Muscle Integrity Myopathy in Registered Welsh Section C and D Cobs:
A Report on Preliminary Findings

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Abstract

These are the initial findings of an ongoing study on Muscle Integrity Myopathy (formally known as Polysaccharide Storage Myopathy (PSSM2)) in Welsh Cobs. The new name has been adapted by Generatio GmbH - Center for Animal Genetics, in consultation with other experts, to differentiate between the current published research on biopsy-based diagnostics and new genetic work in the field. It is based on the latest scientific findings and correctly classifies the disease in the area of exertional myopathy. PSSM stands for polysaccharide storage myopathy and is based on the appearance of muscle biopsy results. PSSM1 is diagnosed in horses through a genetic test for the predisposing *GYS1* mutation. PSSM2 is diagnosed in horses that genetically test negative for this mutation but present either a muscle biopsy or clinical symptoms similar to PSSM1. Because of these biopsy findings, it is assumed that these horses have a mutation in a gene that is involved in metabolism of sugar or storage of sugar in the muscle; however, no new mutations have been identified in genes involved in polysaccharide storage or glycogen metabolism. Many horses are diagnosed with PSSM2 based on clinical symptoms rather than biopsy, and in these horses, mutations have only been identified in genes involved with muscle structure and function. These should therefore be classified as Muscle Integrity Myopathies (MIM), a separate subclassification of exertional myopathy. Importantly, no correlation has been found between published biopsy findings and the MIM variants. The name PSSM2 should be reserved for horses whose biopsy findings are similar to those of PSSM1 horses that are negative for the *GYS1* mutation. Muscle Integrity Myopathy is therefore the classification term for a group of related muscle diseases with similar clinical symptoms, unrelated to PSSM1 or PSSM2. When necessary, we shall write MIM ("PSSM2") for clarification because of the historical use of the term "PSSM2", but this is meant to refer to the genetic tests based on the six so-called "P"and "K" variants now classified as MIM. The data in this report consists of test results of 121 registered Welsh Sec C and D cobs and information about pedigrees, symptoms, and diagnostics obtained from owners. The cobs have been tested for the presence of genetic variants associated with MIM either because they were symptomatic, for breeding purposes, or because a relative had a positive result. The association with the genetic variants with MIM has yet to be confirmed in a peer-reviewed publication. As the test is gaining more exposure, breed specific information about the usefulness and interpretation of test results is needed. The goal of this report is to provide breeders and owners of Welsh Cobs with the information necessary to understand the implications of test results and make informed decisions about their breeding programs and management of their cobs should they choose to do so. Only a subset of the cobs (34 from 121) have been tested for PSSM1; these were tested at Animal Genetics or Generatio, and no horses tested positive for the *GYS1* mutation, so no changes to breeding practices are necessary in this regard. This study has found a significant correlation ($p < 0.0001$) between health and MIM test results in Welsh Cobs (n=121). Out of these animals, 39 horses (32.2%) tested normal (n/n for all variants). 82 cobs (67.7%) had at least one variant: 40.5% tested heterozygous (1.7% homozygous) for a single variant, 14.9% compound heterozygous for two variants (2.5% homozygous for one of those two variants). 6.6% were compound heterozygous for 3 variants, and 0.8 % each for 4 and 5 variants. Five of the currently six testable variants have been found in the breed, with P4 being the most common (allele frequency 0.17 in our non-representative sample). No cobs tested positive for the P2 variant. The data does not support the hypothesis that cobs with multiple variants are more likely to be symptomatic than those with a single variant, but the group size for multiple variants is small and therefore inconclusive. These are only preliminary findings, which therefore limits the conclusions.

Introduction

In recent years, Polysaccharide Storage Myopathy (PSSM) has become a controversial topic of discussion within the horse world; most recently within the Welsh Cob community. Much of the controversy revolves around the commercially offered genetic test for PSSM2 (now termed MIM) – one of two types of PSSM – as its validity has not yet been confirmed in a peer-reviewed publication. Although initially there was no indication that PSSM/MIM is a significant problem within the Welsh breeds, compared to others, it is emerging that there are Welsh Cobs with symptoms of myopathy that test positive for the MIM variants. As there is little known about the prevalence of the currently identified testable variants within the breed, or the correlation, if any, between the variants and symptoms, there is a need to monitor the situation and develop further knowledge especially as Cobs can have a high prevalence of in/line breeding. The Welsh Pony and Cob Society only support the peer reviewed PSSM1 data and advise that muscle biopsy is the only recognized tool for diagnosing PSSM2. (https://wpcs.uk.com/wp-content/uploads/2022/08/PSSM-Paper-R-Piercy.pdf).

The Registered Welsh Pony and Cob Research Group is a small research group of knowledgeable individuals, independent of any official breed association. We collect data from interested owners/breeders to assess the frequencies of genetic variants associated with MIM (PSSM2) within the breed. The research group is interested in pedigrees and breeding lines of cobs, correlations between the identified genetic variants, symptoms and the reaction to changes in diet and exercise. Our goal is to offer advice and support while documenting information such as age of onset and response to treatment protocols. The group has contributors from the UK, Australia, New Zealand, America and Europe. The following report is a summary of our initial findings, based on an analysis of the data we have accumulated over the course of the past two years. The initial data consists of test results of 121 purebred registered Welsh Cobs as well as information about pedigree, symptoms, and diagnostics obtained from their owners.

Background

What is PSSM?

As noted above, there are two types of PSSM: PSSM1 and PSSM2. PSSM1 is a glycogen storage disease that results in the accumulation of abnormal complex sugars in muscle cells, and it is associated with a single gene mutation in *GYS1* that predisposes horses to developing clinical symptoms. The PSSM2 designation is now reserved for cases with muscle biopsies similar to PSSM1. Horses with clinical symptoms, but with biopsies that do not appear similar to PSSM1, or without biopsy, are now referred to as having Muscle Integrity Myopathy (MIM).) MIM is also a form of Exertional Myopathy, in which symptoms are caused by changes in the structure and/or function of the muscle. It is caused by hereditary predispositions combined with various environmental factors (age, feeding, husbandry). Typical symptoms can include unexplained lameness, muscle stiffness, difficulties with gait changes/coordination, reluctance to move, muscle atrophy and/or difficulty building muscle and can affect any breed of horse. Six genetic variants that can disrupt muscle structure and/or function have been identified in horses; these can predispose a horse to developing symptoms of exertional myopathy. These are myopathies which involve muscle pain and weakness, but they have different causes and characteristics.

In the past, PSSM2 was essentially an umbrella term for all cases of PSSM which have tested negative for the *GYS1* mutation. This should now be distinguished between PSSM2: horses with biopsies similar to PSSM1 which are

presumed to have glycogen storage diseases; and those with MIM: normal appearing biopsies but similar clinical symptoms.

Several MIM variants are found in genes which in humans are associated myofibrillar myopathies: MFM3 (*MYOT*, P2), MFM5 (*FLNC*, P3), and MFM8, *PYROXD1*, P8). No disease-associated mutation in humans has yet been identified in *MYOZ3* (P4) although it is being researched for multiple orphan muscle diseases. Bethel and Ullrich Myopathies in humans are caused by defects in the COL6 molecules (COL6A3, K1). The final identified variant, Px (*CACNA2D3*) has been associated with recurrent exertional rhabdomyolysis (RER) in some families of horses. Disease and category names are used differently depending on the source and species, and they may change in the future when further research allows for better differentiation. For simplicity's sake we will be using the term MIM throughout this report.

MIM is a category of disorders and can therefore not be attributed to a single gene or cause; furthermore, muscle disorders are very complex with a high number of factors influencing the outcome, and most of those variables are likely still unknown. MIM is believed to be have a strong genetic element – as symptomatic horses of all breeds are found all over and not in geographic clusters. Horses can be kept under identical conditions and some will have symptoms of MIM while others are asymptomatic. Some horses with copies of the variants will not show any symptoms even when well past the usual age of onset; however, if symptoms were primarily caused by environmental factors alone then entire stables would be affected.

The genetic tests offered by Equiseq and Generatio determine the presence or absence of a genetic variant. Apart from rare cases of human error, these tests are completely accurate. The multifactorial nature of the muscle diseases being researched means that the MIM and PSSM1 genetic variants are not fully penetrant; some horses that are heterozygous for the identified variants will not develop exercise intolerance, which means that the tests are not fully predictive. Homozygous horses are much more likely to be affected.

It is recommended that if a positive result is obtained (that the horse has one or more MIM variants), the owner's veterinarian should be consulted for recommendations. If a negative result for a symptomatic cob is obtained, the veterinarian should still be consulted as there are other causes of exertional myopathy besides the known genetic variants and the best possible diagnosis should always be sought.

Classical symptoms of PSSM1 such as raised muscle enzymes (AST, CK) and so-called "tying up" cramping episodes are less often seen in connection with MIM. More common symptoms of MIM disorders are tight muscles, muscle spasms and divots that come and go, lethargy and/or anxiousness and explosiveness, reluctance to move, camping out in a urinating position, stiffness, rope walking (plaiting), difficulty with canter, tripping, dragging hindlegs, inability to work properly/evading contact, depression, pain face (Dyson Ethogram), headshaking, colic symptoms, breathing issues, difficulty standing for the farrier, and reactivity to season/weather. Many of these symptoms overlap with other conditions (e.g. arthritis in the neck or hocks, stifle and SI issues, ulcers, PPID, EMS, ECVM, kissing spines, Lyme disease, neuro-degenerative diseases, electrolyte imbalances), and accordingly such diagnoses should ALWAYS be ruled out or considered alongside MIM as a possible reason for the horse's discomfort.

Diagnosing PSSM/MIM

There is a scientifically verified and trusted genetic test for PSSM1, but the situation is not as clear-cut when it comes to MIM. There are two options: a muscle biopsy or genetic testing. Biopsy testing is the oldest, and originally the only, method. A muscle biopsy is an invasive procedure which can only describe the momentary status of the muscle. Biopsy

timing, location, sample treatment and analysis can affect the results. Studies (see e.g. Valberg et al., 2016) have shown that biopsy results are not a simple black and white kind of situation: depending on which factor you look at they generally have some variation; abnormalities are just seen more often in PSSM-symptomatic horses than in healthy ones. In other words, even though biopsy testing is at present the most widely accepted method for diagnosing MIM/PSSM2, it has some indisputable limitations for both PSSM1 and MIM.

Seeking an alternative to invasive biopsies and knowing there was likely a genetic component to MIM, the American company EquiSeq developed a genetic test which has been commercially available for some years now. It has been patented, and the German laboratory Generatio GmbH procured an exclusive license for commercial use in Europe. Beginning with one genetic variant (mutation) in 2017, the current test panel includes six variants, and that number is likely to increase. EquiSeq has described the nature of the mutations and why they are believed to be harmful (see www.equiseq.com). However, the MIM test is still at a research stage and its validity has yet to be confirmed in the form of peer-reviewed, empirical evidence. The continued lack of such publications from the EquiSeq researchers is a cause of much debate. There are a number of independent, ongoing studies looking into the correlation of the testable variants and symptoms within and across a wide range of breeds. It is hoped to see a K1 publication later this year.

Studies have been published by a "competing" group who provides PSSM2 testing by biopsies (Valberg et. al. 2020, 2021, 2022). These studies discredit the genetic test by proving a lack of correlation with biopsy results. The study design is, however, based on some rather shocking assumptions, which questions the value of peer reviewed data. A key issue with biopsy-based diagnostic studies is that it is difficult to obtain a large control group of samples from healthy horses. Owners (and scientific review boards) are understandably reluctant to have holes cut into their healthy, active performance horses. As such, the majority of control samples are from breeding stock, i.e. horses that might never have been proven sound in work, or they may simply not be symptomatic at the time of sampling. Horses down to two years of age are used as healthy controls, even though the authors acknowledge that PSSM2 is typically adult-onset. Their description of the age of onset is inconsistent: in one article it is 11 years and in another, published a few months later, it is 7 years (Valberg et al., 2020; 2021). Even worse, symptomatic horses biopsied for diagnostic purposes are promptly declared healthy when the results of the biopsy sample are normal. In one of the articles (Valberg et al., 2021), for the warmblood healthy control group, 81% were obtained in this manner with only 20% of those stated as not having clinical signs of muscle disease. This means the studies compare biopsy results to genetic result – the horse's actual health or lack thereof is questionable. When using working performance horses as healthy controls in a former study (Valberg et al., 2016), one of the healthy horses got one of the worst scores on the biopsy.

It is also important to note that a positive genetic test result is not the same as a diagnosis. There are many apparently asymptomatic horses with the genetic variant which causes PSSM1. However, research has shown that this mutation generates changes in the muscle fibres even in the absence of clinical signs (see Zsoldos et al., 2019). Currently unknown factors may reduce the appearance of symptoms in a breed, or the disease has not yet progressed to a level where symptoms are noticeable. Likewise, there are horses positive for multiple MIM (PSSM2) variants successfully competing at a high level (Aretz, 2021). In other words, when relying on the genetic test as a diagnostic tool, it must always be considered in a context of symptoms and other possible diagnoses. Genetic testing, if proven accurate, is to some extent predictive, in the sense that a positive test result indicates that the horse is at risk of developing symptoms at some stage. However, not enough is currently known about why some horses with the gene variant(s) become symptomatic while others remain healthy. Environmental factors may play a significant role along with genetic

disposition – climate, nutrition, injury or disease throughout the horse's life. The ongoing research projects that were referred to earlier are looking into these issues.

Breeding and Management

With regard to the MIM test as a potential tool for making breeding decisions, a common misconception among many supporters of the test is that a stallion or broodmare is automatically unsuitable for breeding if it tests positive for any of the variants. It must be emphasized that this interpretation is not in line with EquiSeq's recommendations; in fact, they acknowledge that there may be very valid reasons for breeding a horse with any of these variants. Because of the polygenic and multifactorial nature of MIM and the many currently unknown factors which may determine whether a horse will ever develop symptoms, culling all positive tested individuals from the breeding stock would neither be a realistic nor a desirable solution. It would be particularly harmful for the genetic diversity of a breed with a closed studbook like the Welsh Cob. The advice from EquiSeq and Generatio is to not breed symptomatic animals and to use the test constructively in order to avoid breeding multiple variant/homozygous positive individuals so that the level of the variants can be reduced over time. Furthermore, research is ongoing, but not yet published in the scientific literature to provide evidence that the specific mutations are in fact harmful.

When it comes to diet and management of PSSM1 and MIM affected horses, each has different recommendations. Horses with PSSM1 generally benefit from a low starch and sugar diet as well as daily exercise. Horses symptomatic for MIM often benefit from a high protein diet and supplementation of the amino acids lysine, methionine and threonine (although this may not hold true for the K1 variant of COLA6A3). Even though many MIM horses present symptoms of exercise intolerance, daily movement and light exercise are recommended. An open shelter with a track system or access to a larger field may be favorable in this respect. These horses can be sensitive to cold and wet weather conditions, and heavier rugging than normal is often required. It must also be emphasized that the above-mentioned recommendations are general – the dietary needs and athletic abilities of PSSM1 and MIM-affected horses vary greatly and individualised adjustment of diet and management is thus crucial.

Genetic Variants

The PSSM1 test identifies a mutation in the *GYS1* gene which causes an excessive activity of the enzyme glycogen synthetase 1 (see e.g., McCue et al., 2008). The MIM panel tests for six gene variants which have been labeled P2, P3, P4, Px, P8 and K1 (see www.equiseq.com and https://shop.generatio.de/en). These are mutations believed to alter the function of the protein product of their genes. With the exception of P4, mutations in the comparable genes have been linked to human myopathies. However, genetic mutations are common and the controversy pertains to whether these particular mutations are causative of MIM. P2, P3 and P4 are in structural proteins in the building blocks of muscle fibres. Px is in a subunit of a calcium channel which functions in signaling. P8 has a function in protection against oxidative stress. K1 is in a collagen in the connective tissue around muscles. For the genomic region containing Px, a peer reviewed article (Fritz et al., 2012) has shown a correlation with RER in one family of thoroughbred horses but not in a second, wider sample. The current theory is that Px interacts with at least one yet unknown gene (Szauter, 2020b). Horses testing positive for Px can be seemingly perfectly healthy. Until more is known, breeding decisions for Px horses should be based on the presence or absence of symptoms, not the genetic status.

MIM Variants ("PSSM2")

Six genetic variants that can affect muscle structure and/or function have been identified in horses with symptoms of exertional myopathy. These so-called "P variants" and the "K1 variant", previously described as subtypes of PSSM2, are now properly classified as muscle integrity myopathies. (https://generatio.de/en/expertise/mim-pssm2-horse/exertional-myopathy/mim-variants-pssm2)

MYOT (P2)

The *MYOT* gene encodes the structural protein myotilin, which plays an important role in the stability of the thin filaments during muscle contraction. Myotilin binds F-actin and crosslinks actin filaments. In humans, mutations in myotilin lead to MFM3 (myofibrillar myopathy 3). The disease is very rare, with onset reported between the ages of 50 and 77 years, and the main symptoms are progressive distal muscle weakness and peripheral neuropathy. In the horse, the MYOT-S232P mutation in the orthologous gene causes an amino acid change from a serine to a proline in a serine-rich region of the protein. Five of the seven pathogenic alleles of *MYOT* in human patients are missense alleles of serine at various positions in this region.

FLNC (P3)

The filamin C gene (*FLNC*, equine mutation P3, FLNC-E73K + FLNC-A1207T) encodes an actin-binding protein that is involved in the connection of the actin filament and the Z-disk. The Z-disk delimits the sarcomeres within a myofibril. The mixed presence of the defective gene leads to a partial loss of function. In humans, mutations in the *FLNC* gene are associated with myofibrillar myopathy 5 (MFM5), an adult-onset disease characterized by progressive skeletal muscle weakness that sometimes also affects the diaphragm and heart. The changes in the biopsy are highly dependent on the muscle being examined and the stage of the disease.

MYOZ3 (P4)

Myozenins are considered intracellular binding proteins for the linkage of other proteins that are active at the Z-disk (a-alpha-actin, g-filamine, TCAP/telethonin, LDB3/ZASP). In addition, myozenins play an important role in the action of calcineurin on the sarcomere. So far, no mutations have been described in humans that would lead to a change in the encoded protein. The mutation in horses, P4, is MYOZ3-S42L. At the 20th Anniversary Symposium for the Genome Sciences Department at the University of Washington in November 2022, Dr. Paul Szauter reported that MYOZ3 is required for muscle development and function in zebrafish. EquiSeq funded work by Dr. Robert Bryson-Richardson of Monash University, who developed antisense RNAs against *MYOZ3a* and *MYOZ3b* in zebrafish (the gene is duplicated in zebrafish). Embryos injected with antisense RNA at a level that showed a complete absence of the *MYOZ3* transcript by PCR fail to complete development, exhibiting muscle contractures. If the dose of antisense RNA is reduced to a level where some individuals are able to complete development, they exhibit exercise-induced defects in muscle structure. Together with the evidence from evolutionary conservation that shows that MYOZ3-S42L is not tolerated, and the evidence from affected horses, this work makes a strong case that the equine MYOZ3-S24L allele is pathogenic.

PYROXD1 (P8)

The *PYROXD1* gene (pyridine nucleotide disulfide oxidoreductase domain 1) encodes a protein important in defence against oxygen radicals (reactive oxygen species, ROS). Such free radicals are generated continuously during normal

cell metabolism and as part of intercellular signaling. In order for these ROS not to damage the DNA, they must be intercepted and neutralized. The protein encoded by the gene *PYROXD1* is such an antioxidant molecule and therefore an essential part of the defence system to reduce oxidative stress.

In humans, mutations in *PYROXD1* lead to myofibrillar myopathy 8 (MFM8), which can occur in both children and adults. It is characterized by slowly progressive proximal muscle wasting and weakness. The horse mutation (chr6:48,924,749 G/C) causes an amino acid substitution at a highly conserved site in the PYROXD1 protein, reducing its functionality.

The *PYROXD1* gene encodes a thiol reductase that can partially substitute for glutathione reductase in yeast. Yeast that have had the glutathione reductase gene knocked out cannot grow in the presence of a concentration of hydrogen peroxide tolerated by normal yeast. If the human *PYROXD1* gene is introduced into yeast cells deficient in glutathione reductase, they are partially rescued and can grow in the presence of hydrogen peroxide. If the human *PYROXD1* gene is altered to carry missense alleles found in human patients, the human gene no longer rescues glutathione reductase-deficient yeast cells grown in the presence of hydrogen peroxide. The equine S42L mutation eliminates the ability of the human *PYROXD1* gene to rescue glutathione reductase-deficient yeast cells, showing that the equine allele affects PYROXD1 protein function in the same way as pathogenic human alleles.

CACNA2D3 (Px)

The gene *CACNA2D3* (Calcium voltage-gated channel auxiliary subunit alpha2 delta 3 (equine mutation Px: CACNA2D3-A525A) encodes a protein that is part of a regulatory subunit of the calcium channel DHPR (dihydropyridine receptor) that controls signaling to trigger muscle contractions. In the context of MIM (PSSM2), the Px variant appears to exacerbate the effects of other variants.

The Px mutation causes no change in the encoded protein; either a direct effect via the modulation of splicing mechanisms is suspected, or the Px mutation could be an indirect marker of a coupled pathogenic mutation. Px is considered a risk factor for recurrent exercise-related rhabdomyolysis (RER) in several Thoroughbred and Arabian horse families.

COLA6A3 (K1)

The *COLA6A3* gene encodes the protein collagen type 6, alpha 3. The chain-like molecule combines with two similarly structured proteins to form a collagen type 6 (COL6) molecule. COL6 is a primary structural protein of the extracellular matrix throughout the body, synthesized primarily in fibroblasts. In muscles, COL6 is a key endomysial protein. The clinical symptoms of a COL6 defect depend on the effect of any mutations in the COL6 genes.

A large number of hereditary diseases are known in humans which are caused by defects in the COL6 collagen, including Bethlem myopathy and congenital Ullrich muscular dystrophy, in which, like the K1 variant in horses, the gene *COL6A3* is defective.

Human patients with glycine substitutions in the triple helical region exhibit a range of severity of symptoms, depending on the position of the glycine substitution and the amino acid substituted. There is a critical region near the N-terminus of the triple helical region in which any substitution produces severe symptoms. Substitutions further from this region

are associated with milder symptoms. Human patients with severe symptoms have disruptions of muscle structure that are easily seen on muscle biopsy. Human patients with milder symptoms show no abnormalities on muscle biopsy.

A fibroblast assay shows that human patients with mild symptoms have defective extracellular matrix, of which collagen is an important component. Fibroblasts from mildly affected human patients export a disorganized extracellular matrix. This assay was also carried out on fibroblasts isolated from n/n, n/K1, and K1/K1 horses. Both n/K1 and K1/K1 fibroblasts export a disorganized extracellular matrix, as reported by Dr. Paul Szauter at the 20th Anniversary Symposium for the Genome Sciences Department at the University of Washington in November 2022, and by Kirsten Dimmler, a graduate student in the laboratory of Dr. Molly McCue at the University of Minnesota, at the Plant and Animal Genomes 2023 meeting in January 2023.

A horse can have genetic variants associated with both PSSM1 (although none currently identified in Welsh Cobs) and MIM as well as more than one MIM variant. To date, the P2 variant has not been identified in Welsh Cobs. One of the hypotheses put forth by EquiSeq is that the risk of a horse developing symptoms increases if it is homozygous positive for one variant or when different variants occur in any combination, whether the horse be homozygous or heterozygous positive for those variants.

Materials and Methods

Data Collection

The RWPCRG was formed in the Autumn of 2021, with the aim to collect and analyse breed-specific data. A database was established for the purpose of recording data in a systematic manner. Owners of tested cobs and others have access to this database although some results are held privately within the Group. The participants of our study have mainly been recruited via various PSSM forums and breed groups on Facebook. At the beginning of 2023, a DNA program was created in collaboration with Generatio GmbH, the laboratory which offers MIM testing in Europe. By enrolling their cobs in this program, owners grant RWPCRG access to the test results for research purposes.

As of February 2023, we had a total of 121 purebred registered Welsh Cobs and Ponies of Cob Type in our database. While the majority of the cobs were bred in the UK, cobs originating from other different countries are represented. Both private owners and breeders participate in our study. Some of these cobs are ridden regularly and/or performing at different levels in various equine sports.

Our recording of the health status of each cob has been based on the owner's report – in most cases, we were in touch with the owner before testing and categorized the animal as either "healthy" or "symptomatic" with respect to the list provided by Equiseq, before the test result was known. The majority of the symptomatic cobs have had extensive veterinary examinations; owners typically report that they resorted to MIM testing only after everything else failed to provide an explanation. Despite this, an important caveat pertaining to our data is that we cannot unequivocally conclude that MIM is at play in every case that we have categorized as symptomatic.

Admittedly, there are some limitations to our approach here: since MIM affects horses differently and to varying degrees in terms of clinical symptoms, we cannot completely rule out that those symptomatic involve a myopathy. Nonetheless, given the parameters of our study, this was deemed to be the most pragmatic solution.

Even though both symptomatic and healthy cobs are included in our data, it is important to note that this is not a randomized, representative sample of the breed. While the cobs are mostly unrelated, individuals with extensive health issues are significantly overrepresented compared to the general Welsh Cob population. The results of our analysis must be considered with this in mind.

Statistics

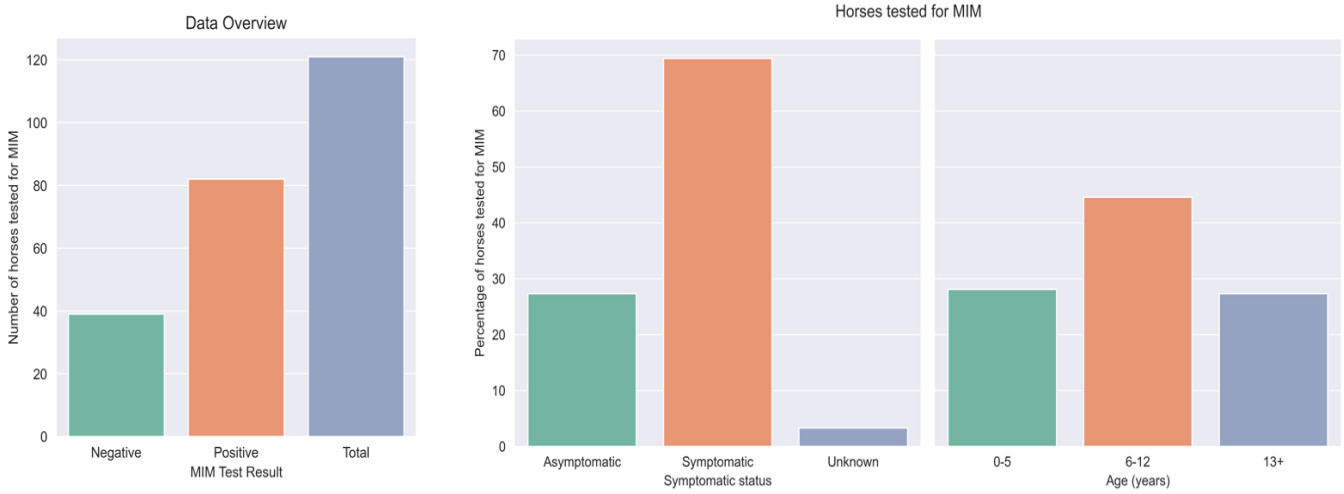
Statistical analysis was applied in a very limited manner and only on the complete data set. Fisher's exact test was used to check for correlation between health and test results. This report works with preliminary data and as such the group sizes become small when subdividing the data. At this stage, therefore, we only look for potential patterns in subdivided data and do not attempt to draw significant conclusions beyond those for the complete data set.

Results and Discussion

The Data

Some of the cobs in our data set have been tested for both PSSM1 and MIM (Figure 1). Because our data has been collected entirely from private testing and every tested cob was included, it was not possible to design a study with optimal group sizes or otherwise affect the selection of horses.

Horses tested for MIM (Figure 1). The percentage of horses tested for MIM (n=121) divided by health and age.

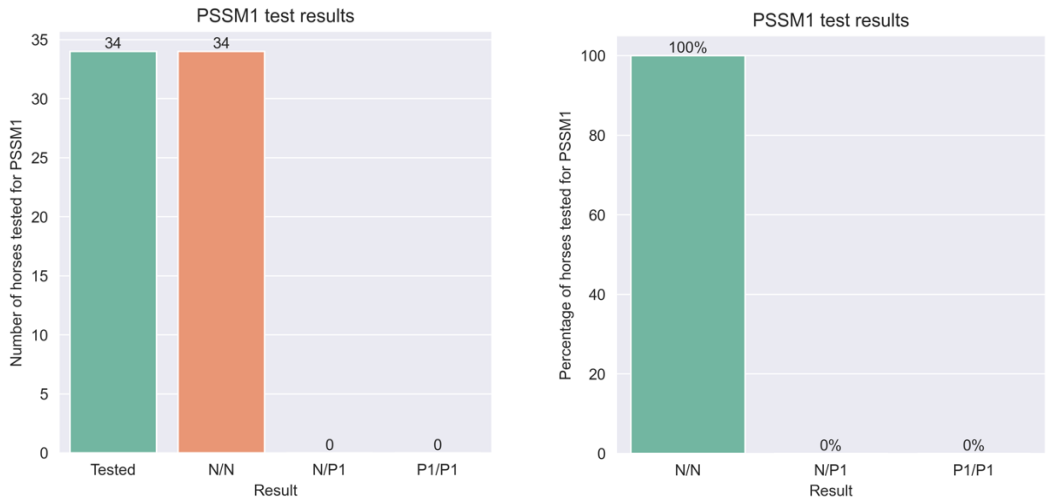


PSSM1 Test Results

34 cobs have been tested for PSSM1. All have tested negative. The tested cobs cover a wide representation of bloodlines and it is the only genetic test recommended by the WPCS. The allele frequency for PSSM1 in our sample is 0 (n=34). We are not aware of any active breeding lines with PSSM1. Furthermore, Generatio reports that they have tested 141 Welsh D and 10 Welsh C for PSSM1 and have never had one of either breed test positive for PSSM1 (Cox, personal communication, April 2023).

At this stage, we can confidently say that PSSM1 is not a significant problem within the breed and systematic testing of breeding stock is not necessary. PSSM1 is unlikely to be found in major breeding lines. It is, however, still possible that PSSM1 could be found in less common lines. Therefore, testing of all symptomatic cobs is still recommended.

Figure 2. PSSM1 test results. **A**) PSSM1 test results listed by number of cobs and genotype. **B**) PSSM1 results shown by genotype in percentage, n=34.



MIM Test Results

121 cobs have been tested for MIM. The overall test results are shown in Figure 3. 32% of the tested cobs are negative for all variants with 68% positive for one or more. One cob has tested homozygous positive for P3, two for P4 and one each for K1 and Px. These frequencies are low compared to what is rumoured for other breeds (see e.g., Aretz, 2021; Wackermann, 2022). It must be remembered, however, that according to Generatio, approximately 85% of samples submitted for testing are from symptomatic horses (Cox, private communication); it is therefore expected that the frequency of positive-testing horses will be high in those populations. Only 68.5% of the Welsh cobs submitted for testing in this report were classified as symptomatic, making a direct comparison not possible.

Figure 3. MIM test results in percent, n=121. This figure does not differentiate between the variants in the MIM test panel.

The distribution is of healthy (asymptomatic) (29%), not known (2.5%), and symptomatic (68.5%) cobs in our sample. Clinical symptoms range from mild to severe. Some cobs have intermittent periods of stiffness and substandard

performance, whereas others are unrideable and display significant behavioral and physical problems. To our knowledge, 10 cobs (between the ages of 2-9 years) included in our data set have been humanely euthanized on veterinary advice due to unmanageable MIM symptoms. Many owners report that the MIM diet, tailored to the variant(s) helps to reduce symptoms. Furthermore, cobs that have been retired from riding tend to display few to no symptoms of exertional myopathy when they are not expected to exert themselves. Climate and seasonal weather changes also appear to be consequential factors; more often than not, MIM-symptomatic cobs do fairly well in the summer months but struggle in the winter, and the combination of rain and wind seems to be particularly hard for them. Some however cannot tolerate the grass.

Several of the cobs in our data set have more than one health issue simultaneously, which can make management challenging; for instance, the high protein MIM diet may not be optimal if the cob also has EMS. Ulcers and stifle issues appear to be particularly common among symptomatic cobs in our database, with the owners reporting that clinical signs persist after the cobs have received veterinary treatment and been declared free of these issues. One potential explanation for this, then, is that these conditions might be side-effects of MIM rather than unrelated ailments; i.e., there is a possibility that physical distress caused by muscle pain contributes to ulcers and that weakened muscles generate stress on the joints. Another possibility is that MIM acts as an enhancer for any additional physical problems that the cobs have (see Wackermann, 2022). It must be acknowledged, however, that the fact that many MIM symptoms overlap with those associated with other ailments contributes to uncertainty with respect to a MIM diagnosis in some cases. Accordingly, complete correlation between health and test results is not possible in this study.. Despite these limitations, a statistically significant correlation between health and test results was found (Figure 4), $p<0.0001$ ($p = 1.38x10^{-8}$). This means that statistical analysis indicates that the pattern found is highly unlikely to be the result of random variation. In other words, if the genetic variants were harmless mutations with no impact on health, we should be seeing a more even distribution of the variants across the two groups.

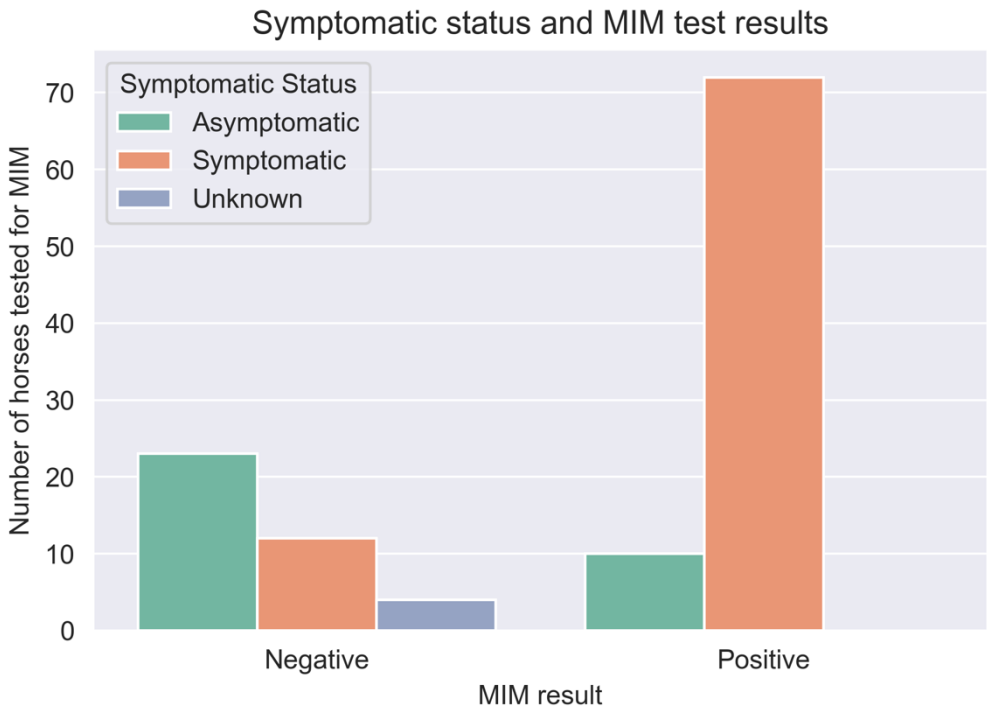


Figure 4. The health status compared to the MIM test results, n=121. Fisher's exact test $p<0.0001$. This figure only differentiates between positive or negative test results, not the number of variants for which a cob tests positive. Horses with an unknown symptomatic status (n=4) were excluded from the statistical calculation.

Single variants

The MIM panel tests for six variants. The percentage of cobs testing heterozygous for each variant are shown in figure 5. Five homozygous positive cobs have been found: two with single variants and three with two variants. However, it must be reiterated that this is not a representative sample of the breed. Our data shows a correlation between symptoms and variants (Figure 4), and symptomatic cobs are massively overrepresented in this sample compared to the general Welsh Cob population. The frequency of variants found in the breed as a whole can therefore be presumed to be lower.

In most cases, the heritability of the variants is independent. An exception to this are the P2 and P4 variants, which are located in genes positioned closely together on the same chromosome (see Szauter, 2020a). A cob with both variants can either have them on separate chromosomes or on the same. If they are on the same chromosome, they will most often be inherited together. The test is unable to differentiate between these two states. Both variants are present in many horse breeds, although no cob has tested positive for P2. Nevertheless, this means that there are four kinds of chromosomes that can be transmitted by a horse to its offspring: clear (n n), P2 (P2 n), P4 (n P4), and both (P2 P4). The genetic linkage of these two variants means that the parental configuration tends to be preserved on an individual chromosome, with only 8% of the gametes (sperm or egg) containing a nonparental configuration of P2 and P4. Horses that are (P2 P4) / (n/n) will give rise to 92% P2 P4 and n/n gametes, while horses that are (P2 n)/ (n P4) will give rise to 92% P2 n and n P4 gametes. There is substantial inbreeding during the development of any breed. Inbreeding in the case of Welsh Cobs appears to have driven out P2 entirely. If it was present in the founding population, as was P4, it was eliminated over time. The linkage of P2 and P4 may have assisted this, as (P2 n)/ (n P4) horses generate mainly parental types. (https://equiseq.com/blog/p2-p4-linkage)

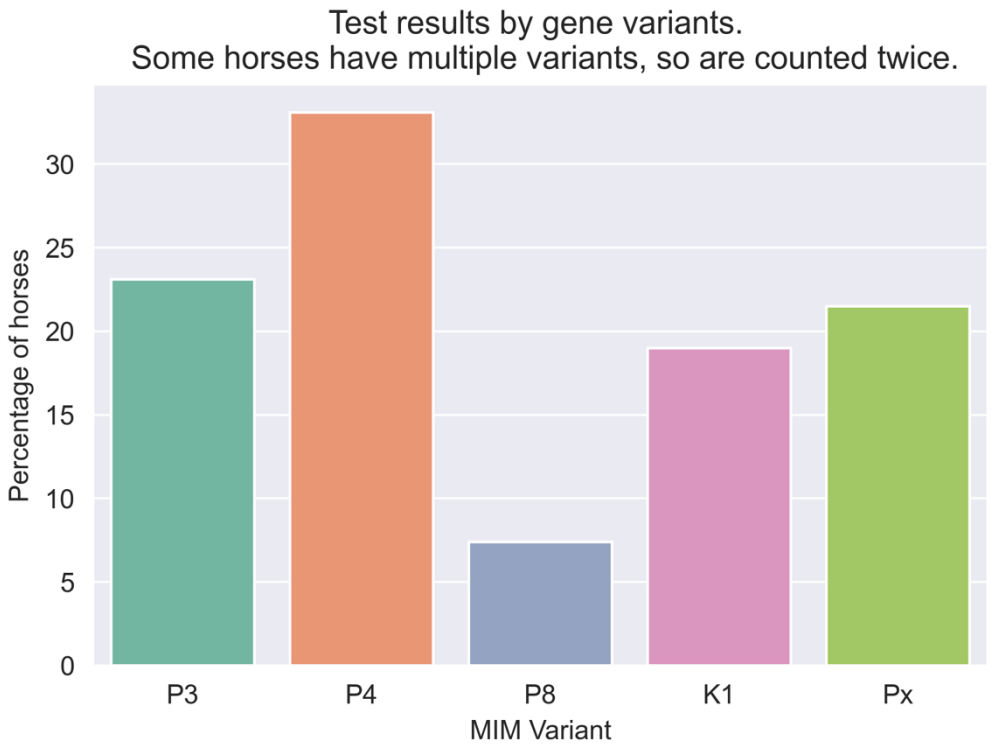
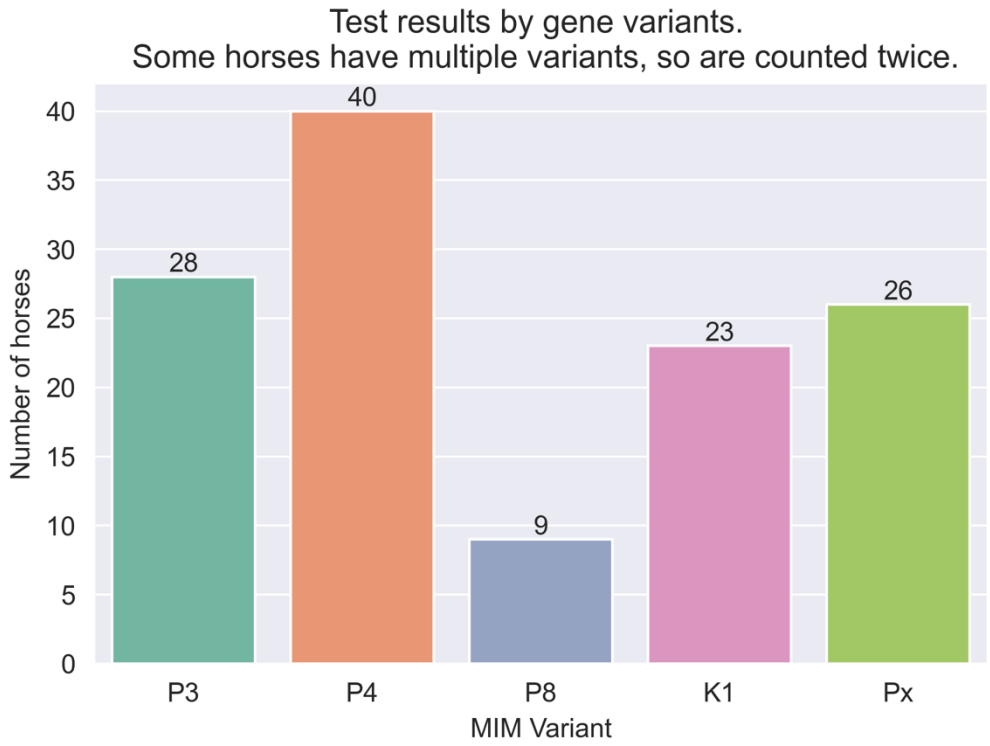


Figure 5. The percentage of cobs (N=82) testing heterozygous for each MIM variant. Five homozygous cobs have been found; one for P3, two for P4 and one each for K1 and Px. Cobs with multiple variants count with each variant they have. n=28 for P3, n=39 for P4, n=9 for P8, n=23for K1 and n=26 for Px



The Effect on Health of the Different MIM Variants

We have shown a correlation between the genetic variants and health (Figure 4). To determine whether any of the five MIM variants found in the breed had more impact on health than others, we looked at the cobs which have tested heterozygous n/P for a single variant only. These cobs (n=49) represent 60% of the positive MIM test results in our data. There was a positive association between horses having a heterozygous single variant (n=42) and a "symptomatic" health status (n=42). Accordingly, only 7 n/P horses were listed as asymptomatic (healthy) at the time of testing (Figure 6). Six of e these cobs range from 2-7 years of age and one is age 13 years. Two horses have two variants each but are asymptomatic at 8 and 10 years of age; one cob with 3 variants and no symptoms is aged 6 years. For these cases there are no doubts about the health status. It is possible that symptoms may be triggered in these horses at a later age, or that they have a protective genetic or environmental factor that reduces the penetrance of the mutation. A theory which has been proposed by a group of German veterinarians and researchers is that hormones delay or prevent the onset of MIM symptoms (see Wackermann, 2022). Stallions, as well as breeding mares, anecdotally have fewer symptoms than geldings and ridden mares. Empirical data supporting this theory has not yet been published. We had one positive asymptomatic brood mare in our dataset and two breeding stallions that were also considered symptomatic.

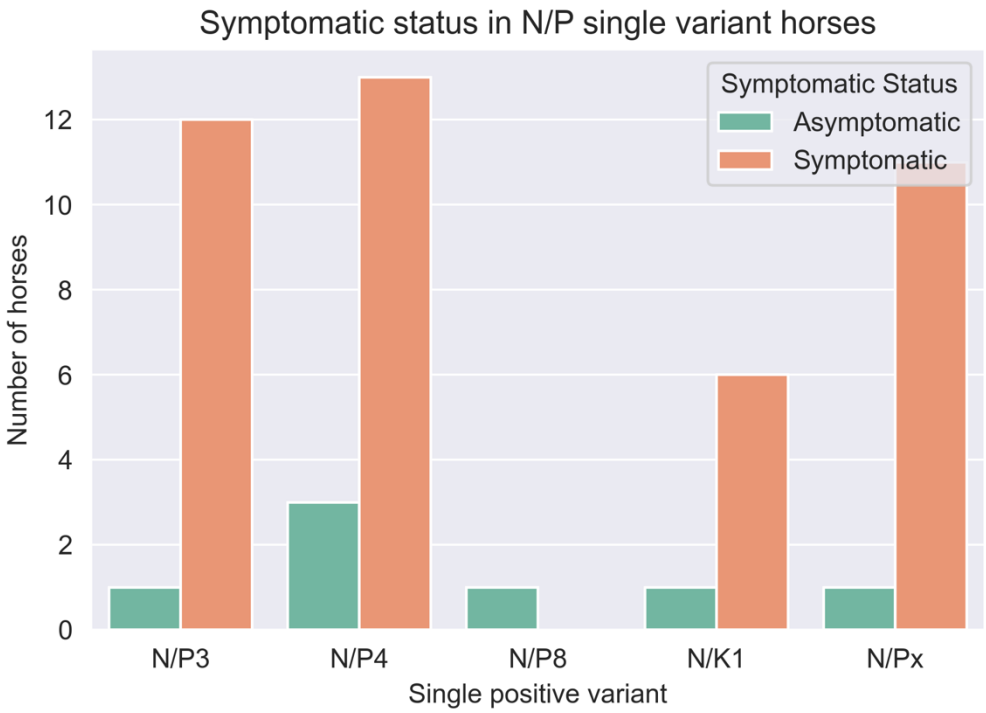


Figure 6. Cobs tested heterozygous for a single MIM variant only, divided by variant and owner reported symptomatic status, n=49.

With that being said, we must again acknowledge some factors which have potentially had an impact on our findings. Due to the mode of inheritance, and the lack of close genetic links between positive-tested cobs in our data set there must statistically be a considerable number of most likely asymptomatic (healthy) or very mildly symptomatic cobs that would test positive. It is fair to assume that symptoms present in a range from insignificant (asymptomatic/healthy) to extremely symptomatic, and our study design has attracted cobs from the extreme end of the scale – often those where conventional veterinary examinations have failed to explain the symptoms. The occurrence of variants in the breed may be low enough that our sample of healthy cobs (asymptomatic) tested (n=35) found seven single variant individuals. A more accurate way of analyzing the effect of the variants would have been to use pairs of matched siblings tested negative and positive, with the same owner and environment. As our data is gathered from private cob owners, no selective design was possible. With few exceptions the tested cobs are not closely related. At this stage, we can conclude that P3 (92%), P4 (81%) and Px (92%) are the variants displaying the most symptoms when heterozygous. The lack of single variant tested cobs in the healthy control group further supports our hypothesis that the overall occurrence of the variants in the breed is low – much lower than the numbers found in this study's non-representative sample (Figure 5).

The P4 variant is that most commonly found in our sample of cobs testing positive for MIM, with 48% testing positive for P4. This gives an allele frequency of 0.17 (Figure 5). While this number may not be representative of the breed, the incidence of this variant in the general Welsh Cob population is also unlikely to be extremely low.

Continuing on the topic of health status in relation to the different genetic variants, there is nothing in our data which indicates that particular symptoms are associated with specific variants. As previously mentioned, the Px variant is on its own unlikely to impact on a cob's health, as it is believed to require an additional unknown genetic factor to cause clinical symptoms (Szauter, 2020b). We cannot rule out the possibility that symptoms of tying up and elevated muscle enzymes are associated with the other genetic variants included in the current MIM test panel. Although the MIM test did not provide any helpful answers for the owners of symptomatic cobs testing negative, (n=12), this part of our data cannot necessarily be used as a basis for arguing a lack of correspondence between the genetic test and the disease state

of MIM, but rather points towards a subtype of MIM whose genetic foundation has yet to be discovered. In humans more than 300 genes have been identified in more than 600 single gene neuromuscular disorders; nevertheless, depending on the study population, only 13-79% of clinically affected humans test positive for known variants (Ng et al., 2022). Therefore, what our admittedly very limited data might suggest, then, is that the MIM test, in its current form, is not yet complete as a diagnostic tool for Welsh Cobs that display particular symptoms.

Multiple Variants

A claim made by the company developing the genetic test for MIM is that the presence of multiple variants causes more severely affected horses. Our data does not support this (Figure 7). It can, however, easily be argued that our data are not suitable to address this hypothesis. The low occurrence of variants in the breed, in combination with our current sample size, means that the group of cobs with multiple variants is too small. A total of ten cobs with multiple (three to five) variants have been found. Of these, seven share the P8 variant; 5 with a similar line from the second and third generation.

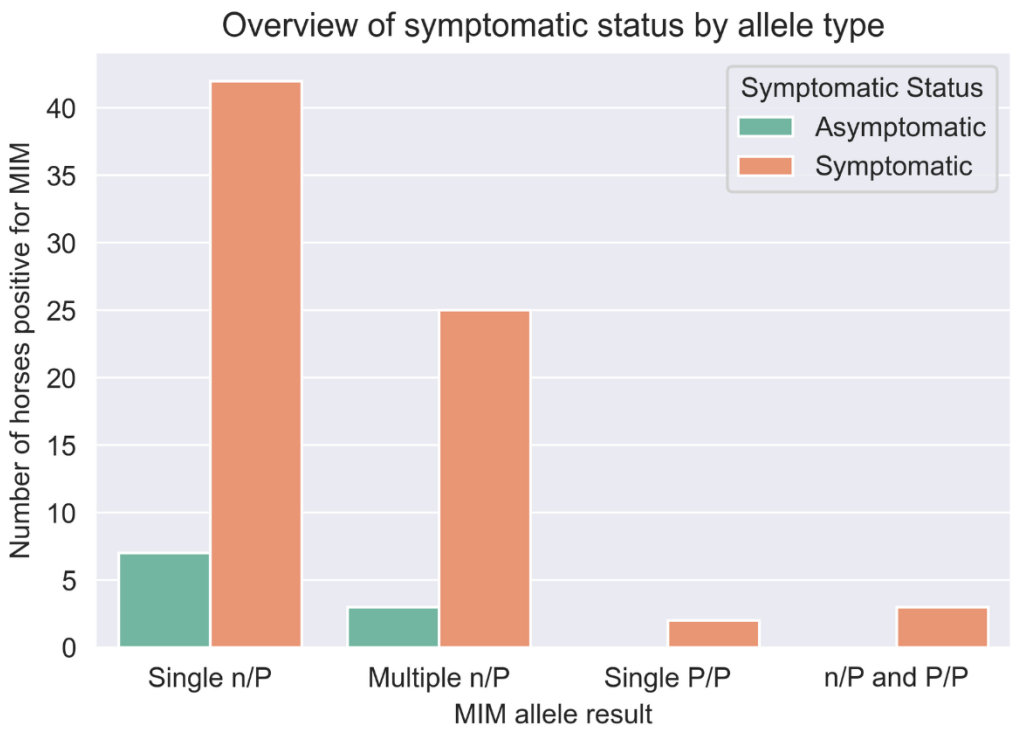


Figure 7. The health status compared to the MIM test results and divided by the number of variants found, n=82.

With 121 cobs tested for MIM, one was homozygous for P3, two for P4, and one each for K1 and Px. None of the variants are embryonically lethal when homozygous. The current theory is that homozygous horses are among the most severely affected, more so than horses with multiple different variants. This is not demonstrated here. As previously mentioned, if the test is applied to breeding decisions, the recommendation is to avoid combinations which can result in homozygous offspring. The small number of homozygous cobs in our data is noteworthy. 69% of the tested cobs are symptomatic (n=84). Despite a method of data collection which favours identification of severely affected cobs, of which 68% tested positive for one or more variants, five homozygous cobs were found. The simplest theory to explain this is that the frequency of the variants in the breed is so low that our sample becomes too small to find more. More homozygous cobs are highly likely to exist. The variants are proven to be very old, several thousand years for some,

and will not be eliminated by natural selection (Valberg et al., 2021). There have been no reports from other breeds that homozygous individuals should systematically fail to thrive and as such remove themselves from the breeding stock.

MIM and Age

In order to further investigate the connection between the MIM test results and health, the data was divided by age into three groups. A total of 121 cobs have been tested for MIM. The youngest age group was set to 0-5 years (n=34). In this group it is likely cobs are too young to show symptoms. The middle group was set to 6-12 years (n=54). At this age cobs should be healthy athletes and the contrast to cobs with issues is the greatest. MIM is adult-onset and most cases will become symptomatic at this age. The last group consists of cobs aged 13 and above (n=33). This group will show more general "wear and tear" and the probability of issues caused by non-MIM related ailments is increased. While MIM symptoms are likely to be noticeable if present, they may also be dismissed as "wear and tear" and therefore not investigated thoroughly.

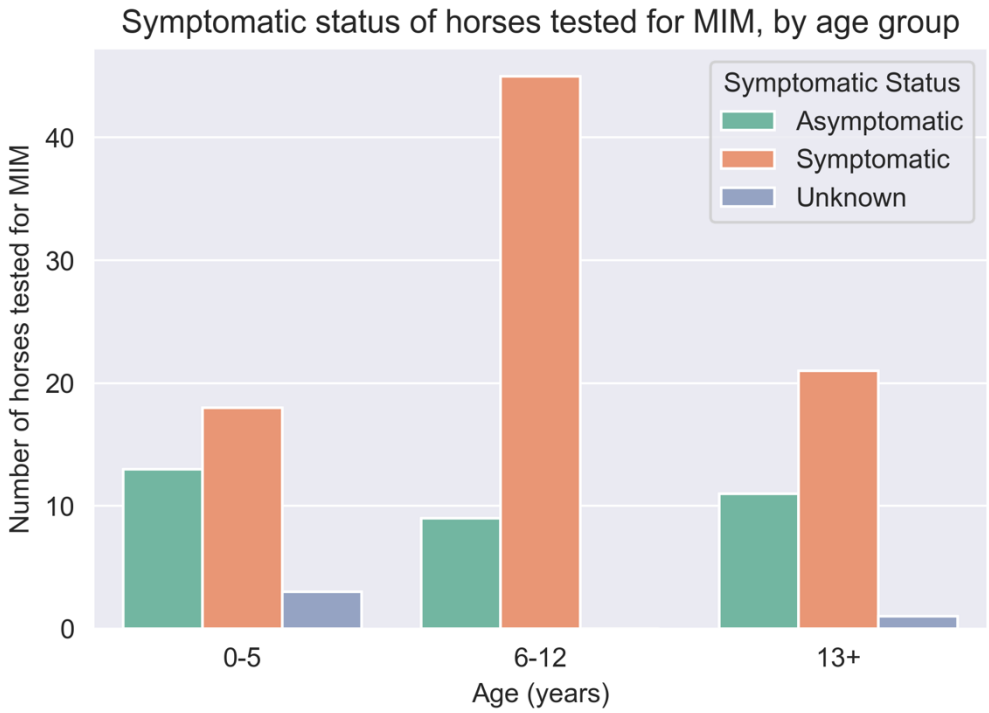


Figure 8. The health status compared to the age group of MIM tested cobs. Test results are not considered in this figure.

The highest number of symptomatic cobs was found in the middle age group (Figure 8). These are typically ridden cobs that have either never quite performed as expected, or they have had a few good years after which their performance inexplicably deteriorated. When further dividing data by test results (Figure 9), it becomes noticeable that the majority of these symptomatic cobs test positive. It also becomes very noticeable that among the older, asymptomatic cobs, a higher number test negative (91%). Only one older cob with a variant is categorized as asymptomatic. (Figure 9)

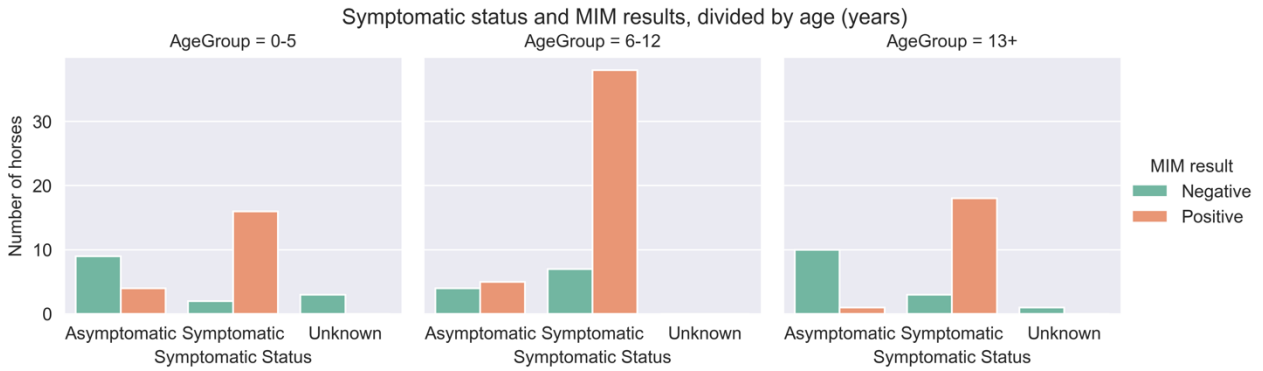


Figure 9. The health status compared to the MIM test results and divided by age. The number of testable variants is not considered in this figure.

Pedigree Studies

The attempt to track the genetic variants beyond more than a single generation has so far been unsuccessful. The lack of homozygous cobs makes tracking the variants difficult. With the exception of some targeted testing following positive test results which have been made public by owners who have a particular blood line, the majority of tested cobs are generally not closely related apart from some with the P4 variant. Assuming pedigrees are accurate for a minimum of two to three generations, the number of cobs that are at least heterozygous for a variant cannot be insignificant. The majority of tested cobs are ridden cobs that do not have any progeny. For those with heterozygous variants, we cannot assume which side of the pedigree the gene came from. At this stage, unless a cob happens to be very closely related to one tested, we cannot predict if it has an increased likeliness of testing positive based on its pedigree. When looking at one of the least frequent variants, the P8 mutation, these cobs (n=9) seem to be linked within the breed. Seven have a line in common, and two nothing in the first three generations. However, the source cannot be determined of the P8 variant, as other variants are present. Six can be linked by their pedigree within 3 generations or less, and all nine have shared ancestors within 6 generations. However, the source of the P8 variant cannot be determined due to the small sample size and relative lack of testing in the breed.

For P4 (48% of positive MIM test variants, n = 40) two horses were homozygous for the allele, indicating that both of their respective sire and dam must be at least heterozygous for the P4 variant, increasing our sample size to n=44. In this group, 49% have the same sire in generations 2-5, and that stallion's sire in 54% of the 5th and 6th generation pedigrees. This was highlighted after two stallions were tested positive for P4 both with the same bloodlines from the 2nd and 3rd generation with offspring also testing positive for the same P4 variant. All P4 positive horses can be linked within 6 generations or fewer, with 59% of these horses being related within 3 generations or fewer. However, some horses (n=24) tested positive for other MIM test variants alongside P4, further complicating attempts to trace the bloodlines of these horses.

The scientific methods (PCR amplification with Sanger Sequencing, qPCR, or Illumina Beadchip) used to test for the variants are commonly used and can be presumed to be accurate. At this stage, the lack of patterns for some of the variants may suggest the possibility that pedigrees could be just slightly inaccurate in the back breeding. Our data is, however, still very limited. There are major breeding lines, stallions with hundreds of foals, for which we have no data.

Patterns may emerge as we continue to gather data.

Conclusion

Due to the limited size of our current data, the findings reported in this report must be regarded as preliminary. When more substantial data has been accumulated, we may be able to explore further dimensions of the material than those which have been addressed here. Trends in our developing data can also be expected to fluctuate. There are major breeding lines for which we have no data. Nevertheless, at this early stage of our study we have been able to uncover some surprisingly convincing patterns.

Our report format mirrors that used by the Connemara Research Group so that comparison within breeds may be made. It should be noted that they also found a very low incidence of PSSM1 but that P2 was the most common variant identified in Connemara ponies.

For PSSM1, the allele frequency has been estimated to be 0 with no known affected active breeding lines. As such, systematic testing of breeding stock is not considered necessary. Testing of all symptomatic cobs would still be recommended.

For MIM, we have shown a significant correlation between symptomatic status and a positive test result. In spite of this, it is too early to develop a test-based breeding strategy. As of yet our data does not support the hypothesis that cobs with multiple variants have increased probability and severity of symptom development, which is a cornerstone of the proposed breeding application of the test. Little is known about the frequency of healthy cobs testing positive as well as why some cobs develop symptoms and others do not. Of the 10 cobs testing positive that are not symptomatic, their ages range from 2-8 (n=8), 10 (n=1), 13(n=1). Symptoms have been shown to manifest later in life often after a trigger from a management or metabolic issue. Younger animals are generally more able to compensate for environmental stressors than that trigger symptoms in adults, at least with correct management. Of those testing negative (n=39), 12 are symptomatic with 3 unknowns so this may be due to other, as yet unidentified, variants. The application of any genetic test to breeding decisions affects the genetic diversity within a breed, and the situation is especially complex when it comes to a multifactorial, polygenic disease like MIM. Numerous ongoing studies are currently looking into all these areas and their results will contribute to a better understanding of this complex issue. It is, however, strongly recommended never to use symptomatic cobs for breeding. There is clearly a genetic element to these disorders, and the presence of one or more of the tested variants are highly predictive of a horse developing symptoms

This study will continue and updates will be published once the sample size increases sufficiently or critical data emerges which changes our preliminary conclusions. If you have chosen to test your cob then please share the results with our research group (https://generatio.de/en/dna-programmes/main-programmes/registered-welsh-pony-and-cob-research-group)

References

Aretz, J. (2021). Was steckt hinter PSSM2 beim Pferd? *Cavallo*, 31.05.2021 (https://www.cavallo.de/medizin/was-steckt-hinter-pssm2/)

Cox, M., Generatio GmbH (April 2023), Personal communication

Fritz, K. L., McCue, M. E., Valberg, S. J., Rendahl, A. K. & Michelson, J.F. (2012). Genetic mapping of recurrent exertional rhabdomyolysis in a population of American Thoroughbreds. *Anim Genet.*, 43 (6): 730738.

McCue, M., Valberg, S. J., Lucio, M. & Mickelson, J. R. (2008). Glycogen synthase 1 (GYS1) mutation in diverse breeds with polysaccharide storage myopathy. *J Vet Intern Med*, 22: 1228-1233.

Ng, K.W.P. (2022). Using gene panels in the diagnosis of neuromuscular disorders: a mini-review. *Frontiers in Neurology*, doi: 10.3389/fneur.2022.997551.

Royal Veterinary College (2019). New study on tying-up in horses and ponies (https://www.rvc.ac.uk/equine-vet/news/new-study-on-tying-up-in-horses-and-ponies).

Szauter, P.(2020a). ThelinkageofP2andP4.EquiSeqblogpost,20.01.2020

(http://equiseq.com/blog/p2http://equiseq.com/blog/p2-p4-linkagep4-linkage)

Szauter, P. (2020b). The PX allele of CACNA2D3 may affect splicing of a component of the voltage-gated calcium channel. EquiSeq blog post, 08.02.2020 (http://equiseq.com/blog/px-allele-of-cacna2d3).

Valberg, S. J., McKenzie, E. C., Eyrich, L. V., Shivers, J., Barnes, N. E. & Finno, C. J. (2016). Suspected myofibrillar myopathy in Arabian horses with a history of exertional rhabdomyolysis. *Equine Veterinary Journal*, 48 (5): 548-556.

Valberg, S. J., McKenzie, E. C., Eyrich, L. V., Shivers, J., Barnes, N. E. & Finno, C. J. (2020). Candidate gene expression and coding sequence variants in Warmblood horses with myofibrillar myopathy. *Equine Veterinary Journal*, 53 (2): 306-315.

Valberg, S. J., Finno, C. J., Henry, M. L., Schott, Velez-Irizarry, D., Peng, S., McKenzie, E. & Petersen, J. L. (2021). Commercial genetic testing for polysaccharide storage myopathy and myofibrillar myopathy does not correspond to a histopathological diagnosis. *Equine Veterinary Journal*, 53 (4): 690-700.

Valberg, S. J., Henry, M. L., Herrick, K. L., Velez-Irizarry. D., Finno, C. J. & Petersen, J. L. (2022). Absence of myofibrillar myopathy in Quarter Horses with a histopathological diagnosis of type 2 polysaccharide storage myopathy and lack of association with genetic tests. *Equine Veterinary Journal*, 00: 1-9.

Dyson, Sue, et al. "Development of an ethogram for a pain scoring system in ridden horses and its application to determine the presence of musculoskeletal pain." *Journal of Veterinary Behavior* 23 (2018): 47-57.

Wackermann, K. (2022). PSSM2 beim Pferd – die Trainings-Intoleranz. *St. Georg*, 10.03.2022 (https://www.st-georg.de/wissen/pssm2-beim-pferd-die-trainings-intoleranz/).

Zsoldoz, R., Khayatzadeh, N., Soelkner, H. & Licka, T. (2019). Breed specifics of muscle function changes in the Austrian Haflinger and Austrian Noriker horses with polysaccharide storage myopathy (PSSM). *Neuromuscular Disorders*, 29 (1).

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https://generatio.de/en/dna-programmes/main-programmes/connemara-pony-pssm-research-group