Validation of the Dysgen Hip Dysplasia DNA test in the Danish population of Labrador Retrievers.

Main supervisor: Professor Ph.D., Dr.Vet.Sci., Merete Fredholm. Department of Veterinary Clinical and Animal Science, University of Copenhagen.
Co-supervisor: Specialekonsulent, Ph.D, Helle Friis Proschowsky. Dansk Kennel Klub
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Name of department: Department of Veterinary Clinical and Animal Science, University of Copenhagen.

Authors: Anna Bank (rfd395)  
Fredriksbergsgata 7, 212 11 Malmö, Sweden.  
Anna Ström (qnc917)  
Skräddarebyn 5A, 218 42 Bunkeflostrand, Sweden.

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Academic advisors: Main supervisor: Professor Ph.D., Dr.Vet.Sci. Merete Fredholm. Department of Veterinary Clinical and Animal Science, University of Copenhagen.  
Co-supervisor: Specialekonsulent, Ph.D, Helle Friis Proschowsky. Dansk Kennel Klub

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Anna Bank rfd395

Anna Ström qnc917
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Abstract

Canine Hip Dysplasia is a widely known orthopedic disease, affecting all types of dogs all around the world. It is a big problem among the large and giant breeds, such as Labrador retriever, the studied breed in this paper. Canine Hip Dysplasia, or CHD, is characterized by abnormal development and laxity of the coxofemoral joint, which lead to variable degrees of clinical discomfort, in juvenile dogs (definition 5-12 months) the pain is due to joint laxity leading to stretching of the soft tissue surrounding the joint and abrasion of the articular cartilage, which eventually exposes the pain sensors located in the subchondral bone. In older dogs the pain is mostly present as a result of secondary osteoarthritic changes.

The specific causes of CHD are to this day not completely understood but it has been accepted that the manifestation of the disease occurs in genetically predisposed animals exposed to environmental factors that enhance expression of the genetic weakness. For many years the goal has been to minimize the prevalence of the disease in troubled breeds and today most countries diagnoses CHD using phenotypic radiographs at 1 or 2 years of age and asses the risk of CHD in every individual based on the phenotype. Thereafter dogs with bad hips are excluded from the genetic pool and only offspring from lower risk parents are allowed to breed. Even though a lot of effort has been put into breeding programs like this, the results are not convincing and the disease is still prevalent. Better methods of diagnosing or methods complimenting todays procedure to diagnose CHD is necessary.

Many scientists have in recent years tried to map out the dogs genome and thereafter to connect specific genes to CHD and other diseases. Through extensive efforts by researchers at Bioiberica in collaboration with the University of Barcelona and Progenica Inc it has been possible to recognize 7 specific SNPs linked directly to the development of canine hip dysplasia in Labrador retrievers and on the basis of these SNPs they have developed a genetic test, where a single blood sample should be able to predict the individuals probability for developing canine hip dysplasia throughout life. Dysgen sections dogs into 4 different categories by estimated risk for developing CHD sometime during life. The estimate is given in a percentage and specific recommendations and restrictions are tailored for each group.

The intention with this article is to validate the Dysgen test in the Danish population of Labrador retrievers and to see if there is a correlation between the individual Labradors Dysgen status and their earlier phenotypic radiographic grade. The validation trial consists of a total of 51 Danish Labrador Retrievers. DNA was extracted from blood samples and sent to Bioiberica in Spain for the Dysgen analysis. The results were then compared to the phenotypic FCI radiographic scores. The correlation was evaluated using several statistical analyses with a chi-square in focus.
Contrary to the previous validation trial no correlation between Dysgen genetic score and phenotypic radiographic grade could be supported with any statistical evidence using a 95% confidence interval.

The prediction value of DNA is increasingly appreciated and the field is constantly being refined and under constant development, which is promising for the future.
Resume

Höftledsdysplasi, HD, hos hundar är en välkänd ortopedisk sjukdom. HD kan drabba hundar av alla sorter men är framförallt ett problem hos stora och gigantiska raser därribland Labrador retrivern som vi fokuserat på i detta arbete. Höftledsdysplasi är karakteriserad av en onormal utveckling av höfterna och en slapphet i den coxofemorala leden, vilket leder till olika grader av besvär hos hundar i olika åldrar. Hos unga hundar (ca 5-12 månader) beror smärtan främst på onormal töjning och sträckning av mjuka strukturer kring leden och på abrasion av ledbrocket vilket leder till exponering av underliggande småreceptorer. Hos äldre hundar beror smärtan förknippad med HD framförallt på sekundära osteoarthritiska förändringar.

De specifika orsakerna till HD hos hundar är idag inte fullkomligt förklarade, men man har accepterat teorin om att sjukdomen uppkommer hos genetiskt predisponerade hundar som utsätts för vissa miljöfaktorer under livet. I många år har rasklubbar I samarbete med veterinärer och forskare arbetat mot målet att minska prevalens av HD i populationer där sjukdomen är ett stort problem. Detta har gjorts genom att höftledsröntga alla hundar vid 1 eller 2 års ålder, och sedan med hjälp av fenotypen bedöma individernas risk att utveckla HD. Hundar med dåliga höfter exkluderas från aveln och endast hundar med låg risk får användas till avel. Även om mycket tid och energi har lagts på avelarbete som detta är sjukdomen fortfarande prevalent inom många raser. Bättre metoder att diagnostisera och förutsäga vilka hundar som kommer föra höftledsdysplasin vidare alternativt metoder som kan komplettera den fenotypiska bedömningen som görs idag är nödvändigt att utveckla.

Under senare åren har många forskare arbetat för att kartlägga hundens genom, och sedan för att finna specifika gener som är inblandade i HD och i andra sjukdomar. Genom omfattande forskning har man vid Bioiberica I samarbete med the University of Barcelona och Progenica Inc funnit 7 specifika SNPs som är direkt kopplade till utvecklingen av höftledsdysplasi hos Labradorer. Dessa 7 SNPs har sedan används för att utveckla ett test som på enda blodprov ska kunna förutsäga individens risk att utveckla HD under livet. Dysgen fördelar hundar i 4 olika kategorier efter den estimerade risken att de kommer utveckla HD under livet. Estimatet ges i procent och specifika rekommendationer och restriktioner finns för varje riskgrupp.

Syftet med denna artikel är att validera Dysgen HD test i den Danska populationen av Labradorer och undersöka om det finns någon korrelation mellan det genetiska testresultatet och den fenotypiska röntgendiagnosen. I försöket ingick 51 Danska labradorer. Deras DNA extraherades ur blodprover och skickades till Bioiberica i Spanien för att analyseras med Dysgen testet. Resultaten jämfördes sedan med hundarnas röntgenscore och korrelation utvärderades med hjälp av flera statistiska analyser med fokus på en chi-square analys. I motsats till föregående valideringsförsök
kunde ingen korrelation mellan den genetiska diagnosen och den fenotypiska röntgendiagnosen hittas med 95% statistiskt konfidensintervall. Att använda genetiska tester för att diagnostisera sjukdomar är ett relativt nytt men mycket användbart område som utvecklas snabbt vilket är lovande för framtiden.
Foreword and acknowledgements

This veterinary master’s thesis was written as part of the Veterinary Medicine master program at the Faculty of Health and Medical Science at Copenhagen University in Denmark. The project was carried out in collaboration with the inventors of the Dysgen genetic blood test, Bioiberica and the University of Barcelona and Progenica Inc, as well as the academic advisors Merete Fredholm and Helle Friis Proschowsky.

This thesis targets veterinarians working with small animals, breeders, dog owners and producers and analyzers of the Dysgen test.

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List of abbreviations

- BCS – Body Condition Score
- BVA - British Veterinary Association
- CCL - Cranial cruciate ligament
- CHD – Canine Hip Dysplasia
- CS – Chondroitin sulfate
- DKK – Dansk Kennel Klub (The Danish Kennel Club)
- DI – Distraction Index
- DJD – Degenerative Joint Disease
- EBV – Estimated breeding value
- FCI - Fédération Cynologique Internationale
- GWAS – Genome Wide Association Study
- HD – Hüftledsdysplasi
- NA - Norberg Angle
- OA – Osteoarthrosis
- OFA - Orthopedic Foundation for Animals
- QTL – Quantitative trait locus/loci
- ROM – Range Of Motion
- SNP – Single nucleotide polymorphism
- TBV – True breeding value
Theoretical background

Hip Dysplasia in dogs

Canine Hip Dysplasia (CHD) is an orthopedic disease that is characterized by abnormal development and laxity of the coxofemoral joint, which lead to variable degrees of clinical discomfort. It can affect any breed of dog but is most commonly reported in large and giant breeds, such as Rottweilers, German Shepherds, Golden Retrievers, Saint Bernards and Labrador Retrievers (Smith et al 2012). The disease was first recognized in the middle of 1930 by Gerry Schnelle and was reported in the American kennel Gazette as the new disease (Schnelle 1935). Back then, it was thought that a young dog diagnosed with a dysplastic hip definitely would develop an incapacitating osteoarthritis, leading to an early death. Today it is recognized as one of the most common orthopedic diseases of large and giant breed dogs and many treatment options are available. The diagnosis of CHD is important both when it comes to selective breeding and to decide on the best treatment approach for the individual. For many years breeding programs based on individual phenotypes have been used but since the prevalence has not declined vigorously, it is an ineffective process and a selection procedure based on breeding value estimation, later referred to as EBV, is more often recommended. Some breeds have a prevalence of hip dysplasia up to 70% of screened individuals (Bartolome et al. 2015; Paster et al 2005; OFA ranking). In Labrador Retrievers, the study population in this paper, a prevalence of 18.5% is announced by OFA, the Orthopedic Foundation for Animals, putting the breed in the 91st place out of 173 on their current hip dysplasia ranking.

Despite over 70 years of meticulous investigation the diagnosis and treatment of Canine Hip Dysplasia still remain controversial.

Pathogenesis

The disease is thought to be inherited as a complex polygenic trait, where different environmental factors such as weight and exercise can remodel the gene expression in the individual. The estimated heritability is 0.2-0.6 depending on study population (Marcias et al The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). Many pathogenic theories have been proposed but now days it is agreed that the disease is biomechanical, meaning that the laxity of the hip joint eventually results in subluxation and secondary OA changes (Carlson et al 2012; Fossum et al Diseases of the joints, in, Small Animal Surgery). During weight bearing, the femoral head subluxates out of the acetabulum which changes the forces acting on the cartilage and bone and focuses the pressure to the medial aspect of the femoral head and dorsal rim of the acetabulum.
which in young puppies results in a delayed ossification process in these areas (Alexander et al. 1992; Fries et al. 1995) and contrariwise speeding the ossification in areas with less pressure, ventromedial acetabulum and lateral femoral head (Brass 1989; Fries et al. 1995). This results in a convex dorsal rim and shallower acetabulum (Alexander et al. 1992; Fries et al. 1995). The abnormal weight bearing that develops due to the skeletal changes in the pelvis leads to multiple micro fractures in the subchondral bone in the areas with increased pressure, the dorsal acetabular rim and femoral head. With the following healing of these micro fractures, the bone gets thicker and harder which leads to a decrease in capacity to absorb shocks. The increased wear and tear at these same areas leads to degeneration of the cartilage and eventually exposure of the subchondral bone (Fries et al. 1995; Brass 1989). This is defined as OA, osteoarthritis and the beginning of a cycle of degenerative joint disease.

![Figure 1; Showing normal hips, dysplastic hips and dysplastic hips with OA](image)

It is thought that no lesions are present at birth (Carlson et al. 2012; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders; Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics; Riser et al. 1966), and the first signs develop around the age of 2 weeks with stretching of the joint capsule and ligament of the femoral head (Alexander et al. 1992; Shepherd et al. 1986; Fries et al. 1995; Riser et al. Hip Dysplasia, in Textbook of Small Animal Orthopedics). At 4 weeks fair proliferative, nonsuppurative synovitis with edema and fibroplasia of the ligaments of the femoral head and joint effusion are present (Fries et al. 1995; Riser et al. Hip Dysplasia, in Textbook of Small Animal Orthopedics). The palpable or clinical Ortolani (will be explained further down in this text) sign is noticeable at 4 to 8 weeks of age and the Bardens (will be explained further down in this text) should optimally be tested on
puppies 8 to 9 weeks old (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics). The first radiographic changes can be identified at 7 weeks of age as delayed ossification of the craniodorsal rim of the acetabulum (Carlson et al 2012; Riser et al. Hip Dysplasia, in Textbook of Small Animal Orthopedics) and at 12 weeks it is possible to see changes in both the synovium and the articular cartilage (Lust et al 1981). From day 60 to 90 the degree of subluxation increases and significant radiographic changes are evident. Gross pathology at that time reveals stretching and thickening of the ligaments and joint capsule which enables the femoral head to be displaced laterally, and in the most severe cases dorso-laterally (Smith et al 2012).

Riser et al reported in 1967 a positive correlation between pelvic muscle mass and prevalence of hip dysplasia. The study showed an evident conjunction between low muscle strength of the pelvic muscles, rapid weight gain in older dogs, rapid growth in puppies and the development of CHD. It was also demonstrated that the muscle mass of dysplastic breeds, like the German Shepherd, were less than that of nondysplastic breeds like the Greyhound (Riser et al 1967).

It has been proposed that hormones play a role in the development (Steinetz et al 1987). Estrogen and relaxin both contribute to the relaxation of the pelvic and coxofemoral ligaments, which is an important precursor to partum, but a study by Hassinger et al in 1997 showed no coherence between hormone concentrations and hip laxity (measured by DI and NA) during a normal estrous cycle in 9 bitches. A positive relation between high levels of relaxin and prevalence of hip dysplasia was found in Labs correlated to Beagles (Steinetz et al 1987).

Culprits such as a lack of collagen I and pectineal muscle myopathy were suggested earlier, but they are now regarded as disclaimed (Madsen et al 1997; Cardinet et al 1974). The most compelling theories are the ones associated with an increased synovial fluid volume, leading to early laxity in puppies (Smith et al 1990), however it is hard to completely disregard the fact that it could just be a consequence to the subluxation, i.e an effect, not an causal factor (Wallace et al 1987). This so-called theorized hip-joint capsule mechanoreceptor feedback loop implies that the low intracapsular pressure generates an early invagination and thereby stretching of the joint capsule during swing phase subluxation (see further down). This stretching of the capsule in its turn triggers the mechano receptors to enroll the appropriate periarticular muscles, which counteract the femoral head from luxation out of the acetabulum (Smith et al 1990; Smith et al 2012).

\[1 \text{ Distraction index and Norberg angle}\]
The conclusion is that the specific causes of CHD are to this day not completely understood but it has been accepted that the manifestation of the disease phenotype occurs in genetically predisposed animals exposed to environmental factors that enhance expression of the genetic weakness (Smith et al 2012). However, there are some indications that onset of the disease has a linear progression over time (Smith et al 2006, Smith et al 2012), meaning the risk is equally prevalent throughout the dogs life span. That statement is controversial, because today most countries diagnoses CHD using phenotypic radiographs at 1 or 2 years of age and by saying that the risk for developing hip dysplasia is just as high in grown and geriatric dogs as it is in juveniles, it invalidates this diagnostic procedure to some extent.

The pain associated with hip dysplasia is in juvenile dogs (definition 5-12 months) due to to joint laxaity leading to stretching of the soft tissue surrounding the joint and causes abrasion of the articular cartilage, which eventually exposes the pain fibers located in the subchondral bone and (Fossum et al, Diseases of the joints; in, Small Animal Surgery; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders; Rieser et al 1985). In older dogs the pain is mostly coherent with the secondary OA changes. (Fossum et al, Diseases of the joints; in, Small Animal Surgery; Smith et al Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders)

Dogs with a lower body condition score (BCS) due to a restricted diet have a significantly later onset of both clinical and radiographic lesions and signs of hip dysplasia and DJD (Smith et al 2012). A study by Smith et al 2012 showed that Labradors over 1 year of age and fed ad libitum had a tendency to weigh 25% more than dogs fed a restricted diet. That same study concludes that Labs fed a restricted diet lived 1.8 years longer than dogs fed ad libitum. Other factors that are related to canine hip dysplasia are rapid weight gain, excessive nutritional intake and mild repeated trauma. The latter causes synovial inflammation which leads to an increased volume of joint fluid and revoke the natural joint stability (Fossum et al, Diseases of the joints; in, Small Animal Surgery)

Signs and symptoms

A distinction is often made between dysplasia in juvenile and mature dogs. That is because the pathogenesis differs between the two; young individuals have problems with primary laxity and older individuals with secondary OA (Fossum et al, Diseases of the joints; in, Small Animal Surgery).

Young dogs and puppies often show exercise intolerance, difficulty rising, bunny-hopping and intermittent or continual unilateral or bilateral lameness (Fossum et al, Diseases of the joints; in,
Small Animal Surgery, Smith et al, Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). In addition to these symptoms, older dogs often develop DJD and muscle atrophy and eventually show signs of problem with gait (Fossum et al, Diseases of the joints; in, Small Animal Surgery; Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). One often sees bilateral muscle hypertrophy on the front legs due to compensation and conscious weight changes from the hind legs by extension of both the tarsal and stifle joints alongside with a stiff and short stridden gait (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal).

The most common symptom of canine hip dysplasia, regardless of age, is exercise intolerance (Fossum et al, Diseases of the joints; in, Small Animal Surgery).

For individuals with functional subluxation, it is not known if the hip tends to luxate during the weight bearing phase or during the swing bearing phase. There are several mechanical justifications for the latter; when the leg is weight bearing the big muscles responsible for propulsion are acting\(^2\) and because of the way they are oriented (opposite to each other) the femoral head is reduced into the acetabulum as they co-contract (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). During the swing phase, other, more parallel-orientated muscles are working\(^3\), releasing a more vertical force on the hip and therefore making it more prone for subluxation (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). There are also histopathological evidence of that statement, as the wear and tear are focused on the areas subject to swing forces, not weight bearing powers (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal).

When examined, young dogs show signs of pain related to extension, external rotation and abduction of the hip joint, and sometimes poorly developed hind muscles. If further investigated under general anesthesia, increased laxity is expected (Fossum et al, Diseases of the joints; in, Small Animal Surgery, Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). As the dog gets older, scar tissue forms around the joint, 

\(^2\) Muscles responsible for weight baring and locomotion; gluteus, adductor magnus et brevis, biceps femoris, semimembranosus and semitendinosus.

\(^3\) Muscles working during swing phase rectus femoris, sartorious and iliopsoas.
preventing it from luxating. That is why clinical findings in older dogs most often do not include laxity of the hip joint but instead reduced ROM and possibly crepitation. Pain during hip extension and muscle atrophy are common signs (Fossum et al, Diseases of the joints; in, Small Animal Surgery).

**Diagnostics**

**Clinical signs**

The clinical examination should always start with a gentle palpation of the muscles surrounding the pelvis (Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). In young animals the next step includes flexion of the hind limb into a position where the knee is ventral to the abdomen, followed by an abduction where the knee is pulled out from the body in a flexed positioning. If that motion is associated with crepitation or if ROM differs from the normal 160 degrees⁴, laxity or discomfort in the hip joint should be suspected (Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). In more mature animals, at risk of developing OA, the examination should include that same flexion of the hind limb, with the limb just ventral to the abdomen, then with proximal pressure force the caput in to the acetabulum at the same time as the leg is abducted in a flexed position. In addition, the hind legs should be simultaneously extended backwards, this motion stretches the pelvic and if dolens is obtained during any of these exercises the hip joint should once again be suspected as the origin of pain (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). In the chronic cases, the gluteal muscles should be palpated and the degree of atrophy should be assessed (Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). It is important to understand that clinical signs of CHD and radiographic findings do not always correspond and the diagnosis should be based upon several things; age, breed, history, clinical symptoms and findings and radiographic results (Fossum et al, Diseases of the joints; in, Small Animal Surgery).

There are 3 clinical tests that can help diagnose Canine Hip Dysplasia, they are the Ortolani, the Barlow and the Barden tests. They can be performed on a conscious animal but can be very painful

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⁴ Range of motion is often limited by pain and periarticular fibrosis.
and best outcome is obtained when the dog is heavily sedated (Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). The Barlow test is the first part of the Ortolani where the dog is placed in either dorsal or a lateral recumbence and the hind leg is in an adducted position. The clinician places one hand on the distal aspect of the stifle and the other hand on dorsum and with a gentle proximal force can luxate the hip dorsally. The second part is the so-called Ortolani part, where the hip is to be reduced back in to the acetabulum again by abducting the leg. A “clunk”-sound or a noticeable luxation and reduction of the hip is considered a positive Ortolani sign and is suggestive of a coxofemoral laxity (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders; Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics).

The Barden involves having the dog in lateral recumbence and putting the femur in a position where it is perpendicular to the plane of the pelvis. One hand is placed on the great trochanter of the proximal femur and the other hand is placed medial to the femur and with that a lateral forced without abduction of the leg and any movement of the greater trochanter more than 0,6 cm (1/4 inch) will result in a positive Barden sign and be indicative for hip laxity (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). However, these tests are not to stand alone in diagnosing hip dysplasia. Studies have shown that these palpation techniques have both low sensitivity and specificity when compared to radiographic imaging (Powers et al 2005).

It is important to rule out other differential diagnoses before attributing certain symptoms like lameness to hip dysplasia. In young animals those could be neurological problems or other orthopedic diseases like panosteitis, hypertrophic osteodystrophy, physeal separation, partial cranial cruciate ligament (CCL) injury, septic arthritis, myopathies, myasthenia gravis and osteochondritis (Fossum et al, Diseases of the joints; in, Small Animal Surgery; Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). One study compiled in 2005 by Powers et al showed that 32% of dogs referred to hospitals for treatment of CHD really had signs attributed to CCL rupture.

In older dogs it is important to differentiate between pain originating from L7-S1 and the pelvic joints, as well as the neurological problem cauda equina and orthopedic diseases like CCL rupture, neoplasia and polyarthritis (Fossum et al, Diseases of the joints, in, Small Animal Surgery; Hedberg ortopedi in Vademecum; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders).
Radiographic signs

Today, the diagnosis is based on a ventrodorsal radiograph of the pelvis, with the dog sedated or under light anesthesia to enable correct positioning. The dog should be placed on its back with the rear legs fully extended, femurs parallel and almost pronated to both each other as well as to the spine, the patellas centered or superimposed to the trochlear groove and the pelvis completely symmetrical (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics).

The dog is considered as dysplastic when the femoral head conforms poorly within the acetabulum, with or without signs of secondary OA changes (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics). A study by Jessen and Spurrel in 1972 shows a 16-32% success rate when using the phenotypic x-ray evaluation at 6 months of age, meaning that the dogs examined were correctly diagnosed as dysplastic at that time when compared to radiographs taken at 5 years of age. That success rate skyrockets to 92-95% at 2 years of age. The later statement is the reason why the OFA has limited their screening age to a minimum 2 years in the US (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). The Jessen and Spurrel study however abruptly stops at the age of 5 years, but a study by Smith et al in 2012 followed a group of Labradors for life and determined that the prevalence of OA changes increases linearly throughout life, which undermines the Jessen et Spurrel study greatly. Others, such as the Australian, British and our own European system has a minimum age set at 1
year for the screening, which has resulted in a minimum error of diagnosis of 30% (Smith et al., Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). This method has been the golden standard for 50 years but is being increasingly questioned since it is not a normal position for the dog and the hind limb extension actually tightens the joint capsule and the surrounding muscles, which can result in a false negative phenotypic result. The method also reflects an opinion since the radiographs are evaluated somewhat subjectively (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics).

**Ultrasound signs**

Ultrasoundographic evaluations have been found to have a high rate of false-positive diagnoses in human neonates (Smith et al., Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). Because of the femoral head ossification occurring at around 8 weeks of age ultrasound is precluded as a diagnostic tool for assessing morphology and integrity of the joint in older puppies and dogs. Dynamic ultrasound is usable in dogs 8-16 weeks old for assessing laxity but is mainly based on subjective scoring since there are no implemented reference ranges with which to compare (Smith et al., Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). A study by Fischer et al in 2010 shows no correlation between ultrasonic assessment between 16 and 49 days and phenotypic radiographic signs at 1 and 2 years of age. The conclusion that follows is that ultrasound should not routinely be used as a screening or a diagnostic tool for Canine Hip Dysplasia (Smith et al., Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal).

**Computed tomography and Magnetic resonance imaging signs**

In human medicine both CT and MRI are being used more frequently when assessing immature and mature hips, but few studies have been done on dogs. One of the advantages of the MRI is the ability to assess the amount of synovial fluid within the joint (Smith et al., Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal) but a study by Ginja et al. in 2009 shows bad correlation between hip joint laxity at 7-9 weeks compared to laxity later in life. The procedures are both expensive and time-consuming but could possibly be improved in the future.

**Prevention**

Preventative measures are taken both on a superior and genetic level, using breeding, as well as at an individual dog level. Since CHD is polygenetic trait, also known as a quantitative trait, it has by definition both a genetic and an environmental aspect and by manipulating the latter it is possible to
decrease risk or delay the clinical symptoms of hip dysplasia. Smith et al compiled a lifetime cohort study in 2012 using 48 Labradors, where they focused on several x-factors, one of them being weight in relation to CHD. The study divided the dogs into 2 groups, one receiving restricted feeding and the other group getting fed ad libitum and the results where conspicuous; the group getting ad libitum showed signs of dysplasia 6 years earlier than their littermates with lower BCS. Earlier it was believed that mega doses of ascorbic acid, popularly referred to as Vitamin C, counteracted scurvy\(^5\) and therefore it was given to pregnant bitches and puppies during their first 2 years of life (Belfield 1976). However, overdoses of ascorbic acid can lead to hypocalcaemia, which paradoxically causes delayed bone remodeling and cartilage maturation (Hazewinkel \textit{et al} 1985). Current studies has shown that by injecting puppies intramuscularly with polysulfated glucosaminoglycan twice a week at ages between 6 weeks and 8 months it is possible to limit the severity of hip dysplasia by reducing subluxation scores and histopathologic evidence of arthritis at 8 months of age, compared to untreated littermates (Lust \textit{et al} 1992). Other hot topics being discussed are the use of pharmaceuticals, nutraceuticals, omega 3 fatty acid diets and specific training, including rehabilitation regimens, but longitudinal cohort trials on these strategies are currently lacking but could potentially open new and interesting doors in the future (Smith \textit{et al}, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal).

**Treatment**

When deciding on a treatment plan for the individual patient it is very important to include the patients physical status, activity, environment and the owners expectations. According to Bergh and Budsberg 2014, there is no optimal treatment and therapeutic recommendations are often made up by personal preference and comfort level.

\(^5\) A deficiency disorder, due to lack of Vitamin C resulting in improper collagen formation, resulting in defective bone formation.
Medical

A medical, non-surgical approach is often recommended to patients with mild signs or initially when the problems occur as a first course of action. This conservative approach can be divided in to three major categories; nutritional, exercise modified physical therapy and pharmacologic management (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal).

The nutritional part focuses on supplementing with omega-3 fatty acids and to promote weight loss, the latter having a huge impact on clinical signs (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). As mentioned earlier in this article, Smith et al did in 2012 a study using Labradors and got convincing results that dogs with a lower BCS due to a restricted diet has a significantly later onset of both clinical and radiographic lesions and signs of hip dysplasia and DJD. A precursor to the Smith study concluded that predisposed growing individuals fed a restricted diet had 50% less risk of showing radiographic signs at 2 years of age, than individuals fed ad libitum (Kealy et al 1992). A follow-up study showed that

predisposed animals had less progression of secondary OA when fed a restricted diet, compared to dogs with free access to food 8 years into the disease (Kealy et al 1997).

Non-steroidal anti-inflammatory drugs or NSAIDs are a group of drugs that inhibit cyclooxygenase enzyme(s) in the body for the purpose of relieving pain, reducing fever and suppressing inflammation. In the CHD treatment the main purpose is to relieve the pain originating from the secondary OA changes in the joint or joints. Studies have shown that aspirin have some protective effect against the breakdown of articular cartilage (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics). When using NSAIDs long-term it is important to be aware of the adverse effects associated with the drugs, such as GI irritation, renal and liver intoxication and disturbed coagulation (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics).

A specific exercise regimen should be designed individually. Even though exercise and movement should be kept to the minimum in the acute and painful phases, low impact fitness such as swimming and walks, can help in the long term disease process. Such exercise strengthens muscles, increases ROM and even alleviates pain (Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders).

Corticosteroids are used similarly to NSAIDs, for relief of pain of secondary OA changes and for inflammation within the joint. When injected intra-articular the noxious effects on the articular cartilage are well documented (Altman 1989; Mankin, 1974; Mankin, et al 1966) even after a single injection. One study (Mankin, et al 1966) done on rabbits shows that a single injection with an intra-articular corticosteroid inhibits the synthesis of proteoglycans to the point where it resembles chondromalacia, in literature often referred to as charcot arthropathy (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics). Septic arthritis is another possible complication when using local corticosteroids. Intra-articular corticosteroids, like Depo-medrol should not be for longtime use, and should be viewed as a treatment of last resort when surgery is not an option. The lowest effective dose should always be used (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics).

Glycosaminoglycans have several positive local results on the joint cartilage. It has a chondroprotective effect, retards the catabolic process, decreases inflammation and increases synovial fluid viscosity. When used, it is crucial that there is viable articular cartilage present for these effects to be practicable (Olmstead, Non fracture related orthopedic problems, in, Small Animal Orthopedics).
Surgical

For younger, skeletally immature dogs the main purpose is prophylactic prevention of clinical signs or to prevent and decrease secondary OA changes. For older individuals with mature bones the reason for treatment is pain relief by replacing the joint, or parts of it and thereby reducing the osteoarthritis (Smith et al, Surgical Therapy of Canine Hip Dysplasia, in, Veterinary Surgery Small Animal). In young dogs the surgical options are juvenile pubic symphysiodesis and triple pelvic osteotomy. In dogs with mature skeletons the alternatives are total hip arthroplasty and femoral head and neck ostectomy. However, in the Bergh and Budsberg article from 2014 no surgical treatment was concluded to be superior, meaning that no procedure could consistently allow a return to normal function in dogs with diagnosed CHD.

Juvenile pubic symphysiodesis is an option for young dogs 11-16 weeks and is considered minimally invasive. A pubic symphysiodesis involves premature closure of the growth plate using thermal destruction, which allows for an increased ventral rotation of the acetabulum and thereby a reduced laxity in the joint (Smith et al, Surgical Therapy of Canine Hip Dysplasia, in, Veterinary Surgery Small Animal).

Pelvic osteotomy, or triple pelvic osteotomy is recommended for dogs under 1 year of age and involves the pelvis being surgically cut in three different places and thereby changing the angles and letting the acetabulum fall deeper into the fossae. The surgery is proffered when the dog has minimal osteoarthritis and should therefore be considered as a prophylactic treatment. (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal).

Contrary to the TPO mentioned above, the femoral head and neck ostectomy is a salvage procedure where the main goal is to eliminate pain from laxity in the immature dog or secondary OA in the older mature patient. It can theoretically be performed in any dog but best outcomes will be obtained with patients weighing less than 20 kilograms. The purpose is to eliminate bone to bone contact and permit the formation of a pseudoarthrosis, compiled out of dense fibrous connective tissue, lined by a synovial membrane, which will completely eliminate the problems associated with the previous CHD. (Smith et al, Surgical Therapy of Canine Hip Dysplasia, in, Veterinary Surgery Small Animal).

Total hip arthroplasty is also considered as a salvage procedure and involves replacing the ill femoral head and acetabulum with manufactured implants and hopefully bring the dog back to near normal function, including athletic standard. The technique is best suited for mature individuals of large and giant breeds (Smith et al, Surgical Therapy of Canine Hip Dysplasia, in, Veterinary Surgery Small Animal).
**Hip dysplasia and genetics**

It has been established many times that canine hip dysplasia is a multifactorial disease. Both genetic and environmental factors play a role in the development and the severity of the disease (Wilson *et al.* 2011).

**Screening systems and eradication of CHD**

Programs to control and decrease the prevalence of CHD by selective breeding in popular dog breeds have been used for decades. The aim of control schemes like these is to increase canine health and welfare by reducing the prevalence of CHD in a long term and to minimize the severity of the disease in affected individuals. The main way of doing this has been by estimating the severity of CHD on some radiographic traits associated to CHD pathology, as described below, and thereafter exclude the most severely affected individuals from breeding programs (Wilson *et al.* 2011). By selecting breeding animals on a phenotypic basis, we assume that CHD phenotype is strongly associated with genes predisposing or protecting against CHD. Before we have mapped and identified all contributing genes in a breed, phenotypic screening of individual dogs is the best way we have to assess and select for or against a disease (L. Zhu *et al.* 2009).

**X-ray evaluation**

There are three different models commonly use worldwide to assess the degree of CHD in individual dogs, the FCI (Fédération Cynologique Internationale) model, the OFA (Orthopedic Foundation for Animals) model, and the BVA/KC (British Veterinary Association/The Kennel Club) model. Common for all methods is that the evaluations are performed on a ventrodorsal radiograph of the hip, taken while the animal is deeply sedated. The hind legs are extended caudally so that the femurs are parallel to the spine, in line with each other and the patella is centered over the femoral bone (Fluckinger, 2007).

The FCI model is used in most European countries, South America and Asia and uses the letter A (No sign of hip dysplasia) to E (Severe hip dysplasia) to score the degree of dysplastic changes. The score is then based on the shape and depth of the acetabulum, the degree of subluxation, the Norberg angle (NA) and the level of secondary osteoarthritic changes. The dog has to be over one year old before the screening and if the dog is older the severity of secondary changes to the joint is evaluated in relation to the age of the dog (Fluckinger, 2007).

The OFA model is only used in USA and Canada and dogs has to be over two years to be scored. Normal hips are scored as either excellent, good or fair while dysplastic hips are scored as mild CHD, moderate CHD or severe CHD. There is also a seventh score called borderline, used for hips
that are neither normal nor dysplastic. The OFA model does not use the NA and is mostly focusing on the shape of the femoral head in relation to the acetabulum, the presence of secondary changes and the degree of subluxation of the joint (Fluckinger, 2007).

The BVA/KC model is used in England, Ireland, Australia and New Zealand and each joint is evaluated at 9 criteria and each criterion gets a point from 0 to 6 where 6 is the worst. The points are then added up and both hips together get a total score of 0-106 points. The properties evaluated are NA, subluxation of the joint, the shape and depth of the acetabulum and signs of secondary changes to the joint (Fluckinger, 2007).

**Distraction index**

The distraction index, or DI, is a number ranging from 0 to 1 that describes the maximum hip joint laxity. A DI of for example 0.43 means that 43% of the femoral head is displaced from the acetabulum in its most lax position. PennHIP is a commercially available method developed by Antech Imaging Services which is used under deep sedation to put the hips in a position of maximum laxity. A radiograph is taken and the DI is measured. It is described as a quantitative way to measure hip joint laxity and gives each hip measured an index (a number) instead of more subjectively assigned category like the above described x-ray methods (www.pennhip.org/). DI was found to be the most significantly correlated risk factor for development of CHD-associated OA in a study performed on 4349 dogs of 4 large dog breeds (Runge et al. 2010). Another study found that DI and weight was significant risk factors for CHD-associated OA in 4 other popular dog breeds (Smith et al. 1990).

**EBV, estimated breeding value**

When examining the phenotype of a specific trait (CHD) in an individual and giving them a score, we are actually making an estimate of the animals breeding value for that trait based on one or more selection criteria (the phenotype in this case). Unfortunately, in most complex genetic diseases it is not that simple, and the estimated breeding value (EBV) we get from scoring only one individual on its phenotype is often far from its true breeding value. Collecting information from all available relatives of the specific individual can therefore be helpful when trying to estimate a breeding value as close to the TBV as possible. So when estimating a dogs breeding value when selecting against CHD the hip score of the individual is put together with the hip scores of siblings, parents and offspring’s. Genes that might be inherited but not expressed in the screened individual’s phenotype can therefore be corrected for and we get a more accurate estimate of the true breeding value (Nicholas, Selection within population; in, Introduction to veterinary genetics). When we calculate EBV using information from relatives we can possibly also correct for more aspects influencing the
single dogs phenotypic score such as measurement errors and difference between radiographic examiners and the technicians taking the radiographs and positioning the dogs (Wilson et al 2011). We can also correct for the age of the dog at screening (Wood et al 2003). It has also been proposed that one of the advantages with using EBV for CHD is that in a population where screening of dogs for the disease is voluntarily (which is usually the case, unless it is a population used for research only) we can estimate breeding values for individuals that was never screened (Wilson et al 2011). Moreover, as the genetics underlying CHD are being discovered it is fairly simple to incorporate results of a genetic test in to the estimation of breeding values. Individual EBVs can therefore be more and more accurately calculated on both phenotype and genotype (Zhu et al 2009, Wilson et al 2011).

Effectiveness and results
The effect of these selective breeding programs based on phenotypic traits have been evaluated by several authors, Wilson et al. (2006) compiled the results of these studies and found some reporting little or no improvement while others reported favorable phenotypical or genetic trends. They discuss that the results are not surprising, since the section pressure and dedication by breeders play a major role. From the study they conclude that genetic improvement by phenotypic selection against CHD is possible, but not guaranteed only because a control scheme is in use. Studies suggest that there is big room for improvement if estimated breeding values calculated on the both the individual and its relatives were added to the control schemes instead of only using phenotypic traits of only one individual when choosing breeding animals (Zhu et al 2009; Hou et al 2010; Lewis et al 2010). Some authors has also in the past few years suggested in their studies that selective control schemes against CHD pathology based on genetic information instead of or combined with phenotype are better than schemes based on phenotypic selection alone (Guo et al 2011; Sanchez-Molano 2013).

Searching for the genes behind CHD
CHD is a problem in many dog breeds, and the prevalence is still high after several decades of selection against it. Research indicates that CHD is a complex genetic trait, or a quantitative trait, if we were to identify the specific mutations that contribute to the development of CHD our understanding of the disease and the biochemical changes that lead to disease development we could also develop more effective control schemes (Zhu et al. 2009).

A genetic study with microsatellites published in 2005 led to the identification of quantitative trait loci for CHD by crossbreeding CHD free greyhounds and dysplastic Labrador retrievers (Todhunter et al 2005). A quantitative trait locus (QTL) is a chromosomal region harboring one or a group of
genes with influence on phenotypic expression of a quantitative or multifactorial genetic trait, for example CHD, and the biggest challenge with these QTL is to identify the individual genes in each locus (Zhu et al. 2009). The chances of finding a QTL when present depends on many factors such as size and effect of the QTL, frequency of the QTL alleles, trait heritability, method of analysis, sample size and degree of variation in the population. Found QTLs can therefore differ between studies. One major locus contributing to CHD development was located though, in several different studies (Leighton 1997; Todhunter et al. 2003; Janutta et al. 2006). A QTL is considered major if it contributes to 20% of the total phenotypic variance (Zhu et al. 2009). After finding a QTL the next step is to identify the underlying genes and find mutations therein. This is not always an easy process, however since 2005 there is a map of over 2.5 million characterized SNPs available, and the whole dog genome is sequenced (Lindblad-Toh et al. 2005). This breakthrough made it possible to develop new genotyping tools, and paved the way for further research and localisation of specific genes behind multifactorial diseases such as CHD.

Using SNP chips makes the genotyping a lot faster than with the previous microsatellite methods, and allows mapping of specific genes in much smaller families (Bahlo et al. 2006). Association mapping can thereafter be used to statistically link a specific SNPs with a phenotypic trait or disease. If unrelated individuals with the same phenotype (in this case, show signs of CHD) share a specific SNP, and the same SNP is not present in unaffected individuals, the gene affected by that SNP becomes a candidate gene. Found candidate genes are thereafter screened to see if they are somehow connected to the development of CHD. Genes involved in bone, cartilage and joint formation and health, fibrous connective tissue are obviously of importance but all other genes somehow connected to CHD, or genes of unknown function must also be considered (Zhu et al. 2009; Goldsmith et al. 1994).

Several studies trying to map specific SNPs as genetic markers for CHD had been published lately. The results seem to reveal useful markers both in specific breeds (mostly German Shepherd and Labrador Retriever) and in the general dog population. The most extensive study was published this year, and using a genome wide high density SNP genotyping microarray studying 170 000 SNPs throughout the dog genome, they found 7 SNPs that are said to predict hip dysplasia development in Labrador Retrievers (Bartolome et al. 2015).

**Dysgen Hip Dysplasia DNA test**

Researchers at Bioiberica have in collaboration with the University of Barcelona and Progenica Inc. developed a genetic test, where a single blood sample can predict the individual's probability for
developing canine hip dysplasia. One of the huge advantages with the test is that it is not dependent on age, meaning that it can be tested already on juveniles. The test has an official accuracy of 85%, a mean sensitivity of 80% and mean specificity of 78% (Bioberica). When conjugated with the phenotypic results from x-rays those numbers increase and can then be applied in breeding programs or registries to reduce the incidence of the trait.

It is important to clarify that the test does not diagnose CHD but the genetic traits associated with the disease; the presence or absence of 7 single nucleotide polymorphisms, which, as earlier mentioned, have been linked to hip dysplasia in Labrador Retrievers (Bartolome et al. 2015).

**Theory and development of the test**

775 Labrador Retrievers were initially recruited for the development of the test. A 1 ml EDTA blood sample and a ventro-dorsal hip radiograph were taken from each individual. According to the dogs FCI CHD score they got divided into 2 separate groups; Control group (scoring A/B) and Dysplasia group (scoring D/E). Dogs scoring a C were simply excluded. The studied dogs differed in age, see table 1 and 2.

The study population was compiled of 240 Labrador Retrievers and out of those individuals a genetic characterization was made using a genome wide association study (GWAS) to locate SNPs. Another five dogs fell through due to missing genotypes making the final population 235 dogs. This genetic characterization enabled a case-control population association study. Another 114 dogs were afterwards tested with the final product and used to validate the test.

<table>
<thead>
<tr>
<th>Development cohort</th>
<th>FCI grade</th>
<th>n</th>
<th>Mean age ± SD</th>
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<tbody>
<tr>
<td>Controls</td>
<td>A</td>
<td>99</td>
<td>34 ± 18</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>30</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>Cases</td>
<td>D</td>
<td>64</td>
<td>37 ± 21</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>47</td>
<td>45 ± 28</td>
</tr>
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</table>

Table 1: Distribution of FCI grades in development trial.

<table>
<thead>
<tr>
<th>Validation cohort</th>
<th>FCI grade</th>
<th>n</th>
<th>Mean age ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>A</td>
<td>44</td>
<td>21 ± 12</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>14</td>
<td>64 ± 24</td>
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<tr>
<td>Cases</td>
<td>D</td>
<td>26</td>
<td>48 ± 37</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>30</td>
<td>48 ± 48</td>
</tr>
</tbody>
</table>

Table 2: Distribution of FCI grades in validation trial.

A GWAS was performed using the Canine HD Genotyping BeadChip (Illumina) that assays 172,155 SNPs per sample. In the candidate gene study, 768 custom SNPs in candidate genes and quantitative trait loci (QTL) for CHD were genotyped with the Illumina Golden Gate genotyping
platform. Initially 250 markers were identified as correlateable with CHD, but the final product, a mathematical model, was assembled out of the 7 most consistently matching SNPs, using a logistic regression. This model was then validated using an independent population of 114 dogs, showing an accuracy of 85% (Bartolome et al. 2015).

**Test results**

Dysgen sections dogs into 4 different categories and thereby gives an estimated risk for developing CHD sometime during life. The estimate is given in a percentage and specific recommendations and restrictions are tailored for each group; Group 1 with minimum predisposition has an estimated 3% risk for developing CHD, which is 6.7 times less than the average of the general population\(^6\).

Even though there is a minimum risk of developing the disease, it is important to keep the dog lean and to promote moderate exercise. A clinical examination and supplementary radiographic study should be performed at 8 months of age. In dogs with special genetic value, making the dog further predisposed, the owner is encouraged to perform the OFA x-ray at 24 months. When it comes to breeding it is always preferable to breed with other animals out of this same group but no restrictions apply.

Group 2 with low predisposition has an estimated risk of 16 %, which is almost equivalent to the average of the general population. Despite the low risk of developing CHD, preventing the animal from becoming overweight and advocating moderate exercise, mainly focused on the hind legs, are important. No high intensive exercise before 9-10 months and good, well composed food for the growing individual is preferred. A clinical examination and supplementary radiographic study should be performed already at 6 months of age. In dogs with special genetic value, making the dog further predisposed, the owner is encouraged to perform the OFA x-ray at 24 months. Advise to breed with dogs from minimum or low predisposition.

Group 3 with a medium predisposition, 45% risk for developing canine hip dysplasia, corresponding with 2.3 times the average risk for the general population of Labradors. General preventive measures:

\(^6\) The average of the general population is set to 20% in Labrador Retrievers.
Due to the medium risk of developing CHP, it is very important to prevent this dog from becoming overweight. Avoid intense exercise and promote moderate exercise to improve ROM, maintain cartilage in good condition and increase muscle tone. Appropriate type of food should be chosen for the growing animal and supplementation of products that promote joint health is recommended.

A clinical examination and supplementary radiographic study should be performed already at 6 months of age. If signs of CHD is found, an orthopedic surgeon should be consulted. If no symptoms of CHD is present, the dog should still be followed up regularly during life. Dogs in this category should not be allowed to breed.

**Group 4** with a high predisposition >67% risk of developing canine hip dysplasia. The recommendations for dogs in this group correlates with what is described above for group 3 dogs, but even more restricted regarding exercise and nutrition.

An example of the test result is attached in appendix as attachment 6.

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Figur 4: Illustration borrowed from Bioibericas homepage, showing the different Dysgen categories just as in the testresults. (http://veterinary.bioiberica.com/Joint_Health/Dysgen/Pets.html)

**Specific genes linked to HD in Labrador Retrievers**

As mentioned, seven SNPs are used in the Dysgen Hip Dysplasia DNA test. The surrounding chromosomal region of the found SNPs were run through the Ensemble database (www.ensemble.org) in hope of finding them located close to genes with potential effect on CHD. Three of the SNPs were found to be connected to protein coding genes associated with the extra
cellular matrix synthesis. Some of the SNPs were located in the actual protein coding gene, and others were located up or downstream to the gene, more likely having an effect as a part of a transcription regulatory element. SNP BICF2P772455 was found located upstream the start codon of the CHST3 gene while the SNP BICF2G630339806 was found close to the CHSY1 gene. Both the CHSY1 and CHST3 genes are coding for enzymes with important effects on the biosynthesis of chondroitin sulfate (CS). CS is a component of the extracellular matrix with important effect on cartilage function (Bartolome et al. 2015). In humans, a congenital development disorder of the skeleton has been linked to mutations in the CHST3 gene (Hermanns et al. 2008) and mutations in another gene in the same family as CHST3 have been linked to hip OA also in humans (Zeggini et al. 2012). The same SNP (BICF2G630339806) was also located close to the ADAMTS17, mutations in genes in the ADAMTS family have been associated with metabolic bone diseases, such as osteoporosis and OA. The last of the extracellular matrix related SNPs found was the BICF2S230609 SNP, located close to a gene called fibroblast growth factor 4, the FGF4 gene (Bartolome et al. 2015). Members of the FGF gene family are thought to be involved in embryonic development, tissue repair, production of extracellular matrix and limb bud outgrowth (Boulet et al. 2004). Like the ADAMTS17, there are two more genes found close to the suggested SNPs that are associated with bone metabolism. The BICF2G630227898 SNP is found upstream of the RAB7A gene and the BICF2S2452559 SNP downstream the PKCE gene. Both genes seem to somehow be connected with bone remodeling, growth and osteoclast activity. One SNP, BICF2G630558239, seems on the other hand to be related to muscle mass and muscle atrophy rather than bone or extracellular matrix. It is found upstream of the SMYD3 gene. This gene could also have an impact on CHD, but the authors state that further research is needed (Bartolome et al. 2015). Only for one SNP, the BICF2P548082, no association to any known gene was found.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Nearby coding gene</th>
<th>Gene associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>BICF2P772455</td>
<td>CHST3</td>
<td>CS biosynthesis</td>
</tr>
<tr>
<td>BICF2G630339806</td>
<td>CHSY1</td>
<td>CS biosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADAMTS17</td>
</tr>
<tr>
<td>BICF2S230609</td>
<td>FGF4</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>BICF2G630227898</td>
<td>RAB7A</td>
<td>Osteoclast activity</td>
</tr>
<tr>
<td>BICF2S2452559</td>
<td>PKCE</td>
<td>Osteoclast activity</td>
</tr>
<tr>
<td>BICF2G630558239</td>
<td>SMYD3</td>
<td>Muscle mass and atrophy</td>
</tr>
<tr>
<td>BICF2P548082</td>
<td>No known</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Different genes associated with the 7 SNPs in the Dysgen test.
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Validation of the Dysgen Hip Dysplasia DNA test in the Danish population of Labrador Retrievers.

Abstract

Canine hip dysplasia is a common and well known orthopedic disease usually predicted by phenotypic radiographic assessment and prevented by selecting breeding dogs based on that assessment. Eradication of the disease is slow, and many years after breeding programs was started the disease is still prevalent, better systems are needed to single out dogs giving offspring with high risk of developing CHD. Through extensive research, by Bartolome et al 2015, it has been possible to recognize 7 specific SNPs linked directly to the development of canine hip dysplasia and on the basis of these SNPs, researchers at Bioiberica in collaboration with the University of Barcelona and Progenica Inc. developed a genetic test, where a single blood sample should be able to predict the individuals probability for developing canine hip dysplasia throughout life. The purpose of this trial was to validate the Dysgen CHD genetic test in the Danish population of Labrador Retrievers. A total of 51 dogs with a registered radiographic hip score were recruited and blood samples were collected from each individual. DNA was then purified from the samples using a simple salting out procedure and the samples were sent off to the producers of the Dysgen test in Spain where the test was conducted. Based on the results the correlation between clinical radiographic diagnoses and DNA test diagnoses was calculated using a chi-square test. In this trial no significant statistical evidence could be found, supporting the predictive value of the test. A lot more research is needed to develop an optimal method to predict and prevent canine hip dysplasia in the future.

Key words: Canine hip dysplasia, Labrador Retriever, orthopedic disease, phenotypic radiograph, selective breeding, eradication, validation trial, Dysgen, DNA test, SNP.

Introduction

Hip Dysplasia

Canine hip dysplasia, later referred to as CHD is a well-known orthopedic disease involving the acetabulum and femoral head of the pelvis, resulting in coxofemoral incongruity and secondary osteoarthritis (Fossum et al, Diseases of the joints; in, Small Animal Surgery; Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery: Small Animal; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders). The affected individuals are considered normal at birth (Carlson et al 2012; Marcias et al. The Hip, in

CHD is thought to be inherited as a complex polygenic trait, with an estimated heritability of 0,2-0,6 depending on study population (Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders) and where different environmental factors such as weight and exercise can remodel the gene expression in the individual and make the development of the disease more or less plausible.

Figure 1; Showing normal hips, dysplastic hips and dysplastic hips with OA

The pain associated with hip dysplasia is in juvenile dogs (definition 5-12 months) due to the abrasion of the articular cartilage, which eventually exposes the pain fibers located in the subchondral bone and also because of the stretching of the soft tissue surrounding the joint due to joint laxity (Fossum et al, Diseases of the joints; in, Small Animal Surgery; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders; Rieser et al. 1985). In older dogs the pain is mostly coherent with the secondary OA changes. (Fossum et al, Diseases of the joints; in, Small Animal Surgery; Smith et al. 2012; Marcias et al. The Hip, in BSAVA Manual of Canine and Feline Musculoskeletal Disorders).

The disease can affect any breed but is most commonly reported in large and giant breeds, such as the study population in this article, Labrador Retrievers (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). In Labradors, a prevalence
of 18.5% is announced by the Orthopedic Foundation for Animals, ranking the breed in the 91th place out of 173 on their current hip dysplasia ranking (www.offa.org).

The CHD diagnosis is based on a ventrodorsal radiograph of the pelvis, with the dog sedated or under light anesthesia to enable correction positioning. The dog is considered as dysplastic when the femoral head conforms poorly within the acetabulum, with or without signs of secondary OA changes (Olmstead L.M., Non fracture related orthopedic problems, in, Small Animal Orthopedics). There is a demonstrated success rate of 16-32% when using the phenotypic x-ray evaluation at 6 months of age, which increases to 70% at 1 year\(^7\) and 92-95\(^8\) at 2 years of age (Smith et al, Pathogenesis, diagnosis and control of canine hip dysplasia, in, Veterinary Surgery Small Animal). Contradictory, there is also evidence suggesting that the prevalence of secondary OA changes increases linear throughout life (Smith et al 2012) which greatly undermines the accepted radiographic methods of today.

The diagnosis of CHD is important both when it comes to selective breeding and to optimize and decide on the best treatment approach for the individual. Breeding programs are based on individual phenotypes, but since the prevalence has not declined vigorously (Wilson et al 20011), it is an ineffective process and a selection procedure based on control schemes against CHD pathology composed of genetic information instead of or combined with phenotype are better than selection based on phenotypic selection alone (Guo et al. 2011; Sanchez-Molano 2013).

By crossbreeding CHD free greyhounds and dysplastic Labrador Retrievers it was possible to identify quantitative trait loci for CHD (Todhunter et al. 2005), and subsequently recognize underlying genes and find mutations therein, so-called single nucleotide polymorphism (SNPs), eventually mapping the whole dog genome (Lindblad-Toh et al. 2005). Through association mapping specific SNPs can be linked with a phenotypic trait or disaese and found candidate genes can be screened to look for any association with the development of CHD (Zhu et al. 2009; Goldsmith et al. 1994).

**Dysgen**

In early 2015 an extensive study was published that concludes that 7 specifik SNPs can predict hip dysplasia in Labrado Retreivers (Bartolome et al. 2015). With these 7 specifik SNPs researchers at

\[^7\] FCI (Fédération Cynologique Internationale) used in Europe  
\[^8\] OFA (Orthopedic Foundation for Animals)used in the US
Bioiberica in collaboration with the University of Barcelona and Progenica Inc. developed a genetic test, where a single blood sample should be able to predict the individuals probability for developing canine hip dysplasia throughout life. The test is copy rated at Idexx as Dysgen and 1ml of EDTA stabilized blood is collected and sent to the lab. One of Dysgens main advantages is that it is independent of age. It has an official accuracy of 85%, a mean sensitivity of 80% and mean specificity of 78% but when conjugated with the phenotypic results from x-rays those numbers increase and can then be applied in breeding programs or registries to reduce the incidence of the trait. It is important to clarify that the test does not diagnose CHD but the genetic traits associated with the disease (Bartolome et al. 2015).

Dysgen sections dogs into 4 different categories and thereby gives an estimated risk for developing CHD sometime during life. The estimate is given in a percentage and specific recommendations and restrictions are tailored for each group;

**Group 1** with minimum predisposition has an estimated 3% risk for developing CHD, which is 6.7 times less than the average of the general population. Even though there is a minimum risk of developing the disease, it is important to keep the dog lean and to promote moderate exercise. A clinical examination and supplementary radiographic study should be performed at 8 months of age. In dogs with special genetic value, making the dog further predisposed, the owner is encouraged to perform the OFA x-ray at 24 months. When it comes to breeding it is always preferable to breed with other animals out of this same group but no restrictions apply.

**Group 2** with low predisposition has an estimated risk of 16%, which is almost equivalent to the average of the general population. Despite the low risk of developing CHD, preventing the animal from becoming overweight and advocating moderate exercise, mainly focused on the hind legs, are important. No high intensive exercise before 9-10 months and good, well composed food for the growing individual is preferred. A clinical examination and supplementary radiographic study should be performed already at 6 months of age. In dogs with special genetic value, making the dog further predisposed, the owner is encouraged to perform the OFA x-ray at 24 months. Advise to breed with dogs from minimum or low predisposition.

**Group 3** with a medium predisposition, 45% risk for developing canine hip dysplasia, corresponding with 2.3 times the average risk for the general population of Labradors. General preventive measures: Due to the medium risk of developing CHD, it is very important to prevent

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9 The average of the general population is set to 20% in Labrador Retrievers.
this dog from becoming overweight. Avoid intense exercise and promote moderate exercise to improve ROM, maintain cartilage in good condition and increase muscle tone. Appropriate type of food should be chosen for the growing animal and supplementation of products that promote joint health is recommended. A clinical examination and supplementary radiographic study should be performed already at 6 months of age. If signs of CHD are found, an orthopedic surgeon should be consulted. If no symptoms of CHD are present, the dog should still be followed up regularly during life. Dogs in this category should not be allowed to breed.

Group 4 with a high predisposition >67% risk of developing canine hip dysplasia. The recommendations for dogs in this group correlates with what is described above for group 3 dogs, but even more restricted regarding exercise and nutrition.

**Purpose**

Our intention with the study presented in this paper is to validate the Dysgen test and to see if there is a correlation between the individual Labradors Dysgen status and their phenotypic clinical status in the Danish population of Labradors. By doing this we hope to further improve the possibility to prevent and diagnose canine hip dysplasia within the Labrador breed.

**Materials and Methods**

**Sample collection and DNA extraction**

The owners of 126\(^{10}\), Danish Labrador retrievers with a radiographic hip score registered in the Danish kennel club (DKK) were contacted and asked if they would allow their dogs to participate in the validation trial. Danish dogs are scored using the FCI model where an A hip shows no signs of hip dysplasia, a B hip is very near normal, a C hip has mild signs of hip dysplasia, a D hip has moderate signs of HD and a E hip shows severe HD (Fluckinger, 2007). EDTA stabilized blood samples were then collected from a total of 51 of the dogs\(^{11}\), with their owners consent. DNA from the samples was purified using the simple salting out method in attachment 1, appendix 1 (Miller *et al.* 1988) and the extracted DNA was then quantified using the Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and diluted to the desired concentration. The

\(^{10}\)64 with an A or B score and 62 with a C, D or E score

\(^{11}\)23 A, 3 B, 10 C, 5 D, and 10 E dogs.
samples were then sent to Progenika Biopharma \(^{12}\) for them to perform the Dysgen test on each sample.

![Figure 2: Our validation trial process, from left: a) Blood sample collection. b) DNA extraction. c-d) PCR and sequencing of the 7 SNPs. e) Test results and interpretation.](image)

**Statistical analysis**

To measure the correlation between phenotypic radiographs and Dysgen genotypes a chi-square statistical analyze was performed. As disease marker the FCI phenotypic radiograph categories were used. A and B were defined as no disease present and D and E as disease present. The Dysgen genotypes Minimal and Low were defined as no exposure in the chi-square, and the Dysgen genotypes Moderate and High were defined as exposure. This approach is the same as in the first validation trial by Bartolome et al published in 2015. To allow comparison between our trial and the first validation trial, using the same methods for statistic analysis is crucial. In attempt to get a more multifaceted interpretation of the results we used the LR+ values given to us from Bioiberica for each individual blood sample to perform an ANOVA and a boxplot, plotting the LR+ values in relation to the phenotypic radiographic scores.

When performing the statistical calculations, the Prism graph pad software was used.

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\(^{12}\) Progenica Biopharma SA, Parque Twechnologico de Bizkaia, Edificio 504, E48160 Derio-Bizkaia, Spain
Results

The results of the chi-square were unambiguous, showing no statistical significant correlation between phenotypic scores and genotypic predisposition to CHD (p-value 0.378 using a 95% confident limit). In our sample, a negative correlation trend can be suspected. A RR value of 0.585 indicates a lower risk for developing hip dysplasia when placed in categories defined as exposed, but with a p-value > 0.05 it is not possible to apply these results on the general population of Labrador Retrievers. The chi-square results can be found in Appendix as attachment 3.

In the additional ANOVA and boxplot, no correlation could be detected. The boxplot is shown in the discussion section further down. The ANOVA results can be found in Appendix as attachment 2.

Discussion

If the Dysgen Hip Dysplasia test were to significantly predict the risk of a Labrador retriever developing CHD we would expect to find a significant correlation between dysplastic phenotypic hips (D or E hips) and a high Dysgen score (3 or 4), and likewise a significant correlation between non-dysplastic hips (A or B hips) and a low Dysgen score (1 or 2). In our validation trial we found no such correlation.

Since canine hip dysplasia is a polygenic trait, we expect many genes to play a role in the development of disease. It is possible that more SNPs than the 7 that has been included in the Dysgen test together can provide sufficient risk or protection against CHD for the Dysgen include genes not to give a significant prediction alone in all cases. One can also discuss that there is a small but significant genetic difference between dogs included in the development of the Dysgen test and the Danish Labrador retriever population that prevents us from getting the same result as Bartolome et al. One thing speaking against this theory is the statement that a population containing both American, Spanish and European lineages of Labradors were used in the above mentioned study, but the possibility remains that the dogs used in the Bartolome et al. trial are not genetically representative for the Danish population. Another possibility that should be considered is that the nurture role in CHD might be very important, and dogs with a high risk gene set still can show little phenotypic signs of disease if treated right during their upbringing. It is also highly possible that the failure to show significant disease prediction with the Dysgen test are due to bias in our trial.

One great confounder in this validation trial is the fact that Bartolome et al took new phenotypical radiographs in conjunction with their field test, which we did not have enough grant to do. The fact that we early on in this text refer to studies showing evidence supporting the theory that the onset of hip dysplasia in dogs has a linear progression over time, greatly reduces the reliability of our
conclusion since a radiograph taken at 12 months of age does not necessarily mean that the dogs stay in that category throughout life. The radiographic evaluation can in some sense be viewed as a snapshot, while the genetic blood test is dynamic and prophetic about the future and the two does not always match temporally. A Dysgen result stating Moderate or High risk for CHD associated with a group A or B phenotypic hip may thereby to some extent be explained by a mismatch in time, age and development. However, the Danish kennel club is using estimated breeding value to compliment the radiographic hip score for their Labradors, and as most of the A and B scored dogs in our trial have high EBV scores calculated with good certainty, this fact gives more evidence pointing towards the validity of the phenotypic diagnoses. In appendix, attachment 5 the EBVs can be seen, unfortunately only dogs scored A or B on their radiograph had available EBVs so no comparison can be made to the D and E group.

In the Bartolome trial they used only extremes, where phenotypes A and B where classified as control and D and E were merged as case group, leaving C excluded from the trial. Because we wanted to make part of our study equivalent to theirs we had to do the same and exclude the phenotypic graded C individuals, which reduced our already slim population further and made it harder to extrapolate statistical theories to the general population. From an ethical and economical point of view it is questionable to include C individuals in either of these studies, since it from the start was decided to preclude them from any statistical analyses.

While we in our study only had a final population of 49 dogs (of which 10 were graded C hips and therefore excluded from the chi-square calculations), the Bartolome study included 142 individuals for their validation trial. The small population studied made it harder to find a significant result and makes the decision to merge the phenotypic A-E groups into one CHD free (A+B) and one CHD positive (D+E) questionable, because an ANOVA and a box-plot using all the categories and plotting them against the continuous LR+ value already given in the test results, would give a wider interpretation scale and would allow us to include the dogs with C scored hips.
Since the LR+ values are handed to us without further explanation in the test result sheets for each dog, we do not know exactly how they are calculated from the different genetic combinations and how trustworthy and discriminatory they are down to a decimal form. This may explain the exclusion from the calculations in the original (Bartolome et al 2015) validation trial.

In our study we used a cross-sectional sampling approach, which caused an uneven distribution of the individuals, fx we only have 3 dogs that scored a phenotypic B and 5 that scored a phenotypic D which results in a too small sampling population and thereby very wide confidence limits containing 1. If we had used a case-control approach instead we could easier have controlled the number of individuals sampled in each group and thereby gotten a balanced distribution and more reliable calculations.

**Conclusion**

In this study no significant statistical evidence were found to support the predictive value of the Dysgen test. However, this study could in many ways have been more extensive, fx a larger population of dogs could have been included. The technique of using SNPs as genetic markers for predicting inheritable diseases is still being refined and is under constant development which is promising for the future.
References


- Olmstead M.L., Small animal orthopedics, Non fracture orthopedic problems, Mosby. 348-390 St.Louis, 1995
Appendix

Attatchment 1

2. days DNA extraction from EDTA-blood.

1. day

1. The cooling centrifuge in B209 is turned on and set at 4° C.

In the fume hood triton lysis buffer is added to the blood samples, use gloves.

- 10 ml blood = 40 ml tritonlysisbuffer
- 5 ml blood = 20 ml tritonlysisbuffer
- 2,5 ml blood = 10 ml tritonlysisbuffer
- 1,25 ml blood = 5 ml tritonlysisbuffer

2. The samples are turned over several times and then putted in to the fridge for 30 min. During the 30 min turn each sample 3-5 times. Remember to turn on the cooling centrifuge.

3. Centrifug the samples for 15 min at 2500 rpm, 4ºc. (or 3000 rpm. If old/coagulated samples)

4. The supernatant is discarded in the beaker, and then the sink in the fume hood. Use a fume hood and wear gloves. Be aware that the pellet may be loose.

5. Add 0,9 % NaCl:

- 10 ml blood = 2 ml 0,9 % NaCl
- 5 ml blood = 1 ml 0,9 % NaCl
- 2,5 ml blood = 0,5 ml 0, 9% NaCl
- 1,25 ml blood = 0,25 ml 0, 9% NaCl

6. Vortex until the pellet is reasonably dissolved.

7. Centrifug10 min at 2500 rpm, 4ºc. (or 3000rpm if the pellet vad dissolved after the first centrifug)

8. Take pronase (20 mg / ml) from freezer and prepare pronase mixture to the number of samples you have.

9. Discard the supernatant in the sink.
Carefully pour and make sure the pellet is not dislodged by the fluid return! The pellet may be loose. If possible, the tubes should be placed upside-down on a piece of paper, to remove as much of the liquid as possible.

10. Add nuclei lysis buffer:

\[
\begin{align*}
10 \text{ ml blood} &= 3 \text{ ml} \\
5 \text{ ml blood} &= 1.5 \text{ ml} \\
2.5 \text{ ml blood} &= 0.75 \text{ ml} \\
1.25 \text{ ml blood} &= 0.375 \text{ ml}
\end{align*}
\]

11. Add pronase-mixture:

\[
\begin{align*}
10 \text{ ml blood} &= 1.1 \text{ ml} \\
5 \text{ ml blood} &= 0.55 \text{ ml} \\
2.5 \text{ ml blood} &= 0.275 \text{ ml} \\
1.25 \text{ ml blood} &= 0.1375 \text{ ml}
\end{align*}
\]

12. Shake up the pellet and set the tests on the "shake table" in B212 overnight (at room temp.)

2. day.

1. Turn on the cooling centrifuge in B209, 4°C and press "fast temp".

2. Pick up the tests from the shaking table. Make sure that the pellet is dissolved by turning the tube slowly from side to side. If there is a lump, add more pronase-mixture and let the samples stay on the shaking table fora few more hours. If the liquid is homogeneous continued on to point 3.

3. Add saturated NaCl (6M):

\[
\begin{align*}
10 \text{ ml blood} &= 1.0 \text{ ml} \\
5 \text{ ml blood} &= 0.5 \text{ ml} \\
2.5 \text{ ml blood} &= 0.25 \text{ ml} \\
1.25 \text{ ml blood} &= 0.125 \text{ ml}
\end{align*}
\]
5. Vortex the samples at full speed for 15 seconds. (should be skimming)

7. Transfer the supernatant to a new tube without getting the precipitated part with. If there is dirt on top of the liquid, the liquid is sucked up with a pipette. In that case clip the end of the tip of the P1000 so that the thick liquid can be easily transferred to new tubes.

8. DNA precipitated with 1 vol. Isopropanol, shake / swirl tube well, so the two liquids are mixed well.

9. Wrap it up on the ball by a "fishing rod", then the DNA are not too far up and not passed over into the tube with TE buffer.

**fume hood and gloves !! Waste is collected in labeled waste bottle C2!!**

9. Dissolve the DNA in a suitable amount of TE buffer (40-200uL) and use 1.5 ml tubes with screw cap.

10. Take the tubes to the bonding apparatus in the cold room overnight in the basement.

11. Measure UD and mark the pipes with concentration, date and initials.

12. Store the samples.

*Manual explaining how to perform the simple salting out method used during DNA extraction in our trial.*
Excel sheet summary of the 49 dogs included in our validation trial.

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Chi-square showing no correlation between phenotypic radiographs and genetics. Disease is defined as FCI scores D and E. Exposure is defined as Dysgen categories high and moderate.
One-way ANOVA performed in Prism Graphpad Software showing no statistical correlation (p-value >0.05) between LR+ and phenotypic radiographic score.

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### Attachment 5

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<th>EBV</th>
<th>Reliability</th>
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<td>HD_L_020</td>
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</tbody>
</table>

Excel sheet summary of dogs with phenotypic radiographic scores A and B, also given estimated breeding values and associated reliability estimates. Corresponding estimates are missing for dogs scored C, D and E.
Attachment 6

Copy for the veterinarian

Report Issue Date: 03 December 2015

- Sample ID: HD_L_001
- Sample code (Laboratory): DG_DO_151
- Requesting veterinarian: --
- Veterinary Centre: --
- Name of dog owner: --
- Dog details:
  - Name: --
  - Breed: Labrador retriever
  - Microchip No.: --
  - Tattoo ID No.: --

RESULT

Genetic predisposition of the dog to develop hip dysplasia: **LOW**

INTERPRETATION OF RESULTS

The test includes information on seven genetic markers and classifies the animals in a risk group (genetic predisposition to develop hip dysplasia), defined by the Positive Likelihood Ratio index (LPR):

<table>
<thead>
<tr>
<th>Genetic Marker</th>
<th>Patient Genotype*</th>
<th>Odds ratio† CI (95%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker 1</td>
<td>AJ (heterozygous, protective)</td>
<td>0.32 (0.13-0.77)</td>
<td>0.012</td>
</tr>
<tr>
<td>Marker 2</td>
<td>GS (homozygous, protective)</td>
<td>0.24 (0.11-0.56)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 3</td>
<td>GS (homozygous, protective)</td>
<td>0.18 (0.07-0.45)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 4</td>
<td>GG (homozygous, protective)</td>
<td>0.34 (0.17-0.71)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 5</td>
<td>AU (heterozygous, risk variant)</td>
<td>2.2 (1.20-4.3)</td>
<td>0.017</td>
</tr>
<tr>
<td>Marker 6</td>
<td>AA (homozygous, risk variant)</td>
<td>2.8 (1.50-5.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 7</td>
<td>AX (homozygous, protective)</td>
<td>0.46 (0.22-0.99)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

* The presence of the risk phenotype is highlighted in red.
† The odds ratio is defined as the ratio between the probability of the phenotype occurring in the presence of the risk variant and the probability of the phenotype occurring in the absence of the risk variant.

Genetic predisposition to develop hip dysplasia
Positive Likelihood Ratio=3.2

MINIMAL LOW MODERATE HIGH

Dysgen Test® is a trademark of Bioreerca. Worldwide patents pending.
Prognostic Information

LOW (LR+ 2.4:1) The genetic predisposition of this dog to develop hip dysplasia is similar to the average (20%) of the general population of Labrador retrievers. 16% of dogs in this risk group develop hip dysplasia.

(*)Without additional data on other possible risk factors, the probability of developing hip dysplasia in the general population of Labrador retrievers is the same as the prevalence: 20%. (Lopanrind and Balonriko, 1999. Preventive Veterinary Medicine 42, 121-131).
TECHNICAL DATA

- Prognostic information on the results of the multicentre clinical validation study on Dysgen.

For product development, 700 Labrador retrievers were recruited. Dogs with extreme phenotypes were used (grade A, B, D and E) in accordance with the FCI scale (Fédération Cynologique Internationale) for the creation of a model for predicting hip dysplasia. The product was developed in an initial population of 235 Labrador retrievers (117 grade AB and 118 grade DE) and was validated on a second independent population of 114 animals (58 grade AB y 56 grade DE) validation population). The 340 dogs had a mean age of 45 months and were representative of the main American and European lines of Labrador retriever. The result was the development of a predictive mathematical model for genetic predisposition for canine hip dysplasia that includes information on seven genetic markers.

- The risk groups or groups with genetic predisposition to develop hip dysplasia were defined based on the positive likelihood ratio (LR+), the measurement that compares the concepts of sensitivity and specificity (LR+ = sensitivity/1 specificity) (page 4 of the report):

  LR+ ≥ 8: high predisposition to develop hip dysplasia
  5 ≤ LR+ < 8: moderate predisposition to develop hip dysplasia
  2 ≤ LR+ < 5: low predisposition to develop hip dysplasia
  1 ≤ LR+ < 2: minimal predisposition to develop hip dysplasia

- Dysgen present mean sensitivity\(^1\) and specificity\(^2\) values of 86% and 79%, respectively. Below are the sensitivity and specificity values for the points of LR+ = 2, 5 y 8:

  LR+ = 2: Sensitivity 53%; Specificity 58%
  LR+ = 5: Sensitivity 70%; Specificity 88%
  LR+ = 8: Sensitivity 42%; Specificity 95%

\(^1\)Sensitivity: Proportion of dogs with hip dysplasia correctly identified by the test
\(^2\)Specificity: Proportion of dogs without hip dysplasia correctly identified by the test

- The overall interpretation of the report is at the criteria of the clinician, who will always perform an assessment with the overall context of the animal.

- DNA Extraction was performed by the IDEXX RealPCR™ Laboratory.

- Genetic analysis was carried out by Progenika Biopharma, SA.

Lourdes Oceña
Diagnostic Services Manager
Progenika Biopharma S.A.

Diego Teyjador
Manager of the Genetic Diagnostic Laboratory
Progenika Biopharma S.A.
Report Issue Date: 03 December 2015

- Sample ID: HD_I_001
- Sample code (Laboratory): DS_DG_151
- Requesting veterinarian: --
- Veterinary Centre: --
- Name of dog owner: --
- Dog details:
  - Name: --
  - Breed: Labrador retriever
  - Microchip No.: --
  - Tattoo ID No.: --

Date of receipt of sample: 05 November 2015

Type of sample: DNA
Type of analysis: Dysgen genotyping

RESULT

Genetic predisposition of the dog to develop hip dysplasia: LOW

INTERPRETATION OF RESULTS

The test includes information on seven genetic markers and classifies the animals in a risk group (genetic predisposition to develop hip dysplasia), defined by the Positive Likelihood Ratio index (LR+).

<table>
<thead>
<tr>
<th>Genetic Marker</th>
<th>Patient Genotype*</th>
<th>Odds Ratio† (95%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker 1</td>
<td>AC (heterozygous, protective)</td>
<td>0.52 (0.13-2.07)</td>
<td>0.12</td>
</tr>
<tr>
<td>Marker 2</td>
<td>GG (homozygous, protective)</td>
<td>0.24 (0.11-0.56)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 3</td>
<td>GG (homozygous, protective)</td>
<td>0.18 (0.07-0.53)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 4</td>
<td>GG (homozygous, protective)</td>
<td>0.34 (0.17-0.71)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 5</td>
<td>AA (homozygous, risk variant)</td>
<td>2.2 (1.20-4.4)</td>
<td>0.017</td>
</tr>
<tr>
<td>Marker 6</td>
<td>AA (homozygous, risk variant)</td>
<td>2.3 (1.56-7.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Marker 7</td>
<td>AA (homozygous, protective)</td>
<td>0.48 (0.22-1.09)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

* The presence of the risk genotype is highlighted in red.
† The odds ratio is defined as the ratio between the probability of the phenotype occurring in the presence of the risk variant and the probability of the phenotype occurring in the absence of the risk variant.

Genetic predisposition to develop hip dysplasia

Positive Likelihood Ratio=3.2
Prognostic information

LOW (LR+ ≥ 4.0) The genetic predisposition of this dog to develop hip dysplasia is similar to the average (20%) of the general population of Labrador retrievers. 16% of dogs in this risk group develop hip dysplasia.

(*) Without additional data on other possible risk factors, the probability of developing hip dysplasia in the general population of Labrador retrievers is the same as the prevalence: 20% (Lepannen and Salonen, 1989, Preventive Veterinary Medicine 42, 127-131).
TECHNICAL DATA

- Prognostic information on the results of the multicentre clinical validation study on Dysgen.

For product development, 700 Labrador retrievers were recruited. Dogs with extreme phenotypes were used (grade A, B, D and E) in accordance with the FCI code (Fédération Cynologique Internationale) for the creation of a model for predicting hip dysplasia. The project was developed in an initial population of 235 Labrador retrievers (127 grade AB and 108 grade DE) and was validated on a second independent population of 114 animals (56 grade AB y 56 grade DE) (validation population). The 349 dogs had a mean age of 45 months and were representative of the main American and European lines of Labrador retriever. The result was the development of a predictive mathematical model for genetic predisposition for canine hip dysplasia that includes information on seven genetic markers.

- The risk groups or groups with genetic predisposition to develop hip dysplasia were defined based on the positive likelihood ratio (LR+), the measurement that compares the concepts of sensitivity and specificity (LR+ = sensitivity / 1 - specificity) (page 1 of the report):

  1. LR+ > 28: high predisposition to develop hip dysplasia
  2. LR+ = 5: moderate predisposition to develop hip dysplasia
  3. LR+ = 2: low predisposition to develop hip dysplasia
  4. LR+ = 1: minimal predisposition to develop hip dysplasia

- Dysgen presents mean sensitivity * and specificity * values of 80% and 78%, respectively. Below are the sensitivity and specificity values for the points of LR+ = 2, 5, and 8:

  - LR+ = 2: Sensitivity 91%, Specificity 89%
  - LR+ = 5: Sensitivity 70%, Specificity 86%
  - LR+ = 8: Sensitivity 42%, Specificity 95%

  *Sensitivity: Proportion of dogs with hip dysplasia correctly identified by the test
  *Specificity: Proportion of dogs without hip dysplasia correctly identified by the test

- The overall interpretation of the report is at the criteria of the clinician, who will always perform an assessment with the overall context of the animal.

- DNA Extraction was performed by the IDEXX RealPCR™ Laboratory.

- Genetic analysis was carried out by Progenika Biopharma, S.A.
Example of test result from the Idexx Laboratorie in Spain.