardiomegaly in addition to skeletal muscle atrophy and associated symptoms including exercise intolerance, shortness of breath and pulmonary edema. Hepatomegaly and subclinical neuropathy were present in some cases and cardiac failure resulting in death was reported in two patients.

Tissue biopsy and autopsy analysis demonstrated PB accumulation throughout patient tissues (especially in cardiomyocytes) and an increased quantity of glycogen in heart and skeletal muscle tissue. GBE1 activity was reported to be absent. The consensus
hypothesis is that the reduced severity of hepatic symptoms (for unknown reasons) resulted in the later onset of cardiac and skeletal muscle symptoms. Heart transplant is a rarely-implemented therapeutic option for these patients and liver transplant could also be considered given the above-described extrahepatic improvements post-liver transplant (Aksu et al., 2012; Ewert et al., 1999).

1.2.3.5 Adult polyglucosan body disease

APBD (OMIM 263570), a primary focus of this thesis, is an adult-onset variant of GSD IV that presents as a progressive neurological disorder with central and peripheral nervous system involvement. Symptoms typically onset around 50 years of age and include neurogenic bladder, distal sensory abnormalities (paresthesia and hypoesthesia) and gait disturbance (pyramidal paraplegia; Hellmann et al., 2015; Mochel et al., 2012). Patients are usually wheelchair dependent by 60 years of age and have a reduced life expectancy of 70 years of age. Cognitive decline involving memory and attention deficits are observed in approximately 50% of patients.

Neurological tests confirm central and peripheral nervous system dysfunction in APBD. Magnetic resonance imaging consistently shows medullary and spinal cord atrophy in addition to white matter lesions (Lossos et al., 1991; Mochel et al., 2012). Proton magnetic resonance spectroscopic imaging depicted abnormal metabolic activity (reduced N-acetylaspartate to creatine ratio and increased lactate), hypothesized to be potentially indicative of neuroaxonal damage and/or gliosis and infiltrating macrophages (Massa et al., 2008). EMG and nerve conduction velocity tests demonstrate reduced action potentials and conduction velocities indicative of a sensorimotor polyneuropathy (Lossos et al., 1991).

APBD occurs at a higher prevalence in the Ashkenazi Jewish population, where the majority of affected individuals are homozygous for the mutation p.Y329S (c.986A>C in exon 7; Lossos et al., 1998; Mochel et al., 2012). Tyr329 is a highly conserved amino acid in the GBE1 catalytic domain and recombinant expression of p.Y329S GBE1 protein resulted in reduced amounts of soluble protein (Froese et al., 2015). Structural
analysis predicts the p.Y329S mutation impairs protein folding and results in surface-exposure of hydrophobic residues, thereby destabilizing the protein (Froese et al., 2015). Individuals with homozygous p.Y329S mutations have 18% GBE1 activity measured in leukocytes, compared to controls (Akman et al., 2015a). A further 30% of individuals with APBD have heterozygous p.Y329S mutations (Mochel et al., 2012) and this mutation has also been detected in patients with the non-progressive hepatic form of GSD IV but not in the classical progressive hepatic, perinatal or congenital forms (Bao et al., 1996b). The residual activity of p.Y329S GBE1 is thought to underlie the comparatively milder disease course of individuals with this mutation (Bao et al., 1996b).

Until recently, the majority of p.Y329S heterozygous APBD patients were considered manifesting heterozygotes as they were clinically indistinguishable from p.Y329S homozygous patients and lacked a second detectable GBE1 mutation (Mochel et al., 2012; Ubogu et al., 2005). Work by Akman et al. identified these manifesting heterozygous patients to contain a 9 bp deletion replaced by a 20 bp insertion in exon 15 (GBE1-IVS15+5289_5297delGTGTTGGTGGGinsTGTTTTTTACATGACAGGT; Akman et al., 2015a). This mutation introduces an artificial splice acceptor, leading to an ectopic exon 16, a novel termination codon and ultimately an unstable truncated protein. GBE1 activity in compound heterozygous individuals was measured as 8% and the absence of APBD patients homozygous for the IVS15+5289_5297delGTGTTGGTGGGinsTGTTTTTTACATGACAGGT mutation suggests it may result in a severe perinatal disease course.

While the majority of APBD patients are of Ashkenazi Jewish ancestry, APBD has been diagnosed in individuals from a range of other ancestries. Non-Ashkenazi Jewish patients commonly do not have the p.Y329S or IVS15+5289_5297delGTGTTGGTGGGinsTGTTTTTTACATGACAGGT mutation and are more likely to have atypical clinical presentations including transient neurological symptoms (Billot et al., 2013; Colombo et al., 2015; Dainese et al., 2013; Naddaf et al., 2016; Paradas et al., 2014; Sagnelli et al., 2014).
The neuropathology pathognomonic feature of APBD is the presence of PBs in astrocytic and neuronal processes (Robitaille et al., 1980; Suzuki et al., 1971). PBs are particularly prevalent in neuronal axons and disrupt the axon’s integrity, often causing distention and thinning of the surrounding myelin sheath (Robitaille et al., 1980; Vos et al., 1983). Demyelination and gliosis are commonly observed in the CNS (Peress et al., 1979; Suzuki et al., 1971), with loss of myelinated fibers in the PNS (Cafferty et al., 1991; Vos et al., 1983). PBs are found throughout patient tissues and are sometimes associated with histological abnormalities (e.g., liver fibrosis, cardiomyocyte enlargement), but non-neurological clinical manifestations in APBD cases are rarely observed (Gray et al., 2011; Peress et al., 1979; Suzuki et al., 1971). Glycogen measurements have confirmed increased amounts of glycogen in brain and heart tissue (Peress et al., 1979; Robitaille et al., 1980).

Similar to other GSD IV variants, the gold standard for diagnosing APBD involves identification of GBE1 mutations and confirmation of a reduction in GBE1 activity. Sural nerve biopsies are also commonly employed to test for intra-axonal PBs and positive findings are considered diagnostic (Vos et al., 1983). The presence of PBs in other tissues biopsied, e.g., in intramuscular nerve branches, could support testing for APBD but are not diagnostic given their association with aging and other diseases (Bernsen et al., 1989; Furtado et al., 2016).

Given the rarity and lack of awareness of APBD, in addition to its clinical similarity to other conditions (especially in the early stages), the average time to diagnosis for APBD patients is 6.8 years (Hellmann et al., 2015). The most common misdiagnoses are cerebral small vessel disease, peripheral neuropathies, multiple sclerosis and amyotrophic lateral sclerosis (Hellmann et al., 2015; Segers et al., 2012). In males, the initial urinary symptoms are also commonly first attributed to prostate disorders (Hellmann et al., 2015). The prevalence of postmortem diagnoses and retrospective inquiry into deceased siblings of probands in the literature are indicative of significant under-diagnosis of APBD. Increased use of genetic testing and inclusion of GBE1 in Ashkenazi Jewish genetic panels will hopefully serve to shorten the diagnostic odyssey for patients. There are presently no treatments to halt or reverse disease progression in APBD and clinical care is focused on symptom management.
1.2.3.6 Animal models of GSD IV

1.2.3.6.1 Naturally occurring GSD IV in non-human animals

GSD IV has also been documented to naturally occur in Quarter horses and the related Paint horse breed (Render et al., 1999; Valberg et al., 2001) as well as in Norwegian forest cats (Fyfe et al., 1992).

1.2.3.6.1.1 Quarter and Paint horses

GSD IV was initially suspected in the Quarter horse breed, prized as short-distance sprinters, after detection of PAS-positive inclusions in the tissues of an aborted fetus, a stillborn foal and a 1-month-old foal that had to be euthanized due to severe weakness (Render et al., 1999). Genetic mapping and sequencing subsequently identified a nonsense mutation, p.Y34X, that resulted in no detectable GBE1 protein or activity when homozygous (Valberg et al., 2001; Ward et al., 2003, 2004). Further investigation confirmed the GSD IV phenotype to range from abortion to stillbirth to death prior to 2 months of age, with foals that do survive birth presenting with symptoms including hypoglycemia, seizures, progressive weakness, cardiac or respiratory failure and sudden death (Valberg et al., 2001). In accordance with the human GSD IV phenotype, PAS-positive inclusions were shown to accumulate in an age-dependent manner in skeletal muscle, heart, liver and CNS tissues, and glycogen was poorly-branched (Render et al., 1999; Valberg et al., 2001). The GSD IV symptoms and cause of death in Quarter horses have been proposed to be due to an insufficiency of glucose due to a reduced ability to breakdown poorly-branched glycogen as well as potential cytotoxicity of the PAS-positive inclusions (Valberg et al., 2001). Carrier horses heterozygous for the p.Y34X mutation are not affected by GSD IV though they show a 50% reduction in GBE1 protein and activity (Valberg et al., 2001).

Paint horses are a pinto-coloured, related breed that inherited the GBE1 p.Y34X mutation from the Quarter horse breed (Wagner et al., 2006). Mutant allele frequencies for Quarter and Paint horses are estimated to be 0.041 and 0.036, respectively, and
most affected or carrier horses can be traced back to one stallion alive in the early 20th century (Wagner et al., 2006).

1.2.3.6.1.2 Norwegian forest cats

Norwegian forest cats are a domestic breed acclimatized to cold conditions and are a second naturally occurring non-human animal affected by GSD IV (Fyfe et al., 1992). Affected cats are homozygous for a complex rearrangement in the GBE1 gene involving a 334 bp insertion in the place of a 6.2 kb deletion which encompasses exon 12 (Fyfe et al., 2007). This mutation results in abnormal splicing, formation of a premature termination codon (PTC) and consequent ablation of GBE1 protein and activity (Fyfe et al., 1992, 2007). While autosomal recessive inheritance of this complex genetic rearrangement accounts for all affected cats, two distinct clinical presentations have been observed.

The most common clinical presentation of affected cats is death within the perinatal period hypothesized to be due to severe hypoglycemia during the birthing process and subsequent cardiac or respiratory failure (Fyfe et al., 1992, 2007). A smaller percentage of affected cats live normally until juvenile-onset of a progressive neuromuscular phenotype including generalized tremors, skeletal muscle atrophy and weakness, and gait abnormalities leading to tetraplegia. These cats also begin to exhibit signs of cardiac disease and sudden death or euthanasia typically occurs before 13 months of age.

Fyfe et al. hypothesized that the phenotype variability, despite GBE1 mutation homogeneity, may be due to genetic modifiers, environmental effects and/or the ability to survive hypoglycemia in a critical perinatal period (Fyfe et al., 1992, 2007). Cats that survived the perinatal period (either naturally or due to experimental intervention with glucose supplementation) developed the juvenile-onset neuromuscular phenotype at which point they were not hypoglycemic but their tissues showed diffuse accumulation of PAS- & PASD-positive inclusions in addition to histopathological indicators of
degeneration in the CNS, skeletal muscle and heart. Genetic screening estimated the mutant allele frequency to be 0.082 in the Norwegian forest cat population.

While breeding programs to eradicate GSD IV from Quarter and Paint horses and Norwegian forest cats are ongoing, affected Norwegian forest cats may ultimately also serve as larger animal models for the development of GSD IV therapeutics.

1.2.3.6.2 Mouse models of GSD IV

Several mouse lines have been generated to recapitulate the spectrum of GSD IV allelic diseases. The perinatal form of GSD IV has been modeled by two different mouse lines containing a chemical mutagenesis-induced nonsense mutation in exon 14 or a targeted deletion of exon 7 (Akman et al., 2011; Lee et al., 2011). Mice homozygous for these mutations have no detectable GBE1 protein and show abnormal cardiac development and perinatal lethality. The childhood form of GSD IV has been modeled by a mouse line containing a phosphoglycerate kinase promoter driven neomycin cassette inserted into Gbe1 intron 7 and resulting in 11-16% GBE1 activity (Akman et al., 2011). Mice homozygous for this mutation accumulate PBs throughout brain, muscle, heart and liver, and have a shortened life span of less than 10 months of age.

APBD has been accurately modeled by the generation of a mouse line containing the p.Y329S mutation (Akman et al., 2015b). Mice homozygous for the p.Y329S mutation retain 16-21% GBE1 activity in muscle, brain and heart tissue compared to wild-type mice and 37% GBE1 activity in the liver. These mice accumulate PBs throughout their tissues and have reduced grip strength, hind limb spasticity with age and a shortened life span of less than 20 months of age.

1.3 Polyglucosan bodies in the nervous system

Despite the central pathogenic role of neuronal and astrocytic PBs in APBD, many aspects pertaining to PB formation and accumulation remain unknown. The presence of PBs, histochemically and ultrastructurally indistinguishable from each other (Robitaille et
al., 1980; Suzuki et al., 1971; Yokota et al., 1987), in a range of human conditions affecting the nervous system broadens and informs the perceived significance of PBs in health and disease.

In 1837, Purkinje first observed the presence of age-related PBs, called corpora amylacea, in the CNS of elderly patients (Catola and Achúcarro, 1906). Corpora amylacea have since been established as a common finding associated with aging in humans and other species, however, their role in protecting from or contributing to age-related decline is debated (Cavanagh, 1999; Duran and Guinovart, 2015). Increased loads of corpora amylacea have been associated with several neurodegenerative diseases, including Alzheimer’s disease and multiple sclerosis (Fleming et al., 1987; Selmaj et al., 2008), and also with temporal lobe epilepsy (Abubakr et al., 2005) and double athetosis (PBs called Bielschowsky bodies; de León, 1974). PBs in these diseases are not considered directly related to the primary cause of disease and it is unclear whether they impart a protective, neutral or deleterious effect.

Neuronal PBs have also been reported in the GSD, Lafora disease, in which they play a central role in pathogenesis (Duran et al., 2014; Lafora and Glueck, 1911). Lafora disease shares several histological and biochemical similarities to APBD but results in a highly divergent clinical presentation, thereby providing comparative insight into the pathogenesis of both diseases.

1.3.1 Lafora disease

Lafora disease is a GSD that manifests as a progressive myoclonus epilepsy in young adolescents (Lafora and Glueck, 1911). As reviewed by Minassian, Lafora disease onsets insidiously with symptoms such as a decline in school performance rapidly progressing to intractable seizures, hallucinations, and dementia (Minassian, 2001). Lafora disease is invariably fatal, typically within 10 years of symptom onset.

Lafora disease is inherited in an autosomal recessive manner with the causative genes being a carbohydrate binding phosphatase, laforin (EPM2A, laforin glucan phosphatase, \textit{EPM2A}, EC 3.1.3.16, EC 3.1.3.48), and a ubiquitin E3 ligase, malin (NHL repeat
containing E3 ubiquitin protein ligase 1, *NHLRC1*, EC 2.3.2.27; Chan et al., 2003; Minassian et al., 1998). Laforin and malin have been shown to regulate enzymes in the glycogen synthesis pathway, including GYS1 and PPP1R3C, in a codependent manner (Vilchez et al., 2007; Worby et al., 2008). Loss of laforin or malin is hypothesized to result in the dysregulation of these proteins and consequent accumulation of PBs, which in Lafora disease are termed Lafora bodies (LBs). Of note, no differences in GBE1 protein or activity levels have been detected in brain or muscle from the EPM2A-deficient Lafora disease mouse model (Tagliabracci et al., 2007, 2008).

Given the intrinsic similarities between PBs in APBD and LBs in Lafora disease, the divergent clinical presentations are thought to be due to different intra- and inter-cellular localizations (Suzuki et al., 1971). Unlike PBs, which occur in both astrocytic and neuronal processes but predominantly in neuronal axons, LBs predominantly localize to neuronal perikarya and dendrites (Robitaille et al., 1980; Suzuki et al., 1971). These different localization patterns could conceivably result in i) PBs in APBD impairing intra-neuron signal transmission resulting in an axonal neuropathy and ii) LBs in Lafora disease impairing inter-neuron signal transmission resulting in a progressive epilepsy. The mechanisms responsible for different PB and LB localization are unknown.

### 1.4 Rational pursuit of therapeutic targets for APBD

A first step towards developing a therapeutic for GSD IV, and specifically for APBD, involves the identification and validation of therapeutic targets. The outlook for identifying therapeutic targets for APBD is promising given its monogenic inheritance, the sole catalytic function of the deficient enzyme and the clear therapeutic aim of reducing PBs. Logically, PBs can be reduced by either preventing their continuous formation or increasing their degradation. It is not known whether PBs can be degraded via interventional or endogenous means and the cellular damage incurred by the PBs, or the mechanism of degradation, may not be reversible. The prevention of PB formation therefore presents a more immediate and proactive opportunity for the development of a therapeutic treatment. Prevention of PB formation may be achievable
by restoring the deficient glycogen branching activity or by manipulating the glycogen synthesis pathway to accommodate for the branching deficiency, as discussed below.

1.4.1 GYG

One therapeutic strategy involving manipulation of the glycogen synthesis pathway could aim to reduce total glycogen synthesized. Given that PBs are predominantly comprised of polyglucans thought to derive from previously soluble glycogen, one could hypothesize that a reduction in total glycogen could result in an analogous reduction in PBs. Given GYG’s role in initiating glycogen molecule synthesis, manipulation of GYG protein levels would be a rational approach to alter glycogen accumulation. Evidence supporting this approach was demonstrated via in vitro experiments in which overexpression of GYG2 or knockdown of GYG1 resulted in an increase and decrease in glycogen levels respectively (Douillard-Guilloux et al., 2008; Mu and Roach, 1998).

Further in vitro and in vivo analyses have, however, demonstrated that the relationship between quantity of GYG protein and glycogen is not consistent. In vitro overexpression of GYG1 did not lead to an increase in glycogen levels but rather an increase in the total number of glycogen particles and a shift towards smaller particle sizes (Skurat et al., 1997); a phenomenon that has also been noted in vivo (Shearer et al., 2005). The lack of an association between GYG deficiency in GSD XV patients and mouse models and reduced glycogen levels further demonstrates that GYG is at present a poor therapeutic target for sensitive manipulation and guaranteed reduction of glycogen levels.

1.4.2 GYS1 and PPP1R3C

Manipulation of total GYS1 activity, either via changes in protein levels or activation state, has been shown to result in corresponding shifts in glycogen levels and degree of branching (Douillard-Guilloux et al., 2008; Duran et al., 2012; Manchester et al., 1996; McCue et al., 2008; Pederson et al., 2003, 2004; Raben et al., 2001). Reduction of total GYS1 activity could therefore prove therapeutic by reducing total glycogen synthesized
and also by increasing the relative glycogen branch frequency. Support for targeting GYS1 in the APBD context was demonstrated via the reduction of accumulated glycogen in neuronal cell culture transduced by lentiviruses expressing $Gbe1$-targeted short hairpin RNAs and exposed to rapamycin or starvation, both of which reduce GYS1 activity among other things (Kakhlon et al., 2013).

Targeting of GYS1 could involve direction inhibition or indirect inhibition through targeting proteins involved in GYS1 regulation. As previously discussed, kinase-mediated phosphorylation and PP1-mediated dephosphorylation of GYS1 results in its respective inhibition and activation. The implicated kinases and PP1 catalytic subunits are involved in a myriad of cellular pathways and are therefore not optimal therapeutic targets. The PP1 regulatory subunits, on the other hand, provide an opportunity for a targeted therapeutic intervention. Of the seven identified glycogen-targeting PP1 regulatory subunits, PPP1R3C is the most thoroughly characterized subunit with expression in the brain. Overexpression of PPP1R3C in neuronal and astrocytic cell culture has been shown to increase the GYS1 activation state and glycogen accumulation (Ruchti et al., 2016; Vilchez et al., 2007). Overexpression of PPP1R3C in vivo has been shown to result in the formation of PBs in the murine brain, not unlike the consequences of GYS1 overexpression (Duran et al., 2014). Congruently, PPP1R3C knockout in mice has been shown to reduce brain glycogen levels (Turnbull et al., 2014). Beyond PPP1R3C’s role in binding and targeting the PP1 catalytic subunit to glycogen, the glycogenic consequences of PPP1R3C overexpression and knockout may be further mediated by PPP1R3C’s demonstrated interaction with other enzymes involved in glycogen metabolism including GYS, PYG, AGL, PHK and laforin, and its proposed role as a molecular scaffold (Fernández-Sánchez et al., 2003; Fong et al., 2000; Printen et al., 1997).

Furthermore, the mono- and bi-allelic knockout of GYS1 or PPP1R3C in the Lafora disease mouse models reduces or, in the case of Gys1 knockout, entirely prevents the accumulation of LBs (Duran et al., 2014; Pederson et al., 2013; Turnbull et al., 2011a, 2014; Figures 2-3), suggesting a similar therapeutic response may be possible in regard to PBs in APBD. Wholly, this body of evidence supports the in vivo interrogation of GYS1 and PPP1R3C as potential therapeutic targets for APBD.
Figure 2. Mono-allelic Gys1 knockout in the Epm2a<sup>−/−</sup> Lafora disease mouse model. 

PASD staining (A-C) and anti-GFAP (D-F) and anti-AIF1 (G-I) IHC depict reduced hippocampal LB accumulation, astrogliosis and microgliosis in 23-month-old Epm2a<sup>−/−</sup>Gys1<sup>+/−</sup> mice (C, F, I) compared to Epm2a<sup>−/−</sup> mice (B, E, H) and wild-type (WT) control mice (A, D, G). Scale bars represent 50 µm. LB quantification (J), measured as the percent LB area of the total hippocampal area, and measurement of brain glycogen in 15-month-old mice (K) similarly depict a respective reduction in LB and glycogen accumulation in Epm2a<sup>−/−</sup>Gys1<sup>+/−</sup> mice compared to Epm2a<sup>−/−</sup> mice (n = 4-7). Data presented as mean ± SEM. One-way ANOVA: F (2, 14) = 63.62, P ≤ 0.0001 (J); F (2, 13) = 45.26, P ≤ 0.0001 (K). Tukey-Kramer multiple comparisons test: a vs. b P ≤ 0.0001, a, b vs. c P ≤ 0.001 (J); a, c vs. b P ≤ 0.0001, a vs. c P ≤ 0.05 (K).
Figure 3. Mono-allelic Ppp1r3c knockout in the Nhlrc1<sup>−/−</sup> Lafora disease mouse model.
PASD staining (A-C) and anti-GFAP (D-F) and anti-AIF1 (G-I) IHC depict reduced hippocampal LB accumulation, astrogliosis and microgliosis in 10-12-month-old Nhlrc1<sup>−/−</sup> Ppp1r3c<sup>−/−</sup> mice (C, F, I) compared to Nhlrc1<sup>−/−</sup> mice (B, E, H) and wild-type (WT) control mice (A, D, G). Scale bars represent 50 µm. LB quantification (J), measured as the percent LB area of the total hippocampal area, and measurement of brain glycogen in 9-12-month-old mice (K) similarly depict a respective reduction in LB and glycogen accumulation in Nhlrc1<sup>−/−</sup> Ppp1r3c<sup>−/−</sup> mice compared to Nhlrc1<sup>−/−</sup> mice (n = 5-6). Data presented as mean ± SEM. One-way ANOVA: F (2, 14) = 27.18, P ≤ 0.0001 (J); F (2, 14) = 62.74, P ≤ 0.0001 (K). Tukey-Kramer multiple comparisons test: a vs. b P ≤ 0.0001, a vs. c P ≤ 0.01, b vs. c P ≤ 0.05 (J); a vs. b P ≤ 0.0001 (K).

1.4.3 GBE1

An alternate therapeutic approach for GSD IV is to specifically resolve the GBE1 deficiency through deoxyribonucleic acid (DNA), mRNA or protein targeted interventions. In regard to genetic interventions, Yi et al. recently demonstrated partial rescue of the murine APBD phenotype using adeno-associated virus serotype nine vector-mediated delivery of the human GBE1 gene (Yi et al., 2016). GSD IV is a promising disease candidate for gene therapy because GBE1 can fit within the AAV packaging constraints and expression of GBE1 at non-endogenous levels is unlikely to be deleterious. The pursuit of a gene therapy treatment for APBD would however face substantial challenges in clinical translation given the older age of the patient population. As reviewed by Choudhury et al., some of these challenges include immune responses to AAV capsid proteins and the difficulty of achieving widespread...
transduction and stable gene expression throughout the adult CNS (Choudhury et al., 2017). GBE1 transgene immunogenicity would, however, likely not be a concern given GBE1 expression in APBD patients. Nuclease-mediated gene editing for directed mutation repair is not currently a viable therapeutic approach when targeting post-mitotic cells such as neurons because this method requires homology-directed repair, which is predominantly active during the S/G2 phase of the cell cycle (Chapman et al., 2012; Cox et al., 2015). Identification of factors or delivery vehicles that enhance homology-directed repair in post-mitotic cells is an area of ongoing research (Canny et al., 2016; Nishiyama et al., 2017).

An alternate transcript-targeted approach to repair aberrant splicing present in individuals carrying the GBE1-IVS15+5289_5297delGTGTGTTGGinsTGGTTTTTACATGACAGGT mutation could prove therapeutic for approximately 30% of APBD patients (Akman et al., 2015a; Mochel et al., 2012). As reviewed by Havens and Hastings, this approach involves use of antisense oligonucleotides (ASOs) that bind pre-mRNA at the errant splice site to block recognition by the spliceosome (Havens and Hastings, 2016).

Pharmacologic interventions targeting mutant GBE1 proteins are also in development. A small peptide designed to stabilize GBE1 protein containing the p.Y329S mutation was shown to increase GBE1 p.Y329S activity by 10-15% in vitro (Froese et al., 2015).

The multi-pronged pursuit of therapeutic interventions for APBD patients, including knockdown of therapeutic targets and amelioration of the GBE1 deficiency at the DNA, mRNA and protein level, increases the likelihood of clinical success, potentially via combination therapy to individualize care and optimize patient outcomes.
Chapter 2: Aims and Hypotheses

The aim of this thesis was to characterize the effect of a GYS1 and a PPP1R3C deficiency on the APBD mouse model disease phenotype. Using a genetic pharmacotherapy strategy, the larger aim of this thesis was to contribute to the identification and in vivo validation of effective therapeutic targets for APBD patients and potentially other GSD IV variants. Given the pathway manipulation inherent to this project, a secondary aim was to glean insight into glycogen metabolism and mechanisms involved in PB formation.

The specific hypotheses being tested were:

i) Mono-allelic Gys1 knockout impedes disease progression in the p.Y329S APBD mouse model as measured by morphological and behavioural parameters and histological and biochemical analyses.

ii) Mono- and bi-allelic Ppp1r3c knockout impedes disease progression in the p.Y329S APBD mouse model as measured by morphological and behavioural parameters and histological and biochemical analyses.
Chapter 3: Methods

3.1 Mice

Mice modeling APBD were crossed with mice deficient in either GYS1 or PPP1R3C resulting in two independent mouse lines housed at Ball State University (Muncie, Indiana, USA) and The Centre for Phenogenomics (Toronto, ON, CA), respectively. The APBD mouse model, referred to here as *Gbe1*<sup>y/y</sup> and described in Section 1.2.3.6.2, has homozygous *Gbe1* c.986A>C (p.Y329S) mutations (MGI: *Gbe1*<sup>tm2.1Hoa</sup>) and was received from Dr. Akman (Akman et al., 2015b). GYS1-deficient mice (*Gys1*<sup>+/−</sup>), described in Section 1.2.2.1, were heterozygous for a null allele (MGI: *Gys1*<sup>Gt[OST3395]Lex</sup>; Pederson et al., 2004) and PPP1R3C-deficient (*Ppp1r3c<sup>+/−</sup> and *Ppp1r3c<sup>−/−</sup>* mice contain a null allele (MGI: *Ppp1r3c*<sup>tm1Adpr</sup>) missing almost the entire coding DNA sequence (Turnbull et al., 2011a; Zhai et al., 2007). *Gys1<sup>−/−</sup>* mice were not studied given their embryonic lethal phenotype (Pederson et al., 2004).

Experimental cohorts included male and female littermates generated by breeding *Gbe1*<sup>y/y</sup> *Gys1*<sup>+/−</sup> or *Gbe1*<sup>y/y</sup> *Ppp1r3c<sup>+/−</sup>* mice and were maintained under standard laboratory conditions. Mice were sacrificed via cervical dislocation with harvested brain, muscle, heart and liver immediately frozen in liquid nitrogen or fixed in 10% neutral buffered formalin to minimize post-mortem glycogen degradation (Geddes and Rapson, 1973). All experimental procedures were conducted in accordance with federal guidelines (Public Health Service, Canadian Council on Animal Care) and approved by institutional Animal Care Committees.

Genotyping of the *Gbe1* allele was conducted using the forward and reverse primers 5’-AGTGACCATGATTGGCTAGCTT-3’ and 5’-GTCTATGTCCAGCACAGTATTAAGGA-3’, respectively (*Gbe1*<sup>y</sup> product: 321 bp, wild-type product: 256 bp). Genotyping of *Gys1* was conducted using a multiplex polymerase chain reaction (F: 5’-GGGAAAGGGTTTCTTGGATATCATGCTTCTC-3’, R: 5’-GGCTCATAGTAGGAGGGGAAGA-3’, R: 5’-ATAAGTTGCTGGCCAGCTTACCTCAGGGAAGA-3’) to detect the wild-type (product: 293 bp).
bp) and null (product: 750 bp) alleles. Genotyping of Ppp1r3c was conducted using primer pairs to detect the wild-type allele (F: 5’-GAGCTGTGTCAGACTTGTTCAGATAGAG-3’, R: 5’-TTGAAAACCATTGTAAGGACCCAGGAAA-3’, product: 401 bp) or the null allele (F: 5’-AGATCTCATCACCCCCAGTGC-3’, R: 5’-TAGTTCCCAGGCTGCTCTG-3’, product: 192 bp).

The Gbe1ys Gys1- and Gbe1ys Ppp1r3c- mouse lines were on mixed and slightly different backgrounds. Gbe1ys Gys1- were 50% C57BL/6NTac;129/Ola and 50% C57BL/6J;129SvJ. Gbe1ys Ppp1r3c- mice were 50% C57BL/6NTac;129/Ola and 50% C57BL/6J.

3.2 Life span and morphology analysis

Mouse life span was measured as days until humane endpoint or 24 months of age. As per experimental animal care guidelines, endpoint was defined as the stage in disease progression at which point humane euthanasia was conducted to terminate mouse distress. Body mass was measured at 7 and 9 months of age in the Gbe1ys Gys1- mouse line and at 7, 9 and 12 months of age in the Gbe1ys Ppp1r3c- mouse line. Body composition was characterized in Gbe1ys Ppp1r3c- mice between 12-14.5 months of age using nuclear magnetic resonance imaging-based EchoMRI technology (Echo Medical Systems, Houston, TX, USA).

3.3 Behavioural analysis

Total activity was measured in Gbe1ys Gys1- mice by video recording mice in a chamber (32 cm x 26 cm) for 30 min. FreezeScan software (Clever Sys Inc., Reston, VA, USA) was used to quantify pixel change between consecutive frames as an indicator of mouse movement (reported as pixel change/s). Locomotor activity level was measured in Gbe1ys Ppp1r3c- mice by video recording mice in a chamber (47.5 cm x 36 cm) for 10 min. The chamber floor overlay a 15-square grid and the number of squares in which a
mouse entered with all four limbs was counted. Data were binned such that counts less than 10 received a score of one, counts less than 20 received a score of two, etc., up to 99 at which point counts greater than 99 received a score of 11.

Hindfeet step length in 9-month-old Gbe1<sup>Y5</sup> Gys1<sup>−</sup> mice was measured as the distance between homolateral hindfoot steps after painting the hindfeet of mice and having them walk along a piece of white parchment paper. Automated gait analysis was conducted for 12-14.5-month-old mice from the Gbe1<sup>Y5</sup> Ppp1r3c<sup>−</sup> mouse line using the ExerGait (XL) treadmill (Columbus Instruments, Columbus, OH, USA) and TreadScan analysis software (Clever Sys Inc., Reston, VA, USA). Treadmill speed was 19 cm/s with 20 s of video footage analyzed (100 frames/s). Parameters reported include mouse body length, distance from front of treadmill, homolateral feet distance and maximum foot distance from transverse plane. Homolateral feet distance is the distance between the stride midpoints of front and rear feet (distances for left feet and right feet were average). Maximum foot distance from transverse plane was averaged between front left and right feet and rear left and right feet.

Balance beam testing was conducted in 8.5-14-month-old Gbe1<sup>Y5</sup> Gys1<sup>−</sup> mice by measuring their time to traverse 60 cm across a 2 cm-wide beam. A maximum time of 60 s was allowed. Post-training, 4-5 consecutive trials separated by 10 s were completed and scores were averaged (highest and lowest scores were removed). Trials were video recorded and the number of footfalls quantified. Mice received a score of zero if they were unable to cross the beam within the allotted time, one if they crossed the beam with footfalls and two if they crossed the beam with no footfalls.

Rotarod testing was conducted at 9 months of age in both GYS1- and PPP1R3C-deficient APBD mouse lines. Latency to fall or to complete two passive rotations was measured across a 300 s trial in which the rod continuously accelerated from 4 to 40 rotations per min. Mice underwent four rotarod trials separated by 15 min inter-trial intervals. Trial scores were averaged per mouse.

Maximal forelimb strength was measured in five consecutive trials at 9 months of age in the Gbe1<sup>Y5</sup> Gys1<sup>−</sup> mouse line and at 12-14.5 months of age in the Gbe1<sup>Y5</sup> Ppp1r3c<sup>−</sup> mouse line. Trial scores were averaged per mouse.
3.4 Histological analysis

Formalin-fixed tissues were embedded in paraffin and PASD stained. Slides were scanned by a Pannoramic digital slide scanner (20x objective; 3DHistech, Budapest, Hungary) and PBs were quantified using a method established in the image analysis program HistoQuant (3DHistech, Budapest, Hungary) in which PBs were defined based on pixel colour. Brain and liver PB quantification values are presented as the percent area covered by PBs in coronal hippocampal and liver sections, respectively. The hippocampus was selected for PB quantification as consistent delineation of this brain structure is possible and its high glycogen content compared to other brain regions (Oe et al., 2016) likely contributes to the high PB quantity. Liver PBs were classified as having a minimum area of 50 $\mu$m$^2$.

Anti-glial fibrillary acid protein (GFAP) and anti-allograft inflammatory factor 1 (AIF1) immunohistochemistry (IHC) was conducted on formalin-fixed brain sections. Rabbit polyclonal anti-GFAP (1:2000; BioLegend, San Diego, CA, USA) and anti-AIF1 (1:2000; Wako Chemicals, Richmond, VA, USA) antibodies were used.

3.5 Biochemical analysis

Tissues from age-matched cohorts of male and female mice were used for glycogen quantification. Cohorts from the $Gbe1^{+/-}$ Gys1$^{-}$ and $Gbe1^{+/-}$ Ppp1r3c$^{-}$ mouse lines were 8.5-10.5 and 12-14.5 months of age, respectively. Total brain, muscle and liver glycogen content was measured as described by Suzuki et al. (Suzuki et al., 2001). Briefly, frozen ground tissue was boiled in 30% (wt/vol) potassium hydroxide and precipitated three times using 67% (vol/vol) ethanol and 15 mM lithium chloride. Pellets were resuspended in water and glycogen was digested using amylglucosidase (Megazyme, Bray, Ireland) in 80 mM sodium acetate buffer (pH 4.5). An aliquot was used to enzymatically quantify glucose concentration in a solution of approximately 175 mM tricine-potassium hydroxide (pH 8), 9 mM magnesium chloride, 1 mM adenosine triphosphate and 0.7 mM $\beta$-nicotinamide adenine dinucleotide phosphate. Glucose-6-phosphate dehydrogenase and hexokinase (Roche Diagnostic GmbH, Mannheim,
Germany) were added sequentially with absorbance (340 nm) recorded prior to and post addition of hexokinase. Glycogen content is reported as µmol glucose/g of tissue (fresh weight).

Quantification of degradation-resistant liver glycogen was conducted by initially incubating 200 mg of ground frozen tissue at 37 °C for 2 h to facilitate degradation of metabolically active glycogen by endogenous enzymes. Samples were subsequently homogenized using a TissueRuptor (Qiagen, Valencia, CA, USA) in 2 mL of a glycogen isolation buffer (150 mM sodium chloride, 50 mM tris [pH 8], 50 mM sodium fluoride, 5 mM tetrasodium pyrophosphate and 2 mM ethylenediaminetetraacetic acid) and then centrifuged at 13 000 g for 10 min. The supernatant was discarded and the pellet was resuspended in 1.5 mL of the glycogen isolation buffer. The cycle of centrifugation, supernatant disposal and pellet resuspension was repeated an additional four times. An aliquot of the final suspension underwent amyloglucosidase-mediated degradation and glucose concentration determination as per the above-described steps.

3.6 Statistical analysis

One- or two-way analysis of variance (ANOVA) and post-test Tukey-Kramer multiple comparisons tests were performed for morphological and behavioural parameters and PB and glycogen quantification. An exception was the use of an unpaired, two-tailed t-test to compare quantification of liver PBs between Gbe1ys/ys and Gbe1ys/ys Gys1+/− mice. A significance threshold of 0.05 was used with P values reported as ≤ 0.05, ≤ 0.01, ≤0.001 or ≤ 0.0001. Log-rank test and log-rank pairwise comparisons with Bonferroni-adjusted significance thresholds were used to compare survival curves. Male and female mice were included in all analyses and were pooled when no sex effect was detected. The results of pairwise comparisons are depicted on graphs using letters (e.g., “a” is significantly different than “b” while “ab” is not significantly different than “a” or “b”). Numbers affiliated with these letters (e.g., “a1n”) are used to enable reporting of pairwise levels of significance (e.g., “a1n” and “a2n” are significantly different than “b” but to different levels of significance). All statistical calculations were performed using
GraphPad Prism 7.0b (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard error of the mean (SEM).
Chapter 4: Results

4.1 Deficiency of GYS1 or of PPP1R3C improves the APBD mouse model life span and morphological phenotype

4.1.1 Mono-allelic knockout of Gys1 rescues the reduced life span and loss of body mass in \(Gbe1^{ys/ys}\) mice

Mono-allelic knockout of \(Gys1\) in the APBD mouse model, \(Gbe1^{ys/ys}\), improved the disease-associated life span reduction and morphological phenotype. The median life span of \(Gbe1^{ys/ys}\) mice was 10.8 months of age compared to a median life span of greater than 24 months in \(Gbe1^{ys/ys} Gys1^{+/−}\) mice. The survival curves for wild-type, \(Gbe1^{ys/ys}\), \(Gbe1^{ys/ys} Gys1^{+/−}\) and \(Gys1^{+/−}\) mice were significantly different (\(P \leq 0.0001\)) with rescue of the shortened \(Gbe1^{ys/ys}\) mouse survival curve in \(Gbe1^{ys/ys} Gys1^{+/−}\) mice (Figure 4A). Of note, qualitative observations of the overt \(Gbe1^{ys/ys} Gys1^{+/−}\) mouse phenotype indicated disease progression despite the rescued life span.

Mouse body mass, measured at 7 and 9 months of age, decreased in male and female \(Gbe1^{ys/ys}\) mice, plateaued in \(Gbe1^{ys/ys} Gys1^{+/−}\) mice, and increased in control mice over this time period (Figure 4B-C). In male mice, the mean body mass of 9-month-old \(Gbe1^{ys/ys} Gys1^{+/−}\) mice was significantly higher than that of \(Gbe1^{ys/ys}\) mice (\(P \leq 0.001\)) and similar to that of wild-type mice. Female body masses showed analogous trends, however, genotype was not found to be a significant source of variation. While both male and female \(Gbe1^{ys/ys}\) mice reached a low mean mass of 25 g, the upper mean mass was 30% higher in males than in females.
**Figure 4. Mono-allelic *Gys1* knockout rescues *Gbe1*ys/ys mouse life span and reduces disease-associated loss of body mass.**

Survival curves for wild-type (WT), *Gbe1*ys/ys, *Gbe1*ys/ys *Gys1*+/− and *Gys1*+/− mice (n = 15-25; A). Log-rank test found the curves to be significantly different (P ≤ 0.0001). Pairwise comparisons using the log-rank test with a Bonferroni-corrected significance threshold (P ≤ 0.008): a vs. b P ≤ 0.0001. Body mass was measured at 7 and 9 months of age in male (n = 8-18; B) and female (n = 7-14; C) mice. Data presented as mean ± SEM (B-C). Two-way ANOVA (effect of genotype): F (3, 96) = 17.36, P ≤ 0.0001 (B); F (3, 73) = 2.20, P > 0.05 (C). Tukey-Kramer multiple comparisons test (comparisons within each time point, statistical relationships for 9-month time point presented on graph): a vs. b P ≤ 0.01, a, ab vs. c P ≤ 0.0001, b vs. c P ≤ 0.001 (B).

### 4.1.2 Mono- and bi-allelic *Ppp1r3c* knockout improves life span, loss of male body mass and kyphosis phenotype in *Gbe1*ys/ys mice

PPP1R3C deficiency due to mono- or bi-allelic knockout increased the median APBD mouse model life span from 12.4 months of age to 13.2 and 21.6 months in *Gbe1*ys/ys *Ppp1r3c*+/− and *Gbe1*ys/ys *Ppp1r3c*+/− mice, respectively. Comparison of the survival curves for these mice and controls found *Gbe1*ys/ys and *Gbe1*ys/ys *Ppp1r3c*+/− mice to have shorter survival curves than *Gbe1*ys/ys *Ppp1r3c*+/− (P ≤ 0.0001), *Ppp1r3c*+/− (P ≤ 0.0001) and wild-type (P ≤ 0.0001, 0.001, respectively) mice (Figure 5A). *Gbe1*ys/ys *Ppp1r3c*+/− mice lived nearly twice as long as *Gbe1*ys/ys mice, however, their survival curve was still significantly different than *Ppp1r3c*+/− (P ≤ 0.05) and wild-type (P ≤ 0.01) mice.

Body mass was measured in 7-, 9- and 12-month-old mice from the *Gbe1*ys *Ppp1r3c*− mouse line (Figure 5B-C). Genotypes did not significantly differ in body mass among male or female mice at 7 months of age. By 12 months of age, male *Gbe1*ys/ys mice had
significantly less body mass than $Gbe1^{ys/ys}$ $Ppp1r3c^{+/+}$ ($P \leq 0.01$), $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ ($P \leq 0.001$) and $Ppp1r3c^{-/-}$ and wild-type ($P \leq 0.0001$) mice. Furthermore, $Gbe1^{ys/ys}$ $Ppp1r3c^{+/+}$ and $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ mice did not significantly differ in body mass compared to wild-type mice. At 12 months of age in female mice, $Gbe1^{ys/ys}$, $Gbe1^{ys/ys}$ $Ppp1r3c^{+/+}$ and $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ mice all had significantly lower body masses than control $Ppp1r3c^{-/-}$ ($P \leq 0.0001$) and wild-type ($P \leq 0.05, 0.01, 0.05$, respectively) mice.

**Figure 5.** Mono- and bi-allelic $Ppp1r3c$ knockout improves $Gbe1^{ys/ys}$ mouse lifespan and disease-associated loss of body mass in males.

Survival curves for wild-type (WT), $Gbe1^{ys/ys}$, $Gbe1^{ys/ys}$ $Ppp1r3c^{+/+}$, $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ and $Ppp1r3c^{-/-}$ mice ($n = 6-18$; A). Log-rank test found the curves to be significantly different ($P \leq 0.0001$). Pairwise comparisons using the log-rank test with a Bonferroni-corrected significance threshold ($P \leq 0.005$): $a$ vs $b$, $P \leq 0.005$; $a$ vs. $c_1$, $P \leq 0.001$; $a$ vs. $c_2$, $P \leq 0.0001$; $ab$ vs. $c_1$, $c_2$, $P \leq 0.0001$. Body mass was measured at 7, 9 and 12 months of age in male ($n = 5-13$; B) and female ($n = 5-10$, except $n = 2$ for $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ and 9 months of age; C) mice. Data presented as mean ± SEM (B-C). Two-way ANOVA (effect of genotype): $F(4, 96) = 16.58$, $P \leq 0.0001$ (B); $F(4, 82) = 17.99$, $P \leq 0.0001$ (C). Tukey-Kramer multiple comparisons test (comparisons within each time point, statistical relationships for 9-month time point presented on graph): $a$ vs. $b$, $P \leq 0.05$; $a$, $ab$ vs. $c$, $P \leq 0.0001$; $ab_2$ vs. $c$, $P \leq 0.01$; $b$ vs. $c$, $P \leq 0.001$ (B); $a_1$, $a_2$, $b_1$ vs. $b_2$, $P \leq 0.0001$; $a_2$ vs. $b_1$, $P \leq 0.05$; $a_2$ vs. $b_2$, $P \leq 0.01$ (C).

Body composition analysis of $Gbe1^{ys/ys}$, $Gbe1^{ys/ys}$ $Ppp1r3c^{+/+}$, $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$, $Ppp1r3c^{-/-}$ and wild-type mice was conducted at 12-14.5 months of age (Figure 6). In both males and females, $Gbe1^{ys/ys}$ mice had reduced amounts of body fat compared to wild-type mice ($P \leq 0.05, 0.01$, respectively), a trend observed in $Gbe1^{ys/ys}$ $Ppp1r3c^{+/+}$ and $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ mice as well. Male mice had approximately 37% more lean
mass (includes organs) than female mice, with the exception of male Gbe1ys/ys mice whose lean mass was reduced to levels similar to that of female mice.

Figure 6. Body composition analysis depicts Gbe1ys/ys-associated fat loss. Body composition measurements in male (n = 3-7; A) and female (n = 3-6; B) 12-14.5-month old mice. Data presented as mean ± SEM. One-way ANOVA: F (4, 19) = 7.64, P ≤ 0.001 (fat; A); F (4, 19) = 6.94, P ≤ 0.01 (lean; A); F (4, 19) = 15.63, P ≤ 0.0001 (fat; B); F (4, 19) = 0.28, P > 0.05 (lean; B). Tukey-Kramer multiple comparisons test: ab vs. c P ≤ 0.05, b vs. c P ≤ 0.01, ac vs. b P ≤ 0.05 (fat; A); d1 vs. e P ≤ 0.01, d2 vs. e P ≤ 0.05, d3 vs. e P ≤ 0.001 (lean; A); ab vs. c P ≤ 0.01, b vs. c P ≤ 0.0001, ac vs. b P ≤ 0.01 (fat; B). WT = wild-type.

Gbe1ys/ys mice displayed the overt morphological phenotype of progressive kyphosis (Figure 7A). Kyphosis, first observable in Gbe1ys/ys mice around 4 months of age, progressed to such severity by 12-14.5 months of age that it was the likely cause of a significant reduction in Gbe1ys/ys mouse body length compared to Gbe1ys/ys Ppp1r3c+/− (P ≤ 0.05), Gbe1ys/ys Ppp1r3c−/− (P ≤ 0.01), Ppp1r3c−/− (P ≤ 0.0001) and wild-type (P ≤ 0.01) mice (Figure 8). Neither Gbe1ys/ys Ppp1r3c+/− nor Gbe1ys/ys Ppp1r3c−/− mice were significantly different than wild-type mice at this time point. Gbe1ys/ys Ppp1r3c−/− mice did, however, consistently developed a similar kyphotic phenotype with age and disease progression. Gbe1ys/ys Ppp1r3c−/− mice similarly developed kyphosis but not always to the same extent of severity before endpoint or sacrifice (Figure 7B).
Figure 7. Kyphosis phenotype in Gbe1<sup>ys/ys</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mice. Photos of a 12.5-month-old Gbe1<sup>ys/ys</sup> mouse (A) and a 20-month-old Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mouse (B).

Figure 8. Reduced Gbe1<sup>ys/ys</sup> mouse body length is rescued in PPP1R3C-deficient mice. Body length was measured in 12-14.5-month-old mice (n = 7-9). Data presented as mean ± SEM. One-way ANOVA: F (4, 33) = 11.76, P ≤ 0.0001. Tukey-Kramer multiple comparisons test: a vs. b P ≤ 0.01, a vs. c P ≤ 0.05, ab vs. c P ≤ 0.01, b vs. c P ≤ 0.0001. WT = wild-type.

4.2 Deficiency of GYS1 or of PPP1R3C improves behavioural deficits in the APBD mouse model

Gbe1<sup>ys/ys</sup> mice deficient in GYS1 or PPP1R3C underwent analogous behavioural tests to assay physical abilities sensitive to neuromuscular perturbation, such as locomotion,
coordination, balance and muscle strength. Differences in methods used and parameters reported are present between the two mouse lines.

4.2.1 Mono-allelic Gys1 knockout normalizes activity, gait and balance abnormalities in Gbe1<sup>ys/ys</sup> mice

At 9 months of age, total spontaneous activity was measured in Gbe1<sup>ys</sup> Gys<sup>+</sup> mice and was significantly reduced in Gbe1<sup>ys/ys</sup> mice compared to Gbe1<sup>ys/ys</sup> Gys<sup>1<sup>+/−</sup></sup> (P ≤ 0.05) and control (P ≤ 0.001) mice (Figure 9A). Total activity of Gbe1<sup>ys/ys</sup> Gys<sup>1<sup>+/−</sup></sup> mice was rescued to the activity level of control mice. Measurement of hindfeet step length found Gbe1<sup>ys/ys</sup> mice to have significantly reduced hindfeet step lengths compared to Gbe1<sup>ys/ys</sup> Gys<sup>1<sup>+/−</sup></sup> and control mice (P ≤ 0.05; Figure 9B). Capacity to cross a balance beam differed among genotypes in a cohort of 8.5-14.5-month-old mice (Figure 9C). Gbe1<sup>ys/ys</sup> mice received a score of zero given their inability to cross the beam in under 60 s (P ≤ 0.0001) with no significant differences in capacity to cross the beam with or without footfalls in Gbe1<sup>ys/ys</sup> Gys<sup>1<sup>+/−</sup></sup>, Gys<sup>1<sup>+/−</sup></sup> and wild-type mice.

**Figure 9.** Mono-allelic Gys1 knockout improves Gbe1<sup>ys/ys</sup> mouse behavioural impairments.
Total activity (n = 10-15; A) and hindfeet step length (n = 15-17; B) was quantified in 9-month-old mice. Balance beam testing was conducted in 8.5-14-month-old mice (n = 7-10; C). Data presented as mean ± SEM. One-way ANOVA: F (3, 47) = 9.16, P ≤ 0.0001 (A); F (3, 61) = 4.71, P ≤ 0.01 (B); F (3, 27) = 19.44, P ≤ 0.0001 (C). Tukey-Kramer multiple comparisons test: a<sup>1</sup> vs. b P ≤ 0.0001, a<sup>2</sup> vs. b P ≤ 0.05 (A); a vs. b P ≤ 0.05 (B); a vs. b P ≤ 0.0001 (C). WT = wild-type.
No disease phenotype was detected in latency to fall on the rotarod (Figure 10A) or in maximal forelimb grip strength measurement (Figure 10B) in $Gbe1^{ys/ys}$ mice from the $Gbe1^{ys}$ Gys1’ mouse line.

![Figure 10](image)

**Figure 10. Failure to detect a $Gbe1^{ys/ys}$ mouse disease phenotype by rotarod testing and forelimb grip strength measurement.**
Latency to fall during rotarod testing ($n = 11-22$; A) and forelimb grip strength measurement ($n = 15-21$; B) in 9-month-old mice from the $Gbe1^{ys}$ Gys1’ mouse line. Data presented as mean ± SEM. One-way ANOVA: F (3, 64) = 0.40, $P > 0.05$ (A); F (3, 70) = 2.31, $P > 0.05$ (B). WT = wild-type.

### 4.2.2 Mono- and bi-allelic $Ppp1r3c$ knockout normalizes activity, locomotor and gait abnormalities in $Gbe1^{ys/ys}$ mice

At 12-14.5 months of age in the $Gbe1^{ys}$ $Ppp1r3c^{-/-}$ mouse line, spontaneous locomotor activity was scored based on the number of grid squares entered by mice (Figure 11A). $Gbe1^{ys/ys}$ mice showed significantly reduced locomotor activity compared to $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ ($P \leq 0.05$), $Ppp1r3c^{-/-}$ ($P \leq 0.05$) and wild-type ($P \leq 0.01$) mice. Automated analysis of mouse gait on a treadmill identified several abnormalities indicative of impaired locomotor functioning. Distance from the front of the treadmill, indicative of the mouse’s ability to maintain pace with the treadmill speed, was increased in $Gbe1^{ys/ys}$ mice compared to wild-type mice ($P \leq 0.05$; Figure 11B). This indicator of impaired locomotion was rescued in $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ and $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ mice. Measurement of homolateral feet distance found $Gbe1^{ys/ys}$ mice to have a reduced
distance between front and rear stride midpoints compared to $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ ($P \leq 0.01$), $Ppp1r3c^{-/-}$ ($P \leq 0.0001$) and wild-type ($P \leq 0.01$) mice (Figure 11C). Maximum distance from transverse plane was similar for front feet across genotypes, but significantly reduced in rear feet of $Gbe1^{ys/ys}$ mice (Figure 11D). Furthermore, $Gbe1^{ys/ys}$ mice were the sole genotype to have significantly different front and rear maximum distances from the transverse plane ($P \leq 0.0001$), supporting visual observations and previous reports (Akman et al., 2015b) of progressive hindlimb dysfunction and paralysis in these mice. Of note, progressive generalized body tremors and myoclonus-like head jerks were also observed in $Gbe1^{ys/ys}$, $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ and $Gbe1^{ys/ys}$ $Ppp1r3c^{-/-}$ mice as they neared endpoint.
Figure 11. Mono- and bi-allelic *Ppp1r3c* deficiency in *Gbe1* 
ys/ys mice improves behavioral impairments.
Scoring of locomotor activity (n = 8-16; A) and analysis of gait, including the parameters distance from front of treadmill (B), homolateral feet distance (C) and maximum foot distance from transverse plane (D; n = 7-9) in 12-14.5-month-old mice. Data presented as mean ± SEM. One-way ANOVA: F (4, 50) = 4.27, P ≤ 0.01 (A); F (4, 33) = 5.63, P ≤ 0.01 (B); F (4, 33) = 8.63, P ≤ .0001 (C). Tukey-Kramer multiple comparisons test: a₁ vs. b P ≤ 0.01, a₂ vs. b P ≤ 0.05 (A); a₁ vs. b P ≤ 0.05, a₂ vs. b P ≤ 0.01 (B); a₁ vs. b P ≤ 0.01, a₂ vs. b P ≤ 0.0001 (C). Two-way ANOVA (effect of genotype): F (4, 66) = 3.37, P ≤ 0.05 (D). Tukey-Kramer multiple comparisons test: a₁ vs. b P ≤ 0.05, a₂ vs. b P ≤ 0.01, a₃ vs. b P ≤ 0.001 (D). WT = wild-type.

No disease phenotype was detected in latency to fall on the rotarod (Figure 12A) or in maximal forelimb grip strength measurement (Figure 12B) in *Gbe1* 
ys/ys mice from the *Gbe1* 
ys Ppp1r3c⁻ mouse line.
4.3 Deficiency of GYS1 or of PPP1R3C reduces PB and glycogen accumulation in the APBD mouse model

4.3.1 Mono-allelic Gys1 knockout reduces gliosis and PB accumulation in brain, skeletal muscle, heart and liver of Gbe1ys/ys mice

PASD staining of brain tissue from 10.5-month-old wild-type, Gbe1ys/ys, Gbe1ys/ys Gys1+/− and Gys1+− mice showed a reduction in PBs in the brains of Gbe1ys/ys Gys1+/− mice (Figure 13A-D, 14). Quantification of this reduction, specifically of the hippocampal area covered by PBs, similarly showed a greater than 50% reduction in Gbe1ys/ys Gys1+/− PB accumulation (P ≤ 0.0001; Figure 15A). Quantification of total brain glycogen, including PB and non-PB glycogen, showed a reduction in Gbe1ys/ys Gys1+/− glycogen to a level similar to wild-type mice (Figure 15B). Gys1+− mice showed an almost 50% reduction in brain glycogen levels compared to wild-type mice (P ≤ 0.01). Anti-GFAP and anti-AIF1 IHC depicted increased astrogliosis and microgliosis, respectively, in the brains of Gbe1ys/ys mice (Figure 13E-L). Reduced gliosis was observed in Gbe1ys/ys Gys1+/− mice.
Figure 13. Mono-allelic Gys1 knockout improves the neurohistological disease phenotype of Gbe1^{ys/ys} mice.
Hippocampal PASD staining (A-D), and anti-GFAP (E-H) and anti-AIF1 (I-L) IHC of 10.5-month-old wild-type (A, E, I), Gbe1^{ys/ys} (B, F, J), Gbe1^{ys/ys} Gys1^{+/−} (C, G, K) and Gys1^{+/−} (D, H, L) mice. Scale bars represent 50 µm.
Figure 14. Mono-allelic Gys1 knockout reduces PB accumulation in the cerebellum and motor cortex.

PASD staining of cerebellum (A-D) and motor cortex (E-H) in 10.5-month-old wild-type (A, E), Gbe1<sup>ys/ys</sup> (B, F), Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> (C, G) and Gys1<sup>+/−</sup> (D, H) mice. Scale bars represent 50 µm (A-D) and 100 µm (E-H).

PASD staining of skeletal and cardiac muscle similarly depicted a reduction in Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> mouse PB accumulation (Figure 16A-H). Skeletal muscle PBs were dramatically reduced and almost no PBs were detectable in cardiac muscle from Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> mice. Measurement of total glycogen in skeletal muscle confirmed a reduction in Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> compared to the glycogen accumulated in Gbe1<sup>ys/ys</sup> mice (P ≤ 0.0001), and also found a reduction in Gys1<sup>+/−</sup> mice compared to wild-type mice (P ≤ 0.01; Figure 15C). Heterogeneity in Gbe1<sup>ys/ys</sup> skeletal muscle fibers affected and prevalence of PBs across the skeletal muscle section were observed.

Unexpectedly, PASD staining of liver tissue showed a reduction in PB accumulation in Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> compared to Gbe1<sup>ys/ys</sup> mice (Figure 16I-L). Quantification of PBs in the liver supported this observation (Figure 15D). While measurement of total liver glycogen in fed mice did not show a difference between genotypes (Figure 15E), measurement of degradation-resistant liver glycogen showed increased degradation-
resistant glycogen in \( Gbe1^{ys/ys} \) mice compared to wild-type mice (\( P \leq 0.0001 \)) and a reduction in \( Gbe1^{ys/ys} Gys1^{+/-} \) mice compared to \( Gbe1^{ys/ys} \) mice (\( P \leq 0.05 \); Figure 15F).

**Figure 15.** Mono-allelic \( Gys1 \) knockout reduces PB and disease-associated glycogen accumulation in brain, skeletal muscle and liver of \( Gbe1^{ys/ys} \) mice. Quantification of hippocampal area covered by PBs (A), and of total brain (B) and muscle (C) glycogen. Quantification of liver area covered by PBs (D), and of total (E) and degradation-resistant (F) liver glycogen in fed mice. Mice are age-matched cohorts of 8.5-10.5-month-old mice (\( n = 5-7 \)). Data presented as mean ± SEM. One-way ANOVA: \( F (3, 20) = 131.7, P \leq 0.0001 \) (A); \( F (3, 21) = 173.9, P \leq 0.0001 \) (B); \( F (3, 23) = 384.8, P \leq 0.0001 \) (C). Tukey-Kramer multiple comparisons test: \( a \) vs. \( b \) vs. \( c \) \( P \leq 0.0001 \) (A); \( a \) vs. \( b \) \( P \leq 0.0001 \), \( b \) vs. \( c \) \( P \leq 0.0001 \), \( a \) vs. \( c \) \( P \leq 0.01 \) (B); \( a^{1}, a^{2} \) vs. \( b \) \( P \leq 0.0001 \), \( a^{1} \) vs. \( c \) \( P \leq 0.01 \), \( a^{2}, b \) vs. \( c \) \( P \leq 0.0001 \) (C). Unpaired two-tailed t-test: \( t (10) = 4.81, a \) vs. \( b \) \( P \leq 0.001 \) (D). One-way ANOVA: \( F (3, 20) = 1.31, P > 0.05 \) (E); \( F (3, 20) = 30.98, P \leq 0.0001 \) (F). Tukey-Kramer multiple comparisons test: \( a \) vs. \( b \) \( P \leq 0.0001 \), \( a \) vs. \( c \) \( P \leq 0.001 \), \( b \) vs. \( c \) \( P \leq 0.05 \) (F). WT = wild-type.
Figure 16. Mono-allelic Gys1 knockout reduces PB accumulation in murine Gbe1<sup>ys/ys</sup> skeletal muscle, heart and liver.
PASD staining of skeletal muscle (A-D), heart (E-H) and liver (I-L) from 10.5-month-old wild-type (A, E, I), Gbe1<sup>ys/ys</sup> (B, F, J), Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> (C, G, K) and Gys1<sup>+/−</sup> (D, H, L) mice. Scale bars represent 200 µm (A-D, I-L) and 100 µm (E-H).

PASD staining of brain, skeletal muscle, heart and liver tissue from 26-28-month-old mice depicted PBs had continued to accumulate in brain, skeletal muscle and liver since the 10.5-month time point (Figure 17). Heart tissue was an exception in that it still contained few to no PBs even at this older time point. Additionally, Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> livers were found to almost all contain tumours at this time point unlike Gys1<sup>+/−</sup> and wild-type livers at this age.
Figure 17. Mono-allelic Gys1 knockout slows rate of PB accumulation in Gbe1<sup>ys/ys</sup> mice.
PASD staining of hippocampus (A), skeletal muscle (B), heart (C) and liver (D) of 26-28-month-old Gbe1<sup>ys/ys</sup> Gys1<sup>+/-</sup> mice shows PB accumulation in these mice was progressive. Scale bars represent 50 µm (A), 200 µm (B, D) and 100 µm (C).

4.3.2 Mono- and bi-allelic Ppp1r3c knockout reduces PB accumulation in brain with limited effects in skeletal muscle and liver

PASD staining of brain tissue from wild-type, Gbe1<sup>ys/ys</sup>, Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/-</sup>, Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>-/-</sup> and Ppp1r3c<sup>-/-</sup> mice showed a reduction in PBs in Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/-</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>-/-</sup> mice compared to Gbe1<sup>ys/ys</sup> mice (Figure 18A-E, 19). Quantification of hippocampal PBs confirmed reductions of approximately 25% and 55% in Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/-</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>-/-</sup> mice, respectively (Figure 20A), with quantification of total brain glycogen confirming these findings (Figure 20B). PBs in Gbe1<sup>ys/ys</sup> mice from this line and the Gbe1<sup>ys</sup> Gys1<sup>-</sup> line frequently appeared to have been engulfed by what are presumed to be microglia. PBs in Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>-/-</sup> mice appeared more homogenous in texture and circular shape, which comparison to 3- and 6-month-old Gbe1<sup>ys/ys</sup> brain histology indicated was not simply a consequence of reduced amounts of PBs (Figure 21). Similar to findings in the Gbe1<sup>ys</sup> Gys1<sup>-</sup> mouse line, astrogliosis and microgliosis were increased in Gbe1<sup>ys/ys</sup> mice and reduced in Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/-</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>-/-</sup> mice (Figure 18F-O).
Figure 18. Mono- and bi-allelic Ppp1r3c knockout improves the neurohistological disease phenotype of Gbe1<sup>ys/ys</sup> mice.

Hippocampal PASD staining (A-E), and anti-GFAP (F-J) and anti-AIF1 (K-Q) IHC of 13.5-month-old wild-type (A, F, K), Gbe1<sup>ys/ys</sup> (B, G, L), Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/−</sup> (C, H, M), Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> (D, I, N) and Ppp1r3c<sup>−/−</sup> (E, J, O) mice. Scale bars represent 50 µm.
Figure 19. Mono- and bi-allelic Ppp1r3c knockout reduces PB accumulation in the cerebellum and motor cortex.

PASD staining of cerebellum (A-E) and motor cortex (F-J) in 13.5-month-old wild-type (A, F), Gbe1<sup>ys/ys</sup> (B, G), Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/−</sup> (C, H), Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> (D, I) and Ppp1r3c<sup>−/−</sup> (E, J) mice. Scale bars represent 50 µm (A-E) and 100 µm (F-J).

PASD staining of skeletal muscle found mono-allelic Ppp1r3c knockout to have no observable effect on PB accumulation, while bi-allelic Ppp1r3c knockout did reduce skeletal muscle PBs (Figure 22A-E). These observations were confirmed by analogous results in skeletal muscle glycogen quantification (Figure 20C). No effect of PPP1R3C deficiency was detected on cardiac muscle PB accumulation.

PASD staining of liver tissue (Figure 22K-O) and PB quantification (Figure 20D) demonstrated that PPP1R3C deficiency also did not affect PB accumulation in the liver. This finding was biochemically confirmed via quantification of degradation-resistant glycogen in the livers of fed mice from the Gbe1<sup>ys</sup> Ppp1r3c<sup>−</sup> mouse line (Figure 20F). These measurements showed increased and similar levels of degradation-resistant glycogen in Gbe1<sup>ys/ys</sup>, Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/−</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mice compared to controls. Quantification of total liver glycogen, however, depicted a different pattern with significant reduction in Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>+/−</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mouse livers compared to wild-type mice (Figure 20E). PASD staining of liver tissue also showed portal-associated PB localization and lymphocytic infiltrate.
Figure 20. Mono- and bi-allelic Ppp1r3c knockout reduces PBs and disease-associated glycogen accumulation in brain, with restricted effects on skeletal muscle and liver glycogen levels.

Quantification of hippocampal area covered by PBs (A), and of total brain (B) and muscle (C) glycogen. Quantification of liver area covered by PBs (D), and of total (E) and degradation-resistant (F) liver glycogen in fed mice. Mice are age-matched cohorts of 12-14.5-month-old mice (n = 4-6). Data presented as mean ± SEM. One-way ANOVA: F (4, 23) = 111.7, P ≤ 0.0001 (A); F (4, 20) = 111.6, P ≤ 0.0001 (B); F (4, 22) = 80.67, P ≤ 0.0001 (C); F (2, 12) = 0.48, P > 0.05 (D); F (4, 23) = 13.61, P ≤ 0.0001 (E); F (4, 23) = 29.82, P ≤ 0.0001 (F). Tukey-Kramer multiple comparisons test: a vs. b, c, d P ≤ 0.0001, b vs. d P ≤ 0.0001, c vs. b, d P ≤ 0.01 (A); a vs. b vs. c P ≤ 0.0001 (B); a1, a2 vs. b P ≤ 0.0001, a1 vs. c P ≤ 0.001, a2 vs. c P ≤ 0.0001 (C); a vs. ba, bc P ≤ 0.01, a vs. c P ≤ 0.001, ab1 vs. c, c P ≤ 0.01, ab2 vs. c P ≤ 0.001 (E); a vs. b P ≤ 0.0001 (F). WT = wild-type.
Figure 21. Time-dependent accumulation of PBs in Gbe1^{ys/ys} mouse brain. PASD staining of 3- (A-B) and 6-month-old (C-D) wild-type (A, C) and Gbe1^{ys/ys} (B, D) mouse hippocampus. Mouse background was C57BL/6NTac;129/Ola. Scale bars represent 50 µm.

Figure 22. Ppp1r3c knockout reduces PB accumulation in murine Gbe1^{ys/ys} skeletal muscle but not heart or liver. PASD staining of skeletal muscle (A-E), heart (F-J) and liver (K-O) from 13.5-month-old wild-type (A, F, K), Gbe1^{ys/ys} (B, G, L), Gbe1^{ys/ys} Ppp1r3c^{+/+} (C, H, M), Gbe1^{ys/ys} Ppp1r3c^{−/−} (D, I, N) and Ppp1r3c^{−/−} (E, J, O) mice. Scale bars represent 200 µm (A-E, K-O) and 100 µm (F-J).
Similar to older Gbe1<sup>ys/ys</sup> Gys<sup>1−/−</sup> mice, PASD staining of 22-24-month-old brain, skeletal muscle, heart and liver from Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mice indicated that PBs had continued to accumulate since the 13.5-month time point (Figure 23). Brain PBs maintained their above-noted unique characteristics including i) homogenous texture and circular shape, ii) lack of an association with engulfing cells (presumed to be microglia) and, iii) lack of profusion of small PBs in the stratum lacunosum moleculare (SLM) of the hippocampus. These older Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mice also consistently presented with liver tumours; a phenotype not observed in older control mice.

![Figure 23. Ppp1r3c knockout slows rate of PB accumulation in Gbe1<sup>ys/ys</sup> mice.](image)
PASD staining of hippocampus (A), skeletal muscle (B), heart (C) and liver (D) of 22-24-month-old Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mice shows progressive PB accumulation. Scale bars represent 50 μm (A), 200 μm (B, D) and 100 μm (C).
Chapter 5: Discussion

The primary aim of this thesis was to evaluate the efficacy of GYS1 and PPP1R3C as therapeutic targets for the adult-onset GSD IV variant, APBD. Characterization of the effect of mono-allelic knockout of Gys1 and Ppp1r3c and bi-allelic knockout of the latter in the Gbe1<sup>ys/ys</sup> APBD mouse model demonstrated clear behavioural rescue and improvements in the neurological disease phenotype as per histological analyses and glycogen quantification. Further evaluation of skeletal muscle, heart and liver found mono-allelic Gys1 knockout to reduce PB accumulation in all three tissues and bi-allelic Ppp1r3c knockout to reduce PB accumulation in skeletal muscle. These results validate the development of GYS1- and PPP1R3C-targeted therapeutics for APBD and contribute novel insight into the in vivo consequences of GBE1, GYS1 and PPP1R3C deficiencies.

5.1 Effect of GYS1 and PPP1R3C deficiency on life span and morphological and behavioural phenotypes

Deficiency of GYS1 and PPP1R3C in the Gbe1<sup>ys/ys</sup> mouse model was found to significantly improve life span and disease-associated morphological and behavioural phenotypes. The Gbe1<sup>ys/ys</sup> mouse model contains the most common APBD mutation (p.Y329S) and existing data indicate the model is a faithful recapitulation of the human disease, however, acknowledgment of the inherent limitations of a disease model and consequent caution in phenotype interpretation is required. In their respective lines, mono-allelic Gys1 knockout rescued Gbe1<sup>ys/ys</sup> mouse life span to that of control mice and bi-allelic Ppp1r3c knockout almost doubled Gbe1<sup>ys/ys</sup> mouse life span. While the cause of death in Gbe1<sup>ys/ys</sup> mice is unknown, doubling of the Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>-/-</sup> mouse life span in the absence of cardiac or hepatic PB reduction suggests the cause of death is likely due to complications of neurological and/or muscular origin. Rescued Gbe1<sup>ys/ys</sup> Gys1<sup>-/-</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>-/-</sup> mice that lived beyond 20 months of age developed a similar disease phenotype as compared to younger Gbe1<sup>ys/ys</sup> mice, suggesting the disease phenotype and cause of death in these mice was similar to that of younger Gbe1<sup>ys/ys</sup> mice, with the potential contribution of liver tumours.
A second characteristic of the \(Gbe1^{ys/ys}\) mouse phenotype was the loss of body mass with disease progression. Male \(Gbe1^{ys/ys}\) mice lost more body mass than females and accordingly, rescue due to GYS1 or PPP1R3C deficiency was more apparent in males than females. Body composition measurements in the \(Gbe1^{ys}\ Ppp1r3c^{-}\) mouse line indicated that male mice had more lean mass than females and loss of this additional lean mass accounted for the greater male \(Gbe1^{ys/ys}\) mass loss. In both lines, male and female \(Gbe1^{ys/ys}\) mice reached the same lower mass thresholds cumulatively suggesting mass loss in males may have been due to loss of non-essential skeletal muscle mass, potentially due to muscle atrophy as has been reported in several GSD IV variants including APBD (Suzuki et al., 1971). Body composition measurement also found a trend towards reduced body fat in mice containing the \(Gbe1^{ys/ys}\) mutation. To speculate, reduced body fat could be due to increased fatty acid oxidation given impaired glycolysis of the abnormally structured, precipitated PBs, which are presumed to be metabolically inert.

Overt characteristics of the \(Gbe1^{ys/ys}\) murine disease phenotype improved in GYS1- and PPP1R3C-deficient mice included kyphosis, reduced spontaneous activity and abnormal gait. While reduced spontaneous total or locomotor activity are gross phenotypes, inability to cross a balance beam and position at the back of the treadmill in \(Gbe1^{ys/ys}\) mice from the \(Gbe1^{ys}\ Gys1^{-}\) and \(Gbe1^{ys}\ Ppp1r3c^{-}\) mouse lines, respectively, indicated balance and locomotor impairment. Further analysis of gait found reduced homolateral feet distance in \(Gbe1^{ys/ys}\) mice and reduced maximal distance from the transverse plane of hind but not forefeet. Reduced homolateral feet distance may be a function of the reduced body length of \(Gbe1^{ys/ys}\) mice, however, greater impairment of hindfeet distance from the transverse plane compared to forefeet is indicative of hindlimb dysfunction. Reduced hindfoot step length was also observed in \(Gbe1^{ys/ys}\) mice from the \(Gbe1^{ys}\ Gys1^{-}\) mouse line, and corresponds with qualitative observations of mice and previous reports of hindlimb stiffness and spasticity (Akman et al., 2015b). Lack of impairment in \(Gbe1^{ys/ys}\) mouse performance on the rotarod and forelimb grip strength test further supported the hypothesis of a hindlimb specific disease phenotype, which would be suggestive of an underlying axonal neuropathy rather than exclusively a myopathy. Lower limb paraplegia and impaired balance are common symptoms of
APBD patients and rescue of a similar phenotype with GYS1 and PPP1R3C deficiency in the APBD mouse model supports the clinical relevance of the murine results. Cognitive behavioural testing was not pursued as cognitive decline is not a consistent symptom in APBD patients and most cognitive behavioural tests in mice (e.g., novel object recognition) rely on mouse activity and motor function, both of which are impaired in Gbe1ys/ys mice.

5.2 Effect of GYS1 and PPP1R3C deficiency on brain histology and glycogen levels

Comparison of the effect of GYS1 and PPP1R3C deficiency on PB and glycogen reduction in the brain shows GYS1 is a more sensitive therapeutic target. While direct comparison of mice from the Gbe1ys Gys1- and Gbe1ys Ppp1r3c- mouse lines is not appropriate given their different genetic backgrounds and environments, the extent of PB and glycogen reduction in the brains of rescued mice compared to disease model mice within each line was similar between Gbe1ys/ys mice with only mono-allelic Gys1 knockout compared to bi-allelic Ppp1r3c knockout.

For further interpretation of the brain histology and glycogen quantification results it is important to note the gross nature of these findings. PASD staining of brain tissue is an effective method to visualize PBs and was sufficient to clearly determine the therapeutic efficacy of GYS1 and PPP1R3C deficiency in reducing PBs. PASD staining does not, however, inform the detection of differences in PB cell-type specificity, nor does it enable specific interpretation of PB intracellular localization. While PBs have been shown to occur in both neurons and astrocytes in APBD patients (Robitaille et al., 1980; Suzuki et al., 1971) and Gys1 and Ppp1r3c are expressed in both of these cell types (Allaman et al., 2000; Inoue et al., 1988), it is possible GYS1 and PPP1R3C deficiency driven PB reduction was not homogenous across cell types and intracellular pools of glycogen. Furthermore, brain glycogen measurements depict the sum of non-PB and PB glycogen. Because the brain has comparatively little glycogen, total glycogen measurements are thought to be largely indicative of the accumulated PBs as can be seen in the multi-fold increase in Gbe1ys/ys brain glycogen compared to wild-type brain.
glycogen. This of course relies on the assumption that $Gbe^{ys/ys}$ does not result in significantly increased amounts of non-PB glycogen. The reduction of brain glycogen levels in $Gbe^{ys/ys}$ $Gys1^{+/−}$ and $Gbe^{ys/ys}$ $Ppp1r3c^{−/−}$ mice to levels similar to wild-type mice, despite the presence of some PBs, is likely reflective of a significant reduction in PB glycogen and partial reduction in non-PB glycogen.

$Gbe^{ys/ys}$ mice were found to have increased astro- and micro-gliosis. Astroglial gliosis has been reported in APBD CNS tissue (Peress et al., 1979) and macrophage infiltration in liver and skeletal muscle tissue has been reported in the $Gbe^{ys/ys}$ mouse (Yi et al., 2016). PBs localized within macrophages in APBD CNS tissue have also been reported (Peress et al., 1979), however, their presence as a consequence of endogenous microglia metabolism or of PB engulfment in addition to the potential protective and/or deleterious role of microglia in APBD pathogenesis remains unexplored. Reduction of astro- and micro-gliosis in GYS1- and PPP1R3C-deficient $Gbe^{ys/ys}$ brain tissue is likely a consequence of reduced PB accumulation, however, a secondary positive effect of GYS1 or PPP1R3C deficiency on gliosis cannot be ruled out.

### 5.3 Effect of GYS1 and PPP1R3C deficiency on skeletal and cardiac muscle PBs and glycogen levels

While the primary aim of this work was to evaluate the effect of GYS1 and PPP1R3C deficiency on the $Gbe^{ys/ys}$ mouse neurological phenotype, analysis of skeletal muscle, heart and liver, which are important glycogen storage sites and are affected in GSD IV clinical variants, enables: i) comprehensive understanding of the APBD mouse model results particularly in regard to the interpretation of longevity, morphology and behaviour, ii) evaluation of the systemic effects of GYS1- and PPP1R3C-knockdown in anticipation of the development of potentially non-CNS-restricted therapeutics, iii) evaluation of the efficacy of GYS1 and PPP1R3C as potential therapeutic targets for GSD IV variants presenting with myopathy, cardiomyopathy or liver disease, and iv) acquisition of novel insight into the tissue-specific roles of GBE1, GYS1 and PPP1R3C in glycogen metabolism. Of note, $Gys1^{+/−}$ mice have been previously confirmed to have approximately 50% GYS1 protein in skeletal and cardiac muscle compared to wild-type...
controls (Pederson et al., 2004) and analysis of PPP1R3C protein is obstructed by the absence of a working antibody.

The PBs in \textit{Gbe1}^{ys/ys} skeletal muscle varied across muscle fibers and the tissue section. Muscle fibers appeared i) completely filled with PBs, ii) containing sporadic PBs, or iii) completely void of PBs suggesting that this variation might be dependent on fiber type. Investigation of an association between PBs and muscle fiber types in APBD tissue has not been conducted, with mixed reports of a PB-fiber type association in cases of childhood GSD IV (Reusche et al., 1992; Schröder et al., 1993a; Servidei et al., 1987). As a comparator, LBs in Lafora disease skeletal muscle localize to type IIb (fast-twitch, glycolytic) fibers (Turnbull et al., 2011b). PB variation across muscle tissue sections may be due to variation between different muscle groups, potentially due to their different fiber type compositions. Analysis of protein abundance in rat muscle fibers from the predominantly fast-twitch extensor digitorum longus compared to the predominantly slow-twitch soleus identified a four-fold increase in GBE1 protein in the slow-twitch soleus fibers (Murphy et al., 2012). Determination of a PB-fiber type association may lend insight into the molecular and metabolic mechanisms that lead to PB accumulation.

Compared to their respective \textit{Gbe1}^{ys/ys} littermates, GYS1 deficiency resulted in a greater reduction in PB and disease-associated glycogen accumulation in skeletal muscle compared to PPP1R3C deficiency. No effect of mono-allelic \textit{Ppp1r3c} knockout was observed, suggesting PPP1R3C may be haplosufficient in skeletal muscle. This reduced effect of PPP1R3C knockdown in skeletal muscle is not entirely unexpected given the expression of several other glycogen-targeting PP1 regulatory subunits in skeletal muscle. PPP1R3A is a dominant regulatory subunit in skeletal muscle with knockout in mice resulting in a 90% reduction in skeletal muscle glycogen (Suzuki et al., 2001), and human and mouse model carriers of a stop mutation having an approximately 50% reduction in skeletal muscle glycogen (Savage et al., 2008). PPP1R3D has also been shown to be expressed in human skeletal muscle (Armstrong et al., 1997), while PPP1R3B, PPP1R3E and PPP1R3F have high skeletal muscle expression in humans but low expression in rodents (Kelsall et al., 2011; Munro et al.,
No such differences in expression patterns between rodents and humans have been reported for PPP1R3C.

PASD staining of heart tissue demonstrated mono-allelic Gys1 knockout to be an extremely efficacious target in regard to PB reduction. Almost no PBs were detectable in Gbe1ys/ys Gys1+/− young or old heart tissue. In contrast, PPP1R3C deficiency had no detectable effect on cardiac PBs, however, inter-mouse variability in cardiac PBs could have obscured detection of a subtle effect. Crosson et al. previously found a significant reduction in heart glycogen of Ppp1r3c+/− mice, however, Ppp1r3c−/− mice in this line displayed an embryonic lethal phenotype that was lost with backcrossing and these results should therefore be relied upon with caution (Crosson et al., 2003; Lu et al., 2014). Further research is needed to better understand the roles of glycogen-targeting PP1 regulatory subunits in heart glycogen metabolism.

5.4 Effect of GYS1 and PPP1R3C deficiency on liver PBs and glycogen levels

Analysis of liver PBs and glycogen measurements in Gbe1ys/ys mice and in Gbe1ys/ys mice deficient in GYS1 and PPP1R3C yielded several unexpected results. As has been previously shown, Gbe1ys/ys mice accumulate PBs throughout their liver with age (Akman et al., 2015b). Despite accumulation of liver PBs, to varying extents, across GSD IV human variants and naturally-occurring animal models, quantification of glycogen content is typically within the normal range of controls and is often towards the lower end of this range (though liver glycogen content has not been reported in APBD patients; Bannayan et al., 1976; Das et al., 2005; Fyfe et al., 1992; Greene et al., 1988; Schröder et al., 1993b; Servidei et al., 1987; Tang et al., 1994; Valberg et al., 2001). Along these lines, total glycogen content in mice from both the Gbe1ys Gys1+ and Gbe1ys Ppp1r3c− mouse lines in this study was found to be similar between wild-type and Gbe1ys/ys mice. In contrast, measurement of liver glycogen content in the juvenile GSD IV mouse model homozygous for the Gbe1lm1HoA allele (described in Section 1.2.3.6.2) and also in the Gbe1ys/ys APBD mouse model was previously reported to be significantly
higher than in wild-type controls (in both fed and fasted states; Akman et al., 2011, 2015b; Yi et al., 2016).

As previously discussed, total glycogen measurements include quantification of PB and non-PB glycogen. In light of the counter-intuitive findings of similar glycogen levels between wild-type and Gbe1\(^{ys/ys}\) mice despite the mass accumulation of liver PBs, degradation-resistant glycogen was quantified as an effort to approximate the quantity of PBs in the liver. Measurement of degradation-resistant glycogen depicted near complete degradation of glycogen in wild-type liver compared to retained significantly higher amounts of glycogen in Gbe1\(^{ys/ys}\) mice. Theoretically, ex vivo degradation of non-resistant glycogen should have an effect analogous to fasting mice prior to tissue harvest.

These results are suggestive of two novel findings. Firstly, while liver contains much higher levels of glycogen than brain or muscle, if the ratio between glycogen synthesis and PB accumulation (PB formation minus any potential PB clearance) was similar in liver compared to brain and muscle, multi-fold higher total liver glycogen would be expected in Gbe1\(^{ys/ys}\) mice compared to wild-type mice. Given that total liver glycogen was similar between Gbe1\(^{ys/ys}\) and wild-type mice, this suggests that there is less PB accumulation compared to synthesis in the liver (either due to reduced formation or increased clearance). It is, however, also possible that this finding is a technical artifact that could be explained if, e.g., the rate of post-mortem glycogen degradation is higher in brain and muscle compared to liver. Secondly, assuming the degradation-resistant glycogen is analogous to the metabolically inert PBs, subtraction of the amounts of degradation-resistant glycogen from the total glycogen amounts suggests that there is reduced non-PB (i.e., metabolically active) glycogen in Gbe1\(^{ys/ys}\) liver. This could also be the case in other tissues such as brain and muscle tissue.

In regard to the effect of GYS1 deficiency on liver glycogen, no effect of mono-allelic Gys1 knockout was observed on total glycogen measurements. While an increase in total liver glycogen in GYS1-deficient mice could have been expected as a compensatory site for glucose disposal, previous studies have shown this not to be the case with Gys1 knockout mice maintaining normal liver glycogen levels and actually
having improved glucose tolerance (Pederson et al., 2005a, 2005b); results that have since been challenged in a Cre recombinase-mediated muscle-specific Gys1 knockout mouse model (Xirouchaki et al., 2016).

The subsequent findings of reduced liver PBs in Gbe1\(^{+/ys}\) Gys1\(^{+/−}\) mice based on histological quantification and biochemical quantification of degradation-resistant glycogen were unexpected given the reported lack of GYS1 in murine liver. Kaslow et al. first reported an absence of GYS1 in rat liver, which was confirmed in mouse tissue by Irimia et al. (data not shown) and by Cifuentes et al., however, the GYS1 blot in the latter showed faint bands despite the reported absence (Cifuentes et al., 2008; Irimia et al., 2010; Kaslow and Lesikar, 1984). Mass spectrometry of proteins associated with liver glycogen identified GYS1 in mice but not rats, with the authors citing potential contamination of the preparation with other cell types or low levels of hepatic Gys1 expression (Stapleton et al., 2010). Knockout of Gys2 in mice resulted in a 95% reduction in liver glycogen, with all evidence pointing to incomplete Cre recombinase-mediated gene knockout as the cause for the residual 5% of glycogen (rather than Gys1 expression; Irimia et al., 2010). In humans, Gys1 complementary DNA has been detected in liver tissue (Kollberg et al., 2007), however, its potential contribution to hepatic glycogen metabolism and to the retained levels of hepatic glycogen in GSD 0A (GYS2-deficient) patients has not, to the best of my knowledge, been investigated (as discussed in Section 1.2.2.2). Cumulatively, these findings highlight a need to confirm the reported absence of GYS1 in murine liver as well as to characterize the role of GYS1 in human liver. No major perturbations have been reported in the systemic metabolism of Gys1 knockout mice that could directly explain the reduction in Gbe1\(^{+/ys}\) Gys1\(^{+/−}\) liver PBs.

In contrast to GYS1 deficiency, Ppp1r3c knockout resulted in a trend towards lower total glycogen in liver. Both GBE1 impairment and PPP1R3C deficiency in mice appeared to have an additive effect resulting in significant liver glycogen reduction. Even though Ppp1r3c is one of several glycogen-targeting PP1 regulatory subunits expressed in liver, its knockout has been previously shown to reduce total liver glycogen in mice (Lu et al., 2014). Furthermore, PPP1R3C’s contribution to liver glycogen metabolism has been repeatedly demonstrated through the accumulation of glycogen in liver cell lines
and mice overexpressing PPP1R3C (Berman et al., 1998; Cifuentes et al., 2008; Gasa et al., 2000; Lu et al., 2014; O’Doherty et al., 2000; Yang et al., 2015). Unexpectedly, however, this reduction in total glycogen did not have an analogous effect on PB accumulation. Overall, these liver results present a unique scenario in which the total glycogen amounts do not correspond to PB amount.

Further work will be required to validate that these data are indicative of a ‘true’ biological phenomenon and to as well investigate whether the GBE1, GYS1 and PPP1R3C deficiency-induced changes in liver glycogen and PBs were due to perturbation of liver glycogen metabolism and/or systemic metabolism. Lastly, these results highlight the importance of continued acknowledgment that total glycogen measurements in GSDs with PB accumulation include multiple pools of glycogen (e.g., degradable and degradation-resistant).

The observed liver tumours in old Gbe1<sup>ys/ys</sup> Gys1<sup>+/−</sup> and Gbe1<sup>ys/ys</sup> Ppp1r3c<sup>−/−</sup> mice may be a time-dependent complication of GSD IV. A hepatic adenoma has been reported in the case of an 11-month-old infant with the classical progressive hepatic variant of GSD IV (Alshak et al., 1994) and hepatocellular carcinoma has been reported in a 13-year-old child with intermittent periods of hepatic GSD IV progression (de Moor et al., 2000). Liver tumours in mice were rarely observed at the 10- and 13.5-month time points and did not associate with specific genotypes. The literature currently does not support a role of GYS1 or PPP1R3C knockdown in tumour development and rather supports the development of GYS1- and PPP1R3C-targeted cancer therapeutics (Ardourel et al., 2007; Bhanot et al., 2015; Yang et al., 2015).

5.5 Comparative efficacy of therapeutic targets

In the context of APBD, these data support the conclusion that GYS1 is a more sensitive and therefore effective therapeutic target than PPP1R3C. Mono-allelic Gys1 knockout was more effective than mono-allelic Ppp1r3c knockout in reducing PBs in the brain and improving murine behavioural deficits likely of neurological origin. PPP1R3C could, however, prove favorable if aiming to limit the pharmacological effect of a
therapeutic to the nervous system using a route of administration resulting in systemic bioavailability. Of note, the current understanding of APBD pathology positions PB accumulation in the nervous system as the primary driver of disease symptomatology and progression. If novel drivers of disease pathogenesis are identified, e.g., microglia activation, re-evaluation of the comparative efficacy of GYS1 and PPP1R3C as therapeutic targets will be required.

While this study was conducted in a mouse model of APBD, the similar genetic cause of all GSD IV variants and consequent likelihood of similar mechanisms of PB formation support the translation of these results to other GSD IV variants in regard to PB reduction. These results suggest that GYS1 would be an effective therapeutic target, and preferable to PPP1R3C, in GSD IV cases presenting with myopathy and/or cardiomyopathy (most common in the childhood variant). These results also support targeting GYS1 in hepatic GSD IV variants, however, further research would be needed to validate these results and gain some insight into the mechanism of action responsible for hepatic PB reduction. Clinical intervention in the fatal perinatal and congenital variants of GSD IV will remain challenging given their in utero onset, however, it is possible GYS1- or PPP1R3C-targeting ideally in combination with amelioration of the GBE1 deficiency may be therapeutic particularly if the disease course can be reversed.

As discussed in Section 1.4.2, the interrogation of GYS1 and PPP1R3C as therapeutic targets for APBD was in large part based on their efficacy in preventing LB formation in the Lafora disease mouse models (Duran et al., 2014; Pederson et al., 2013; Turnbull et al., 2011a, 2014; Figures 2-3). The effectiveness of these therapeutic targets in reducing LBs in Lafora disease and PBs in APBD supports the broader utility of GYS1- and PPP1R3C-targeting in reducing PBs regardless of the underlying impairment. One such disease candidate could be the recently identified myopathy and cardiomyopathy due to RANBP2-type and C3HC4-type zinc finger containing 1 (RBCK1)-deficiency (OMIM 615895), in which CNS PB formation has also been identified in the mouse model (Figure 24) and uninvestigated in patients (Fanin et al., 2015; Nilsson et al., 2012).
Figure 24. PB accumulation in Rbck1<sup>-/-</sup> mouse brain.
PASD staining of hippocampus (A-B) and cerebellum (C-D) of 6-month-old wild-type (A, C) and Rbck1<sup>-/-</sup> (B, D) mice. Scale bars represent 50 µm.

5.6 Anticipated safety profile of GYS1 or PPP1R3C knockdown in humans

A primary question in the consideration of GYS1 and PPP1R3C as therapeutic targets is whether knockdown in humans will have any unanticipated side effects, particularly given the critical nature of GYS1 in glycogen synthesis. As seen in GSD 0B patients (GY5S1 deficiency, discussed in Section 1.2.2.1), the primary consequence of GYS1 knockout is development of cardiomyopathy, which has had severe consequences when undiagnosed but should be manageable with cardio-protective medication once diagnosed (Cameron et al., 2009; Kollberg et al., 2007; Sukigara et al., 2012). Muscle weakness and exercise intolerance has also been reported in several GSD 0B patients and it is possible that dietary management including frequent meals could help mitigate reduced glycogen storage in skeletal muscle and other non-hepatic tissues. Knockout of GYS1 or impaired utilization of glycogen-derived glucose and lactate has been shown to impair memory formation and learning in mice, chickens and rats (Duran et al., 2013; Gibbs et al., 2006; Newman et al., 2011; Suzuki et al., 2011), however, no cognitive symptoms have been reported in GSD 0B patients.

Critically, however, 100% GYS1 knockout would not be a therapeutic aim nor would it be possible with current pharmacological methods. Given the significant effect of 50% GYS1 knockdown on PB accumulation in the APBD mouse model and the onset of
APBD symptoms in midlife, even partial GYS1 knockdown is likely to delay symptom onset and slow disease progression. In this regard, the parents of GSD 0B patients whom carry one null GYS1 allele are completely asymptomatic. Furthermore, cardiac and skeletal consequences of GYS1 knockout would not be a concern with nervous system-restricted GYS1 knockdown.

Homozygous PPP1R3C mutations have not been associated with any human disease and heterozygous PPP1R3C mutations in two Lafora disease patients have been associated with a milder disease course (Guerrero et al., 2011). To summarize, without minimizing the risk inherent to inhibition of any novel therapeutic target in humans, current evidence does not predict deleterious side effects of pharmacological knockdown of GYS1 or PPP1R3C.
Chapter 6: Future Directions

6.1 Development of GYS1- and PPP1R3C-targeted therapeutics

The most immediate next step dictated by this work is the development of GYS1- and PPP1R3C-targeted therapeutics for APBD patients. As per the discussion in Section 1.4.3, knockdown of GYS1 or PPP1R3C could target their respective DNA sequences or aim to reduce mRNA or protein levels. In regard to DNA targeting, despite the currently poor feasibility of nuclease-mediated directed sequence alteration in post-mitotic cells, gene knockout via non-homologous end joining-induced indel and PTC formation is possible. While nuclease-mediated gene therapy is likely to continue to witness rapid advancements over the years to come, some of the current challenges in clinical translation, and particularly in the treatment of neurological diseases, include surpassing efficacy thresholds to induce a therapeutic effect and minimizing the risk of deleterious consequences, as reviewed by Cox et al. (Cox et al., 2015). To induce a therapeutic effect, a sufficient percentage of target cells and number of alleles per cells will have to have not only undergone editing but also to have led to the desired consequence (e.g., PTC formation). Known potential deleterious consequences include immunogenicity of the vehicle or transgenic nuclease and off-target editing. Advancements in non-viral methods to deliver gene editing nucleases offer one approach to broadening the pool of candidates for treatment (e.g., not limited to adults without neutralizing antibodies), enabling repeat administration and reducing the likelihood of deleterious off-target editing. Reducing levels of mRNA using ASOs or protein using small molecules are alternate clinically viable approaches to GYS1 or PPP1R3C knockdown. Screening of small molecule GYS1 inhibitors would likely aim to reduce total GYS1 activity and the small molecules could therefore act through a range of mechanisms, such as stabilizing less active conformations, blocking G6P-mediated allosteric activation or inhibiting the catalytic site. Small molecule impairment of PPP1R3C could be mediated by blocking binding sites for PP1 catalytic subunits or glycogen and glycogen metabolizing enzymes. Blocking PPP1R3C’s binding site on the PP1 catalytic subunits would not be recommended given evidence of shared regulatory
subunit binding sites and the role of PP1 catalytic subunits in a multitude of pathways in the cell (Heroes et al., 2013).

6.2 Cellular and subcellular differences in PB formation

An important area of APBD pathogenesis that was not explored in this thesis is the characterization of PBs at a cellular and subcellular level. Electron microscopic analysis of post-mortem brain tissue from APBD and Lafora disease patients has identified different cell-type specificities and intracellular localization patterns of PBs compared to LBs (Robitaille et al., 1980; Suzuki et al., 1971). These features are thought to be primary determinants of the clinical manifestation, however, the molecular mechanisms behind different localizations remains unknown.

Review of PB and LB cell-type specificities in the context of normal brain glycogen metabolism does not provide clarity. Astrocytes have long been viewed as the main glycogen storage sites in the brain and until recently, neurons were thought to lack active glycogen metabolisms (Saez et al., 2014), deriving much of their energy from the proposed astrocyte-neuron lactate shuttle. In light of these hypotheses, the finding of PBs in neuronal and astrocytic processes of APBD patients is somewhat unexpected given the absence of predominantly astrocytic PBs, but it is not particularly remarkable. The finding of LBs in neuronal perikarya and dendrites is unexpected in their neuronal cell-type specificity and the different intracellular location compared to PBs. Hypotheses to rationalize the neuronal cell-type specificity of LBs have been proposed including a role of laforin and malin (absent in Lafora disease) in suppressing neuronal glycogen synthesis (Vilchez et al., 2007). However, such hypotheses in the sole context of APBD or LD brain glycogen metabolism often fail to accommodate each other and the intersection of these diseases therefore provides a touchstone upon which to scrutinize novel hypotheses.

An additional layer of complexity, beyond acknowledgement of the crude classification of cells as either neurons or astrocytes, involves the spatial distribution of cells and their proximity to energy sources and sinks. IHC analysis of a microwave-fixed mouse brain
shows there to be glycogen-poor and glycogen-rich astrocytes (Oe et al., 2016). Three-dimensional modeling from serial electron microscopy sections further demonstrated the non-random spatial distribution of glycogen particles, observing an association with excitatory synapses and proximity to blood vessels (which supply glucose; Cali et al., 2016).

The different intracellular localization of PBs and LBs highlights another gap in our understanding of brain glycogen metabolism. It is possible that different intracellular localizations are a function of synthesis from pools of glycogen with different characteristics or a function of post-synthesis transport. There is some evidence from the study of brain and muscle glycogen that different cellular pools of glycogen vary in characteristics such as particle size and rate of turnover (Marchand et al., 2007; Oe et al., 2016). It seems likely that these characteristics could affect the consequences of enzyme deficiencies and the likelihood of glycogen particle precipitation, e.g., is the effect of a GBE1 deficiency correlated with synthesis rate and the number of non-reducing termini to act upon? And does particle size contribute to the likelihood of particle precipitation?

Further research into the inter- and intra-cellular differences of PBs and LBs is evidently critical for improved understandings of these diseases. Challenges in these inquiries include i) the technical limitations of dissecting in vivo brain processes at the cellular and subcellular level, ii) the artifacts introduced by rapid changes that occur in post-mortem brain tissue and, iii) the absence of faithful in vitro brain models. Improvements in technologies, such as imaging, in vitro brain modeling and the accessibility and scale of technologies such as electron microscopy and patch-clamping, will be needed to move beyond incremental and hypothesis-driven steps in understanding brain metabolism and its perturbation in pathogenesis.
6.3 Mechanisms of GYS1 and PPP1R3C deficiency-mediated PB reduction

As discussed in Section 1.4.2, the theoretical rationale behind targeting GYS1- and PPP1R3C- was to reduce total glycogen synthesized and to re-balance the ratio between chain elongation and branching. While this study clearly indicates that both GYS1 and PPP1R3C are effective therapeutic targets to reduce PB accumulation in the APBD mouse model brain, the mechanism responsible for this effect was not interrogated. Measurement of the non-PB glycogen and the glycogen chain length distribution from experimental brain tissues would ascertain if a reduction in total glycogen and/or an increase in the relative branch frequency contributed to the reduction in PBs.

While the above measurements would assess the effect of GYS1- and PPP1R3C-deficiency on the glycogen molecule, the mechanisms responsible for these changes would require further interrogation. A reduction in GYS1 is assumed to have the sole effect of reducing total GYS1 activity, which could rather simply account for changes to total glycogen amounts or branch patterns. A reduction in PPP1R3C is, however, likely to have more nuanced and complex effects. Beyond PPP1R3C’s role in targeting PP1 to glycogen and the consequent effects on the phosphorylation and activity of interacting glycogen-associated enzymes, the consequences of the loss of PPP1R3C’s role as a scaffold protein remain unclear. The likelihood of a more nuanced effect of PPP1R3C-deficiency on PB accumulation is underscored by i) the altered appearance of PBs in Gbe1ys/ys Ppp1r3c−/− brain tissue and ii) the greater efficacy of PPP1R3C as a therapeutic target in Lafora disease.

To speculate, the altered appearance of PBs in Gbe1ys/ys Ppp1r3c−/− brain could be indicative of a variety of phenomena. The darker staining and increased homogeneity in texture and circular shape could indicate a fundamental structural difference or a role for PPP1R3C in targeting PBs for degradation. The altered localization, with a reduction of small PBs in the SLM of the hippocampus could indicate cell-type and/or intracellular specificity in PPP1R3C’s localization. Observations of altered PB appearance in Gbe1ys/ys Ppp1r3c−/− brain is supported by similar findings of reduced small LBs in the
neuropil compared to perikarya in Epm2a<sup>−/−</sup> Ppp1r3c<sup>−/−</sup> brain (Turnbull et al., 2011b) and a seemingly opposite pattern of PB accumulation in the SLM of mice overexpressing PPP1R3C (Duran et al., 2014).

Comparison of the effect of Ppp1r3c knockout on PBs in Gbe<sub>1<sup>−/−</sup></sub> Ppp1r3c<sup>−/−</sup> brain and on LBs in Epm2a<sup>−/−</sup> Ppp1r3c<sup>−/−</sup> and Nhlrc1<sup>−/−</sup> Ppp1r3c<sup>−/−</sup> brain indicates PPP1R3C is a more effective therapeutic target in the Lafora disease mouse models than in the APBD mouse model. Given PPP1R3C’s proven interaction with the Lafora disease gene products, laforin and malin, but not with GBE1, it is possible that knockout of PPP1R3C more specifically addresses the consequences of laforin or malin deficiency. Further research is required to better understand the contribution of PPP1R3C, and other glycogen-targeting PP1 regulatory subunits, to glycogen metabolism in a cell- and tissue-dependent manner.

6.4 Mechanisms of PB degradation

A key question in the study of APBD and the pursuit of therapeutics is whether PBs can be cleared via endogenous or interventional mechanisms. Given that the net rate of PB accumulation is positive in APBD it has been difficult to ascertain whether PB degradation and/or clearance occurs. Two degradation pathways in the brain for which there is some evidence to support involvement in APBD are microglia engulfment and lysosomal degradation.

PBs have been reported in macrophages in the childhood and APBD variants of GSD IV (Peress et al., 1979; Schröder et al., 1993b), however, it is not clear if their presence is due to the endogenous cellular metabolism or due to PB engulfment (which may or may not lead to degradation). The clear activation of microglia in the APBD mouse model identified here in addition to increasing evidence of a deleterious role for microglia (e.g., synapse pruning) in neurodegenerative diseases (Hong et al., 2016) supports the assessment of microglia activation in post-mortem brain tissue from patients with APBD.

The lysosomal pathway for glycogen degradation remains poorly understood, with the potential lysosomal degradation of PBs being no exception. Activation of the autophagy-
lysosomal pathway has been noted in non-CNS tissues from GSD IV patients based on increased acid phosphatase staining and increased numbers of autophagic vacuoles (Nambu et al., 2003; Nolte et al., 2008; Tay et al., 2004). PBs within autophagosomes have also been reported in APBD sural nerve tissue and lymphocytes (Sampaolo et al., 2015). Furthermore, Yi et al. demonstrated a reduction in liver glycogen in fasted \textit{Gbe1}^{ys/ys} mice after administration of recombinant human GAA, however, without further tissue analysis and controls it is unclear if this enzyme replacement therapy had any effect on PB accumulation (Yi et al., 2016). The targeting of glycogen, and specifically of poorly branched glycogen, to the autophagy-lysosomal pathway remains poorly understood, as does its relevance or lack thereof to GSD IV. It is tempting to speculate that targeting of malconstructed glycogen to the autophagy-lysosomal pathway with subsequent retrograde axonal transport could contribute to PB localization and that PB-induced axonal transport impairment could contribute to APBD pathogenesis, however, determination of the involvement of the autophagy-lysosomal pathway in APBD is a basic first step.

Inquiry into the degradation of PBs can be addressed using existing methods in \textit{in vitro} and \textit{in vivo} disease modeling (e.g., further analysis of the GAA-deficient \textit{Epm2a}^{−/−}, \textit{Nhlrc1}^{−/−}, \textit{Stbd1}^{−/−} and \textit{Ppp1r3c}^{−/−} mouse lines generated by Conway) in addition to further IHC and electron microscopic analysis of disease model and patient tissues (Conway, 2015). Novel methods may need to be developed to interrogate lysosomal glycogen and its structure. An improved understanding of mechanisms or lack thereof of PB degradation will lend important insight into disease pathogenesis and alternate therapeutic approaches.

### 6.5 Genotype-phenotype linkage in GSDs

An outstanding question in the clinical diagnosis, prognosis and treatment selection of many GSD patients is the linkage between genotype and phenotype. Several GSDs, and particularly GSD IV, are characterized by a currently inexplicable amount of heterogeneity in the age of onset and tissues affected in spite of their monogenic inheritance. The small patient populations of many GSDs further impede determination
of whether unique symptoms are manifestations of the GSD or of a different pathogenic cause. Continued improvements in global disease awareness and diagnostics are critical to reduce the prolonged diagnostic odyssey commonly experienced by individuals with rare genetic diseases and will also aid in establishing genotype-phenotype linkages for these diseases.

An interesting observation in the literature was the occasional anecdotal reporting of subclinical or mild symptoms in parents of GSD patients. Another retrospective study that sequenced GBE1 in adult muscle biopsies that showed PBs in intramuscular nerve branches (a non-specific finding) found four out of 15 samples to have heterozygous novel missense mutations (Furtado et al., 2016). While determination of the frequency of GBE1 heterozygous missense mutations in a control cohort was not conducted, this fraction seems disproportionately high and lends to speculation that broader sequencing may lead to re-evaluation of genes exclusively associated with autosomal recessive conditions. Continued advancements in the ease and accuracy of in silico, in vitro and in vivo mutation modeling in addition to clinical integration of whole genome sequencing, comprehensive patient data collection (e.g., metabolomics analysis, symptom tracking with wearable technology) and machine learning are likely to contribute to the establishment of genotype-phenotype linkages and the implementation of genomic medicine.
References


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