PSSM in Connemara ponies: A report on preliminary findings

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Abstract

This article reports on the preliminary findings of an ongoing study on Polysaccharide Storage Myopathy (PSSM) in the Connemara breed. Data consists of test results of 100 purebred Connemara ponies and information about pedigree, symptoms/diagnostics, diet and management obtained from owners. The ponies have been tested for PSSM1 and/or PSSM2, and both healthy and symptomatic ponies are represented. The validity of the genetic test for PSSM2 has yet to be confirmed in a peer-reviewed publication. As the test is gaining traction, breed specific information about the usefulness and interpretation of test results is needed. The purpose of this article is to provide breeders and owners of Connemara ponies with the knowledge necessary to understand the implications of test results and make informed decisions about their breeding program. The occurrence of PSSM1 was found to be very low (allele frequency <0.01, n=72) and no changes to breeding practices are necessary in this regard. This study has found a significant correlation ($p < 0.0001$) between health and PSSM2 test results in Connemara ponies (n=75). With a near equal distribution of PSSM2 tested healthy (n=38) and symptomatic (n=37) ponies in our sample, 57% tested negative, 32% tested heterozygous for a single variant and 11% had more than one variant. Four of the currently six testable variants have been found in the breed, with P2 being the most common (allele frequency 0.14 in our non-representative sample). The data does not support the hypothesis that ponies 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. Pedigree tracking of variants has been unsuccessful so far. These are only preliminary findings, which limits the conclusions.

Introduction

In recent years, Polysaccharide Storage Myopathy (PSSM) has become a hot and rather controversial topic of discussion among horse people worldwide, including in the Connemara pony community. Much of the controversy revolves around the genetic test for PSSM2 – one of two types of PSSM – which is offered commercially even though its validity has yet to be confirmed in a peer-reviewed publication. Moreover, while there is no indication that PSSM is a significant problem within the Connemara breed compared to other breeds, the fact that there are Connemara ponies with myopathies cannot be disputed. Since little is known about the prevalence of the testable variants within the breed as well as the correlation, if any, between the variants and symptoms, there is a need to monitor the situation and develop further knowledge. The Connemara Pony PSSM Research Group (CPPRG), a small international group of knowledgeable

Connemara-people independent of any breed society, is keeping up to date with the ongoing research as well as gathering breed-specific data. Our aim is to give breeders and owners of Connemara ponies a well-informed and nuanced understanding of the situation. The following report is a summary of our preliminary findings, based on an analysis of the data we have accumulated over the course of the past two years. The data consists of test results of 100 purebred Connemara ponies as well as information about pedigree, symptoms/diagnostics, diet and management obtained from their owners.

Background

What is PSSM?

As noted above, there are two types of PSSM: PSSM1 and PSSM2. Both are myopathies which involve muscle pain and weakness, but they have different causes and characteristics. PSSM1 is a glycogen storage disease that results in the accumulation of abnormal complex sugars in muscle cells. It is associated with a single gene. PSSM2, on the other hand, is essentially a catch all diagnosis for all cases of PSSM which test negative for the PSSM1 mutation. Despite the name, PSSM2 is not a glycogen storage disease but rather a collective term for a variety of muscle disorders which cause similar clinical symptoms, with two subtypes described as Myofibrillar Myopathy (MFM) and Recurrent Exertional Rhabdomyolysis (RER). These names are used differently depending on the source, and they may change in the future when further research allows for better differentiation. For simplicity's sake we will be using the term PSSM2 throughout this paper.

PSSM2 cannot be linked to a single gene or cause; it is very complex with a high number of factors influencing the outcome, and most of those variables are likely still unknown. PSSM2 is believed to be genetic – or at least have a genetic element – as symptomatic horses are found all over and not in clusters. Horses can be kept under identical conditions and some will have PSSM2 while others are healthy. If it had been caused by environmental factors alone then entire stables would be affected.

With regard to symptoms, raised muscle enzymes and so-called "tying up" episodes are frequently associated with PSSM1 but are less often seen in connection with PSSM2. Other common symptoms are tight muscles, muscle spasms and divots that come and go, lethargy and/or anxiousness and explosiveness, reluctance to move, camping out in peeing position, stiffness, rope walking, difficulty with canter, dragging hindlegs, inability to work properly/evading contact, depression, pain face, headshaking, colic symptoms, breathing issues, difficulty standing for the farrier, 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 be ruled out or considered alongside PSSM as a possible reason for the horse's discomfort.

Diagnosing PSSM

How, then, is PSSM diagnosed? There is a scientifically verified and trusted genetic test for PSSM1, but the situation is not as clear-cut when it comes to PSSM2. 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 PSSM2, it has some indisputable limitations.

Seeking an alternative to invasive biopsies and knowing there was likely a genetic component to PSSM2, the American company EquiSeq developed a genetic test which has been commercially available for some years now. It has been licensed for commercial use in Europe by the German laboratory Generatio GmbH. 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 PSSM2 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, yet these are likely years from publication.

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 makes you question 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 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 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 actually healthy 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 prevalence 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 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 known at this point in time about why some horses with the gene(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 PSSM2 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 genes. Because of the multigenetic nature of PSSM2 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 Connemara pony. The advice from EquiSeq 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. However, before any breeding decisions are made based on PSSM2 test results, it is necessary to have sufficient evidence that these specific mutations are, in fact, harmful. Such evidence is not yet available, though the research is ongoing.

When it comes to diet and management of PSSM affected horses, there are different recommendations for type 1 and 2. Horses with PSSM1 generally benefit from a low starch and sugar diet as well as daily exercise. Horses with PSSM2 often benefit from a high protein diet and supplementation of the amino acids

lysine, methionine and threonine. Even though many PSSM2 horses present symptoms of exercise intolerance, daily movement and light exercise are recommended. An open stall with a track system or access to a larger field may be favourable in this respect. These horses can be sensitive to cold and wet weather conditions, and heavier blanketing than normal is often required. It must also be emphasized that the above-mentioned recommendations are general – the dietary needs and athletic abilities of PSSM-affected horses vary greatly and individualized 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 PSSM2 panel tests for six gene variants which have been labeled P2, P3, P4, PX, P8 and K1 (see www.equiseq.com). 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 PSSM2. P2, P3 and P4 are in structural proteins in the muscles. 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 PSSM2 in one family of thoroughbred horses but not in a second, wider sample. The current theory is that PX interacts with a second as of yet unknown gene (Szauter, 2020b). Horses testing positive for PX can be 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.

A horse can have genetic variants associated with both PSSM1 and PSSM2 as well as more than one PSSM2 variant. 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

CPPRG was formed in the spring of 2020, with the aim to collect and analyze breed-specific data. A database was established for the purpose of recording data in a systematic manner. During the early stages of the data collection process, owners of tested ponies had access to this database. However, in order to protect the privacy of the participants as well as to avoid unfounded rumours about affected bloodlines, access was eventually restricted to the administrative members of the research group. The participants of our study have mainly been recruited via various Connemara pony and PSSM forums on Facebook. At the beginning of

2022, a DNA program was created in collaboration with Generatio GmbH, the laboratory which offers PSSM2 testing in Europe. By enrolling their ponies in this program, owners grant CPPRG access to the test results for research purposes.

As of 24 May 2022, we had a total of 100 purebred ponies in our database[1]. While the majority of ponies were bred in Ireland, ponies originating from eight different countries are represented. They are currently located in eight different countries across two continents, thereby ensuring geographic variation in the data. Furthermore, data has mainly been obtained from unassociated sources, with the exception of 10 ponies which were tested due to their close relation to a confirmed positive stallion. Both private owners and breeders participate in our study. In this connection, it must be noted that breeders in Finland have been particularly supportive of the research project and they are the primary source of healthy ponies tested for PSSM2. Many of these ponies are ridden regularly and/or performing at different levels in various equine sports.

Our recording of the health status of each pony 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" before the test result was known. The majority of the symptomatic ponies have had extensive veterinary examinations; owners typically report that they resorted to PSSM testing only after everything else failed to provide an explanation. In spite of this, an important caveat pertaining to our data is that we cannot unequivocally conclude that PSSM is at play in every case that we have categorized as symptomatic. However, in order to reduce the risk of mislabeling "anything and everything" as a symptom of PSSM, ambiguous cases have been categorized as healthy. This was, for instance, done if all of the pony's symptoms aligned with an alternative diagnosis confirmed by a vet or if other circumstances provided a more likely explanation for the issues. Admittedly, there are some limitations to our approach here: since PSSM affects horses differently and to varying degrees in terms of clinical symptoms, we cannot completely rule out that these ambiguous cases 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 ponies are included in our data, it is important to note that this is not a randomized, representative sample of the breed. While the ponies are mostly unrelated, individuals with extensive health issues are significantly overrepresented compared to the general Connemara pony population. The results of our analysis must be considered with this in mind.

[1] There are also a handful of partbred ponies, but these have not been included in the present analysis.

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

Many of the ponies in our data set have been tested for both PSSM1 and PSSM2 (Figure 1). Because our data has been collected entirely from private testing and every tested pony was included, it was not possible to design a study with optimal group sizes or otherwise affect the selection of ponies. Even so, the data has turned out surprisingly solid with an almost equal distribution of healthy and symptomatic ponies tested for PSSM2 (Figure 1).

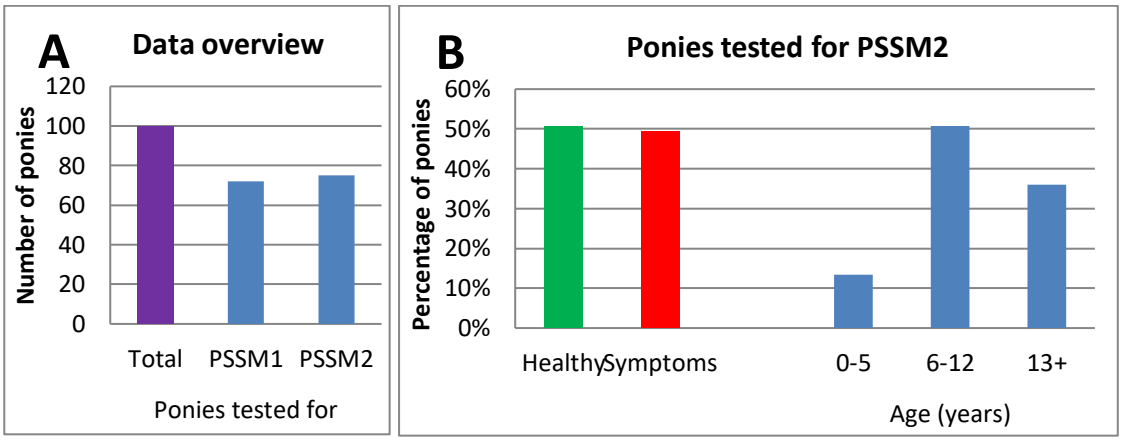

Figure 1. Data overview. **A**) The total number of ponies in the study and which test they have had. Many have been tested for both. **B**) The percentage of ponies tested for PSSM2 (n=75) divided by health and age.

PSSM1 test results

72 ponies have been tested for PSSM1. All but one pony have tested negative (Figure 2). The tested ponies represent lines worldwide, with many breeders having tested their stallions. In addition to breeding stock, symptomatic ponies (n=28) have been tested for diagnostic purposes. The allele frequency for PSSM1 in our sample is 0.007 (n=72). Due to the high number of symptomatic ponies, our sample is not representative of the breed. The allele frequency in the breed can be assumed to be even lower. The one pony that has tested heterozygous for PSSM1 is symptomatic. This pony's sire has tested negative and the dam is of such old and rare breeding that further tracking of the gene has not been possible. We are not aware of any active breeding lines with PSSM1. 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 will be found in less common lines. Therefore, testing of all symptomatic ponies is still recommended.

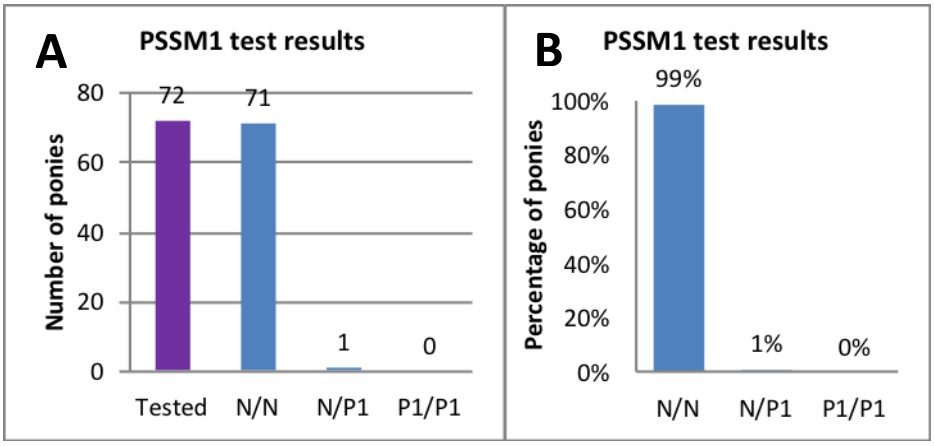

Figure 2. PSSM1 test results. **A**) PSSM1 test results listed by number of ponies and genotype. **B**) PSSM1 results shown by genotype in percentage, n=72.

PSSM2 test results

75 ponies have been tested for PSSM2. The overall test results are shown in Figure 3. 57% of the tested ponies are negative for all variants. No ponies homozygous positive for any variant have been found. 11% of ponies are heterozygous for multiple variants. No ponies with more than three variants have been found. These frequencies are low compared to what is rumoured for other breeds (see e.g., Aretz, 2021; Wackermann, 2022).

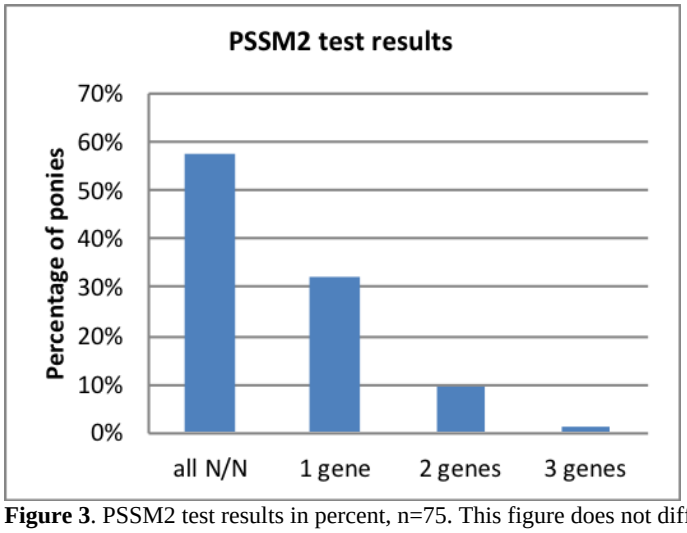

Figure 3. PSSM2 test results in percent, n=75. This figure does not differentiate between the variants in the PSSM2 test panel.

There is a more or less equal distribution of healthy (51%) and symptomatic (49%) ponies in our sample. Clinical symptoms range from mild to severe. Some ponies merely go through intermittent periods of stiffness and substandard performance, whereas others are unrideable and display significant behavioural and

physical problems. To our knowledge, only one pony included in our data set has been euthanized due to PSSM2 (at the age of 12, having been diagnosed several years prior). Most owners report that the PSSM2 diet helps reduce symptoms. Furthermore, ponies that have been retired from riding tend to display few to no symptoms. Climate and seasonal weather changes also appear to be consequential factors; more often than not, PSSM2-symptomatic ponies 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.

Several of the ponies in our data set have more than one health issue going on at once, which can make management challenging; for instance, the high protein PSSM2 diet may not be optimal if the pony also has EMS. Ulcers and stifle issues appear to be particularly common among the symptomatic ponies in our database, with the owners reporting that clinical signs persist after the ponies have received veterinary treatment and been declared free of these issues. One potential explanation for this, then, is that these conditions might be side-products of PSSM2 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 PSSM2 acts as an enhancer for any additional physical problems that the ponies have (see Wackermann, 2022). It must be acknowledged, however, that the fact that many PSSM2 symptoms overlap with those associated with other ailments contributes to uncertainty with respect to a PSSM2 diagnosis in some cases. Accordingly, complete correlation between health and test results is not possible in this study, even if the testing proves accurate. Despite these limitations of our study, a statistically significant correlation between health and test results was found (Figure 4), $p<0.0001$. 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.

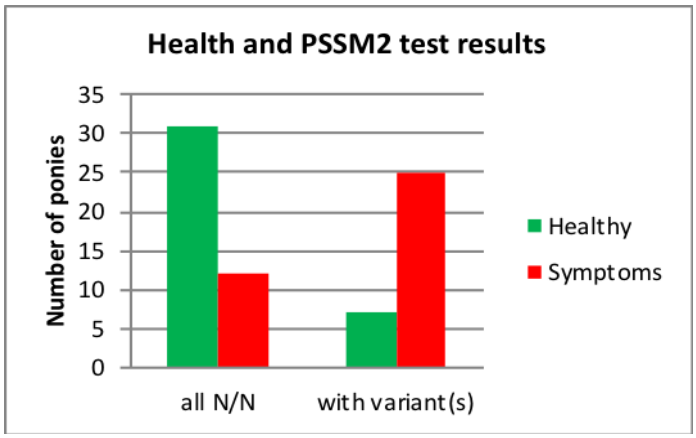

Figure 4. The health status compared to the PSSM2 test results, n=75. Fisher's exact test $p<0.0001$. This figure only differentiates between positive or negative test results, not the number of variants a pony tests positive for.

Single variants

The PSSM2 panel tests for six variants. The percentage of ponies testing heterozygous for each variant are shown in figure 5. No homozygous positive ponies have been found. 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 ponies are massively overrepresented in this sample compared to the general Connemara pony population. The percentage of variants found in the breed can therefore be presumed to be lower.

At the beginning of the data collection process, the European PSSM2 test panel had four variants (P2, P3, P4, Px), with the two additional variants (P8, K1) being added in the fall of 2020. Of the 75 ponies tested for PSSM2, 29 are tested for only four variants. As the P8 and K1 variants have not been found in any tested pony, it has not been necessary to differentiate between ponies tested for four or six variants. The laboratory offering the test in Europe does claim that these variants have been found in the breed, at a very low level. We are limited to the data which has been sent to us by pony owners. It currently counts 46 ponies testing N/N for both of these variants.

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 (see Szauter, 2020a). A pony 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. Early testing revealed a pony with N/P2 and N/P4. Targeted testing of another 10 closely related ponies with a 50% chance of having these variants does affect the frequency of P2 and P4 found. However, even if we subtract all but the originally found pony with both variants, the percentage only goes down about 5%. Accordingly, this targeted testing does not change the fact that P2 is the most frequently found variant in the breed, followed by P4.

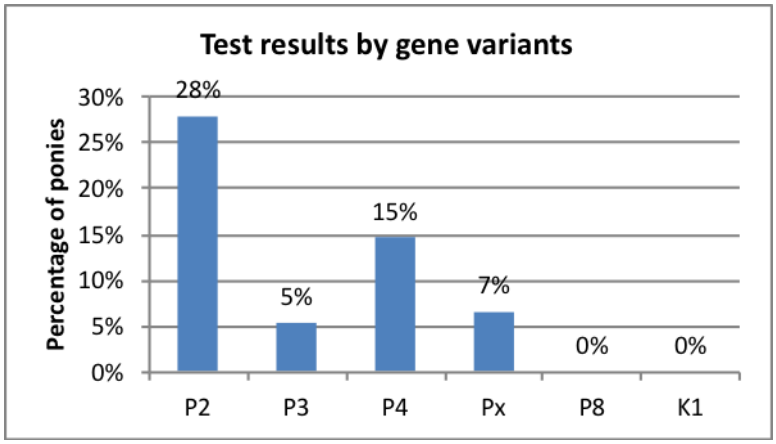

Figure 5. The percentage of ponies testing heterozygous for each PSSM2 variant. No homozygous ponies have been found. Ponies with multiple variants count with each variant they have. n=75 for P2, P3, P4, Px. n=46 for P8, K1.

The effect on health of the different PSSM2 variants

We have shown a correlation between the genetic variants and health (Figure 4). To determine whether any of the four PSSM2 variants found in the breed had more impact on health than others, we looked at the ponies which have tested heterozygous N/P for a single variant only. These ponies (n=24) represent 75% of the positive PSSM2 test results in our data. The correlation between a heterozygous single variant test result and a health status as symptomatic was nearly complete with only two of the 24 N/P ponies being healthy (Figure 6). Both of these healthy ponies tested N/P2 and are stallions. One has had a long and successful performance career, the other is regularly ridden. In both cases there are no doubts about the health status. A theory which has been proposed by a group of German veterinarians and researchers is that hormones delay or prevent the onset of PSSM2 symptoms (see Wackermann, 2022). Stallions as well as mares used for breeding supposedly do better than geldings and ridden mares. Empirical data supporting this theory has not yet been published, but it provides a possible explanation for why only these particular single variant N/P ponies in our data set are asymptomatic. Indeed, the statistics regarding this subgroup border on perfection, which should not have been possible within the setup of this study. While the group sizes for each gene are small, the total of a 22 out of 24 match is not simply dismissible as random variation.

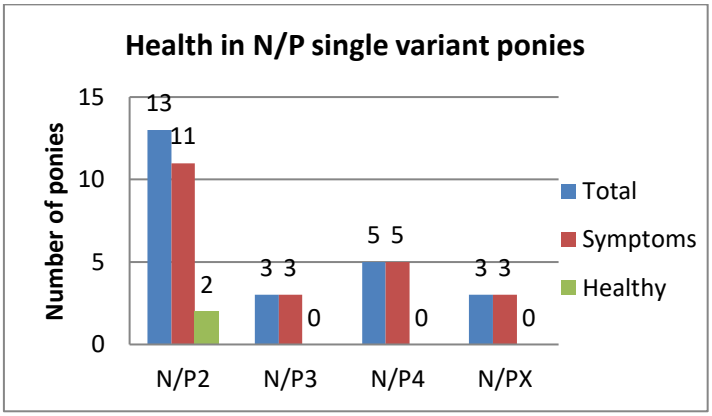

Figure 6. Ponies tested heterozygous for a single PSSM2 variant only, divided by variant and health, n=24.

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 ponies in our data set tested positive with each variant, there must, on a larger scale of things, be a considerable number of most likely healthy ponies that would test positive. It is fair to assume that symptoms present in a range from insignificant (healthy) to extreme, and our study design has attracted ponies 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 ponies tested (n=38) did not find more than two 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. As our data is gathered from private pony owners, no selective design was possible. With few exceptions the tested ponies are not closely

related. At this stage, we can conclude that no single variant appears to have a greater impact on health than others. The lack of single variant tested ponies 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 P2 variant is by far the most commonly found in our sample with 28% of ponies testing heterozygous for P2, which gives an allele frequency of 0.14 (Figure 5). While this number is not representative of the breed, the incidence of this variant in the general Connemara pony population is also unlikely to be extremely low. P2 has been described as one of the less harmful variants by EquiSeq. In fact, P2 as a standalone variant may not be consequential. When combined with other variants, it may may act as an enhancer for symptoms. While all of these theories have yet to be proven by way of empirical data, EquiSeq has publicized indirect evidence in the form of test results from an ongoing study on American Standardbred horses. This breed has an extremely high incidence of P2 with an allele frequency around 0.5 (equal to about 25% N/N, 50% N/P2, 25% P2/P2). As this breed is almost exclusively bred for racing, such a high incidence of the variant would not have occurred if it had an inhibiting effect on performance. Racehorses are, however, generally very young during their careers, and symptoms which only become noticeable later may go undetected. The only other variant found at a reasonable level in the Standardbred is K1. In horses with K1 the frequency of P2 drops very noticeably, suggesting that performance is now affected. For Connemaras, these data leave the possibility that most P2 ponies would be healthy. Indeed, there must be a considerable number of likely healthy ponies with P2 to explain the high occurrence of mostly unrelated N/P2 ponies found in this study. However, of the 13 N/P2 single variant ponies in our current data, 11 are symptomatic. This could be a result of the study design, which attracts highly symptomatic ponies. Owners of severely affected ponies, for which conventional veterinary examinations have failed to explain the symptoms, are more likely to seek answers elsewhere and come into contact with our study. It could also indicate that Connemaras are not as unaffected by P2 as Standardbreds appear to be. The effect of the genetic variant associated with PSSM1 has been shown to vary across different breeds (see e.g., Zsoldos, 2019), and this is also likely to be the case with the PSSM2 variants. The symptoms reported for the P2 ponies are comparable to those reported for other symptomatic ponies in our data set. It is also possible that these P2 individuals have an additional untestable variant or that they may have experienced environmental factors at some stage of their life which enhanced the process of symptom development. With the current data no more can be concluded. We will continue to gather data and may in the future be able to provide better answers with regard to the frequency of P2 and healthy ponies with P2.

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. However, we find it relevant to address an issue pertaining to a handful of ponies for which the owners have reported severe and

recurrent tying up episodes and/or very high levels of serum CK and AST. These ponies have tested N/N for the PSSM2 variants (n=4) or N/PX (n=1). As previously mentioned, the PX variant is on its own unlikely to impact on a pony'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 PSSM2 test panel, but these symptoms have, so far, rarely been reported by owners of Connemara ponies that have the P2, P3 and/or P4 genes. The high incidence of ponies with tying up and heightened CK/AST levels in the group of symptomatic ponies testing negative (n=12) is therefore striking: 33% in this group display these clinical signs. Since tying up is frequently seen in relation with PSSM1, it was particularly relevant to rule out this diagnosis as a potential explanation for these ponies' issues. Two of the ponies have tested N/N for PSSM1 whereas a third one has a confirmed PSSM2 diagnosis by biopsy. PSSM1 results are still pending for the fourth pony in this group, however, given the low occurrence of the PSSM1 mutation in our data set as a whole, a negative test result is the most likely outcome. Although the PSSM2 test did not provide any helpful answers for the owners of these particular symptomatic ponies, 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 PSSM2, but rather points towards a subtype of PSSM2 whose genetic foundation has yet to be discovered. What our admittedly very limited data might suggest, then, is that the PSSM2 test, in its current form, is not optimal as a diagnostic tool for Connemara ponies that display these particular symptoms. We are only aware of a few Connemaras with recurrent episodes of tying up at present, but we will continue to observe this issue as our database of tested ponies grows. An ongoing study by researchers in the UK will hopefully also provide further insight on the matter: specifically investigating Connemaras with clinical signs of tying up, the study aims to identify genetic risk factors associated with RER (see Royal Veterinary College, 2019).

Multiple variants

A claim made by the company developing the genetic test for PSSM2 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 ponies with multiple variants is too small. A total of eight ponies with multiple variants have been found. Of these, six are closely related. At the very beginning of the data collection process, a symptomatic pony with the N/P2 and N/P4 genotype was found. Consequent testing of the pony's deceased sire revealed that he had both variants. This revelation, which was publicized by the last owner of the stallion, resulted in targeted testing of another nine offspring. Test results proved that the stallion had both variants on one chromosome: five offspring have tested all N/N and the other five have inherited both N/P2 and N/P4. All but the original symptomatic pony are healthy. They are, however, also relatively young (average 7.5 years) and to our knowledge only one of

the N/P2 N/P4 ponies is currently being ridden. One of the N/P2 N/P4 offspring has an additional variant inherited from the dam. This pony is the only one found with three variants. The pony is healthy but only three years old. The stallion himself lived to old age but did not have optimal performance results in his teenage years and was rarely ridden the last 10 years of his life. We have, however, not been able to link this specifically to PSSM, and this pony has therefore been categorized as healthy. The two remaining unrelated ponies with two variants are both highly symptomatic.

The only reason we have the current number of ponies with multiple variants is the targeted testing for a chromosome with two variants. Such a chromosome is unlikely to be common in the breed and we have not seen other signs of it. In order to have such a "double chromosome", the stallion must have inherited both genes from one of either the sire or dam. That pony may, however, have had the variants on separate chromosomes, with the recombination occurring only in the stallion himself. With the current data it was not possible to track these variants further.

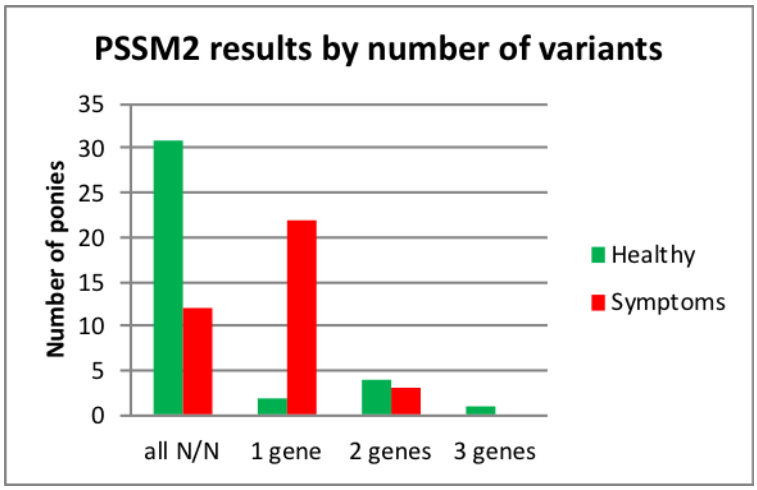

Figure 7. The health status compared to the PSSM2 test results and divided by the number of variants found, n=75.

Ponies homozygous for the variants

With 75 ponies tested for PSSM2, none were homozygous for any variant. None of the variants are embryonically lethal when homozygous. The proposed theory is that homozygous horses are among the most severely affected, more so than horses with multiple different variants. As previously mentioned, if the test is applied to breeding decisions, the recommendation is to avoid combinations which can result in homozygous offspring. The lack of homozygous ponies in our data is noteworthy. Roughly half of the tested ponies are symptomatic (n=37). Despite a method of data collection which favours identification of severely affected ponies, of which 68% tested positive for one or more variants, no homozygous ponies 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 any. Homozygous ponies 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.

PSSM2 and age

In order to further investigate the connection between the PSSM2 test results and health, the data was divided by age into three groups. A total of 75 ponies have been tested for PSSM2. The youngest age group was set to 0-5 years (n=10). In this group it is likely ponies are too young to show symptoms. The middle group was set to 6-12 years (n=38). At this age ponies should be healthy athletes and the contrast to ponies with issues is the greatest. Most cases of PSSM2 will become noticeable at this age. The last group consists of ponies aged 13 and above (n=27). This group will show more general "wear and tear" and the probability of issues caused by non-PSSM2 related ailments is increased. While PSSM2 symptoms are likely to be noticeable if present, they may also be dismissed as "wear and tear" and therefore not investigated thoroughly.

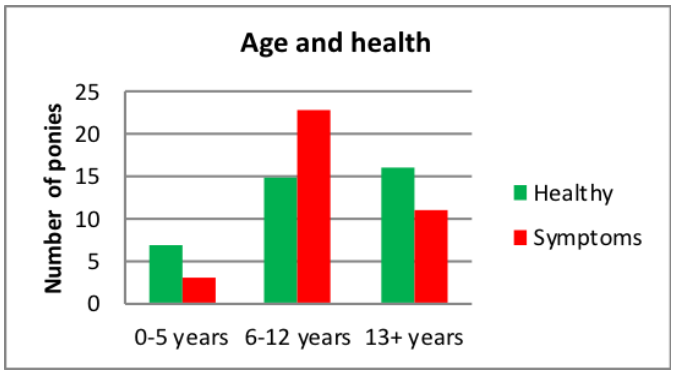

Figure 8. The health status compared to the age group of PSSM2 tested ponies. Test results are not considered in this figure.

The highest number of symptomatic ponies was found in the middle age group (figure 8). These are typically ridden ponies that have either never quite performed as expected, or they have had a few good years after which their performance inexplicably deteriorated. When dividing data further by test results (figure 9), it becomes noticeable that the majority of these symptomatic ponies test positive. It also becomes very noticeable that among the older, healthy ponies, a very high number test negative (88%). Only two older ponies with variant(s) are categorized as healthy. Both are stallions. The potential impact of hormones delaying the onset of symptoms in a stallion has been discussed previously in this report. Of the two older Connemaras with variants categorized as healthy, one had some issues which could not be linked specifically to PSSM. The other pony had a long and successful performance career and was only tested due to a symptomatic progeny testing positive. As both ponies are now deceased, from reasons reportedly unrelated to PSSM, no further observations are possible.

Unlike the younger groups, the group of older symptomatic ponies shows an almost equal number of ponies testing negative and positive (Figure 9). As previously noted, we have no way of verifying that these

symptomatic ponies actually have PSSM2. It is likely, however, that this pattern is due to age related physical decline.

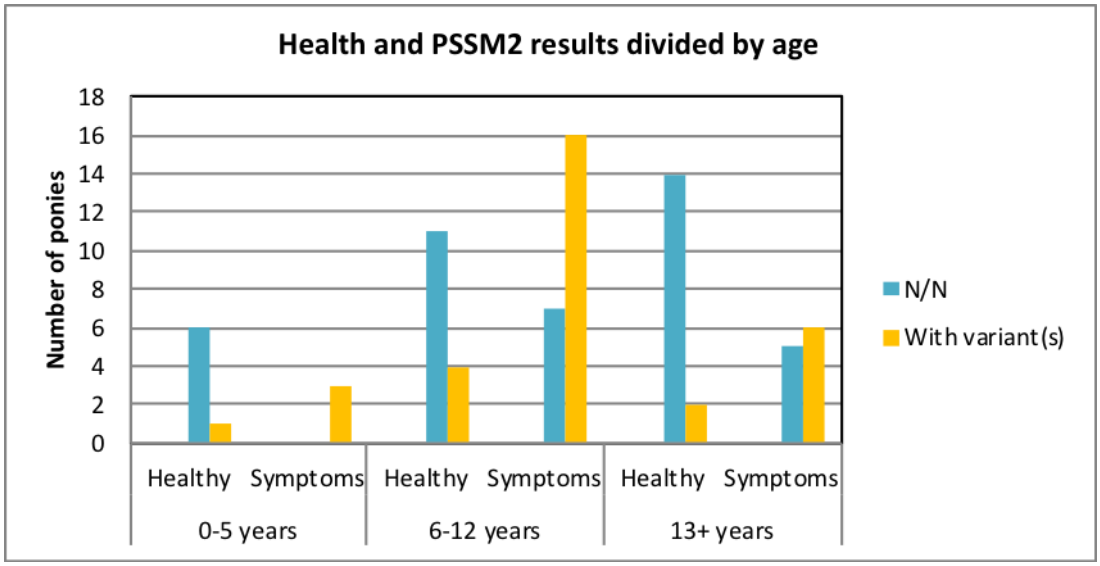

Figure 9. The health status compared to the PSSM2 test results and divided by age. The number of testable variants is not considered in this figure.

Pedigree studies

Any attempt to track the genes beyond a single generation has been unsuccessful. The complete lack of homozygous ponies makes tracking the variants very difficult. With the exception of some targeted testing following positive test results which have been made public by owners, the tested ponies are generally not closely related. Assuming pedigrees are accurate for a minimum of two to three generations, the number of ponies that are at least heterozygous for a variant cannot be insignificant. While we do have some working theories, these continually change and so far none can be proven. The majority of tested ponies are ridden ponies that do not have any progeny. For those with variants, we can only guess which side of the pedigree the gene came from, and even that is no guarantee of the other side being negative. At this stage, unless a pony 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 PX mutation, these ponies (n=5) are about as unrelated as possible within the breed. They have nothing in common in the first three generations, with only famous (common) names showing repeats in the backbreeding. Those repeats are unlikely to be the source of the PX variant, as it would have been found in much higher numbers if that were the case. The scientific method used to test for the variants is commonly used and can be presumed to be accurate. At this stage, the lack of patterns for the less common variants does suggest the possibility that pedigrees are more than just slightly inaccurate in the backbreeding. 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 article 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.

For PSSM1 the allele frequency has been estimated to be <0.01 with no known affected active breeding lines. As such, systematic testing of breeding stock is not necessary. Testing of all symptomatic ponies is still recommended.

For PSSM2 we have shown a significant correlation between health 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 ponies with multiple variants have increased probability and severity of symptom development, which is a cornerstone of the proposed breeding application of the test. Too little is known about the frequency of healthy ponies testing positive as well as why some ponies develop symptoms and others do not. We have found evidence of at least one unknown and untestable variable in the breed which causes severe PSSM2 with tying up episodes. 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 multigenetic disease like PSSM2. Targeted reduction of the known variants may cause an increased occurrence of as of yet unidentified undesirable genes. 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 ponies for breeding. There is a genetic element to this disease, and many others, and we have had several owners report problems which have been passed on.

This study will continue and an updated version of this article 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 pony then please share the results with our research group.

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