University of Veterinary Medicine Hannover

Molecular genetic analysis of haemophilia A and B in several dog breeds

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My family

"No somos disparados a la existencia como una bala de fusil cuya trayectoria está absolutamente determinada. Es falso decir que lo que nos determina son las circunstancias. Al contrario, las circunstancias son el dilema ante el cual tenemos que decidirnos. Pero el que decide es nuestro carácter".

José Ortega y Gasset (1883-1955)

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LIST OF ABBREVIATIONS

a	Active form
A	Adenine
APC	Activated protein C
Ag	Antigen
Ala	Alanine
APTT	Activated partial thromboplastin time
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
bp	Base pairs
C	Cytosine
CalDAG-GEFI	Calcium-Diacylglycerol Guanine Nucleotide Exchange Factor I
^{o}C	Degree centigrade
D	Aspartic acid
ddNTPs	Dideoxynucleotide-5'-triphosphates
del	Deletion
DMSO	Dimethylsulfoxide
dNTPs	Deoxynucleoside triphosphate
EGF	Epidermal growth factor
F	Plasma coagulation factor
F	Forward
Fig.	Figure
G	Guanine
Gla	Gamma-carboxyglutamic acid domain
Gln	Glutamine
Gly	Glycine
His	Histidine
kb	Kilo base pairs
<	Less than

Leu	Leucine
Lys	Lysine
m	Month
Met	Methionine
$MgCl_2$	Magnesium chloride
µl	Microliter
µM	Micromolar
ml	Millilitre
mM	Milimolar
N	Asparagine
N	Amount
ng	Nanogram
p.	Page
PCR	Polymerase chain reaction
%	Per cent
Phe	Phenylalanine
R	Reverse
sec.	Seconds
Ser	Serine
SINE	Short interspersed nuclear element
SNP	Single nucleotide polymorphisms
T	Thymine
Ta	Annealing temperature
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Thr	Threonine
Tm	Melting temperature
T-mix	Terminator ready reaction mix
2N	2 Normandy
Trp	Tryptophan
Try	Tyrosine

UTR	Untranslated region
UV	Ultraviolet
Val	Valine
vWD	Von Willebrand’s disease
vWF	Von Willebrand factor
y	Year

1. INTRODUCTION

Canine haemophilia A and B are the most important inherited defects of secondary haemostasis. The incidence of haemophilia B is about three or four times lower than haemophilia A (BROOKS et al. 2003). These bleeding disorders are caused by an absence or dysfunction of the plasma coagulation factor VIII (FVIII) and factor IX (FIX), respectively (BROOKS 1999; STOKOL 2005), and result in delayed blood clotting (MISCHKE 2012). The haemophilic phenotype has been observed in many dog breeds and in mixed breed dogs (BROOKS 1999). Because these haemostasis disorders are inherited in an X chromosomal recessive manner, male dogs are clinically affected while the bitches act as asymptomatic carriers (MISCHKE 2012; STOKOL 2005). The severity of clinical symptoms depends on the FVIII activity and on the size and activity of the dog, with dogs exhibiting more severe signs at a defined residual factor activity when compared to humans (MISCHKE 2012).

Both disorders are caused by a mutation in the FVIII or FIX gene, respectively. The molecular basis of canine haemophilia A and B are diverse, i.e. the phenotype of both disorders is caused by mutational heterogeneity between the breeds and even within breeds (BROOKS et al. 1997, GU et al. 1999, MISCHKE 2012). The molecular defect causing haemophilia A has been identified in an Irish Setter dog colony (LOZIER et al. 2002), a German Shepherd (MISCHKE et al. 2011A), a Havanese family (MISCHKE et al. 2011B), and a mixed-breed dog colony (HOUGH et al. 2002). In contrast to haemophilia A, the underlying mutation responsible for haemophilia B has been identified in more canine breeds, such as Lhasa Apso (MAUSER et al. 1996), Airedale Terrier (SUGAHARA et al. 1996, GU et al. 1999), Labrador Retriever (SUGAHARA et al. 1996, BROOKS et al. 1997), German Wirehaired Pointer (BROOKS et al. 2003), Rhodesian Ridgeback (MISCHKE et al. 2011C), and mixed breed dogs (EVANS et al. 1989A, GU et al. 1999).

The detection of mutations responsible for haemophilic phenotypes has enabled us to create genetic tests, which are especially important for the detection of asymptomatic carriers. Detection of carriers of haemophilia is uncertain based on measurement of the concentration or activity of factors VIII or IX. The identification of the asymptomatic carriers, and consequently, the elimination from further breeding programmes is crucial to the control of

canine haemophilia (LOZIER et al. 2002; HOUGH et al. 2002; MISCHKE et al. 2011A; MISCHKE et al. 2011B).

Therefore, the objective of this genetic study was to identify the causative mutation responsible for different haemophilic patients of the Small Animal Hospital, University of Hannover (Hannover, Germany). This included a male Fila Brasileiro suffering from haemophilia B, as well as three haemophilia A patients: a Wire Haired Dachshund, a Great Dane and a Poodle mix.

2. LITERATURE

2.1 Overview of haemostasis

The haemostatic system is the physiological process, which stops bleeding after vascular damage. Three related mechanisms contribute in this process: the vascular wall, platelets and coagulation factors. Traditionally, the haemostatic system has been divided into primary (platelet thrombus) and secondary haemostasis (blood coagulation). However, these processes act simultaneously and co-ordinately to form the blood clot, which is initially composed of platelets and some fibrin, and later it develops into a cross-linked fibrin clot (GALE 2011).

The primary haemostasis represents immediate formation of the platelet plug, via interactions between the disrupted vascular wall and platelets. This mechanism involves vascular wall constriction, as well as adhesion, activation and aggregation of platelets (VARGA-SZABO et al. 2008; LÖWENBERG et al. 2010; RUMBAUT et al. 2010). This process requires the presence of the von Willebrand factor (vWF) (SAVAGE et al. 1996; DE LUCA et al. 2000), collagen (SARRATT et al. 2005) and the exposure of the platelet receptors or glycoproteins (CRUZ et al. 1993; NIESWANDT et al. 2001; CRUZ et al. 2005; MA et al 2007). When the continuity of endothelium is disrupted, platelets rapidly adhere to the site of vascular injury (RUMBAUT et al. 2010). This adhesion is mediated by the interaction between several platelet receptors, the exposed elements in the sub-endothelial matrix (mainly collagen) and the vWF, which is immobilized on the sub-endothelial matrix (VARGA-SZABO et al. 2008; GALE 2011). Once a layer of platelets has adhered to the exposed sub-endothelial matrix, the platelets are activated by several agonists such as adenosine diphosphate and collagen (RUMBAUT et al. 2010). This process is critical to recruiting more platelets at the site of vascular injury during the aggregation phase, which mainly involves the binding between two αIIbβ3 receptors on adjacent platelets and the same fibrinogen molecule (MA et al. 2007), and results in the formation of the platelet plug (RUMBAUT et al. 2010; GALE 2011). In addition, the activated platelets promote a catalytic surface for the activation of coagulation factors, and binding sites for enzyme and cofactors that participate in the known secondary haemostasis of the coagulation system (RUMBAUT et al. 2010).

The secondary haemostasis involves calcium, platelets and coagulation factors (GALE 2011), which circulate in an inactive state until they are sequentially activated through proteolysis by

an upstream factor (RAU et al. 2007) on the surface of the activated platelet (LÖWENBERG et al. 2010; RUMBAUT et al. 2010). The coordinated activity of these coagulation factors leads to the stabilization of the generated platelet plug by the deposition of a cross-linked fibrin clot at the site of injury (GALE 2011).

The clot formation is initiated when tissue factor (TF), a transmembrane glycoprotein (KASTHURI et al. 2010), binds to the active form (a) of plasma coagulation factor (F) VII (FVIIa) in blood plasma, forming the FVIIa-TF complex also known as extrinsic FXase complex (OHKUBO et al. 2010), which is the initiator of the coagulation process (KIRCHHOFER et al 1996). This complex activates FX and FIX (OSTERUD et al 1977; KASTHURI et al. 2010). FXa combines with FVa and calcium to form the prothrombinase complex, which converts prothrombin to thrombin (MANN et al. 2003; RAU et al. 2007). This early amount of thrombin initiates the amplification and propagation of the coagulation system by activating platelets and FV, FVIII, FXI and FXIII (LANE et al. 2005). FXIa also activates FIX (SMITH et al. 2008). Once FVIII is activated, FIXa interacts with it to form the intrinsic FXase complex, which leads to the activation of FX (BLOSTEIN et al. 2003), resulting in an increase greater than 96 per cent (%) of thrombin formation (MANN et al. 2003; LANE et al. 2005), which sustains the coagulation process (GAILANI 2000). After the massive thrombin generation, the soluble fibrinogen is cleaved by thrombin to generate fibrin monomers at the site of vascular injury. Consequently, these fibrin monomers spontaneously polymerise to fibrin fibres. Finally, fibrin is cross-linked by FXIIIa, resulting in the clot formation (LANE et al. 2005) (Figure (Fig.) 1, see p. 15).

The haemostasis is regulated by serine protease inhibitors, such as antithrombin, activated protein C, and tissue factor pathway inhibitor, which prevent the excessive and uncontrolled clot formation at the site of vascular injury (PIKE et al. 2005; RAU et al. 2007; KASTHURI et al. 2010).

Once the vascular damage is repaired, the fibrinolytic system is in charge of degrading the fibrin clot, thereby maintaining the vascular lumen (CESARMAN-MAUS et al. 2005).

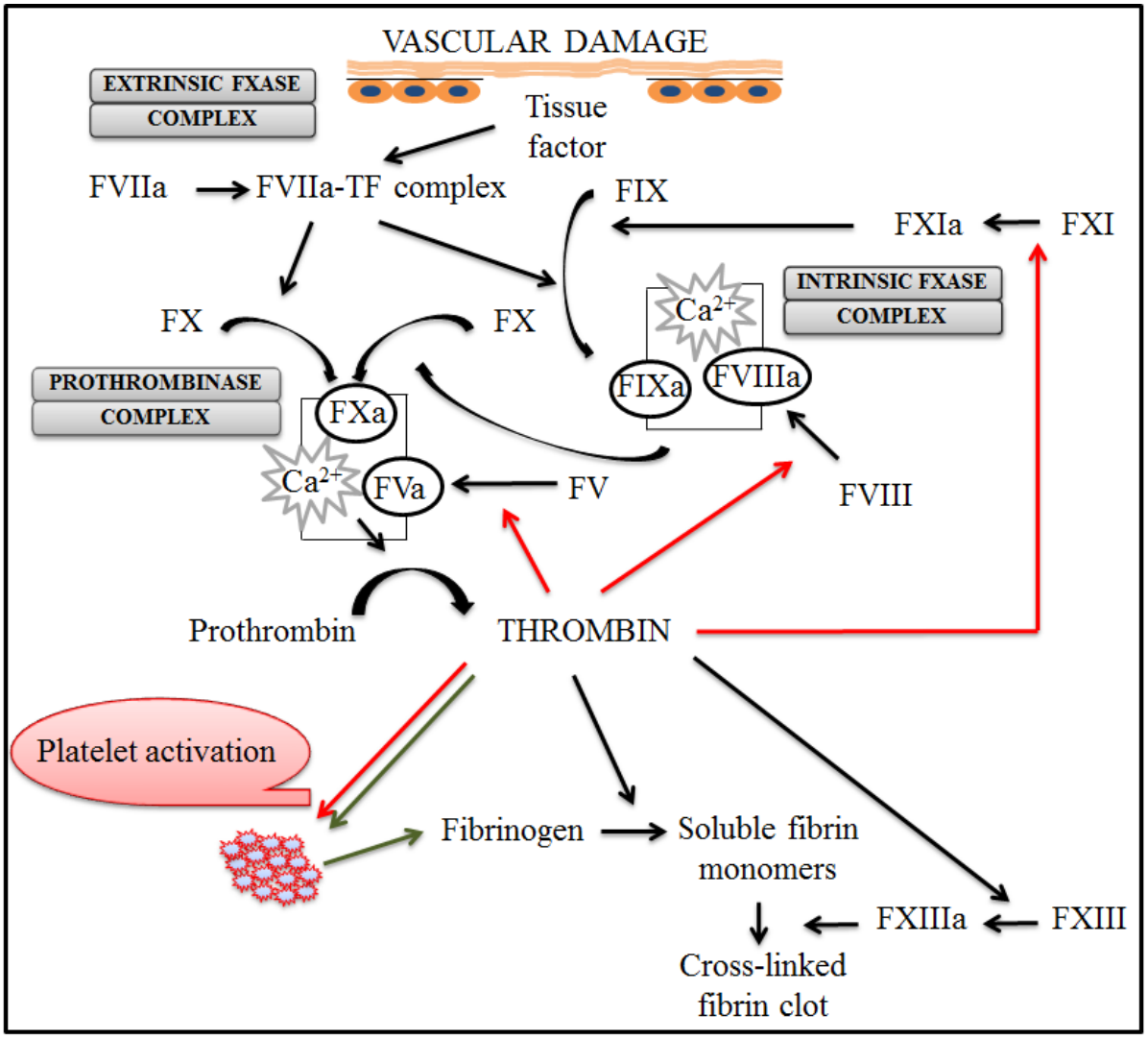

Figure 1: Schematic diagram of the coagulation cascade. *After vascular damage the exposed TF binds to FVIIa forming the FVIIa-TF complex, which activates FX and FIX. FXa combines with FVa and calcium in the known prothrombinase complex to convert prothrombin to thrombin. Consequently, the positive feedback loop by thrombin activates FV, FVIII, FXI, FXIII and platelets, resulting in an amplification of the coagulation. Finally, thrombin cleaves fibrinogen into fibrin molecules, which are cross-linked by FXIIIa. Black arrows correspond to the course of the coagulation cascade, from the initiator (FVIIa-TF complex) to the formation of the cross-linked fibrin clot. Red arrows indicate the positive feedback by thrombin. Green arrows show the link to primary haemostasis (modified from Smith et al. [2005])*

Abbreviations: Active form [a], calcium [Ca^{2+}], plasma coagulation factor [F], Tissue factor [TF]

2.2 Role of factor VIII and factor IX in the coagulation system

The importance of FVIII and FIX in the coagulation system is illustrated by the fact that low circulating levels of FVIII or FIX complicate the assembly of the intrinsic factor Xase complex and impedes the propagation phase of the coagulation system (MANN et al. 2003), resulting in X-linked disorders known as haemophilia A and B, respectively (STOKOL 2005; BROOKS 1999).

The glycoprotein FVIII circulates in plasma as an inactive, pro-cofactor form in a non-covalent complex with the vWF (NICHOLS et al. 1993; SADLER 1998; FAY 2004; FAY et al. 2005). The binding site on canine FVIII for vWF involves the amino acid residues 1666 to 1676 in the A3 domain of the canine FVIII (CAMERON et al. 1998). In dogs, vWF stabilizes the structure of FVIII in plasma. However, canine FVIII does not appear to be as dependent on vWF for stabilization as human FVIII (TURECEK et al. 1997; DENIS et al 1999). Canine vWF also protects FVIII from proteolysis by activated protein C (APC) (NICHOLS et al. 2010). These functions of vWF have been also documented in humans (KOEDAM et al. 1988; KOEDAM et al. 1990; LENTING et al. 1998; SAENKO et al. 1999; CAMIRE et al. 2009). In addition, human VWF also prolongs the half-life of the FVIII (SAENKO et al. 1999) and transports it to the site of bleeding (LACROIX-DESMAZES et al. 2008). In contrast, FIX is a vitamin K-dependent coagulation factor (MATHUR et al. 1997) that circulates as a single-chain zymogene in blood plasma (LACOBELLI 2008).

During blood coagulation, activation of FIX is catalysed in presence of calcium on the phospholipid membrane of the activated platelets. FVIIa-TF complex (OSTERUD et al. 1977; REES et al. 1988) and FXIa (FUJIKAWA et al. 1974; LINDQUIST et al. 1978; GAILANI 2000, GAILANI et al. 2001) are involved in this process. FIX appears to be the primary substrate of the FVIIa-TF complex (LU et al. 2004). FIX activation by FVIIa-TF occurs early in the course of the fibrin formation (OSTERUD et al. 1977; LAWSON et al. 1991) and it finishes when the FVIIa-TF complex is inhibited by the tissue factor pathway inhibitor (TFPI) (MONROE et al. 2007). In this situation, FXIa supplements the activation of FIX (MANN et al. 2003; SMITH et al. 2008). Activation mediated by FXIa appears to be more important in maintaining the integrity of the generated clot on the surface of the activated platelets (GAILANI 2000). In dogs, activation sites on FIX for FXIa have been located at arginine[146]

(Arg)-alanine^{147} (Ala) and Arg^{178}-valine^{179} (Val) in the activation peptide domain of the FIX molecule (AXELROD et al. 1990). In humans, this activation involves simultaneous or sequential cleavage of two identical activation sites, which are situated at Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} and result in the release of the activation peptide (SMITH et al. 2008). Factor Xa participates in the FIX activation during the first proteolytic step (cleavage at Arg^{145}) (LAWSON et al. 1991).

During the propagation of coagulation, coagulant FVIII is activated by early generated thrombin in a positive feedback loop (LANE et al. 2005). Thrombin is an extremely efficient activator of FVIII (ESMON et al. 1996). In dogs, thrombin cleaves three peptide bonds at Arg^{366}-serine^{367} (Ser), Arg^{734}-Ser^{735} and Arg^{1681}-Ser^{1682} during the FVIII activation (CAMERON et al. 1998). These cleaving sites are identical to the human FVIII, but in humans, they are situated at Arg^{372}, Arg^{740} and Arg^{1689} (EATON et al. 1986). Initially, thrombin cleaves FVIII at Arg^{740} (BOS et al. 2010), which dissociates the B domain from the rest of the molecule (PITTMAN et al. 1988). This cleavage facilitates the subsequent proteolysis at Arg^{1689} and Arg^{372} (NEWELL et al. 2007). Cleavage at Arg^{1689} results in conformational changes in the light chain of the FVIII, which lead to the dissociation of FVIII from vWF, allowing its association to the platelet membrane (HAMER et al. 1987; HILL-EUBANKS et al. 1989; LACROIX-DESMAZES et al. 2008). Cleavage at Arg^{372} is the key for the pro-cofactor activation (MYLES et al. 2002; CAMIRE et al. 2009; BOS et al. 2010). It is probably also for the exposition of a functional binding site for FIXa (FAY et al. 2001) and for the change in the FVIII conformation, which is essential for its function (CAMIRE et al. 2009; BOS et al. 2010). In addition, FXa appears to be involved in the activation of FVIII (FAY 2004). There are no reports with respect to canine FXa cleavage sites. In humans, FXa appears to bind to the same cleavage sites as thrombin (NOGAMI et al. 2002).

Once both factors are activated, the dissociated FVIIIa forms a complex with FIXa and FX on the activated platelet surface (LACROIX-DESMAZES et al. 2008). Activated platelets expose binding sites for FVIIIa (AHMAD et al. 2000), FIXa (AHMAD et al. 1989) and FX (SCANDURA et al 1996; AHMAD et al. 2003A). In this complex, known as intrinsic FXase complex (BLOSTEIN et al. 2003), the cofactor FVIIIa presents FX to the protease enzyme FIXa (AHMAD et al. 2003B). Thereby, the catalytic efficiency of FX activation is increased

by 106-fold and the dissociation constant for its interaction with FIXa, as well as the Michaelis-Menten constant for the FX is reduced (VAN DIEIJEN et al. 1981; MANN et al. 1990; FAY 2004). Thus, FVIII restricts the activation of FX to the place of vascular injury (LACROIX-DESMAZES et al. 2008). The main function of FIXa in this complex is to catalyse the proteolytic conversion of FX to FXa in the presence of calcium (VAN DIEIJEN et al. 1981; MATHUR et al. 1997) and to maintain the FX activation after the inhibition of the FVIIa/TF complex by the TFPI (SMITH et al. 2008). FIXa also stabilizes FVIIIa (LENTING et al. 1998) and protects it from APC (BERTINA et al. 1984; REGAN et al 1994). In addition, FIXa is involved in the inactivation of FVIIIa (O'BRIEN et al. 1992), together with the enzymes APC (GALE et al. 2008) and FXa (FAY 2004). Finally, the intrinsic FXase complex has been considered to be responsible for most of the FXa produced during the coagulation cascade (MANN et al. 2003), being critical for the propagation phase of coagulation at the site of vascular injury (FAY 2004).

2.3 Disorders of haemostasis

Disorders of haemostasis are associated with an abnormal function or integrity of some of the elements that contribute in the haemostatic process. These disorders can be classified as primary or secondary haemostatic disorders (STOKOL 2005). Haemostatic disorders can also be classified according to their origin, that is, acquired or inherited:

- Acquired disorders in haemostasis can affect all animals, but normally they appear in older animals and are caused by underlying diseases (STOKOL 2005).

- Inherited disorders are common in young animals with recurrent bleeding episodes or a known family history. Inherited deficiencies in primary and secondary haemostasis have been recognized in several dog breeds (BROOKS 1999; SARGAN 2011).

2.3.1 Inherited disorders of primary haemostasis

The most relevant inherited haemostatic disorder appears to be the von Willebrand's Disease (vWD). This term refers to a heterogeneous bleeding disorder of primary haemostasis, characterized by abnormalities of vWF. This disease has been divided into three categories according to its phenotype (MATTOSO et al. 2010) (Table 1, see p. 21):

- vWD type I is the most common and the less severe type. This disorder is characterized by a partial deficiency in plasma vWF (STOKOL 2005). This variant is found in high prevalence in Dobermans and in many other dog breeds (JOHNSON et al. 1988). It is inherited as an autosomal dominant trait with incomplete penetrance (DENIS et al. 1999). A single nucleotide substitution, c.7541G>A in the donor splice site of exon 43 of the vWF gene has been determined as the causative mutation for vWD type I in Doberman Pinschers and other dog breeds (VENTA et al. 2000A).

- vWD type II results in more severe bleeding than type I, and it is associated with a deficiency of the high-molecular weight multimeric form of vWF (STOKOL 2005). This rare type is transmitted as an autosomal recessive trait. In German Shorthaired Pointers and German Wirehaired Pointers, an adenine (A) to guanine (G) nucleotide substitution has been identified at nucleotide position 5041, c. 5041A>G in exon 28 of the vWF gene (KRAMER et al. 2004). In addition, a thymine (T) to G nucleotide exchange has been found at nucleotide position 1761, c.1761T>G in exon 14 of the vWF gene of German Wirehaired Pointers (LOOHUIS et al. 2004).

- vWD type III is the most severe type inherited as an autosomal recessive trait (STOKOL 2005). Three mutations have been identified in the vWF gene. In Dutch Kooikers, a c.2290+1G>A has been identified at the donor splice site of exon 16 (RIEGER et al. 1998). In Scottish Terriers, vWD type III is caused by a cytosine (C) deletion (del), c.359delC in exon 4 of vWF gene (VENTA et al. 2000B). Finally, a single deletion, c.839delT in exon 7 of vWF gene has been identified in Shetland Sheepdogs (VENTA et al. 2000A).

Platelet function disorders have also been identified in different dog breeds (Table 1, see p. 21):

- Type I Glanzmann's thrombasthenia is an autosomal recessive bleeding disorder caused by qualitative or quantitative deficiencies of the platelet membrane integrin αIIbβ3, which causes failure of platelet aggregation. Two genetic defects have been identified in the canine αIIb gene. A single nucleotide substitution, c.1193G>C, in exon 12 of the αIIb gene has been found in Otterhounds (BOUDREAUX et al. 2001). In the Great Pyrenees, a fourteen base pair insertion in exon 13 has been identified in the αIIb gene (LIPSCOMB et al. 2000).

- An intrinsic platelet function disorder in the membrane receptors has been reported in Greater Swiss Mountain dogs. In this case, a three base pairs (bp) deletion was identified in the P2Y12 gene (BOUDREAUX et al. 2011).

- In platelets, abnormalities of intracellular signal transduction or secretion have been reported in some dog breeds, in which different genetic causes have been characterized. Familial transmission is mediated via an autosomal recessive trait. In Basset Hounds, a TCT deletion in exon 5 of the Calcium-Diacylglycerol Guanine Nucleotide Exchange Factor I (CalDAG-GEFI) gene has been identified. In Eskimo Spitzes, a single adenine insertion has been detected in the exon 5 of the CalDAG-GEFI gene. In Landseers, a single nucleotide substitution from C to T has been located at nucleotide position 982 in exon 8 of CalDAG-GEFI gene (BOUDREAUX et al. 2007).

- A macro-thrombocytopenia has been diagnosed in Cavalier King Charles Spaniels. An amino acid exchange, from aspartic acid (D) to asparagine (N), D249N, has been reported as the causative mutation responsible for this disease (DAVIS et al. 2008), which is inherited as an autosomal recessive trait (PEDERSEN et al. 2002).

Further platelet disorders, such as Scott syndrome in German Shepherds and storage pool deficiency in American Cocker Spaniels, have also been reported. These disorders are inherited as autosomal recessive traits; however the molecular genetic basis has not been identified (CALLAN et al. 1995; BROOKS et al. 2010).

Table 1: Summary of references on known mutations responsible for inherited disorders of primary haemostasis

DISEASE	MUTATION	BREEDS	REFERENCE
vWD type I	c.7541G>A in exon 43	Many	VENTA et al. (2000A)
vWD type II	c.5041A>G in exon 28	German Shorthaired Pointer	KRAMER et al. (2004)
		German Wirehaired Pointer	KRAMER et al. (2004)
	c.1761T>G in exon 14	German Wirehaired Pointer	LOOHUIS et al. (2004)
vWD type IIII	c.2290+1G>A in exon 16	Dutch Kookier	RIEGER et al. (1998)
	c.359delC in exon 4	Scottish Terrier	VENTA et al. (2000B)
	c.839delT in exon 7	Shetland Sheepdog	VENTA et al. (2000A)
Glanzmann's	c.1193G>C in exon 12	Otterhound	BOUDREAUX et al. (2001)
thrombasthenia	Insertion in exon 13	Great Pyrenee	LIPSCOMB et al. (2000)
Intrinsic platelet disorder	3 base pair deletion	Greater Swiss Mountain	BOUDREAUX et al. (2011)
Signal transduction	TCT deletion in exon 5	Basset Hound	BOUDREAUX et al. (1986)
or secretion disorder	Insertion in exon 5	Eskimo Spitz	BOUDREAUX et al. (1986)
	Substitution in exon 8	Landseer	BOUDREAUX et al. (1986)
Macro-thrombocytopenia	D249N	Cavalier King Charles Spaniel	DAVIS et al. (2008)

Abbreviations: Adenine [A], asparagine [N], aspartic acid [D], cytosine [C], guanine [G], thymine [T], von Willebrand's Disease [vWD]

2.3.2 Inherited disorders of secondary haemostasis

Inherited disorders of secondary haemostasis have been associated with deficiencies of coagulation factors.

Factor VIII (haemophilia A) and factor IX (haemophilia B) deficiencies are common disorders inherited as an X-linked trait. Both diseases are explained in a special paragraph (2.4-2.5).

Autosomal factor deficiencies (Fibrinogen, FII, FV, FVII, FX, FXI, FXII) are uncommon in dogs but have also been diagnosed in several dog breeds (BROOKS 1999). The underlying mutation of these disorders is unknown, except in FVII and FXI deficiencies.

- Hypofibrinogenemia: a severe form (low concentration of fibrinogen) of hypofibrinogenemia has been diagnosed in Saint Bernards and an abnormal function (mild form) in Borzois, Collies and Vizslas (KAMMERMANN et al. 1971; BROOKS 1999).

- Factor II (prothrombin) deficiency is inherited as an autosomal dominant trait, and it has been reported in Boxers (BROOKS 1999) and a Cocker Spaniel (HILL et al. 1982).

- Factor VII (proconvertin) deficiency is an autosomal recessive trait, which has been diagnosed in Beagles (POLLER et al. 1971, SPURLING et al. 1972, CALLAN et al. 2006), an Alaskan Malamute (MILLS et al. 1997), Alaskan Klee Kais (KAAE et al. 2007), and in Mongrels (BROOKS 1999; MACPHERSON et al. 1999). A nucleotide substitution, g.6385G>A in exon 5 of the FVII gene was identified in Beagles (CALLAN et al. 2006) and in Alaskan Klee Kai Dogs (KAAE et al. 2007).

- Factor X (Stuart-Power factor) deficiency has been documented in Cocker Spaniels (BROOKS 1999) and a Jack Russell Terrier (COOK et al. 1993). The inheritance pattern appears to be autosomal dominant with variable penetrance (COOK et al. 1993).

- Factor XI (plasma thromboplastin antecedent) deficiency has been described in English Springer Spaniels (BROOKS 1999), Kerry Blue Terriers (KNOWLER et al. 1994) and Great Pyrenees as an autosomal dominant trait (BROOKS 1999). A 90 bp interspersed nucleotide element insertion has been identified in exon 7 of the FXI gene of Kerry Blue Terrier dogs (TCHERNEVA et al. 2007).

- Factor XII (Hageman factor) deficiency is not associated with bleeding episodes and is frequently diagnosed while performing routine coagulation screening tests. This disorder has been documented in Miniature Poodles (RANDOLPH et al. 1986) and a Shar Pei as an autosomal recessive trait (OTTO et al. 1991).

2.4 Canine haemophilia A

2.4.1 Introduction

Canine haemophilia A is the most common and severe inherited defect of secondary haemostasis. This disease is defined as a bleeding disorder which results from an absence or dysfunction of FVIII (BROOKS 1999; STOKOL 2005). This disease has been documented in different species, such as dog (BROOKS 1999; SARGAN 2011), cattle (HEALY et al. 1984), cat (JOHNSTONE et al. 1987), horse (HENNINGER et al. 1988; LITTLEWOOD et al. 1991), sheep (NEUENSCHWANDER et al. 1994) and human (KEMBALL-COOK et al. 1998).

Haemophilia A shows a sporadic or familiar incidence pattern (BROOKS 1999). Since the genetic defect is transmitted as an X-linked trait, the disorder is transmitted by carrier females to 50 % of their sons and 50 % of their daughters (BENN et al. 1978; MISCHKE 2012), and by affected males to 100 % of their daughters (MISCHKE 2012).

A variable phenotype (severity degree) has been observed in many dog breeds and in mixed breed dogs (Table 2, see pages 24-25). This variability is probably due to the fact that canine haemophilia is mutationally heterogeneous (BROOKS et al. 2008). In dogs, only four mutations have been identified in the canine FVIII gene (LOZIER et al. 2002; HOUGH et al. 2002; MISCHKE et al. 2011A; MISCHKE et al. 2011B).

Table 2: Extract of published dog breeds with haemophilia A

BREED	REFERENCE	BREED	REFERENCE	BREED	REFERENCE
Akita	BROOKS (1999)	**French Bulldog**	SLAPPENDEL (1975)	**Portuguese Water Dog**	BROOKS (1999)
Australian Shepherd	BROOKS (1999)	**German Shepherd**	HEIN (1986); MISCHKE et al. (2011A)	**Pomeranian**	LEWIS et al. (1983)
Basenji	BROOKS (1999)	**German Short-Haired Pointer**	BROOKS (1999); JOSEPH et al. (1996)	**Rough Collie**	BROOKS (1999)
Basset Hound	BROOKS (1999)	**Golden Retriever**	BROOKS (1999); BROOKS et al. (2005)	**Rottweiler**	BROOKS (1999)
Beagle	BROOKS (1999)	**Great Pyrenees**	GOLDEN et al. (1980)	**Schnauzer Miniature**	GILES et al. (1984)
Bichon Frise	BROOKS (1999)	**Havanese**	MISCHKE et al. (2011B)	**Scottish Terrier**	BROOKS (1999)
Blue Heeler	BROOKS (1999)	**Husky**	BROOKS (1999)	**Shar Pei**	BROOKS (1999)
Boxer	BROOKS (1999)	**Irish Setter**	BROOKS (1999); LOZIER et al. (2002)	**Shetland Sheepdog**	BROOKS (1999)
Boykin Spaniel	BROOKS (1999)	**Labrador Retriever**	ARCHER et al. (1959); BROOKS (1999)	**Shiba Inu**	BROOKS (1999)

Table 2: (continuing)

BREED	REFERENCE	BREED	REFERENCE	BREED	REFERENCE
Brittany Spaniel	BROOKS (1999)	**Lhasa Apso**	BROOKS (1999)	**Shih Tzu**	BROOKS (1999)
Cairn Terrier	BROOKS (1999)	**Manchester Terrier**	BROOKS (1999)	**Standard Poodle**	GENTRY et al. (1977)
Chihuahua	BROOKS (1999)	**Miniature Dachshund**	BROOKS (1999)	**Toy Poodle**	BROOKS (1999)
Chow Chow	BROOKS (1999)	**Miniature Schnauzer**	BROOKS (1999)	**Vizsla**	BROOKS (1999)
Cocker Spaniel	BROOKS (1999)	**Miniature Poodle**	MANSELL et al. (1990)	**Weimaraner**	DUNNING et al. (2009)
Collie	BENN et al. (1978)	**Mixed-breed dog**	HOUGH et al. (2002)	**West Highland White Terrier**	BROOKS (1999)
English Bulldog	BROOKS (1999)	**Pit Bull Terrier**	BROOKS (1999)	**Yorkshire Terrier**	IAZBIK et al. (1997); BROOKS (1999)
English Springer Spaniel	BROOKS (1999)	**Pembroke Welsh Corgi**	BROOKS (1999)		

2.4.2 Known mutations in canine haemophilia A

The first described mutation in the canine FVIII gene was in the Irish Setter colony at the University of North Carolina. Severe haemophilic dogs showed prolonged coagulation parameters and a lack of FVIII activity. Initial polymerase chain reaction (PCR) was performed using DNA samples and specific primers of the normal canine sequence; PCR was followed by nested PCR. The PCR fragments were sequenced and compared to the normal canine FVIII gene. All fragments were identical to the wild type. However, analysis of the factor VIII transcript revealed a novel sequence, termed ch8, between exon 22 and exon 23. Ch8 consisted of a 421 nucleotides sequence followed by a polyadenylation signal sequence and a polyA tail, and contained several stop codons. Analysis of the genomic DNA of healthy dogs revealed that ch8 is also located upstream of the FVIII gene. The presence of an upstream DNA element downstream of exon 22 in the haemophilic FVIII transcript indicated an intron 22 inversion of the canine FVIII gene (LOZIER et al. 2002).

The dogs in the colony at Queen's University showed dramatic reductions in plasma FVIII and FVIII antigen less than (<) 10%. After the identification of the causative mutation in Chapel Hill, reverse transcription and nested PCR were performed from exon 22 of the FVIII gene to the 3'end of the ch8. The isolated product was sequenced, and sequence analysis revealed that it consisted of exon 22 juxtaposed to a novel sequence element (NSE), which contained a TGA stop codon. The NSE transcript was compared to the isolated ch8 from the Chapel Hill hemophilic colony, and no differences were observed between them. Southern Blot analysis indicated the presence of at least five copies of this sequence in the canine genome, one of them around the splice donor site of exon 22 and the other one within intron 22. It was concluded that FVIII mRNA of haemophiliac dogs showed an NSE spliced to exon 22. The authors suggested an intrachromosomal recombination event between two of these copies, which results in an intron 22 inversion. In addition, this mutation has important similarities with the common intron 22 inversion found in human beings (HOUGH et al. 2002).

In a haemophilic German Shepherd, a point mutation was identified as the causative mutation for the haemophilic phenotype. The patient suffered from severe form of haemophilia A (FVIII plasma activity < 1 %). Once all exons of the FVIII gene were amplified and

sequenced, sequence analysis revealed a c.98G>A in exon 1 that results in a new stop codon (TAG). The authors predicted that the short transcript would encode a truncated FVIII protein that would be rapidly degraded (MISCHKE et al. 2011A).

The last published mutation was identified in haemophilic Havanese dogs, which had a FVIII activity of 10-15 %. In this case, a short interspersed nuclear element (SINE) was found in exon 14 of the canine FVIII gene. This insertion contained several stop codons. Protein modelling indicated that three out of four FIXa binding sites were present in the truncated FVIII. This finding suggested that the FVIII protein was not completely inhibited and had a residual function that was in accordance with the phenotype (MISCHKE et al. 2011B).

2.4.3 Molecular bases of canine haemophilia A

2.4.3.1 Canine FVIII gene and canine FVIII mRNA

The FVIII gene has been isolated in several species. In chronological order, the human FVIII gene was reported in 1984 (GITSCHIER et al. 1984), followed by the mouse in 1993 (ELDER et al. 1993), swine in 1996 (HEALEY et al. 1996) and finally the sequence of the canine FVIII-encoding gene was characterized in 1998 (CAMERON et al. 1998).

The canine FVIII gene is located on the long arm of the X chromosome at Xq28 (DUTRA et al. 1996; LOZIER et al. 2002). The gene is complex and extremely large; it contains 26 exons and 25 introns. Exons span 7032 bp and they range from 69 bp (exon 5) to 3.1 kilo base pairs (kb) (exon 14) in size. Exon 14 is extremely large compared to the other exons (Gene bank accession number AF016234). Introns span approximately 139 kb. The nucleotide sequence of intron 22 is not completely known (KENT 2002) (Table 3, see p. 29). Other components of the canine FVIII gene are two untranslated regions (UTR). The 5' UTR is located at the upstream end of the first exon of the canine FVIII gene and the 3' UTR is situated at the downstream end of exon 26 (Fig. 2, see p. 30) (CAMERON et al. 1998). In contrast to the canine FVIII gene, the human FVIII spans approximately 186 kb (GITSCHIER et al. 1984). Two small genes (F8B and F8A) have been discovered within intron 22 of the human FVIII gene (LEVINSON et al. 1990; LEVINSON et al. 1992B), both are transcribed from a bidirectional promoter. F8B encodes eight amino acids followed by the amino acid sequence

encoded by exons 23-26 of the human FVIII gene (NAYLOR et al. 1995). F8B has been associated with multiple eye defects in mice (VALLEIX et al. 1999; LEVINSON et a. 1992A). F8A is transcribed in the opposite direction to the FVIII gene and encodes a protein that has been related to Huntington disease (PETERS et al. 2001). Two extragenic copies of the human F8A have been found telomeric to the FVIII gene (int22h2 and int22h3) , and have been related to intrachromosomal recombination with the intron 22 sequence that leads to intron 22 inversion, resulting in two independent mRNA transcripts (LEVINSON et al. 1990; NAYLOR et al. 1995). In dogs, the F8A sequence has also been identified within the FVIII gene and outside the FVIII gene (LOZIER et al. 2002).

Regulation of expression of the canine FVIII gene is hardly known (CAMERON et al. 1998). It has been suggested that the start site of transcription could be approximately 177 bp upstream of the start codon for methionine (CAMERON et al. 1998). In humans, it has been documented at position -170 (GITSCHIER et al. 1984).

The canine FVIII transcript is approximately 8740 bases in length and consists of a 208 bp 5' UTR, a 7032 bp open reading frame (GenBank accession number AF016234), and a 1500 bp 3' UTR, which is followed by two polyadenylation signals (Fig. 2, see p. 30) (CAMERON et al. 1998). UTRs of the canine FVIII transcript were compared to the human UTRs by the group of CAMERON et al. (1998). The study revealed that the known sequence of the canine 5' UTR is highly conserved (identity level of 74 %, CAMERON et al. 1998), but it is shorter than the human 5' UTR, which is 1175 bp long. In addition, human 5' UTR has eleven transcription factor binding sites (A-S) (GITSCHIER et al. 1984; FIGUEIREDO et al. 1995). Site A and B are also conserved in dogs (identity level > 80 %, CAMERON et al. 1998). Site A spans 15 bp and contains a hepatocyte nuclear fibrinogen binding site; site B spans 39 bp and contains two binding sites, one for the transcription factor, nuclear factor kappa B and another one for enhancer binding protein (CAMERON et al. 1998; FIGUEIREDO et al. 1995). The 3' UTR of the canine FVIII gene is poorly conserved (identity level < 15 %) and shorter than the human 3' UTR, which spans 1800 base pairs (GITSCHIER et al. 1984; FIGUEIREDO et al. 1995). Unlike the human sequence, canine 3' UTR has a high content of G and C nucleotides (> 70%) in the initial 700 bases (CAMERON et al. 1998). Although UTRs do not encode for the FVIII protein, important roles have been attributed to these

regions in humans; the 5' UTRs contain cis-regulatory elements that influence the protein translation, as well as binding sites for proteins that promote protein translation (CHATTERJEE et al. 2009). The 3' UTR plays an important role in translation, localization and stability of mRNA (CHATTERJEE et al. 2009).

Table 3: Exon and intron size for canine factor VIII gene. *Each exon of the canine FVIII gene size is expressed in base pairs and as a percentage. The intron size is expressed in base pairs*

FVIII EXON	LENGTH [1] (bp)	%	FVIII INTRON	LENGTH [2] (bp)
1	146	2,1	**1**	22173
2	122	1,7	**2**	2962
3	123	1,7	**3**	7275
4	213	3,0	**4**	9535
5	69	1,0	**5**	9900
6	99	1,4	**6**	2168
7	222	3,2	**7**	2028
8	259	3,7	**8**	284
9	172	2,4	**9**	4115
10	94	1,3	**10**	5319
11	215	3,1	**11**	2103
12	151	2,1	**12**	6711
13	210	3,0	**13**	8350
14	3100	44,1	**14**	16244
15	154	2,2	**15**	4436
16	213	3,0	**16**	491
17	229	3,3	**17**	177
18	183	2,6	**18**	1931
19	117	1,7	**19**	593
20	72	1,0	**20**	1285
21	86	1,2	**21**	4573
22	156	2,2	**22**	App. 16187
23	145	2,1	**23**	395
24	150	2,1	**24**	998
25	176	2,5	**25**	8753
26	156	2,2		
TOTAL	7032	100		138986

[1] = *Gene bank accession number AF016234*
[2] = *KENT (2002)*
Abbreviations: Approximately [App.], base pairs [bp], factor [F], per cent [%]

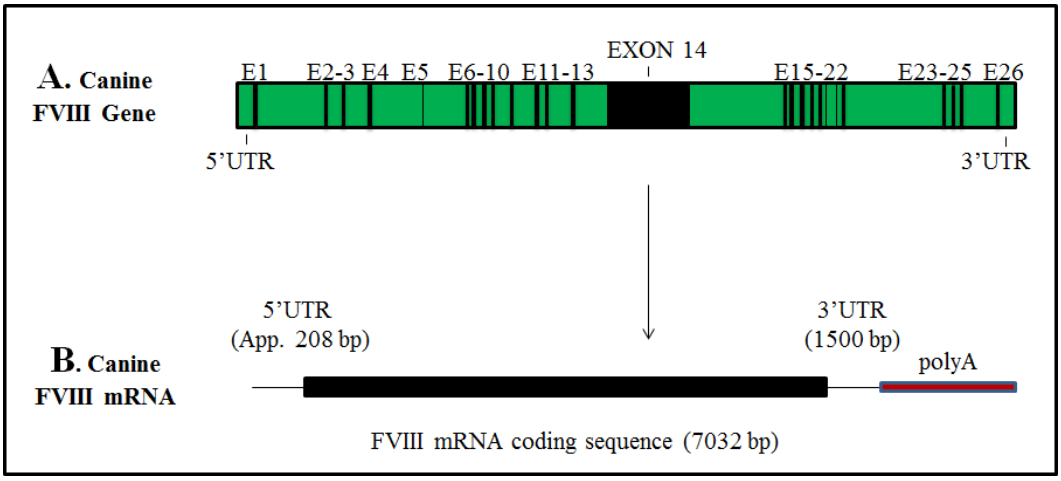

Figure 2: Organization of the canine FVIII gene (A) and FVIII mRNA (B). *The A diagram shows the canine FVIII gene, which consists of 26 exons. Black stripes indicate the exons. Exon 14 is 3100 bp in length and thereby, much larger than the other exons. The B diagram shows the open reading frame of the canine FVIII mRNA, as well as the 5' UTR, the 3' UTR and the polyA. The lengths of these regions are showed in parenthesis (modified from GRAW et al. 2005)*

Abbreviations: *Approximately [App.], base pairs [bp], exon [E], factor [F], untranslated region [UTR]*

2.4.3.2 Structure of canine FVIII protein

The coding region of the canine FVIII gene encodes a 2343 amino acid sequence that is very homologous to the human FVIII protein, but this protein is seven amino acid residues shorter than the human FVIII (2351 amino acids). The translation product comprises a 19 residue N-terminal signal sequence that directs the protein to the blood plasma, followed by the domains A1, A2, B, A3, C1 and C2 (CAMERON et al. 1998). Two acidic regions (a1, a2) are situated between A1-A2 and B-A3, respectively (Fig. 3, see p. 32). In humans, three short acidic regions (a1, a2, and a3) border the A domains (LENTING et at. 1998). Each functional domain of the canine FVIII protein is encoded by several exons of the FVIII gene: exons 1 to 7 encode for the A1 domain, exons 8 to 14 encode for the A2 domain, exon 14 encodes for the B domain, exon 14 to 20 encode for the A3 domain, exons 21 to 22 encode for the C1 domain and exons 23 to 26 encode for the C2 domain (CAMERON et al. 1998).

Maturation steps occur during the FVIII secretion and activation. Prior to secretion, FVIII undergoes intracellular proteolysis, giving rise to a heterodimeric molecule of 2324 amino acids (CAMERON et al. 1998) composed of a heavy chain (A1, A2 and B domains) and a light chain (A3, C1, and C2 domains), connected by a cation, likely copper (Cu^{+}) (FAY 2005) (Fig. 3, see p. 32). The canine B domain spans amino acid residues 687-1621 and possesses 25 N-glycosylation sites (CAMERON et al. 1998). The positions of the other canine domains are not reported.

In humans, mature FVIII protein spans 2332 amino acids. This includes six domains and three acidic regions: A1 (residues 1-336), A2 (residues 373-710), B (residues 741-1648), A3 (residues 1690-2019), C1 (residues 2020-2172) and C2 (residues 2173-2332) (LENTING et al. 1998; GRAW et al. 2005). The a1, a2, and a3 acidic regions contain 35, 29, and 40 amino acids residues, respectively (LENTING et al. 1998).

A sequence alignment for the amino acid sequence of the mature FVIII from different species was supplied by CAMERON et al. (1998). The findings showed that canine A and C domains have a high degree of homology (between 77 and 92 %), at the amino acid level, with the human, porcine and murine FVIII proteins, and a 30 % homology with other proteins (factor V and ceruloplasmin). Alignment for the C domain sequences from different species was also performed by LIU et al. (2000). The canine C1 domain showed a 92 % (CAMERON et al. 1998) or 94 % (LIU et al. 2000) homology (at the amino acid level) with the human C1 domain, and 83 % and 92 % homology with the mouse and porcine C1 domain, respectively (CAMERON et al. 1998). The canine C2 domain showed an 81 %, 84 % and 77 % homology with the human (LIU et al. 2000), mouse and porcine C2 domain, respectively (CAMERON et al. 1998). However, the acidic regions (a1 and a2) and the B domain had only 44 to 70 % homology with the human acidic regions and the B domain. The low conservation of the B domain and its absence in the activated protein could be indicative of its low importance for the activity of the protein (CAMERON et al. 1998).

Studies in human FVIII have reported that the proteolytic cleavage of FVIII by thrombin results in the release of the B domain generating FVIIIa. Therefore, FVIIIa is comprised of a heavy chain (domains A1 and A2) and a light chain (domains A3, C1 and C2), which are connected by calcium (MCCONELL 2000; BOWEN 2002).

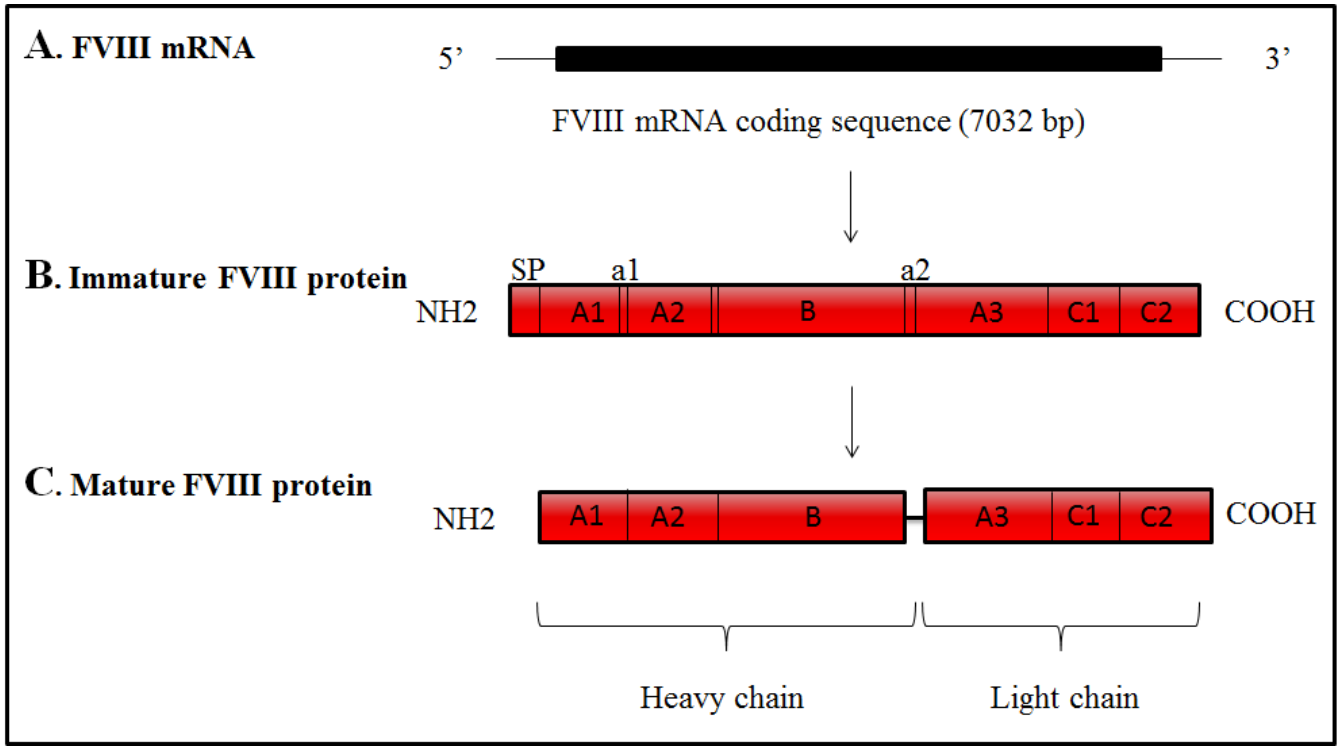

Figure 3: Domain organization of the immature and mature canine FVIII protein. *The FVIII mRNA (shown in A) is translated into a precursor protein (shown in B), which involves a signal peptide (SP), followed by the domains A1, a1, A2, B, a2, A3, C1 and C2. This protein is processed to the mature FVIII protein (show in C), which lacks of SP and it is composed of a heavy chain (domain A1, A2 and B) and a light chain (domain A3, C1 and C2) (modified from GRAW et al. 2005)*

Abbreviations: Base pairs [bp], factor [F], signal sequence [SP]

2.4.3.3 Functional binding sites on canine FVIII protein

A number of significant intermolecular interactions are involved in the life cycle of the FVIII. As in other species, the functional binding sites on canine FVIII are conserved (CAMERON et al. 1998, MISCHKE et al. 2011B). Although not all binding sites on canine FVIII protein are known, these appear to be located at similar positions to the human protein (CAMERON et al. 1998) (Fig. 4, see p. 34):

- Binding site of canine FVIII for vWF is localized at amino acid residues 1666 to 1676 (CAMERON et al. 1998). The equivalent human binding site for vWF involves the a3 acidic region at amino acid residues 1649 to 1689 (LEYTE et al. 1989; FAY 2005). In human beings, the C2 domain at amino acid residues 2181-2243 (HEALEY et al. 1998) and 2303-2332 (SAENKO et al. 1997) is also involved. It has also been suggested that residue Arg^{2150} participates in human FVIII binding to vWF (JACQUEMIN et al. 2000).
- Three canine thrombin cleavage sites are located at amino acid residues Arg^{366}-Ser^{367}, Arg^{734}-Ser^{735}, and Arg^{1681}-Ser^{1682} (CAMERON et al. 1998). Equivalent human cleavage sites have been identified at Arg^{372}, Arg^{740} and Arg^{1689} (EATON et al. 1986; FAY 2005).
- The canine FVIII inactivation by APC results from the cleavage of the heavy chain at Arg^{331}-methionine^{332} (Met) and Arg^{556}-glycine^{557} (Gly) (CAMERON et al. 1998), i.e. at the comparable position as in humans, where it is located at amino acid residues 336 and 562 (FAY et al. 1991).
- There are no reports of canine FXa cleavage sites so far. Human FXa attacks FVIII at identical cleavage sites as thrombin, but binding sites appear to be different (FAY 2005). In the C2 domain, the cleavage site is located at amino acid residues 2253-2270 (NOGAMI et al. 2002).
- The phospholipid binding site has not been reported in dogs yet. In humans, the binding site for phospholipid is located in the C2 domain, at similar amino acid residues like vWF binding site: Met^{2199}- phenylalanine^{2200} (Phe) and leucine^{2251} (Leu) -Leu^{2252} (BARROW et al. 2001; FAY 2005).
- Canine FX binding site has also not been reported yet. The human FX binding site appears to be situated in the C-terminal region of the A1 domain (FAY 2005). LAPAN et al. (1997) initially suggested that amino acid residues 336-372 in the A1 domain of the human FVIII

protein are binding sites for FX. Posteriorly, these authors suggested an electrostatic association between both factors (LAPAN et al. 1998).

- Canine FIXa cleavage site is not known so far. Equivalent human FIXa cleavage sites are located at amino acid residues 484-493, 558-565 and 708-715 in A2 domain (LENTING et al. 1996A; GRAW et al. 2005). Recent mutagenesis studies have proposed amino acid residues 1803-1818 in A3 domain (FAY 2005).

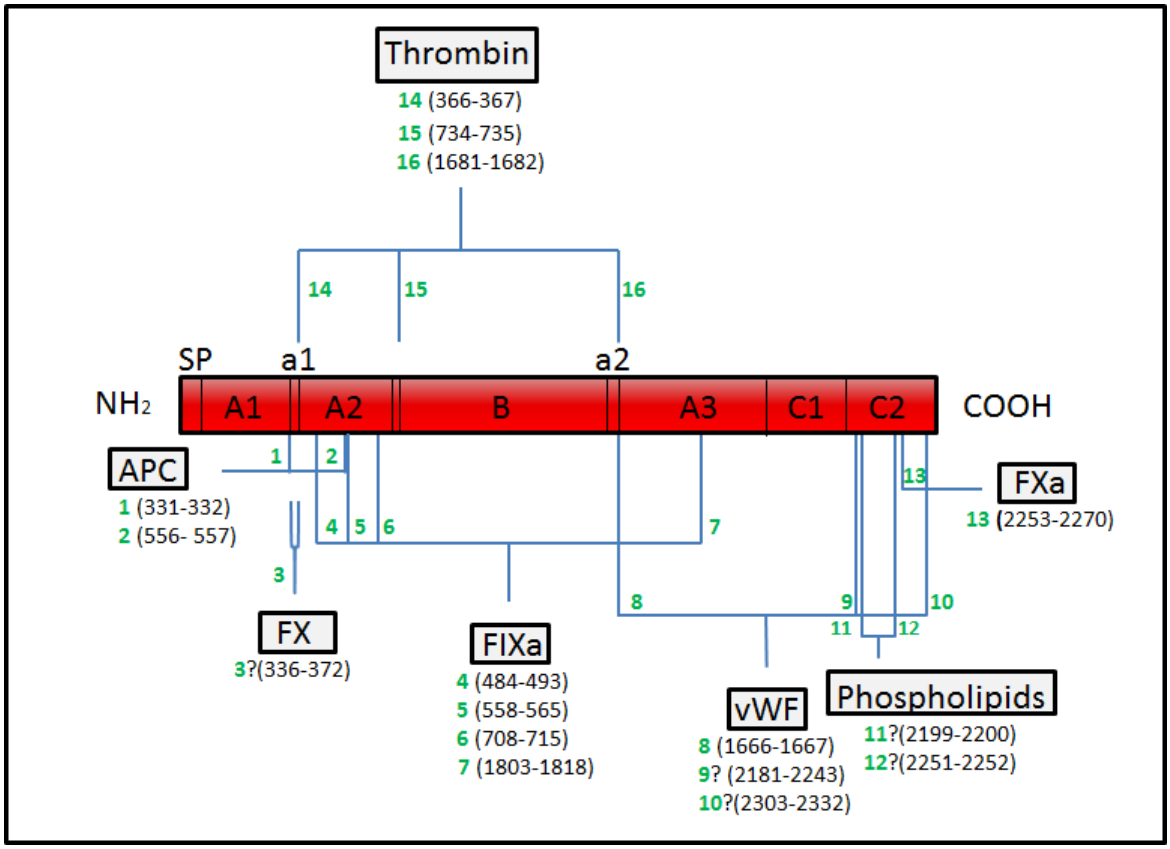

Figure 4: Binding sites on canine FVIII for coagulation proteins. *The signal peptide and the domain structure of FVIII are shown. Numbers in brackets indicate the binding site position on FVIII for each coagulation factor. Green numbers (1-16) symbolize 16 binding sites on FVIII: APC binding sites from 1 to 2; FX binding site 3; FIX binding sites from 4 to 7; vWF binding sites from 8 to 10; phospholipids binding sites from 11 to 12; FXa binding site 13; thrombin binding sites from 14 to 16. The blue lines mark the binding site's position on FVIII for each factor. The interrogation signal marks the equivalent human binding sites (modified from LENTING et al. 1998; MANNUCCI et al. 2001; GRAW et al. 2005)*

Abbreviations: Activate form [a], activated Protein C [APC], factor [F], signal peptide [SP], von Willebrand factor [vWF]

2.4.4 FVIII protein: life cycle

The life cycle of FVIII has been described in detail in humans, and some aspects in dogs. The synthesis of FVIII has been debated for a long time (LOZIER 2006). The liver seems to be the main location of FVIII synthesis (WEBSTER et al. 1971; DO et al. 1999). It has been suggested that sinusoidal endothelial cells express more FVIII than hepatocytes (HOLLESTELLE et al. 2001). An extra-hepatic origin has been demonstrated by normal FVIII levels in dogs transplanted with haemophilic livers (WEBSTER et al. 1971), by FVIII mRNA detection in other tissues such as lymph node, kidney, and spleen (WION et al. 1985; HOLLESTELLE et al. 2005), and by the correction of haemophilia in animal models after spleen or lung transplantation (VELTKAMP et al. 1974; KOBAYASHI et al. 1995). The pulmonary endothelium has been confirmed as a source of the human FVIII (JACQUEMIN et al. 2006).

After FVIII synthesis, the immature protein interacts with several proteins in the cell, such as lectin mannose binding 1 and multiple coagulation factor deficiency 2 protein, that transport FVIII from the endoplasmic reticulum to the Golgi apparatus (GRAW et al. 2005). Once the mature FVIII is secreted into the bloodstream, other proteins interact with FVIII. FVIII circulates as an inactive heterodimeric molecule bound to vWF until its activation by thrombin during the propagation phase of coagulation (LANE et al. 2005). FXa appears to also be involved in this activation (FAY 2004). FVIIIa presents FX to the protease enzyme FIXa in the intrinsic FXase complex (AHMAD et al. 2003B), which leads to the activation of FX. APC in presence of its cofactor, Protein S, inactivates FVIIIa by enzymatic cleavage (WALKER et al. 1987). The liver seems to be the main place for the degradation of FVIII fragments though FVIII cleavage probably also occurs in the spleen (LACROIX-DESMAZES et al. 2008).

2.5 Canine haemophilia B

2.5.1 Introduction

Canine haemophilia B is a specific deficiency or dysfunction of the coagulation FIX caused by a mutation in the FIX gene (BROOKS 1999; STOKOL 2005), which retards or prevents

the interaction between FIXa, FVIIIa, FX, calcium and phospholipids (GU et al. 1999). This disease has been documented in dogs (BROOKS 1999; SARGAN 2011) cats (BROOKS 1999; MAGGIO-PRICE et al. 1993; GOREE et al. 2005), and humans (BOWEN 2002).

Like haemophilia A, haemophilia B is inherited in an X chromosomal recessive manner (FELDMAN et al. 1995), so that mainly male dogs are phenotypically affected (STOKOL 2005).

The incidence of haemophilia B is less that of haemophilia A (BROOKS et al. 2003). The disease has been described as a familiar or sporadic pattern (BROOKS 1999) in many dog breeds and mixed breed dogs (Table 4, see p. 37). The phenotypic variety is probably the result of heterogeneity of mutations in the FIX gene (BROOKS et al. 1997; GU et al. 1999). In contrast to haemophilia A, the underlying mutation responsible for haemophilia B has been identified in more canine breeds (EVANS et al. 1989A; MAUSER et al. 1996; SUGAHARA et al. (1996); BROOKS et al. 1997; GU et al. 1999; BROOKS et al. 2003; MISCHKE et al. 2011C), and in cats (GOREE et al. 2005).

2.5.2 Known mutations of canine haemophilia B

Molecular genetic analyses of canine factor IX gene have revealed diverse molecular mechanisms for haemophilia B in dogs (SUGAHARA et al. 1996, BROOKS et al. 1997):

The first described mutation in the canine FIX gene was a point mutation. Mixed haemophilic dogs from the Chapel Hill colony (originally Cairn Terrier and Beagle mixed breed) suffered from a severe form with no detectable FIX in plasma. No difference in length and amount of FIX DNA and cDNA could be detected using Southern and Northern Blot assays, respectively between healthy and haemophilic dogs. However, a point mutation, c.1477G>A, was identified by sequence analysis and comparison of the coding region of the FIX gene of haemophilic dogs with the wild type FIX gene. The identified mutation leads to an amino acid substitution from glutamic acid to glycine. A protein model showed several structural conflicts and a hydrophobic character disorder of the internal region of the FIX. These findings suggested that the single nucleotide substitution is the causative mutation (EVANS et al. 1989A).

Table 4: Extract of published dog breeds with haemophilia B

BREED	REFERENCE	BREED	REFERENCE
Airedale Terrier	SUGAHARA et al. (1996); BROOKS (1999); GU et al. (1999)	**Lhasa Apso**	MAUSER et al. (1996)
Alaskan Malamute	PETERSON et al. (1979)	**Maltese**	BROOKS (1999); NAKATA et al. (2006)
Beagle	BROOKS (1999)	**Mixed breed**	BROOKS (1999); EVANS et al. (1989A)
Bichon Fries	BROOKS (1999)	**Pit bull Terrier**	BROOKS (1999); GU et al. (1999)
Cairn Terrier	ROWSELL et al. (1960)	**Rhodesian Ridgeback**	BROOKS (1999); MISCHKE et al. (2011C)
Chow Chow	BROOKS (1999)	**Rottweiler**	BROOKS (1999)
Doberman Pinscher	BROOKS (1999)	**Saint Bernard**	BROOKS (1999)
English Sheepdog	SHERDING et al. (1980)	**Scottish Terrier**	BROOKS (1999); CAMPBELL et al. (1983)
German Shepherd	BROOKS (1999); FELDMAN et al. (1995)	**Sealyham Terrier**	BROOKS (1999)
German Wirehaired Pointer	BROOKS (1999); BROOKS et al. (2003)	**Shih Tzu**	BROOKS (1999)
Golden Retriever	BROOKS (1999)	**Weimarane**	BROOKS (1999)
Jack Russell Terrier	BROOKS (1999)	**Wirehaired Fox Terrier**	BROOKS (1999)
Labrador Retriever	BROOKS et al. (1997); BROOKS (1999)		

A deletion mutation was identified in Lhasa Apsos. Studied animals suffered from a severe form of haemophilia B (plasma FIX antigen level < 1 % of normal canine plasma). Northern Blot analyses of total hepatic RNA showed a decreased amount of FIX cDNA in the haemophilic dogs. cDNA was amplified by PCR and sequenced by the dideoxynucleotide chain termination method. The haemophilic sequence was identical to the wild type sequence, with the exception of a 5 bp deletion at nucleotides 772-776 and a C to T transition at nucleotide 777. This mutation leads to a frame shift during the translation and a premature stop codon at amino acid residue 146. Authors concluded that the truncated protein lacks the entire activation peptide and the catalytic domain; therefore, it should not show any enzymatic activity (MAUSER et al. 1996).

Two deletions in exon 8 of the canine FIX gene were identified in two breeds (Airedale Terrier and a Labrador Retriever) at the University of Cornell. Affected dogs suffered from a severe form of haemophilia B (FIX antigen undetectable and FIX activity of less than 1 %). Southern blot analysis failed in the case of the Labrador Retriever. PCR amplification of exon 8 was performed and no PCR product was obtained from these dogs. The authors suggested that a partial and a gross deletion in exon 8 of the canine FIX gene is the mutation responsible for haemophilia A in an Airedale Terrier and a Labrador Retriever, respectively (SUGAHARA et al. 1996).

The complete deletion of the FIX gene in a family of Labrador Retrievers leads to a severe form of haemophilia B. The FIX coagulant activities were not detectable. PCR amplification of all exons of the FIX gene followed by electrophoresis on the polyacrylamide gel failed in the studied haemophilic dog. No hybridization signal was detected by Southern Blot, but it was normal in a second haemophilic Labrador Retriever unrelated to the first one. This finding implies several sporadic mutations in the Labrador Retriever breed as a cause for the same disease (BROOKS et al. 1997).

An insertion in exon 8 was identified in Airedale Terrier dogs. FIX activity was < 1 % and the dogs showed typical signs of severe haemophilia B. Two novel bands of 5.6 kb and of 0.5 kb were identified by the Southern Blot analysis. All exons and introns of the FIX gene were sequenced, and a 5 kb insertion after nucleotide 1471 in exon 8 was detected. This insertion disturbed the splicing of the FIX mRNA resulting in a truncated mature mRNA, which lacks

more than 150 bp of exon 8 and most of the 3' UTR, and encodes an abnormal sequence including a stop codon. As the catalytic domain is encoded by exon 8, a possible disturbance of the binding site to FX was suggested (GU et al. 1999).

A partial gene deletion was identified in Pit Bull Terrier mix dogs, which suffered from a severe form of haemophilia B (FIX:C < 1%). A hybridizing band at 2.6 kb was detected by the Southern Blot analysis. Sequencing of exons and introns of the FIX gene showed a partial gene deletion spanning the region exon 1 to exon 6, which resulted in the complete absence of FIX mRNA (GU et al. 1999).

A large insertion mutation in intron 5 was reported in five generations of German Wirehaired Pointers. Haemophilic dogs had a mild to moderate form of haemophilia B with a FIX activity of about 5 %. A novel band of 5 kb was identified by Southern Blot analysis. The comparison of exon sequences of normal and affected dogs showed no differences. Sequence analyses of introns revealed a 1.5 kb insertion in intron 5 of the FIX gene in the haemophilic males, which contained a 5' truncated canine LINE 1 and a long poly (A) at its 3' end, both flanked by a 15 bp direct repeat. As LINE 1 intronic insertions have been related with aberrant splicing and decreased gene expression levels, the detected canine mutation was suggested to be the causative FIX mutation in German Wirehaired Pointers (BROOKS et al. 2003).

A G to A missense mutation was detected in Rhodesian Ridgebacks. The dogs exhibited a severe form of haemophilia B, and they showed FIX activities <5 %. FIX gene of affected and carrier dogs were sequenced and all exons and exon-intron boundaries were compared to the wild type canine FIX cDNA. This comparison revealed a G to A substitution in exon 7 that caused an amino acid exchange of glycine to glutamic acid in the catalytic domain of the protein. The consequences of this finding were analysed in a structural protein model and amino acid sequence alignment. It was published that the presence of the glutamic acid likely led to a change in conformation, close to the pocket-shaped active site of the trypsin domain, due to the difference in the side and charge between the changed amino acid and the wild type amino acid (MISCHKE et al. 2011C).

2.5.3 Molecular bases of canine haemophilia B

2.5.3.1 Canine FIX gene and canine FIX mRNA

The gene for canine factor IX is located on the long arm of the X chromosome (Xq26.3), unlinked to the gene for factor VIII (DUTRA et al. 1996; BROOKS 1999; BRINKHOUS et al. 1973). The canine FIX gene consists of eight exons and seven introns (KENT 2002; TATSUMI et al. 2008). The coding region of FIX gene ranges from 25 bp (exon 3) to 549 bp (exon 8) in size. Exon 8 is large compared to the other exons (Gene bank accession number NM_001003323) (TATSUMI et al. 2008). Introns span approximately 29 kb. The longest intron is the intron 6, which is approximately a third of the total length (KENT 2002) (Table 5).

Table 5: Exon and intron size for canine factor IX gene. *Each exon size is expressed in base pairs and as a percentage. The intron size is expressed in base pairs.*

FIX EXON	LENGTH (bp)	%	FIX INTRON	LENGTH (bp)
1	67	4,9	**1**	5912
2	164	12,1	**2**	206
3	25	1,8	**3**	4092
4	114	8,4	**4**	6778
5	129	9,5	**5**	2256
6	197	14,5	**6**	9329
7	115	8,5	**7**	628
8	548	40,3		
TOTAL	1359	100		29201

[1] = gene bank accession number NM_001003323. (TATSUMI et al. 2008); [2] = KENT 2002
Abbreviation: Base pairs [bp], factor [F], per cent [%]

The coding region of canine FIX is highly conserved; it shows a 91 % homology with the coding region of human FIX, and the 5' UTR and 3' UTR show 83 % and 72 % identity to the human correlate, respectively (EVANS et al. 1989A; EVANS et al. 1989B).

The first isolated and characterized canine FIX cDNA was 2883 nucleotides in length. The open reading frame was 1356 bp in length and it was flanked by two untranslated regions: a 5' UTR of 224 nucleotides and a 3' UTR of 1303 nucleotides (EVANS et al. 1989B). The latest published canine FIX cDNA is 3257 nucleotides in length with a 3' UTR 1674 bp in length (TATSUMI et al. 2008).

2.5.4 Structure of canine FIX protein

The translation initiation codon has been located 39 amino acids upstream of the triplet which encodes the first amino acid of the mature FIX. The deduced translation product consists of 452 amino acids (EVANS et al. 1989B) and it has a molecular size of 61 kiloDalton (SUGAHARA et al. 1996). Canine FIX contains a leader peptide, which spans 39 amino acid residues (EVANS et al. 1989B). This hydrophobic signal peptide directs the transport of the protein from the hepatocytes into the blood stream (GIANNELLI et al. 1998). The leader peptide is followed by several distinct domains, which are encoded by specific exons of the FIX gene (Table 6, see p. 43) (EVANS et al. 1989B) and possess cofactor and receptor specificity (GIANNELLI et al. 1998):

- The N terminal gamma-carboxyglutamic acid domain (Gla) is located at the N-terminus and spans 45 amino acid residues, from tyrosine^{40} (Tyr) to Tyr^{84} in dogs (EVANS et al. 1989B). This domain contains 12 γ-carboxylglutamyl residues (AXELROD et al. 1990), which are vital for FIX secretion and for the calcium binding of FIX (GIANNELLI et al. 1998; LACOBELLI 2008). In humans, this domain is necessary for binding to phospholipid membrane (PERSSON et al. 2002) and for binding to the light chain of FVIIIa in the tenase complex (BLOSTEIN et al. 2003).
- A short segment from Val^{85} to Asp^{88} (aspartic acid) (EVANS et al. 1989B).
- The epidermal growth factor (EGF)-like domain spans 84 amino acids residues, from glutamine^{89} (Gln) to Gly^{172} in dogs (EVANS et al. 1989B). Two EGFs (EGF1, EGF2) have been described in humans. The first EGF contains carboxylate residues, some of which (Asp^{86}, Asp^{88}, Gln^{89} and Asp^{103}) are calcium binding sites (REES et al. 1988; HANDFORD et al. 1991), which support the interaction with FVIIIa and enhance the activity of the catalyitc domain (LENTING et al. 1996B, CELIE et al. 2002). These residues are also present in dogs (AXELROD et al. 1990). In humans, the role of this domain is very contradictory: it appears

to bind to the FVIIa-TF complex and to promote binding for the A3 domain of FVIIIa (PERSSON et al. 2002). No carboxylate residues are in the second EGF of human FIX (GIANNELLI et al. 1998), which appears to play a role in the orientation of the catalytic domain (PERSSON et al. 2002).

- A short segment from Arg^{173} to Arg^{185} (EVANS et al. 1989B).
- The activation peptide domain spans 32 amino acids residues, from Ala^{186} to Arg^{217} in dogs (EVANS et al. 1989B). Two asparagine (Asn)-linked carbohydrate residues have been located at Asn^{197} and Asn^{207} (AXELROD et al. 1990; SUGAHARA et al. 1996). This domain plays an important role during the FIX activation by FXIa and FVIIa-TF (GIANNELLI et al. 1998). The amino acid residues Arg^{185}-Ala^{186} and Arg^{217}-Val^{218} of the immature FIX protein (Arg^{146}-$Ala^{147}$and Arg^{178}-Val^{179} of the mature FIX) are the cleavage residues during the canine FIX activation (AXELROD et al. 1990). In humans, this activation involves cleavage of Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} (SMITH et al. 2008).
- The catalytic domain consists of 235 amino acid residues (EVANS et al. 1989B) and ends at threonine^{452} (Thr). It contains an Asn-linked carbohydrate residue at Asn^{297} (AXELROD et al. 1990; SUGAHARA et al. 1996). The active site residues involved in the FX activation are located at histidine^{258} (His), Asp^{306} and Ser^{401} (AXELROD et al. 1990). Human studies support that this domain interacts with the A2 domain of FVIIIa (PERSSON et al. 2002).

The predicted synthetized protein is highly conserved in dogs. It possesses an 86 % homology (at the amino acid level) with the human sequence. The Gla domain and the leader peptide are highly conserved; 95 % and 91 %, respectively. The EGF domain and the catalytic domain have an 83 % homology with the human and bovine domains (EVANS et al. 1989B). Moreover, all γ-carboxylglutamyl, β-hydroxyaspartyl and Asn-linked carbohydrate residues are conserved in dogs (AXELROD et al. 1990). However, the activation peptide domain, which is three amino acids shorter than human and bovine one, only shows about 40 % homology (EVANS et al. 1989B), but the cleavage sites revealed to be conserved (EVANS et al. 1989B; AXELROD et al. 1990). Due to this high homology, dogs have been used as a canine model of human haemophilia B (LACOBELLI 2008).

Prior to the FIX secretion, post-translational modifications (gamma-carboxylation, hydroxylation and glycosylation) occur in the liver (AXELROD et al. 1990; MATHUR et al.

1997), resulting in the removal of the 39 amino acids corresponding to the signal peptide (GIANNELLI et al. 1998).

Predicted circulating FIX has a molecular weight of 46609 Dalton. The mature protein contains 413 amino acids and consists of a Gla domain (residues 1-45), a short segment (residues 46-49), EGF-like domains (residues 50-133), a short segment (residues 134-146), activation peptide (residues 147-178) and the catalytic domain (residues 179-413) (EVANS et al. 1989B).

Studies on human FIX have reported that the proteolytic cleavage of FIX during the FIX activation results in the release of the activation peptide generating FIXa, which is composed of two chains linked by a single disulfide bond: the light chain contains Gla and EGFs domains, and the heavy chain comprises protease and catalytic domains (SMITH et al. 2008).

Table 6: Relation between exons of FIX gene and the protein domains of canine FIX

PROTEIN REGION	RESIDUES	EXONS
Leader peptide	1-22	Exon 1
	23-39	Exon 2
Gamma-carboxyglutamic	40-77	Exon 2
acid domain	78-84	Exon 3
Epidermal Growth Factor-	89-123	Exon 4
like domains	124-166	Exon 5
	167-172	Exon 6
Activation peptide	186-217	Exon 6
Catalytic domain	218-232	Exon 6
	233-270	Exon 7
	270-452	Exon 8

2.5.4.1 FIX protein: life cycle

The life cycle of FIX has mostly been described in humans. FIX is a vitamin K-dependent glycoprotein that is synthesized in the liver (EVANS et al. 1989B). During the biosynthesis, it undergoes post-translational modifications, after which the protein is secreted into the bloodstream as a single chain zymogene (MATHUR et al. 1997).

Once FIX is secreted, other proteins interact with FIX. In the course of fibrin clot formation, the FIX activation is initiated by FVIIa-TF (OSTERUD et al. 1977; LU et al. 2004), followed by FXIa (GAILANI 2000). FIXa forms a complex with FVIIIa on the activated platelet surface to activate FX (LACROIX-DESMAZES et al. 2008), which is critical for the synthesis of fibrin (GU et al. 1999). The principal inhibitor of FIX is the serpin antithrombin (PIKE et al. 2005).

2.6 Clinical signs of canine haemophilia A and B

The clinical manifestation of haemophilia A and B is very similar (PETERSON et al. 1979, MISCKE 2012), which is principally due to the fact that the deficiency or dysfunction of both coagulation factors result in an inadequate activity of the FXase complex during the coagulation system (MISCKE 2012).

Since the genetic defect is transmitted as an X-linked trait, male dogs are clinically affected while the bitches normally act as asymptomatic carriers (BENN et al. 1978; JOHNSTONE et al. 1984, MISCHKE et al. 2011C). Bitches can suffer from symptoms if they carry the mutated allele on both X chromosomes (BENN et al. 1978; STOKOL 2005).

The clinical signs in haemophilia are varied (BENN et al. 1978) and these are usually noted at an early age (CAMPBELL et al. 1983). Affected dogs can be in good body conditions (BENN et al. 1978) or can be week and lethargic (GENTRY et al. 1977; VERLANDER et al. 1984; FELDMAN et al. 1995; DUNNING et al. 2009). Commonly, repeated episodes of bleeding occur at birth from the umbilical cord (GENTRY et al. 1977; BENN et al. 1978; JOHNSTONE el al. 1984) and during teeth eruptions (GENTRY et al. 1977; BENN et al. 1978; PETERSON et al. 1979; CAMPBELL et al. 1983; BROOKS et al. 1997; IAZBIK et al. 1997; NAKATA et al. 2006). Affected dogs may also show prolonged bleeding from cutaneous wounds (BROOKS et al. 2003; MISCHKE et al. 2011C) and excessive bleeding after playing with other dogs (BENN et al. 1978), after minimal surgical procedures or medical interventions (GENTRY et al. 1977; VERLANDER et al. 1984; DUNNING et al. 2009; MISCHKE et al. 2011C), and after trauma (GENTRY et al. 1977; BROOKS et al. 2003). Spontaneous musculoskeletal bleeding has been also described in severely affected dogs (GENTRY et al. 1977; GU et al. 1999; HOUGH et al. 2002; MISCHKE et al. 2011C),

particularly if they are large and active animals (JOHNSTONE el al. 1984). In addition, subcutaneous (SUGAHARA et al. 1996; BROOKS et al. 1997; NAKATA et al. 2006) or intramuscular (PETERSON et al. 1979; FELDMAN et al. 1995; NAKATA et al. 2006) haematoma formations are also frequent. Depending on the position and extension of the haemorrhages and haematomas, affected dogs may show different signs, such as epistaxis, haematuria, (BENN et al. 1978; JOHNSTONE el al. 1984), melena (BENN et al. 1978; FELDMAN et al. 1995), disturbances of movement, which are caused by haemorrhages into joints, soft tissues and spinal cord (MISCHKE 2012). Pain in limbs and sporadic lameness has been observed secondary to intramuscular haematoma formation (PETERSON et al. 1979; NAKATA et al. 2006) or haemarthrosis (GENTRY et al. 1977; BENN et al. 1978; IAZBIK et al. 1997). Signs of lower motor neuron paralysis have been described subsequent to intramuscular haematoma formations in limbs (FELDMAN et al. 1995). Signs of cervical pain with posterior paresis (FELDMAN et al. 1995) or progressive quadriparesis (FELDMAN et al. 1995; DUNNING et al. 2009) or quadriplegia (VERLANDER et al. 1984), have been observed in haemophilic dogs, which showed spinal cord haemorrhages.

The severity of haemophilia varies depending on the type of mutation in the FVIII/FIX gene (FELDMAN et al. 1995), on the in vitro measurement of the residual FVIII (JOHNSTONE el al. 1984) or FIX coagulant activity (FELDMAN et al. 1995), and on clinical factors including the size of the breed, the dog activity (MISCHKE et al. 2012), and keeping conditions (NAKATA et al. 2006). Clinical signs of haemorrhage are less frequent in small breeds of dogs (CAMPBELL et al. 1983) and more severe in large dogs (JOHNSTONE el al. 1984; NAKATA et al. 2006). In addition, other factors such as concomitant disorders of haemostasis, thrombocytopenia, and other disease states can compromise the clinical expression of haemophilia (FELDMAN et al. 1995). Some authors classify the severity of haemophilia based on human criteria (BENN et al. 1978; BROOKS et al. 2008, NAKATA et al. 2006; JOHNSTONE et al. 1984), in where only is taken into account the residual FVIII/FIX coagulant activity (LIU et al. 2000; LACOBELLI 2008). However it is known that the haemophilic dogs show more severe signs than haemophilic human at the same reduced FVIII/FIX activity (MISCHKE 2012). For example, severe clinical signs has been observed in German Shepherds with FVIII activity 8 to 10 % (JOHNSTONE el al. 1984), in German Wirehaired Pointers with FIX activity 2.4 to 6.4 % (BROOKS et al. 2003), and in German

Shepherds with FIX activity 7 % and 7.6 % (FELDMAN et al. 1995). In humans, levels of 5 to 10 % of normal are considered as a mild form of haemophilia (LIU et al. 2000) because haemophilic humans tend to be less prone to spontaneous bleeding and they normally bleed after severe trauma or surgery (JOHNSTONE el al. 1984). These differences in the expression of haemophilia between both species have been related to different factors, such as the size of the breed and the dog activity (MISCHKE 2012).

2.7 Diagnosis of canine haemophilia A and B

Haemophilia A and B should be suspected in bitches with a family history of bleeding disorder and/or in male dogs with a bleeding history (FELDMAN et al. 1995). Carrier bitches had usually no signs or history of bleeding (SUGAHARA et al. 1996).

Abnormal hematologic findings can be observed in affected dogs. These findings include low packed cell volume, low total protein (CAMPBELL et al. 1983; FELDMAN et al. 1995; DUNNING et al. 2009), low haemoglobin and high reticulocyte count (CAMPBELL et al. 1983). This regenerative anaemia is compatible with continuous blood loss during the haemorrhage episode (PETERSON et al. 1979; SHERDING et al. 1980; CAMPBELL et al. 1983). Platelets count is frequently within reference range (GENTY et al. 1977; CAMPBELL et al. 1983; DUNNING et al. 2009; MISCHKE 2012) but it is possible to find thrombocytosis with leucocytosis and hyperfibrinogenemia, which is attributed to a compensatory stress response to the haemorrhagic episode (PETERSON et al. 1979). Complete blood counts can also be normal in case of small blood loss (JOHNSTONE et al. 1984).

Affected dogs show normal buccal mucosal bleeding time and prolonged cuticle bleeding time with re-bleeding (SHERDING et al. 1980; BROOKS et al. 1997; NAKATA et al. 2006), caused by the failure of the fibrin stabilization of the platelet plug (SHERDING et al. 1980), which occurs in clotting factor deficiencies such as haemophilia A and B (SHERDING et al. 1980). In contrast, in case of primary haemostatic disorders, the buccal bleeding time is also prolonged (BROOKS et al. 1997).

On examination of blood coagulation profile, which must be performed on citrate plasma, affected dogs normally show prolonged activated partial thromboplastin time (APTT) and

activated clotting time, because reduced factor VIII/FIX activity impairs clot formation via the intrinsic pathway of coagulation (FELDMAN et al. 1995). Results of prothrombin time and thrombin clotting time are usually within reference range (GENTY et al. 1977; CAMPBELL et al. 1983; MAUSER et al. 1996; BROOKS et al. 1997; NAKATA et al. 2006; MISCHKE 2012), unless a second problem, such as vWD or liver disease, coexists (FELDMAN et al. 1995). It should be noted that APTT can be normal due to a stress response to the bleeding crisis, which increases the fibrinogen concentration and shortens the time of clot formation (CAMPBELL et al. 1983, MISCHKE 2000). Some authors have reported normal values of fibrinogen concentration (GENTY et al. 1977; NAKATA et al. 2006; DUNNING et al. 2009), and normal antithrombin III activity in haemophilic dogs (NAKATA et al. 2006).

Due to the fact that the prolongation of APTT occurs in haemophilia A and B (MISCKE et al. 2012), specific measurements of factor activity have to be performed to confirm the diagnosis (BROOKS et al. 2003; MISCHKE 2012). Coagulation FVIII and FIX activities are automatically measured using coagulometric tests, which are optimized for canine sample and are based on commercial human deficient plasma, and a standard curve is generated using serial dilutions of canine pooled plasma (GU et al. 1999; MISCHKE et al. 2010; MISCHKE 2012). Furthermore, photometric assays are also possible (MISCHKE et al. 2012). The reference range for FVIII and FIX activities is 72 to 136 % and 75 to 140 %, respectively (MISCHKE 2012). Affected animal normally show an activity of the haemophilia factor less than 10 %, but in mild forms it can be 10-25 % (MISCHKE 2012).

In haemophilic dogs, FIX concentration can be measured by a radioimmunoassay with polyclonal rabbit anti-canine FIX antibodies, and ELISA with monoclonal and polyclonal anti-human FIX antibodies (BROOKS et al. 1997; GU et al. 1999) In addition, canine FVIII antigen was determined with an ELISA utilizing two monoclonal antibodies to the light chain of the FVIII protein (HOUGH et al. 2002). Particularly important is the fact that in case of a structural change of the protein, the immunologic assay may lead to wrong results, due to the possible alteration of antibody binding sites on the protein (MISCKE 2012).

The diagnosis of asymptomatic carrier is crucial for the control of haemophilia and it should be based on the pedigree analysis and specific genetic tests (MISCHKE 2012). The

calculation of the vWF concentration/FVIII activity ratio, which is usually elevated (> 2.00) in carrier dogs, can help to find the diagnosis (JOHNSTONE et al. 1984; MISCHKE 2012) because the accuracy of specific factor assays is limited (MISCHKE 2012). The APTT assay is not reliable for the detection of carriers due to the additional influence of other clotting factors (JOHNSTONE et al. 1984; MISCHKE 2012). Although carriers normally have about 50 % of normal FVIII activity (BENN et al. 1978; JOHNSTONE et al. 1984; MISCHKE 2012) or less than 50 % (SUGAHARA et al. 1996; MISCHKE 2012), there is an overlap between carrier and healthy dogs, which complicates the diagnose (BENN et al. 1978; JOHNSTONE et al. 1984).

Currently, the identification of mutations in some dog breed have enabled the elaboration of genetic tests to diagnose female carriers of the trait, which allows their elimination from breeding programmes (MISCHKE et al. 2011A; MISCHKE et al. 2011B; MISCHKE et al. 2011C). Alternatively, microsatellites markers and segregation analyses can be helpful for the diagnosis of carriers (BROOKS et al. 2008).

3. MATERIALS AND METHODS

3.1 Study design

A genetic study was performed to define the underlying genetic defect in a Fila Brasileiro with a known haemophilia B status (based on coagulometric FIX activity) and in three dogs with a known haemophilia A status (based on coagulometric FVIII activity): a Wire-Haired Dachshund, a Great Dane and a Poodle mix. The study also included two related Wire-Haired Dachshunds, which were considered suspected carriers of haemophilia A, and five related healthy Wire-Haired Dachshunds. From all these dogs, DNA was isolated from EDTA blood. In the haemophilic Fila Brasileiro, exon screening of the canine FIX gene was performed by using PCR and electrophoresis. Genomic DNA samples from five healthy Argentine Dogos were used as a normal standard. The obtained nucleotide sequences of the FIX gene from the control dog and the patient were compared with the wild type canine FIX mRNA (Gene Bank Accession Number NM_001003323.2) (TATSUMI et al. 2008). Genomic DNA from three healthy Fila Brasileiros was used to validate the results. In addition, the intronic sequences around exon 4 were also analysed. In the patients with haemophilia A and related dogs, the coding region of the canine FVIII gene was amplified and sequenced. Genomic DNA samples of five healthy Spanish Mastiffs were used as a control. The obtained nucleotide sequences were compared with the wild type canine FVIII mRNA (Gene Bank Accession Number AF016234) (CAMERON et al. 1998). To confirm the results, specific exon screenings were carried out with new primer pairs. Genomic DNA from ten random dog breeds and ten Great Danes were used to validate the results. In addition, several protein structure models were performed to prove the functional relevance of the identified mutation in a Great Dane.

3.2 Samples

3.2.1 Material from haemophilic dogs and related animals

Genomic DNA samples from haemophilic dogs and related Wire Haired Dachshund, which were patients of the Small Animal Clinic of the University of Veterinary Medicine Hannover (Hannover), were posted in deep-frozen condition (using dry ice) to Laboklin GmbH & Co. KG (Bad Kissingen, Germany) for investigation of the causative mutation responsible for haemophilia.

After reception, the genomic DNA samples were aliquoted into 0.2 millilitre (ml) tubes, properly labelled with barcodes and stored at -20 degree centigrade (ºC) to avoid degradation of genomic DNA.

Genomic DNA was obtained from a Fila Brasileiro (dog 1) with a known haemophilia B status, as well as from dogs of three breeds with known haemophilia A status: Wire-Haired Dachshund family (dog 2), a Great Dane (dog 10) and a Poodle mix (dog 11). In the Wire-Haired Dachshund family, DNA samples were also obtained from related animals (dogs 3-9) in order to identify possible carriers. The details of the patients are presented in Table 7 and Table 8 (see p. 51). The clinical signs and the relevant laboratory findings, which were found in the four haemophilic patients, are shown in Table 10 (see p. 52).

3.2.2 Control samples

The study included genomic DNA from five healthy Pyrenean Mastiffs in order to optimize the annealing temperature of the primers.

Genomic DNA samples of five healthy Spanish Mastiffs and five healthy Argentine Dogos were used as a normal standard in the FVIII and FIX genes screening, respectively (Table 9, see p. 51). Additional genomic DNA samples from three healthy Fila Brasileiros, were used to validate the genetic analysis in this breed. The study also included genomic DNA from ten healthy dog breeds, which were chosen at random and ten Great Danes with an undefined haemophilia A status, in order to validate the genetic analysis of the Wire-Haired Dachshund family and the Great Dane, respectively.

Table 7: Data on the haemophilic B patient, which was investigated to characterize the underlying genetic defect responsible for haemophilia B

Dog	Internal Number	Age	Sex	Breed	FIX activity[1]	Haemophilia B status
1	P1479	3M	Male	Fila Brasileiro	1 %	Severe form

[1] *= reference range 75-140%*
Abbreviation: Factor [F], month [M], per cent [%]

Table 8: Data concerning haemophilic A patients, which were studied to characterize the underlying genetic defect responsible for haemophilia A

Dog	Internal number	Age	Sex	Breed	FVIII activity[1]	Haemophilia A status
2	P1480	1Y3M	Male	WHD	9 %	Moderate form
3	P1496	7Y3M	Female	WHD	64 %	Suspected carrier
4	P1497	3Y4M	Female	WHD	62 %	Suspected carrier
5	P1498	4Y3M	Female	WHD	N	Healthy
6	P1499	2M	Male	WHD	N	Healthy
7	P1500	2Y4M	Female	WHD	N	Healthy
8	P1501	6M	Male	WHD	N	Healthy
9	P1502	13Y6M	Female	WHD	N	Healthy
10	P1636	7M	Male	Great Dane	4 %	Moderate form
11	P1694	4M	Male	Poodle mix	5 %	Moderate form

[1]= reference range 70-135%
Abbreviation: Factor [F], month [M], per cent [%], within reference range [N], Wire-Haired Dachshund [WHD], year [Y]

Table 9: Healthy dogs used as controls

Breed	n	Purpose
Pyrenean Mastiff	5	Annealing temperature
Spanish Mastiff	5	FVIII screening
Argentine Dogo	5	FIX screening
Fila Brasileiro	3	Validation of the genetic analysis
Random dog breeds	10	Validation of the genetic analysis
Great Dane	10	Validation of the genetic analysis

Abbreviation: Factor [F], amount [n]

Table 10: Clinical signs and relevant laboratory findings in the haemophilic patients

Dog	Internal number	Clinical signs	Relevant laboratory findings[1]
1	P1479	Weakness Haematoma from subcutaneous injection. Oedematous hind legs Spontaneous haemorrhage on right shoulder Generalized papules Free thoracic fluid	Prolonged aPTT[2]: 56.4 sec Hct: 29% FIX activity[3]: 1%
2	P1480	Lively Recurrent haematoma formation in limbs and in the lumbar region Spherical hearth silhouette	Prolonged aPTT[2]: 25,1 sec FVIII activity[3]: 9% vWF[4]: 99%
10	P1636	Depressed, lethargic with difficulties to stand and to move Pale mucous membranes Bleeding during teeth eruptions Haematoma formation after vaccination, and recurrent haematoma formation in the left hind limb Haematuria Related animals had spontaneous haemorrhage and haematomas formation at the vaccination site and in limbs	Hct: 9% Prolonged aPTT[2]: 19.4 sec FVIII activity[3]: 4%
11	P1694	Lively Bleeding after the change of teeth and after an eyelid injury	Prolonged aPPT[2]: 28.5 sec FVIII activity[3]: 5%

[1]*= reference ranges: aPTT: <13.5; Hct: 38-55%; FIX activity: 75-140%; FVIII activity: 70-135%.*

[2]*= semiautomatically measured using a Schnitger and Gross coagulometer and the reagent Boehringer (Amelung, Lemgo, Germany) following the manufacturer's instruction.*

[3]*= automatically measured by using an AMAX Destiny coagulation analyser (ball coagulometer analysis). Citrate plasma was 1:40 diluted with imidazole buffer and it was incubated with 20 microliter (µl) of human FVIII/FIX deficient plasma, and 20 µl of activating reagent (Actin, and all other reagents were purchased from Siemens Healthcare Diagnostics Products, Marburg) for 3 minutes at* 37 ºC. *After that, 3 µl of 25 mmol/L* $CaCl_2$ *solution was added. Time was measured until the clot formation and the factor activity was calculated by using a calibration curve based on different dilutions of a canine pool plasma (n=100).*

[4]*= measured using an immune-turbidimetric method (STA LIATEST vWF) following the manufacturer's instruction.*

Abbreviation: Activated Partial Thromboplastin Time [aPTT], coagulation factor [F], haematocrit value [Hct], per cent [%], seconds [sec], von Willebrand factor [vWF]

3.3 Materials

3.3.1 Laboratory equipment

ABI Applied Biosystems GMBH, Weiterstadt, Germany

- ABI PRISM® 3130 xl Genetic Analyzer (used to perform the capillary electrophoresis)

Consort bvba, Turnhout, Belgium

- UV-Schirm Consort, TFX-20M (ultraviolet light table utilised to view the bands of the PCR products)

Eppendorf AG, Hamburg, Germany

- Mastercycler epgradient S (used during the PCR technique)

Gebr. Liebisch GmbH & Co. KG, Bielefeld, Germany

- Heating block type 50126101 (used during the DNA isolation)

Heidolph Instruments GmbH & Co. KG, Schwabach, Germany

- Vortex-Genie® 2 (vortex mixer used during the DNA isolation, the PCR technique and the purification of cycle sequencing products)

Invitrogen, Karlsruhe, Germany

- E-gel® Mother Base (electrophoresis system used to perform the agarose gel electrophoresis)

LTF Labortechnik GmbH, Wasserburg, Germany

- DNA/RNA UV Cleaner UVC/T-M-AR (ultraviolet (UV) sterilizing cabinet used to prepare the PCR and sequencing reactions)

Nunc GmbH & Co. KG, Wiesbaden, Germany

- Filtair® XL8124 (vertical laminar flow cabinet used to dissolve the sequencing products with formamide)

Thermo Fisher Scientific, Karlsruhe, Germany

- Heraus Pico 17 Centrifuge (used during the purification of PCR products and cycle sequencing products)

3.3.2 Reagents

ABI Applied Biosystems GMBH, Weiterstadt, Germany

- Hi-Di™ Formamide (used to create a denaturing environment during the capillary electrophoresis)
- 3130 POP-7™ Performance Optimized Polymer (used to sieve DNA fragments during the capillary electrophoresis)
- BigDye® Terminator v1.1 Cycle Sequencing Kit (used in the cycle sequencing technique)

Carl Roth GmbH + Co. KG, Karlsruhe, Germany

- Rotipuran® ≥ 99.8%, p.a. Art.-Nr. 9065.4 (ethanol used to reconstitute Buffers AW1, AW2 during the DNA isolation, as well as Buffer PE during the purification of PCR products)

Diagnostica Stago Inc., New Jersey, USA

- STA LIATEST® vWF (used during the quantitative determination of vWF)

Sigma-Aldrich Co., Steinheim, Germany

- 2 - Propanol ≥ 99.8% (GC) 33539-2.5L-R (utilised to precipitate genomic DNA during the DNA isolation)

Eurofins MWG Operon, Ebersberg, Germany

- Oligonucleotides or primers 100 micromolar (µM) (used during the PCR technique and the cycle sequencing)

Roche Diagnostics, Indianapolis, USA

- Deoxynucleoside triphosphate (dNTPs) set PCR Grade. REF 03622614001 (used during the PCR technique)

Finnzymes Oy, Vantaa, Finland

- F-515 100 % DMSO, Phusion Blood Kit: Beta – test version (dimethyl-sulfoxide used to amplify exon 26 of canine FVIII gene)

Genaxxon bioscience, Ulm, Germany

- DF-Taq DNA-Polymerase (DNA-free taq) 5 units/µl M3185.0250 (used during the long PCR reaction)

Invitrogen, Karlsruhe, Germany

- E-Gel® Low Range Quantitative DNA ladder (marker used during the agarose gel electrophoresis)

Qiagen GmbH, Hilden, Germany

- PCR-Buffer 10x containing 15 mM (milimolar) $MgCl_2$. Mat. No. 1005079 (used during the PCR technique)
- $MgCl_2$ 25 mM Mat. No. 1005482 (used during the PCR technique)
- Taq DNA polymerase 5 units/µl Mat. No. 1005476 (used during the PCR technique)
- BioSprint® 15 DNA Blood Kit (used to isolate genomic DNA from whole blood)
- MinElute® PCR Purification Kit (utilized to purify the PCR products)
- DyeEx® 2.0 Spin Kit (used to perform the purification of sequencing reactions)

Sigma-Aldrich Co. LLC, Steinheim, Germany

- Chromasolv® Plus for HPLC 34877-2.SL (sterile distilled water used during the PCR technique, the electrophoresis and the cycle sequencing)

3.3.3 Laboratory consumable materials

ABI Applied Biosystems GMBH, Weiterstadt, Germany

- MicroAmpTM optical 96-well Reaction Plate (used during the capillary electrophoresis)
- Plate septa 96-well (20 PCS/PK) (used during the capillary electrophoresis)

Ansell EMEA Headquarters, Brussels, Belgium

- Touch NTuff® Disposable Nitrile Gloves 92-600 (used during the capillary electrophoresis)

Gilson Inc, Middleton, USA

- Pipetman (10 µl, 100 µl, 1000 µl) (used during the entire course of the study)

Invitrogen, Karlsruhe, Germany

- E-Gel® 48 4 % agarose GP (used for electrophoresis of PCR products)
- E-Gel® EX 1 % agarose gel (used for electrophoresis of a long PCR product)

Roesner-Mautby Meditrade GmbH, Kiefersfelden, Germany

- Vinyl 2000 PF (100Stk/pcs) (gloves used during the entire course of the study)

Sarstedt AG & Co., Nuembrecht, Germany

- Biosphere® Fil. Tip 20 neutral, 100 yellow, 1000 blue (sterile, non-pyrogenic, DNA free, RNase free and ATP free filter tips used during the entire course of the study)
- Micro tube (sterile) 1.5 ml (milliliter) (DNA reaction tubes used during the DNA isolation)

Nerbe plus GmbH, Winsen, Germany

- PCR - Reaktionsgefäss PP natur 0.2 ml, mit anhängendem Deckel (RNase, DNase and pyrogen free tubes used during PCR technique)

3.3.4 Software

ABI Applied Biosystems GMBH, Weiterstadt, Germany

- Sequencing analysis v5.2 (utilized during the capillary electrophoresis to reconstruct the sequence of nucleotides as an electropherogram)

National Center for Biotechnology Information, Bethesda, USA

- Basic Local Alignment Search Tool (used to perform sequence alignment)

UCSC Genome Bioinformatics Group, University of California, USA

- BLAT (utilized to research the exon and intron sequence of the FIX and FVIII genes)

Whitehead Institute, Cambridge, USA and Howard Hughes Medical Institute, Chevy Chase, USA.

- Primer3-web (used to design the PCR primers)

3.4 Methods

3.4.1 DNA isolation

Genomic DNA was isolated from 100 µl of whole blood collected in EDTA or sodium citrate anticoagulant by using a commercially available Biosprint® 15 DNA Blood kit, according to the manufacturer's instructions[1]. The protocol of this kit is based on four basic steps. During the first step, the cell membrane is lysed using Buffer AL and protease K. This step is followed by the genomic DNA precipitation with isopropanol 100 %. During the second step, the genomic DNA binds to the silica surface of MagAttract magnetic particles. After this step, contents such as proteins and polysaccharides are released using wash buffers (AW1, AW2 reconstituted with pure ethanol) and an air-drying step. The last step is the elution of the genomic DNA using Buffer AE (PARK 2007).

3.4.2 Screening of canine FIX and FVIII genes

Screening of the coding region and exon-intron boundaries of both genes in DNA samples of haemophilic and related dogs (Tables 7 and 8, see p. 50-51), as well as in control samples (Table 9, see p. 51), was performed using the following procedure:

- Primer design for PCR
- PCR technique
- Agarose gel electrophoresis
- DNA sequence analysis
- Determination of nucleic acid sequence of interest
- Comparison of the sequencing results with the wild type
- Validation of the genetic analysis

[1] Biosprint 15 DNA Handbook QIAGEN (2006)

3.4.2.1 Primer design for PCR

Canine FIX and FVIII primers, which were used to amplify the coding region and exon-intron boundaries of canine FIX and FVIII genes, were designed based on FIX and FVIII cDNA sequences described by TATSUMI et al. (2008) (Gene Bank Accession Number NM_001003323.2), and CAMERON et al. (1998) (Gene Bank Accession Number AF016234), respectively. Exon and intron sequences of canine FIX and FVIII genes were obtained with a bioinformatics software, BLAT programme (KENT 2002). Primer sequences were designed using the Primer 3 programme (ROZEN et al. 2000). For this design, each exon sequence together with approximately 20-25 bp of the adjacent intronic sequences were entered into the Primer 3 programme, automatically generating two primers (forward [*F*] and reverse [*R*]).

Several criteria were considered in general during the selection of primers to guarantee a successful amplification of the target DNA sequence:

- The selected target region was 100 to 450 bp in length, due to the fact that sequences longer than 1000 bp in length result in difficulties during the DNA amplification (HYNDMAN et al. 2003).

- Primers were located within intron sequences, 20-30 bp before and after the target DNA sequence, in order to amplify the complete exon sequence and exon-intron boundaries (HYNDMAN et al. 2003).

- The selected primer length was from 18 to 28 nucleotides to increase the specificity of the primer hybridization with its target and to equal the annealing temperature (WU et al. 1991). The annealing temperature (Ta) is the temperature at which the two primers bind to the DNA strands during the PCR reaction. The determination of the optimal annealing temperature guarantees an optimal binding of the primers to the template and a minimal binding of them to non-specific sequences. This temperature depends directly on the length and composition of the primers (NITSCHE 2007).

- The G/C content of the primers was established in general between 40 and 60 per cent. For primers with a low GC content, the sequence was extended to keep the optimal melting temperature (Tm) for the primers, and therefore the Ta. These three factors depend on each

other (RYCHLIK et al. 1990). The primer Tm is the temperature at which one-half of a target DNA duplex will dissociate and become single strand DNA. It depends on the primer length, the G/C content, the distribution of the nucleotides, and the reaction composition. Primers should have compatible Tm. This can easily be calculated using the formula of WALLACE et al. (1979), which adds 2 ºC for each A and T and 4ºC for each C and G, Tm= 2(A+T) + 4(G+C) (WALLACE et al. 1979; NITSCHE 2007).

- Complementary sequences between primers were avoided to prevent the hybridization between them and the primer dimer formation. Furthermore, bases repeated sequences were also evaded to prevent inappropriate hybridization to the template. Inverted repeated sequences were also avoided, in order to prevent internal binding that could have led to the formation of a hair-pin loop (HYNDMAN et al. 2003, NITSCHE 2007). Once the primers had been designed, they were ordered from Eurofins MWG Operon (Ebersberg, Germany).

- **Primer designed to amplify the coding region of the canine FIX gene**

Fourteen primers (seven *F* and seven *R*) were designed to amplify exon 1 to exon 7 of the FIX gene. Since exon 8 is 548 bp in length, it was divided into three parts and a pair of specific primers (*F* and *R*) was designed for each part of exon 8. In total, twenty primers (ten *F* and ten *R*) were used to amplify all exons of the canine FIX gene. Primer sequences, primer binding sites in DNA, and the exon target for each primer pair are represented in Table 11 (see p. 60).

- **Primer designed to amplify the coding region of the canine FVIII gene**

To amplify the coding region of the canine FVIII gene, one primer pair was designed for each exon (26 exons), except for exon 14, which was divided into fifteen parts of approximately 200 bp (part 14-1 to part 14-15). A pair of primers was designed for each part of exon 14. In total, eighty primers were designed (forty *F* and forty *R*) of which thirty primers (fifteen *F* and fifteen *R*) were only used to amplify exon 14. Detailed information about the primers is included in Table 12 (see pages 61-62).

Table 11: Canine FIX primers. *Primer sequences, primer binding sites and exon targets are shown in the table*

Primer	Sequence (5' to 3')	Binding site	Exon (bp)
1F	5‘-TGTGTCACTTCCGGCTTCAG-3‘	5’UTR	Exon 1 (67)
1R	5‘-TCTACAGCTAGAAGACAAGCATAC-3‘	Intron 1	
2F	5‘-GATAAATTGGCTTTGGGATTACTTGG-3‘	Intron 1	Exon 2 (164)
2R	5‘-GTACTTTGCATCTGAAGAACATTACG-3‘	Intron 2	
3F	5‘-GCAGTTTTGAAGAAGCACGGG-3‘	Exon 2	Exon 3 (25)
3R	5‘-ACACAGAGAAAAGATACCTAATTCTCA-3‘	Intron 3	
4F	5‘-AAGACAGGGGCATCCCATAATC-3‘	Intron 3	Exon 4 (114)
4R	5‘-CTTCCATTAAGTGTTCCTTACCAC-3‘	Intron 4	
5F	5‘-CTCCCAAGCCTCTTTCCATG-3‘	Intron 4	Exon 5 (129)
5R	5‘-CAAACAGGGTTTATGAAAGTATGTGAAC-3‘	Intron 5	
6F	5‘-CGTTCCGCTTGCCAATGAAAAATA-3‘	Intron 5	Exon 6 (197)
6R	5‘-CTCTGGGCTCCAGTTTTGAC-3‘	Intron 6	
7F	5‘-ACACCCCTGCCTATCAACAG-3‘	Intron 6	Exon 7 (115)
7R	5‘-GCTCCTCTAGCATTAGCCC-3‘	Intron 7	
8_1F	5‘-CCTTGGCAAATACGTTTATGTGTAAG-3‘	Intron 7	Exon 8 (304)
8_1R	5‘-TCATGGAAGCCAGCACAGAAC-3‘	Exon 8	
8_2F	5‘-TTCCTCAAATTTGGGTCTGG-3‘	Exon 8	Exon 8 (110)
8_2R	5‘-CCTAAACGTGTCAACCTTGGA-3‘	Exon 8	
8_3F	5‘-TTCCTCAAATTTGGGTCTGG-3‘	Exon 8	Exon 8 (134)
8_3R	5‘-TGCCAAGGAGGGGAGAATC-3‘	3’UTR	

Abbreviation: Base pairs [bp], forward [F], reverse [R], untranslated region [UTR]

Table 12: Canine FVIII primers. *Primer sequences, primer binding sites and exon targets are shown in the table*

Primer	Sequence (5‘ to 3‘)	Binding site	Exon (bp)
1F	5‘-TTTCTCCTGGGAGCTGAAGA-3‘	5’UTR	Exon 1 (146)
1 R	5‘-CCCTATGGAACACGCCTTTA-3‘	Intron 1	
1nF	5‘-TTTGCCCTTCAGCCTTAGTG-3‘	Exon 1	Exon 1 (109)
1nR	5‘-GCATGCACCTTACCTCAA-3‘	Intron 1	
2 F	5‘-AAAAAGGGTTCACCAAAATAAATG-3‘	Intron1	Exon 2 (122)
2 R	5‘-GGACATCAGTTACTCAGTTGCAT-3‘	Intron 2	
3 F	5‘-ATCACAGGGGCATGTTTTTC-3‘	Intron 2	Exon 3 (123)
3 R	5‘-CCTGAGATGACAGGACAACG-3‘	Intron 3	
4 F	5‘-TCAACAGTGGGTATGGAAAGG-3‘	Intron 3	Exon 4 (213)
4 R	5‘-CTCCCTTATTTCATTTGAGTCCT-3‘	Intron 4	
5 F	5‘-TGATGTCTCCTAGTGTTGATTTCC-3‘	Intron 4	Exon 5 (69)
5 R	5‘-CCAAAGCAGATTGCAGTTCA-3‘	Intron 5	
6 F	5‘-GTCCTCGCCTCCTCTTTCAT-3‘	Intron 5	Exon 6 (99)
6 R	5‘-GACAGAGTCCGAGGCTAACG-3‘	Intron 6	
7 F	5‘-GGTCAATTTGTCCATCCTATCC-3‘	Intron 6	Exon 7 (222)
7 R	5‘-CATTCAAGGTGAAATCTTATACTGG-3	Intron 7	
8 F	5‘-CCCTAACATTGTTTGTTTTGTCC-3‘	Intron 7	Exon 8 (259)
8 R	5‘-CACGGCTTGAGAATTTGTTG-3‘	Intron 8	
9 F	5‘-TTCTCCATCCCAACATCTCA-3‘	Intron 8	Exon 9 (172)
9 R	5‘-ACTCAACCTTACTTCTCTTCTTCAA-3‘	Intron 9	
10 F	5‘-AGGCCACACTTCCTGTTGAT-3‘	Intron 9	Exon 10 (94)
10 R	5‘-CCACTACGATGTCCGTGCTA-3‘	Intron 10	
11 F	5‘-GATTTACCAATATGTGGCTTGTT-3‘	Intron 10	Exon 11 (215)
11 R	5‘-CCATCAAACCGAGAACTTGAA-3‘	Intron 11	
12 F	5‘-CGTGCCATCACTTCCATGT-3‘	Intron 11	Exon 12 (151)
12 R	5‘-TTGGTGGCTTCAATTTTCTTTT-3‘	Intron 12	
13 F	5‘-CAGCTAAAACATCTTGTTCTCCTTT-3‘	Intron 12	Exon 13 (210)
13nF	5‘-TGACAACTTGCAGCTGTCAG-3‘	Exon 13	
13 R	5‘-ACCCTCTGCCACTCTCTCCT-3‘	Intron 13	
13nR	5‘-CATGAAGACAGTTTCTCCTG-3‘	Exon 13	
14-1 F	5‘-GGGACCAGGGTTGTGAGTAA-3‘	Intron 13	Exon 14 (281)
14-1 R	5‘-GTCCCAACAGCATCAACAAA-3‘	Exon 14	
14-2 F	5‘-CAATCTGGAGAAAGAACACAGC-3‘	Exon 14	Exon 14 (200)
14-2 R	5‘-GCAGTTCTGGCTCAGGAGTAA-3‘	Exon 14	
14-3 R	5‘-AAATACAATGGTACCTAAATGACTG-3‘	Exon 14	Exon 14 (200)
14-3 R	5‘-AAATACAATGGTACCTAAATGACTG-3‘	Exon 14	
14-4 F	5‘-CCCCCAAATATGTCAGTTCA-3‘	Exon 14	Exon 14 (200)
14-4 R	5‘-CATTATCTTTGATTAATGAAGCAGG-3‘	Exon 14	
14-5 F	5‘-TGAGAGAAAATGTATTATCAATGGA-3‘	Exon 14	Exon 14 (200)
14-5 R	5‘-GCTGTAGTATTTCTGTCCATAAACG-3‘	Exon 14	
14-6 F	5‘-CAGTACCTCAGTCTGGCAAGATA-3‘	Exon 14	Exon 14 (200)
14-6 R	5‘-ACTGGGCCTTTGCTCAGA-3‘	Exon 14	
14-6nR	5‘-CGTAGTGGCACAGGGTCTTCTTT-3‘	Exon 14	Exon 14 (130)

Table 12: (continuing)

14-7 F	5‘-GGATAAAGACCCATGGCAAG-3‘	Exon 14	Exon 14 (200)
14-7 R	5‘-TTCTTTCTATCTCTTCCGGAGATT-3‘	Exon 14	
14-8 F	5‘-TTGCTAACTTGGCTAATGTCCA-3‘	Exon 14	Exon 14 (200)
14-8 R	5‘-CCCTTCACTATGTGGCGAAT-3‘	Exon 14	
14-9 F	5‘-CAGGTTTAGAAGAGCAGCCATA-3‘	Exon 14	Exon 14 (200)
14-9 R	5‘-CATTTGCAATAACCTTCCTTTCA-3‘	Exon 14	
14-10 F	5‘-AACGTGGTAAGCGGAGTTTG-3‘	Exon 14	Exon 14 (200)
14-10 R	5‘-TTTGCAACGGGTAATGCAG-3‘	Exon 14	
14-11 F	5‘-GAGGAATCATGTCACCATTCAA-3‘	Exon 14	Exon 14 (200)
14-11 R	5‘-TGAACTTTCCTTGCCCTTCA-3‘	Exon 14	
14-12 F	5‘-GAAATAACCTCTCTTTAGCCTTTG-3‘	Exon 14	Exon 14 (200)
14-12 R	5‘-TTTCCCATGAGATCCAGGT-3‘	Exon 14	
14-13 F	5‘-TTTCCCTACAAAAACTAGCAATGA-3‘	Exon 14	Exon 14 (200)
14-13 R	5‘-AAAAGCTGTGTTCGTCTGTGA-3‘	Exon 14	
14-14 F	5‘-TTGCTTGGGATAACCACTATGA-3‘	Exon 14	Exon 14 (200)
14-14 R	5‘-TTTGTCTTCCTCTGGCTGAA-3‘	Exon 14	
14-15 F	5‘-GCTCTCAAAACCCACCAGTC-3‘	Exon 14	Exon 14 (219)
14-15 R	5‘-TCAGCACCTGAAGGCAGAG-3‘	Intron 14	
15 F	5‘-CACCTGGTCCACCTGAAAAT-3‘	Intron 14	Exon 15(154)
15 R	5‘-TTTTCTTATAATTTCATTGTCCTCA-3‘	Intron 15	
16 F	5‘-AAAAGACACTTGAATTTTCTGTAAA-3‘	Intron 15	Exon 16 (213)
16 R	5‘-CCAGTAAGTGGTCAGAGCAATG-3‘	Intron 16	
17 F	5‘-GGGATTGCTGTCTTCTCTCC-3‘	Intron 16	Exon 17 (229)
17 R	5‘-GAGGCTCCCTTGCTGAGG-3‘	Intron 17	
18 F	5‘-TCAACAGCCTTTTCTGTCTTCTC-3‘	Intron 17	Exon 18 (183)
18 R	5‘-GAGGTGGAAGAGGGCACA-3‘	Intron 18	
19 F	5‘-CGTATCTCATGCTCATTGCTTT-3‘	Intron 18	Exon 19 (117)
19R	5‘-AGAAAGGCAACCATGCTAGGA-3	Intron 19	
19 nR	5‘-CTCTTGCTGTACACCAGAAAC-3‘	Exon 19	
20 F	5‘-CTGTCGTCCTCCCCTCCT-3‘	Intron 19	Exon 20 (72)
20 R	5‘-TCAGCTCAGTTCTTCGAGACA-3‘	Intron 20	
21 F	5‘-GACCAACGCAGCTGAATCTA-3‘	Intron 20	Exon 21 (86)
21 R	5‘-GCCATTGAATGTGATGCACT-3‘	Intron 21	
22 F	5‘-CAAGTAACCTCAGAAGGTGGTACA-3‘	Intron 21	Exon 22 (156)
22 R	5‘-AAGATTCATGCATTCTCTTGAAA-3‘	Intron 22	
23 F	5‘-CCTGGCATGTACCTGTGCTA-3‘	Intron 22	Exon 23 (145)
23 R	5‘-GGAAGGGCACGTGATGACT-3‘	Intron 23	
24 F	5‘-GGCTGCCTCTTGTCCTTATG-3‘	Intron 23	Exon 24 (150)
24 R	5‘-GTCGGTTAAGTGGCGAGTGT-3‘	Intron 24	
25 F	5‘-CGGTACTGTTCCCCAGGATA-3‘	Intron 25	Exon 25 (176)
25 R	5‘-TTGTGCCTTTCCCGTGAG-3‘	Intron 26	
26 F	5‘-CCTGGAAGCTGCTGGAAG-3‘	Intron 26	Exon 26 (156)
26 R	5‘-GGAGACAGGGCCGCAGAGG-3‘	3’UTR	

Abbreviation: Base pairs [bp], forward [F], reverse [R], untranslated region [UTR]

3.4.2.2 PCR technique

3.4.2.2.1 Test principle

The PCR is an indispensable tool to produce a selective exponential amplification of a specific genomic DNA sequence. This was developed in 1983 by MULLIS (1990). Two specific oligonucleotide primers (*F* and *R*) are involved in pursuing this goal. Each of the primers is complementary to the 5' and 3' end of one of the strands and they flank the target genomic DNA sequence, which will be amplified. The amplification procedures are based on repeated thermal cycles: denaturation of genomic DNA, annealing of the primers to the complementary target strands, and extension by DNA polymerase. The amplified strand (amplicon) is complementary to the target DNA and is limited by two regions, which are complementary to the primers. When the next cycle of the reaction continues, the amplicon will act as a template, resulting in an exponential amplification of the template strand (2^n results, "n" being the number of the cycles) (MULLIS et al. 1986).

PCR reaction is made up of several essential components and reagents: DNA template (contains the target region), primers (F and R complementary to the 5' and 3' end of the target DNA, respectively), DNA polymerase enzyme, dNTPs (used by DNA polymerase during extension), a buffer solution (to optimize the activity and stability of DNA polymerase) and magnesium (co-factor of DNA polymerase). The used DNA polymerase was purified from thermophilic bacteria Thermus aquaticus, which functions optimally at a temperature of approximately 72 ºC (SAIKI et al. 1988; BARTLETT et al. 2003).

3.4.2.2.2 Test procedure

A total of ten PCR reactions covering all eight exons and exon-intron boundaries of the FIX gene were used for the genetic analysis in dog 1 and forty PCR reactions covering all 26 exons and exon-intron boundaries of the FVIII gene were used for the genetic analysis in dogs 2-11. Genomic DNA from five healthy Spanish Mastiffs and five healthy Argentine Dogos was used as positive control to amplify all exons of the FVIII gene and FIX gene, respectively. Negative controls (distilled water instead of the DNA sample) were included in each PCR test in order to check for contamination.

- **Standard reaction mixture contents**

PCR amplifications of the coding region and exon-intron boundaries of canine FIX and FVIII genes were carried out using a final volume of 50 µl. PCR reactions were generally prepared with the following components: 5 µl of ten times concentrated PCR-Buffer, 2 µl of 25 millimolar (mM) magnesium chloride ($MgCl_2$), 5 µl of 2 mM dNTPs, 1 µl of 10 µM *F* and *R* primers, 33.5 µl of sterile distilled water, 0.5 µl of 5 units/µl of Taq DNA polymerase and 2 µl of approximately 50 nanogram/µl (ng/µl) genomic DNA (Table 13).

Table 13: Standard reaction mixture contents: components, volumes and concentrations used to amplify the coding region of canine FIX and FVIII genes

Component	Volume (µl)	Concentration
PCR-Buffer	5	10 x
$MgCl_2$	2	25 mM
dNTPs	5	2 mM
Forward primer	1	10 µM
Reverse primer	1	10 µM
Sterile distilled water	33.5	-
Taq DNA polymerase	0.5	5 U/µl
Genomic DNA	2	approx. 50 ng/µl
Total	50	

Abbreviation: deoxynucleotide triphosphates [dNTPs], magnesium chloride [$MgCl_2$], micromolar [µM], millimolar [mM], microliter [µl], nanogram [ng], polymerase chain reaction [PCR], times concentrated [x], unit [U]

- **Standard PCR cycling programme**

All the components were pipetted into a 0.2 ml PCR reaction tube. The tubes containing the reaction mixes were then placed in a closed thermal cycler machine (Mastercycler). This machine was used to replicate the temperature changes required for the PCR reaction. The amplification procedures included an initial pre-denaturation step at 95 °C for five minutes, followed by 35 thermal cycles. Each cycle included three steps. First, a denaturation at 95 °C for 30 seconds, during which the hydrogen bonds were broken in the double-stranded DNA to create a single strand of DNA which would serve as a template. The denaturation was

followed by the annealing of the primers to the complementary target strands for 30 seconds (temperature based upon the optimized annealing temperature of primers). The last step of the cycle was the elongation of the annealed primer by the DNA polymerase at 72 °C for one minute. The DNA polymerase adds DNA nucleotides from 5' to 3', reading the template DNA in the 3' to 5' direction. The chain growth involves the formation of a phosphodiester bridge between the 3'-hydroxil group at the growing end of the primer and the 5'-phosphate group of the added dNTP. Finally, an elongation step at 72 °C for 5 minutes was added to ensure that all amplicons had completely been extended. After the PCR amplification, PCR products were maintained in the machine at 4 ºC (Table 14). The total duration of the PCR amplification was one hour and 25 minutes.

Table 14: Standard PCR cycling programme: temperature and times used to amplify the exons of the FIX and FVIII genes

PCR Step	Temperature (ºC)	Time (minutes)	
Pre-denaturation	95	5	
Denaturation	95	0.5	35 cycles
Annealing	Ta of primer	0.5	
Elongation	72	1	
Final elongation	72	10	
Cooling	4	undefined [1]	

[1] = time until collection of tubes from the machine, maximum twelve hours
Abbreviation: Annealing temperature [Ta], degree centigrade [ºC], polymerase chain reaction [PCR]

- **Experimental determination of optimal annealing temperature**

Genomic DNA samples from Pyrenean Mastiffs (Table 9, see p. 51) were used for the experimental determination of the optimal annealing temperature of all primers for PCR. For this purpose, six PCR reaction tubes were utilised for each pair of primers. During the annealing step, each PCR reaction tube was subjected to a specific temperature, between 52 ºC to 62 °C with a temperature difference of two degrees between each one (52 °C, 54 °C, 56 °C, 58 °C, 60 °C and 62 °C). The standard PCR and thermal cycler conditions were used.

After that, PCR products were analysed on agarose gel electrophoresis to select the optimal annealing temperature of the primers. In some cases (exon 6, 10, 13, 17 and 18 of the FVIII gene), this gradient specified above was inadequate to result in a clear band in the gel. Therefore, in these cases the gradient had to be elongated to 68 °C. The optimal annealing temperatures can be found in Table 15 and Table 16 (see pages 67-68).

- **Optimisation of PCR conditions**

The PCR was optimised for the amplification of exons 10, 12, 14 (14-3, 14-6, 14-7, 14-8, 14-10, 14-14, 14-15), 15, 17 and 21 of the canine FVIII gene. For this purpose, deviating from the routine PCR protocol (Table 13, see p. 64), 3 µl of 25 mM $MgCl_2$, 3 µl of genomic DNA and 31.5 µl of sterile distilled water were used (Table 16, see p. 68). The volume of the other PCR components (primers, dNTPs, PCR-Buffer and DNA polymerase) corresponded to the routine protocol (Table 13, see p. 64).

Optimisation of the PCR had to also be performed to amplify exon 13 and 19. Use of originally designed primers (13F+13R, 19F+19R) with the standard PCR protocol or in higher concentration (20 µM) and with variable volumes of 25 mM $MgCl_2$ (2-5 µl), genomic DNA (2-5 µl), sterile distilled water (27.5-33.5 µl) and a temperature gradient of 52 to 66 ºC resulted in various detectable bands using agarose gel electrophoresis. Finally, a clear band was observed by using a new pair of primers, which were manually designed: 13n*F* and 13n*R* for the amplification of exon 13 and 19n*R* for the amplification of exon 19 (Table 12 and Table 16, see pages 61, 62 and 68).

The amplification of exon 26 was impossible using the standard PCR reaction and thermal cycler conditions mentioned before. The analysis of the GC content of exon 26 revealed a GC content of 70 % (30 % AT). A high GC content probably impeded the amplification due to poor denaturation at 96 ºC or primer dimer formation (secondary to the complementary bases in the primers) or mispriming formation (primer binding to an unintended sequence). To solve this low yield and specificity, 5 % dimethylsulfoxide (DMSO) was added to the PCR mixture and the denaturation temperature was increased to 98 ºC in order to enhance the efficacy of PCR in this GC-rich DNA sequence (DUTTON et al. 1993; CHOI et al. 1999; JENSEN et al. 2010). This resulted in the following modified composition of the master mix: 1 µl of 5 % DMSO, 3 µl of 25 mM $MgCl_2$, 3 µl of genomic DNA, and 30.5 µl of sterile distilled water,

maintaining an equal volume of each of the other components (primers, dNTPs, PCR-Buffer and DNA polymerase) (Table 16, see p. 68).

- **Summary of reaction mixture contents and annealing temperature**

The volumes of $MgCl_2$, genomic DNA and sterile distilled water, which were used for each exon during the PCR technique, as well as the specific temperatures used during the annealing step, are shown in Table 15 (FIX) and Table 16 (FVIII).

Table 15: Reaction mixture contents and Ta to amplify the coding region of the canine FIX gene

Exon	Primers	$MgCl_2$ (µl)	DNA (µl)	H_2O (µl)	Ta (°C)
Exon 1	*1F+1R*	2	2	33.5	60
Exon 2	*2F+2R*	2	2	33.5	56
Exon 3	*3F+3R*	2	2	33.5	60
Exon 4	*4F+4R*	2	2	33.5	58
Exon 5	*5F+5R*	2	2	33.5	54
Exon 6	*6F+6R*	2	2	33.5	58
Exon 7	*7F+7R*	2	2	33.5	54
Exon 8-1	*8-1F+8-1R*	2	2	33.5	54
Exon 8-2	*8-2F+8-2R*	2	2	33.5	60
Exon 8-3	*8-3F+8-3R*	2	2	33.5	58

Abbreviation: Annealing temperature [Ta], degree centigrade [°C], forward [F], sterile distilled water [H_2O], magnesium chloride [$MgCl_2$], microliter [µl], reverse [R]

Table 16: Reaction mixture contents and Ta used to amplify the coding region of the canine FVIII gene

Exon	Primers	$MgCl_2$ (µl)	DNA (µl)	H_2O (µl)	Ta (°C)
Exon 1	*1F+1R*	2	2	33.5	55
Exon 2	*2F+2R*	2	2	33.5	56
Exon 3	*3F+3R*	2	2	33.5	59
Exon 4	*4F+4R*	2	2	33.5	58
Exon 5	*5F+5R*	2	2	33.5	62
Exon 6	*6F+6R*	2	2	33.5	67
Exon 7	*7F+7R*	2	2	33.5	60
Exon 8	*8F+8R*	2	2	33.5	62

Table 16: (continuing)

Exon 9	*9F+9R*	2	2	33.5	58
Exon 10	*10F+10R*	3	3	31.5	66
Exon 11	*11F+11R*	2	2	33.5	60
Exon 12	*12F+12R*	3	3	31.5	63
Exon 13	*13F+13nR*	2	2	33.5	60
	13nF+13R	2	2	33.5	63
Exon 14-1	*14-1F+14-1R*	2	2	33.5	60
Exon 14-2	*14-2F+14-2R*	2	2	33.5	60
Exon 14-3	*14-3F+14-3R*	3	3	31.5	62
Exon 14-4	*14-4F+14-4R*	2	2	33.5	58
Exon 14-5	*14-5F+14-5R*	2	2	33.5	61
Exon 14-6	*14-6F+14-6R*	3	3	31.5	62
Exon 14-7	*14-7F+14-7R*	3	3	31.5	56
Exon 14-8	*14-8F+14-8R*	3	3	31.5	57
Exon 14-9	*14-9F+14-9R*	2	2	33.5	60
Exon 14-10	*14-10F+14-10R*	3	3	31.5	58
Exon 14-11	*14-11F+14-11R*	2	2	33.5	57
Exon 14-12	*14-12F+14-12R*	2	2	33.5	57
Exon 14-13	*14-13F+14-13R*	2	2	33.5	59
Exon 14-14	*14-14F+14-14R*	3	3	31.5	59
Exon 14-15	*14-15F+14-15R*	3	3	31.5	62
Exon 15	*15F+15R*	3	3	31.5	56
Exon16	*16F+16R*	2	2	33.5	56
Exon17	*17F+17R*	3	3	31.5	65
Exon18	*18F+18R*	2	2	33.5	63
Exon 19	*19F+19nR*	2	2	33.5	60
Exon 20	*20F+20R*	2	2	33.5	59
Exon 22	*22F+22R*	2	2	33.5	58
Exon 23	*23F+23R*	2	2	33.5	62
Exon 24	*24F+24R*	2	2	33.5	62
Exon 25	*25F+25R*	2	2	33.5	58
Exon 26[1]	*26F+26R*	3	3	30.5	62

[1] *= to amplify exon 26, 5 % DMSO was also added in the PCR mixture.*

Abbreviation: Annealing temperature [Ta], degree centigrade [°C], Dimethyl-sulfoxide [DMSO], f*orward [F], sterile distilled water [H_2O], magnesium chloride [$MgCl_2$], microliter [µl],* percentage [%], *reverse [R]*

3.4.2.3 Agarose gel electrophoresis

All PCR products were analysed on agarose gel electrophoresis (4% E-Gel® 48 agarose gel). E-Gel® 48 agarose gel contains two rows, each with twenty four sample wells and two marker wells. To avoid gel resolution loss, which is associated with high salt levels, PCR products and markers were diluted with sterile distilled water by initially pipetting a defined volume of distilled water and afterwards the residual volume of PCR product or marker into the wells. A volume of 12 µl of sterile distilled water was used to dilute 8 µl of PCR product. The molecular weight standard (E-Gel® Low Range Quantitative DNA Ladder) was also diluted 1:2 (10 µl of sterile distilled water and 10 µl of marker). Dilute markers were pipetted into the four marker wells. The remaining empty wells were loaded with 20 µl of sterile distilled water. Once all PCR products had been pipetted, the gel was placed into the two electrode connections on the electrophoresis system (Mother E-Base) and the system was connected to an electric current. The selected run programme was 17 minutes long, according to the manufacturer's recommendations[2]. During the agarose gel electrophoresis, the influence of an electric field leads to the migration of the negatively charged nucleic acids to the positive electrode of the gel (GUILLIATT 2002). The electrophoretic mobility of the nucleic acids is inversely proportional to the $\log_{10}$ of the number of base pairs, allowing the separation of the PCR product in size (HELLING et al. 1974). E-Gel® 48 contains fluorescent dye ethidium bromide, which intercalates between the bases of the DNA, allowing it to be displayed when given ultraviolet radiation of 260-300 nanometre (GUILLIATT 2002). After the run, the bands of the PCR products were viewed using an UV table. The presence of a marker in the gel, which consists of five linear double-strand DNA fragments (100, 200, 400, 800 and 2000 bp) allowed the identification of the size of the PCR product bands. The visualization of a clear band in the gel with the expected size indicated the possible target sequence amplification. The presence of various bands meant that the primers had non-specifically bound to the target sequences, and the absence of the band indicated that no PCR product had been amplified. In these last cases, the PCR technique was repeated, and in some cases, a

[2]E-Gel® Technical Guide: General information and protocols for using E-Gel® pre-cast agarose gels, INVITROGEN (2008)

PCR optimization was performed or a new pair of primers was designed as previously described.

3.4.2.4 DNA sequence analysis

3.4.2.4.1 Test principle

The "DNA sequence analysis" allows determination of the order of nucleotides in a particular sequence of DNA. In this study, analysis of generated PCR fragments was based on the dye-terminator sequencing method, a variant of the chain-terminator method, also known as Sanger sequencing (SANGER et al. 1977). Dye-terminator sequencing was developed by Leroy Hood in 1986 (SMITH et al. 1986). The underlying method is equivalent to Sanger, but it incorporates some improvements to speed up and automate the sequencing method. Synthesis of the strand is supplied with dNTPs, primers, DNA polymerase and chain terminating dideoxynucleotide-5'-triphosphates (ddNTPs), which were developed by SANGER et al. (1977). These ddNTPs lack the 3'OH group, required for the formation of a phosphodiester bond between the nucleotides during the DNA strand extension. Thus, their incorporation will stop the synthesis of the strand, resulting in DNA fragments of different length in accordance with the moment at which the ddNTP was incorporated. In contrast to Sanger's method, each ddNTP is labelled with a different-coloured fluorescent dye, which allows us to perform the sequencing reaction in a single reaction tube rather than in four, resulting in DNA fragments of different colours. The labelled DNA fragments can be visualised on capillary electrophoresis by using automated sequencers (STIRLING 2003; KUMAR et al. 2007).

3.4.2.4.2 Test procedure

After amplification of the exons of FIX and FVIII genes from the subject animals and the visualization of one single band in the agarose gel electrophoresis, fragments were analysed using the following steps:

- Purification of PCR products
- Cycle sequencing
- Purification of cycle sequencing products
- Capillary electrophoresis

Initially, all PCR products were purified to release the PCR components that had not been used during the PCR reaction (PCR primers, DNA polymerase, dNTPs and $MgCl_2$) and to equal the optimal sequencing component ratios. This step was done by using the MinElute® PCR Purification Kit, according to the manufacturer's recommendations[3]. This kit combines the use of spin columns with a silica membrane to purify amplicons from 70 bp to 4 kb. The kit contains specific buffers (PB, PE and EB), which were used for the amplicon binding to the silica membrane, washing and final elution, respectively.

After purification, the cycle sequencing technique was performed. This step combines PCR technique and dideoxy terminator chemistry (KUMAR et al. 2007). It was carried out using the BigDye® Terminator v1.1 Cycle Sequencing kit, which is a specific kit to sequence short PCR products and is also compatible with all automated capillary electrophoresis machines[4]. It was used according to the manufacturer's instructions[4-5].

Sequencing reactions were set up in a total volume of 10 μl containing the following components: 2 μl of purified PCR product, 3 μl of sterile distilled water, 1 μl of F or R primer and 4 μl of terminator ready reaction mix (T-mix). T-mix includes dNTPs, dye-labelled ddNTPs (ddATP, ddCTP, ddGTP and ddTTP), which each carry a different fluorescent dye colour (green, blue, black and red, respectively) and AmpliTaq DNA polymerase. All reagents added in the sequencing reactions can be found in Table 17 (see p.73). These components were pipetted in two 0.2 ml PCR tubes, one of them contained the specific forward primer and the other tube contained the specific reverse primer (Tables 11 and 12, see pages 60-62).

The sequencing reaction was performed in a closed thermal cycler machine, which was used to replicate the temperature changes required for the cycle sequencing. The cycle sequencing protocol included an initial pre-denaturation step at 96 °C for one minute, followed by 26 thermal cycles. Each cycle included three steps: A rapid denaturation at 96 °C for ten seconds, annealing step for five seconds using the annealing temperature of the primers (Tables 15 and 16, see pages 67-68) and elongation step at 60 °C for four minutes. Finally, a final elongation

[3] MinElute Handbook QIAGEN (2008)

[4] Course material of DNA Sequencing Applications APPLIED BIOSYSTEMS (2009)

[5] BigDye® Terminator v1.1 Cycle Sequencing kit Protocol APPLIED BIOSYSTEMS (2002)

step at 60 °C for one minute was performed. After the cycle sequencing, the sequencing product was maintained in the machine at 4 ºC (Table 18, see p. 73). The total duration of the cycle sequencing was one hour and 51 minutes.

Purification of cycle sequencing products was carried out to remove the unincorporated dye-terminators, which can interfere with the visualization of sequencing results[6]. This step was performed by using DyeEx® 2.0 Spin Kit, following the manufacturer's instructions[7]. This kit contains a pre-hydrated gel in the spin column that retains the dye-terminators, which have not been used during the cycle sequencing, allowing the recovery of the purified sequencing reaction product into a centrifuge tube.

The sequencing products were consequently analysed by capillary electrophoresis in an automated sequencer ABI PRISM® 3130 Genetic Analyzer. The machine is a 16 capillary instrument with a single 96 well plate platform for the automated analysis of up to 96 samples in a single run. The capillary is a tube approximately 50 centimetres long and 50 micrometres in diameter. It contains a performance optimized polymer solution (POP - 7™ polymer), which sieves DNA fragments according to length. In practice, the samples were prepared in a total volume of 13 µl. A volume of 6 µl of each sequencing reaction was put into a 96 sample plate. The samples were dissolved in 7 µl of formamide (Hi-Di Formamide) to create a denaturing environment. The Plate Septa was put on the sample plate and on this, a plate retainer. Afterwards the plate was placed in the ABI PRISM® 3130 Genetic Analyzer to perform the capillary electrophoresis. During the capillary electrophoresis, the negatively charged sequencing products were exposed to a positive voltage that injected them into the capillary (electrokinetic injection). After that, sequencing products were moved with the electrophoretic flow from the negative electrode (cathode) towards the positive electrode (anode). When the fluorescent dye-labelled sequencing products passed the laser, the fluorescence emitted by each of the ddNTPs was collected in a charge-coupled device camera, which provided digital signals (BUTLER 2011). These digital signals were consequently

[6] Course material of DNA Sequencing Applications APPLIED BIOSYSTEMS (2009)
[7] DyeEx 2.0 Spin Kit Handbook QIAGEN (2002)

integrated by computer software (DNA Sequencing Analysis Software v5.1)[8], reconstructing the sequence of nucleotides in the original template as a diagram, known as the electropherogram (BUTLER 2011).

Table 17: Sequencing reactions contain: *components and volumes used to sequence the amplified sequences of the FVIII and FIX genes*

Component	**Volume (µl)**
Purified PCR product	2
Sterile distilled water	3
Forward or reverse primer	1
T-mix	4
Total	10

Abbreviation: microliter [µl], polymerase chain reaction [PCR], terminator ready reaction mix [T-mix]

Table 18: Standard cycle sequencing programme: *Description of the cycle sequencing steps (temperature and times) used to sequence the obtained PCR products from the amplification of the coding regions of the FIX and FVIII genes*

Step	**Temperature (ºC)**	**Time (seconds)**	
Pre-denaturation	96	60	
Denaturation	96	10	26 cycles
Annealing	Ta of primer	5	
Elongation	60	240	
Final elongation	60	60	
Cooling	4	-[1]	

[1] *= PCR products were held at a temperature of 4º C*
Abbreviation: Annealing temperature [Ta], degrees centigrade [ºC], polymerase chain reaction [PCR]

[8] DNA Sequencing Analysis Software version 5.1 for Windows® XP and 2000 Platforms. User Guide APPLIED BIOSYSTEMS (2003)

3.4.2.5 Determination of the nucleotide sequence of interest

The attained electropherograms were used to determine the nucleic acid sequence of the coding region and the exon-intron boundaries of the canine FIX and FVIII genes. As two cycle sequencings for each exon of the FIX and FVIII genes were performed, two electropherograms were obtained for each exon of both genes. In the case of canine FIX gene, a total of twenty electropherograms, ten from forward sequencing and ten from reverse sequencing, were necessary in the haemophilic dog (Table 7, see p. 50) and control dog (Table 9, see p. 51). In contrast, eighty electropherograms, forty from forward sequencing and forty from reverse sequencing, were interpreted for each dog that was a suspected carrier or suffering from haemophilia A, as well as for related healthy dogs (Table 8, see p. 51) and control dog (Table 9, see p. 51).

3.4.2.6 Comparison of the sequencing results with the wild type

The obtained nucleotide sequences of the FIX gene and the FVIII gene from control dogs and patients were compared, as appropriate, with the wild type canine FIX mRNA (Gene Bank Accession Number NM_001003323.2) (TATSUMI et al. 2008) or with the wild type canine FVIII mRNA (Gene Bank Accession Number AF016234) (CAMERON et al. 1998). The sequencing results from the control dogs served to ensure that sequenced fragments from the patients corresponded to the sequences of interest (exons of the canine FIX and FVIII genes). Comparison of the obtained sequences from the patients with the respective wild type genes (TATSUMI et al. 2008; CAMERON et al. 1998) was performed to identify possible mutations in the coding regions or in the exon-intron boundaries of the FIX and FVIII genes. For this purpose, BLAST programme was used (ALTSCHUL et al. 1990).

3.4.2.7 Validation of the genetic analysis

All the genetic findings were validated via phenotype in the subject dogs (Tables 7 and 8, see pages 50-51).

The nucleotide changes identified in the Wire-Haired Dachshund family were validated by using PCR technique. Nucleotide change in exon 1 was validated by using a new pair of primers (*1nF+1nR*). Two PCR reactions were carried out to confirm the finding found in the exon 14 sequence. The pair of primers *14-3F* and *14-4R* was used in the first case and the

primer pair *14-7F* and *14-8R* in the second. In each PCR reaction, genomic DNA from a healthy Spanish Mastiff was used as a control. These validations were performed in accordance with the described above (point 3.3.2.2-3.3.2.6). To determine the annealing temperature of the primers, a gradient of 52 ºC-62 ºC was carried out. The annealing temperatures and the PCR conditions are shown in Table 19 (see p. 76).

In the haemophilic Great Dane, exon 21 amplification was repeated to confirm the results. In addition, the obtained coding region of the canine FVIII gene from the Great Dane was sent to the Biocenter at the University of Wuerzburg (Germany), where several protein structure models were performed to analyse the possible structural or functional effects of the detected mutation. An overlay of very similar models of the human wild type FVIII (molecule names: 2r7eA and 2r7eB) (SHEN et al. 2008) and the canine wild type FVIII was carried out using SWISS-MODEL (ARNOLD et al 2006; KIEFER et al. 2009). In addition, human FVIII binding sites for FIXa, vWF and phospholipids were identified in the model. A protein overlay between the wild type FVIII and the mutated canine FVIII was also elaborated and pocket screening in the C domain of canine coagulation FVIII was performed by using a 3D2Go server. The electrostatic properties of the found pockets were evaluated with APBS software (BAKER et al. 2001). To investigate if the identified mutation results in a change of protein structure, a tunnel was elaborated by using MOLE programme. Tunnels are frequently ion conductance zones of proteins (PETREK et al. 2007). Amino acid sequence identities for the canine FVIII to the human FVIII gene (CAMERON et al. 1998; LIU et al. 2000) allowed us to verify the predictions from these programmes.

In the haemophilic mixed poodle, the exon 14-6 amplification was repeated three more times by using PCR technique. Products were consequently sequenced to perform the electropherogram interpretation, as has previously been indicated (point 3.3.2.2-3.3.2.6). The pair of primers *14*-4F and *14-6R* was used in the first PCR reaction. In the second PCR reaction, *14-6F* primer was combined with *14-7R* primer. To perform the third PCR reaction, a new reverse primer (*14-6nR*) within exon 14-6 was manually designed, and it was used with the *14-6F* primer. The annealing temperature of the primers was determined by performing a gradient of 52 ºC-62 ºC. The annealing temperatures and the PCR conditions are shown in Table 20 (see p. 76)

Table 19: Cycler conditions to validate the genetic analysis in the canine FVIII gene from the Wire-Haired Dachshund family

Dog	Exon	Primers	$MgCl_2$ (µl)	DNA(µl)	H_2O (µl)	Ta (ºC)
2-9	Exon 1	*1nF+1nR*	2	2	33.5	59
	Exon 14-4	*14-3F+14-4R*	2	2	33.5	59
	Exon 14-8	*14-7F+14-8R*	2	2	33.5	56

Abbreviation: Annealing temperature [Ta], degree centigrade [ºC], forward [F], sterile distilled water [H_2O], magnesium chloride [$MgCl_2$], microliter [µl], reverse [R]

Table 20: Cycler conditions used to validate the genetic analysis in the canine FVIII gene from a mixed poodle.

Dog	Exon	Primers	$MgCl_2$ (µl)	DNA(µl)	H_2O (µl)	Ta (ºC)
11	Exon 14-6	*14-4F+14-6R*	3	3	31.5	56
		14-6F+14-7R	3	3	31.5	56
		14-6F+14-6nR	3	3	31.5	58

Abbreviation: Annealing temperature [Ta], degree centigrade [ºC], forward [F], sterile distilled water [H2O], magnesium chloride [$MgCl_2$], microliter [µl], reverse [R]

3.4.2.8 Research of identified changes in control dogs

The results were proved in a total of twenty-three control dogs (table 9, see p. 51) by specific exon screenings of the canine FIX and FVIII gene. The aim was to investigate whether the findings could be related to haemophilia. This study was performed according to the protocol described above (point 3.3.2.2-3.3.2.6). Initially, screening of exon 4 of the canine FIX gene was performed in three control Fila Brasileiros to confirm the finding in this breed. After that, the identified nucleotide changes in the Wire-Haired Dachshund family, which theoretically result in amino acid exchanges, were researched in ten random control dogs. For this purpose, sequences of exon 14 (parts 14-4 and 14-8) from the canine FVIII gene were analysed in these dogs. Finally, genomic DNA from ten healthy Great Danes was used to confirm the finding in this breed. Exon 21 of the canine FVIII gene was amplified and sequenced in all ten dogs.

3.4.3 Analysis of intronic sequences located around exon 4 of the FIX gene

In haemophilic dog 1, intron sequences around exon 4 were analysed. In intron 3, which spans 4092 bp, the 1780 nucleotide sequence upstream of exon 4 was divided into four parts of about 290-421 bp (Fig. 5). PCR amplification reactions were carried out with three pair of primers situated close to the target sequence (Table 21, see p. 78). In intron 4, which is 6778 bp in length, the 1030 nucleotide sequence located downstream of exon 4 was fragmented into two parts of 330 and 372 bp apiece (Fig. 6, see p. 78). Two pairs of primers, which bind to the nucleotide sequence close to the target sequence, were designed (Table 21, see p. 78).

PCR technique was carried out using the method described above (point 3.3.2.2-3.3.2.6). Cycler conditions used to amplify these fragments around exon 4 of the canine FIX gene are shown in Table 22 (see p. 79). DNA from a control dog was used in each PCR amplification reaction. Additionally, a long PCR was performed by using DF-Taq DNA-Polymerase, I_3-2F primer and a manually designed reverse primer (I_4-LR), which was located within intron 4 (4507-4527). Genomic DNA of an Argentine Dogo was used as a control. PCR reaction was performed according to the manufacture's indication, and electrophoresis was performed on 1 % agarose gel according to the manufacture's indication.

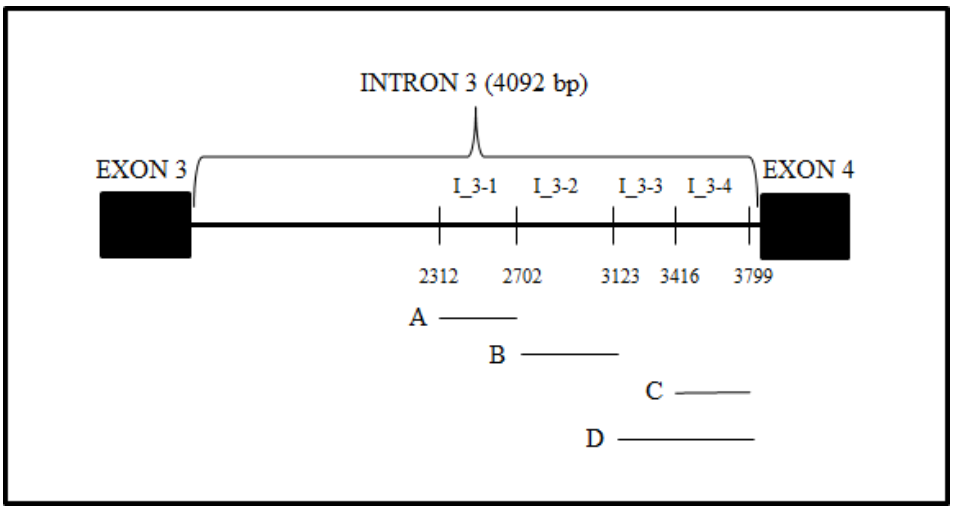

Figure 5: Analysis of intron 3 sequence around exon 4 of the canine FIX. *The intron 3 is 4092 base pairs (bp) in length. The analysed intronic sequence spans 1780 bp. The four parts, in which this sequence was divided, are represented as I_3-1, I_3-2, I_3-3, I_3-4. The lines A-D represent the PCR amplification reactions that were performed.*

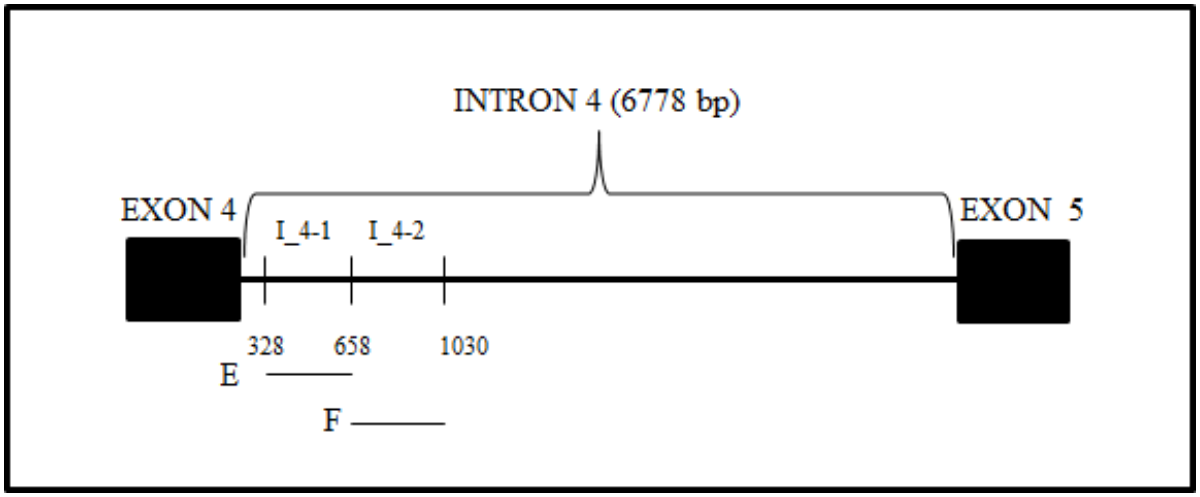

Figure 6: Analysis of intron 4 sequence around exon 4 of the canine FIX. *The intron 4 is 6778 base pairs (bp) in length. The analysed intronic sequence spans from 328 to 1030 nucleotides in intron 4. The two parts, in which this sequence was divided, are represented as I_4-1, I_4-2. The lines E and F show the PCR amplification reactions which were carried out.*

Table 21: Primer sequences, primer binding sites in DNA and target amplicon sizes to amplify the sequence around exon 4 of canine FIX gene.

Primer	Sequence (5' to 3')	Binding site	Intron (bp)
I_3-1F	5‘-AATGAGTTGCTGTGTAGCTG-3‘	Intron 3	Intron 3 (390)
I_3-1R	5‘-GAGCAAAACTCCGTGCCATC-3‘	Intron 3	
I_3-2F	5‘-CCACATCGACACACTAGATG-3‘	Intron 3	Intron 3 (421)
I_3-2R	5‘-AGAAGGTTCTGAAGATACGC-3‘	Intron 3	
I_3-4F	5‘-AGAAGTAGATGCATTCGCTAC-3‘	Intron 3	Intron 3 (383)
I_3-4R	5‘-GCAGGCACTAAACCTCTGAG-3‘	Intron 3	
I_4-1F	5‘-TCTGCCTATTGCAGACACTGAC-3‘	Intron 4	Intron 4 (330)
I_4-1R	5‘-TGGTACTGACCACATACGGTTG-3‘	Intron 4	
I_4-2F	5‘-CAAGAAACTACAGTAGGAGC-3‘	Intron 4	Intron 4 (373)
I_4-2R	5‘-GTGCTCCTAGATTTGCTGTG-3‘	Intron 4	
I_3-2F	5‘-CCACATCGACACACTAGATG-3‘	Intron 3	Intron 3-4 (5909)
I_4-LR	5‘-GGCTCATAGTGCAGATAGGA-3‘	Intron 4	

Abbreviation: Bases pairs [bp], Forward [F], reverse [R]

Table 22: Cycler conditions to amplify part of intron 3 and intron 4 of canine FIX gene.

Dog	Intron	Primers	$MgCl_2$ (µl)	DNA(µl)	H_2O (µl)	Ta (ºC)
1	Intron 3-1	*I_3-1F + I_3-1R*	2	2	33.5	60
	Intron 3-2	*I_3-2F + I_3-2R*	2	2	33.5	64
	Intron 3-3	*I_3-2F + I_3-4R*	2	2	33.5	64
	Intron 3-4	*I_3-4F + I_3-4R*	2	2	33.5	60
	Intron 4-1	*I_4-1F + I_4-1R*	2	2	33.5	58
	Intron 4-2	*I_4-2F + I_4-2R*	4	3	30.5	54

Abbreviation: Annealing temperature [Ta], degree centigrade [ºC], forward [F], sterile distilled water [H_2O], magnesium chloride [$MgCl_2$], microliter [µl], reverse [R]

4. RESULTS

4.1. Male Fila Brasileiro with severe form of haemophilia B

4.1.1. Exon screening of the canine FIX gene

PCR products from the haemophilic Fila Brasileiro (Table 7, see p. 50) and control Argentine Dogo showed the expected size for each exon on agarose gel electrophoresis, except for exon 4 of canine FIX from the haemophilic Fila Brasileiro, in which no band was observed (Fig. 7, see p. 81). Sequence analysis of the PCR products, which were amplified, revealed no differences to the wild type canine FIX cDNA (NM_001003323.2). Unlike the haemophilic Fila Brasileiro, exon 4 could be amplified from genomic DNA of three normal Fila Brasileiros. On agarose gel electrophoresis, a band of the expected size was easily observed.

4.1.2. Intron screening around exon 4 of the canine FIX gene

All PCR reactions (A-F) generated a PCR product of the expected size in a control Argentine Dogo. In the haemophilic Fila Brasileiro, PCR reactions C-F did not yield PCR product. Amplification products with the expected size were only generated in the PCR reactions A and B (Fig. 8, see p. 82). Sequence comparisons between the wild type FIX gene and the genomic DNA from the haemophilic Fila Brasileiro show no differences. In addition, the elaborated long PCR reaction did not generate a PCR product using genomic DNA obtained from the haemophilic dog and from the control Argentine Dogo.

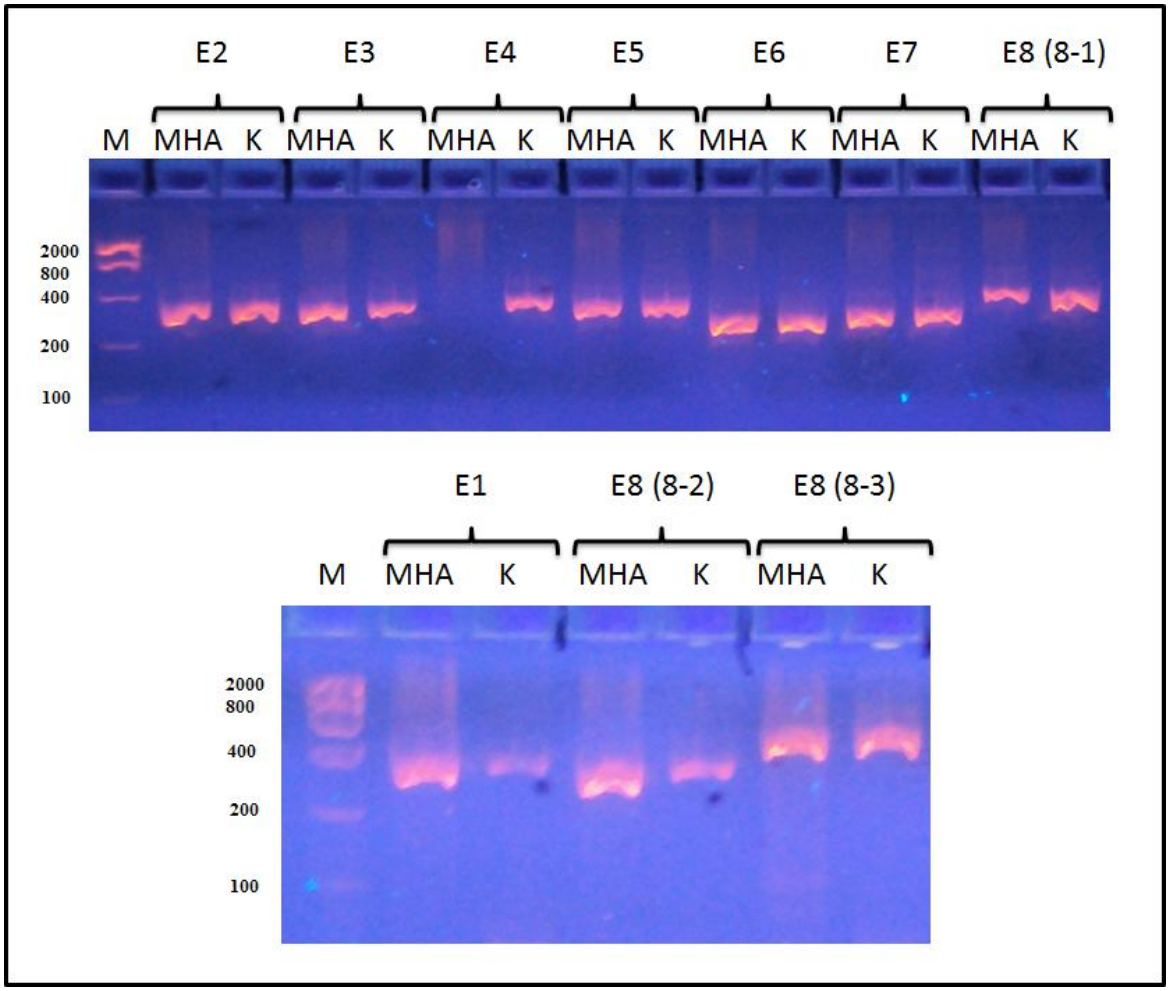

Figure 7: Agarose gel electrophoresis of all exons of the canine FIX gene from a haemophilic Fila Brasileiro. *The image illustrates the bands of PCR products from a haemophilic male Fila Brasileiro (MHA) and a control Argentine Dogo (K). The use of a marker (M) allowed the identification of the size of the bands. In K, all PCR reactions yielded an amplification product of expected size. In MHA, clear bands with the expected size were visualized for each exon (E) except in the case of exon 4, where no PCR product was amplified.*

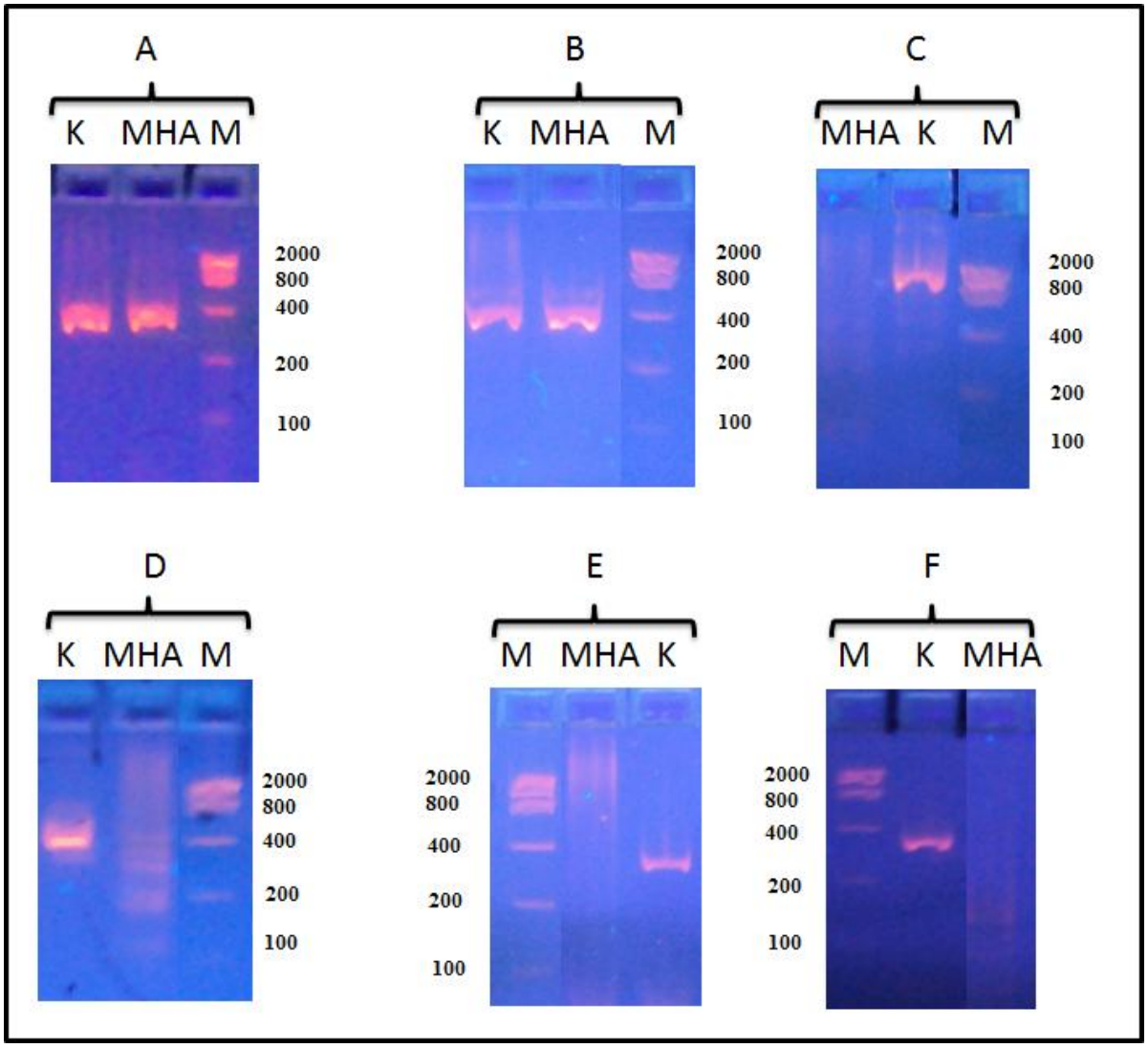

Figure 8: Agarose gel electrophoresis of intron sequences around exon 4 of the canine FIX gene from a haemophilic Fila Brasileiro. *The image illustrates the bands of PCR products from a haemophilic male Fila Brasileiro (MHA) and a control Argentine Dogo (K). The use of a marker (M) allowed the identification of the size of the bands. In K, all PCR reactions yielded an amplification product of expected size. In MHA, PCR reactions A and B* yielded a clear band with the expected size. In contrast, PCR reactions C, D, E and F failed.

4.2. Wire-Haired Dachshund family

When the obtained PCR products were sequenced and compared with the wild type canine FVIII cDNA (AF016234), no significant differences were observed in haemophilic dog 2 (Table 8, see p. 51). Screening of canine FVIII in a suspected carrier of haemophilia A (dog 4) and healthy dogs 5-9 (Table 8, see p. 51) revealed a nucleotide chain in exons 1, 14 and 15. In contrast to dogs 2 and 4-9, the amplification of the coding region of the canine FVIII gene failed on the DNA isolated from the other suspected carrier of haemophilia A (dog 3. Table 8, see p. 51), in which no bands or unspecific bands were visualized on agarose gel electrophoresis. However, some PCR products, which were obtained by PCR amplification of exon 5, 7, 10, 12 and some parts of the exon 14 (14-1, 14-6, 14-8), could be sequenced and compared to the published canine FVIII cDNA (AF016234). Comparisons revealed no significant similarity between the obtained sequences and the wild type. By using the BLAT programme, these sequences were located in chromosomes 19, 12, 5, 1, 5, X (FVIII gene) and 13, respectively, of human genomic DNA.

4.2.1. Single base change at nucleotide position 141 in exon 1 of the FVIII gene

A nucleotide change was found at nucleotide position 141 in exon 1 of canine FVIII cDNA from suspected carrier dog 4 and from healthy dogs 5-9. The change was characterised as a single-base change, c. 141C>T in healthy dogs 5-8. In contrast, the genomic DNA from suspected carrier dog 4 and healthy dog 9 showed two overlapping peaks (C and T) of similar height (Fig. 9, see p. 85). Sequence analysis of exon 1 sequence after using a new primer pair, revealed a nucleotide change at the same position, which confirmed the findings obtained during the first amplification of exon 1 (Fig. 10, see p. 86).

4.2.2. Single base change at nucleotide position 2782, 2943 and 3608 in exon 14

Based on the exon 14 sequence analysis of the genomic DNA from the patients (table 12), three different nucleotide changes were found at position 2782, 2943 and 3608 of the canine FVIII cDNA from suspected carrier dog 4 and healthy dogs 5-9. Two nucleotide changes, c.2943G>A and c.3608C>T were identified in the genomic DNA of four healthy dogs (dogs 5 to 8). Moreover, a nucleotide change, c.2782A>G was found in the genomic DNA of healthy dogs 5 and 8. However, overlapping peaks of similar height were detected in the exon 14

sequences from suspected carrier dog 4 and healthy dog 9: G-A overlapping at position 2782 and 2943 and C-T overlapping at position 3608 (Fig. 11-13, see pages 87-89). Sequence analysis of exon 14 sequences after using two new primer pairs showed same nucleotide changes, confirming the findings obtained during the first PCR reaction (Fig. 14-16, see pages 90-92).

4.2.3. Single base change at nucleotide position 5292 in exon 15 of the FVIII gene

Exon 15 sequence analysis of the genomic DNA from dogs 5-8 revealed a single-base exchange, c.5292C>T. The exon 15 sequence from the suspected carrier dog 4 and healthy dog 9 showed two overlapping peaks (C and T) of similar height (Fig. 17, see p. 93).

4.2.4. Summary of results in the Wire-Haired Dachshund family

All the single base changes found in the canine FVIII gene of the Wire-Haired Dachshund family are summarised in Table 23. None of the detected abnormalities were associated with the haemophilia A phenotype.

Table 23: Single base changes identified in the canine FVIII gene of the Wire-Haired Dachshund family

NUCLEOTIDE POSITION	EXON	PHENOTYPE*							WILD TYPE
		MHA	FC?	FH	MH	FH	MH	FH	
141	1	C	C-T	T	T	T	T	C-T	**C**
2782	14	A	A-G	G	A	A	G	A-G	**A**
2943	14	G	G-A	A	A	A	A	G-A	**G**
3608	14	C	C-T	T	T	T	T	C-T	**C**
5292	15	C	C-T	T	T	T	T	C-T	**C**
		2	**4**	**5**	**6**	**7**	**8**	**9**	
		DOG NUMBER							

** based on coagulation factor VIII activities*
Abbreviations: adenine [A], cytosine [C], female healthy dog [FH] female suspected carrier [FC?], guanine [G], male haemophilic dog [MHA], male healthy dog [MH], thymine [T]

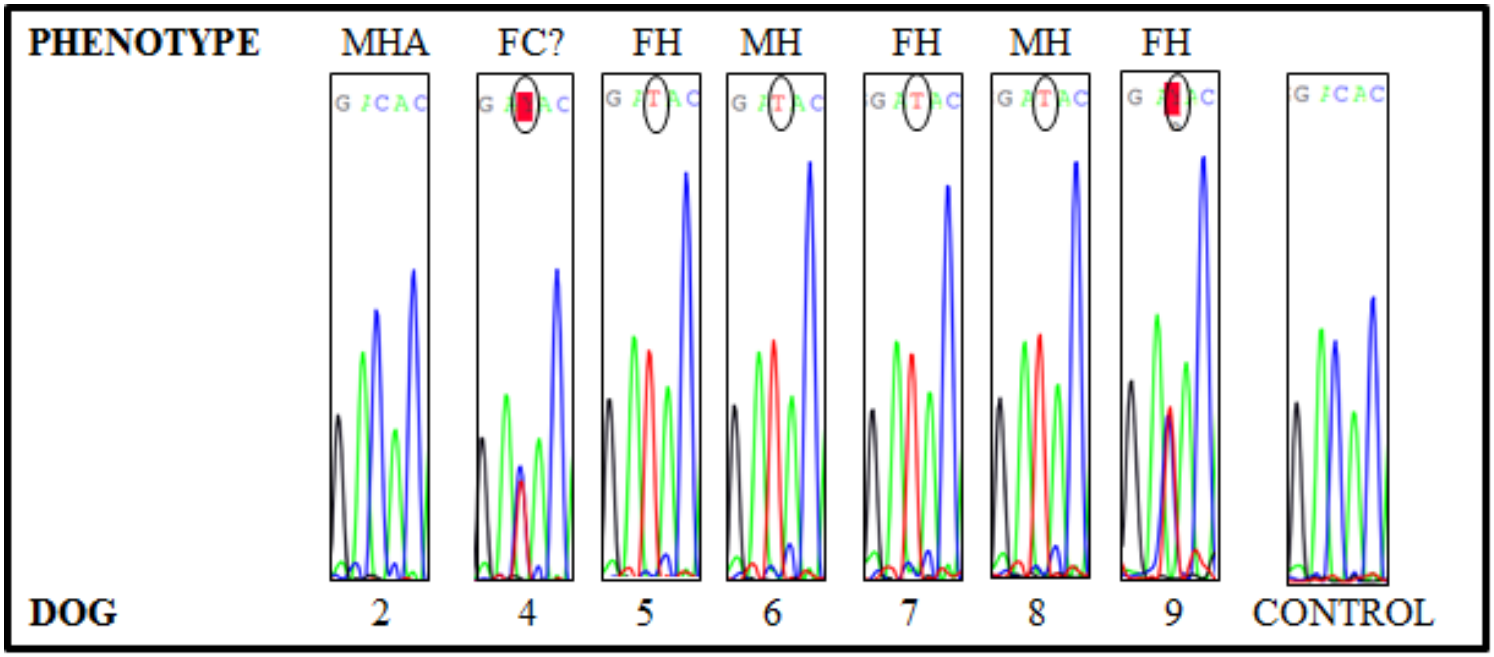

Figure 9: Single base change at position 141 in exon 1 of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms show a part of the nucleotide sequence of exon 4, from nucleotide 139 to 143, from patients (dogs 2, 4-9) and from a control dog (CONTROL). Comparison of exon 1 sequence from the haemophilic male dog 2 (MHA) with the wild type and control dog (shown on the right side) revealed* no significant *differences. However, a single nucleotide change was observed at position 141 of exon 1 from dogs 4-9. A C→T change was identified in two healthy male dogs (MH, dogs 6 and 8) and two healthy female dogs (FH, dogs 5 and 7). An overlap between C and T (letter Y) was observed in exon 1 of a suspected female carrier dog (FC?, dog 4) and a healthy female dog (FH, dog 9). The ellipses indicate the single base change.*

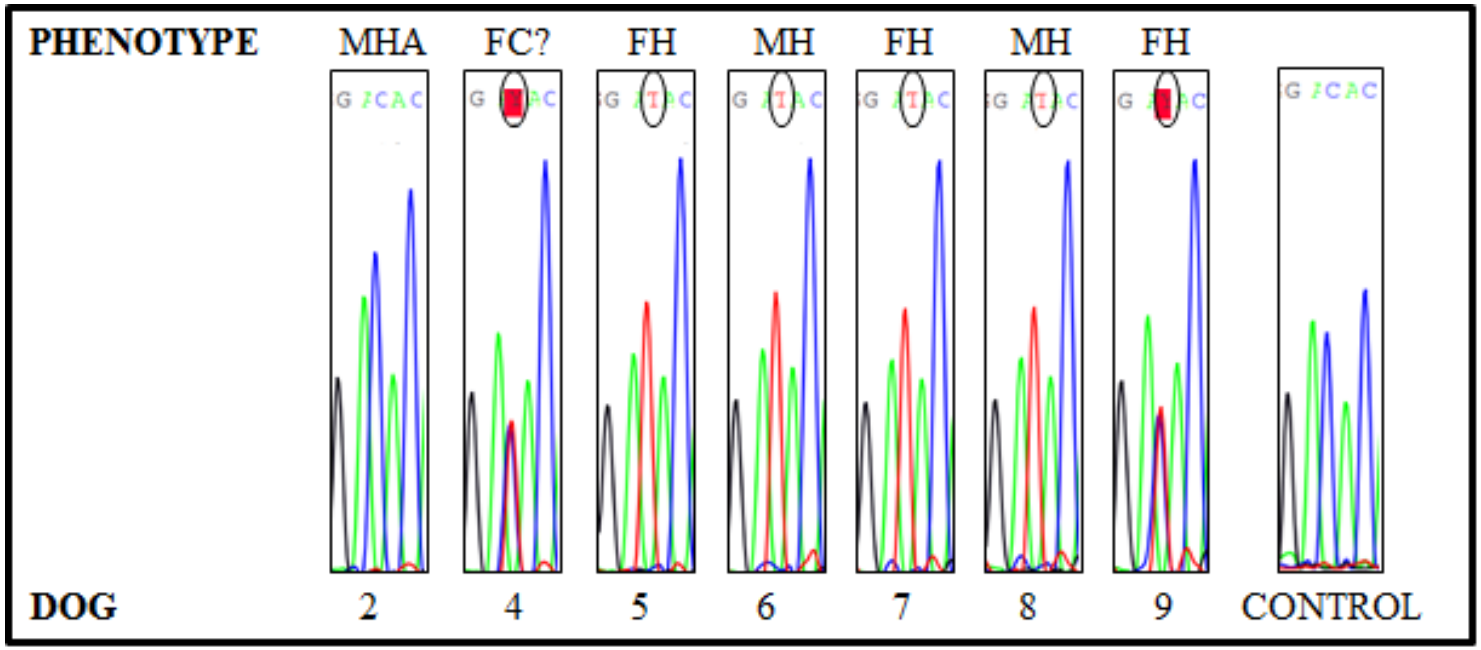

Figure 10: Single base change at position 141 in exon 1 of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms show the forward sequences of a part of exon 1, from nucleotide 139 to 143, from patients (dogs 2, 4-9) and from a control dog (CONTROL). Nucleotide sequences were obtained after the second amplification of exon 1 from patients. Sequence analysis revealed* no significant *difference between the* wild-type, *the control dog and haemophilic male dog 2 (MHA). However, a single nucleotide change was observed at position 141 of exon 1 from dogs 4-9. A C→T change was identified in two healthy male dogs (MH, dogs 6 and 8) and two healthy female dogs (FH, dogs 5 and 7). An overlap between C and T (letter Y) was observed in exon 1 of a suspected female carrier dog (FC?, dog 4) and a healthy female dog (FH, dog 9). The ellipses indicate the single base change.*

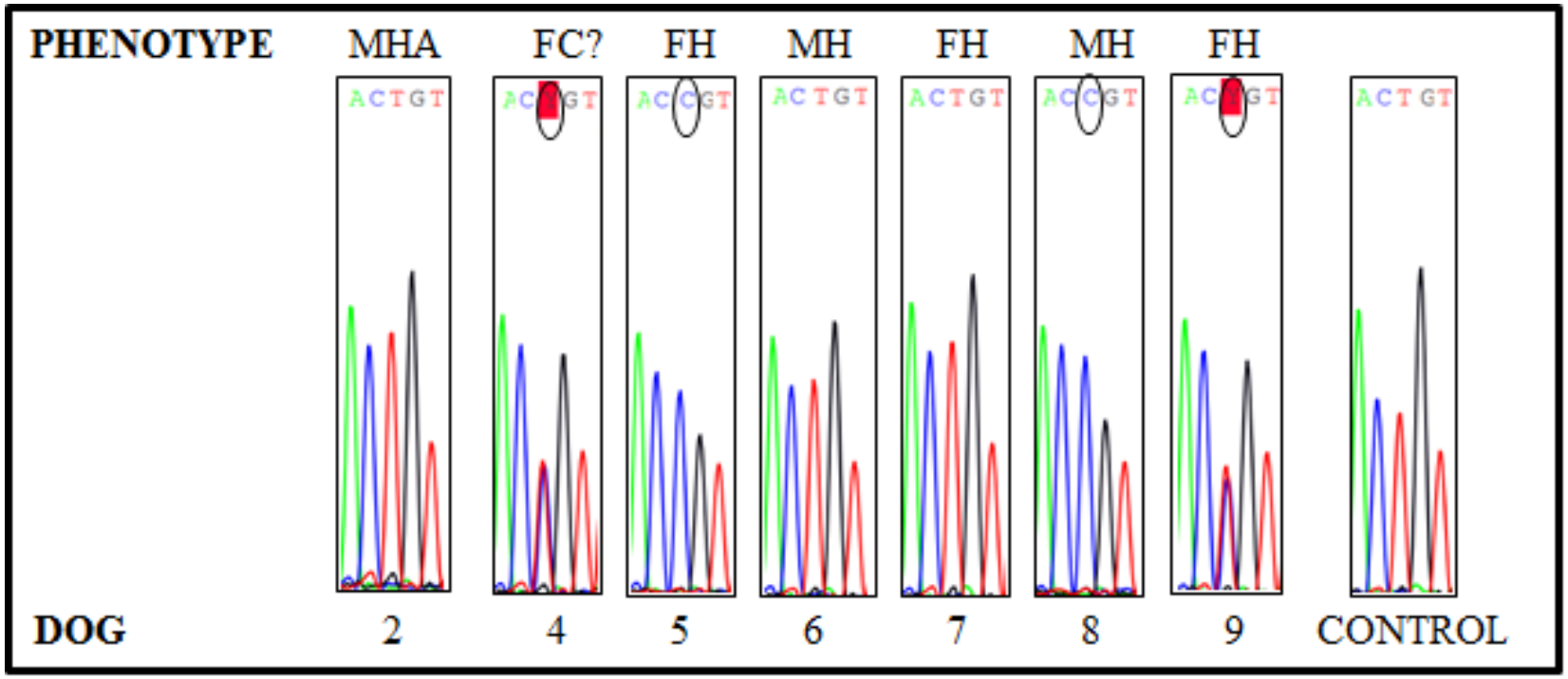

Figure 11: Single base change at position 2782 in exon 14 (part 14-4) of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms represent the forward sequences of a part of exon 14, from nucleotide 2780 to 2784, from patients (dogs 2, 4-9) and from a control dog (CONTROL). The obtained sequences were compared with the published wild type. No difference was observed between the wild type,* control dog*, haemophilic male dog 2 (MHA) and healthy dogs 6 and 7. A nucleotide exchange (T →C) was identified in a healthy female dog (FH, dog 5) and in a healthy male dog (MH, dog 8). A T-C overlap (letter Y) was found in a suspected female carrier dog (FC?, dog 4) and in a healthy female dog (FH, dog 9). The ellipses exhibit the single base change.*

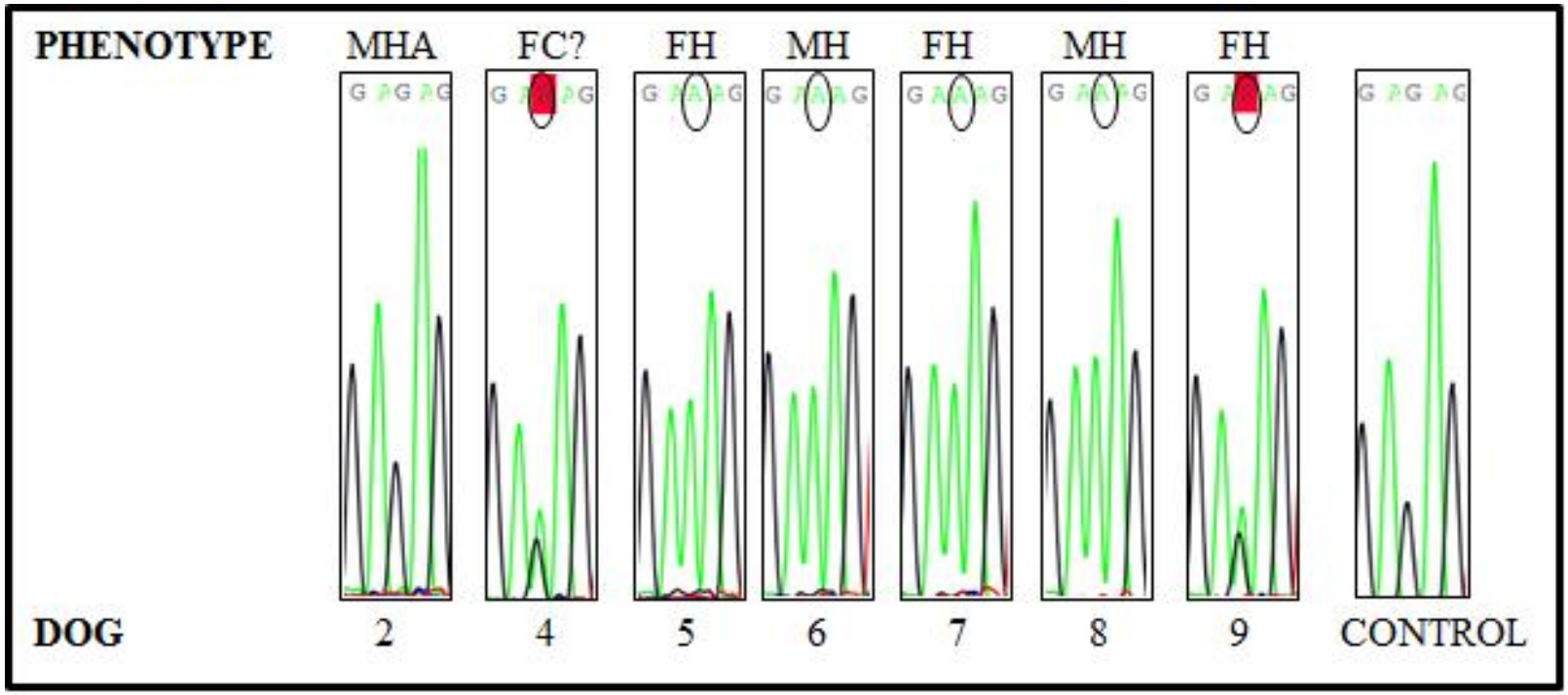

Figure 12: Single base change at position 2943 in exon 14 (part 14-4) of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms show the forward sequences of a part of exon 14, from nucleotide 2941 to 2945, from patients (dogs 2, 4-9) and from a control dog (CONTROL). The obtained sequences were compared with the published wild type. No differences were observed between the wild type and exon 14 (part 14-4) from haemophilic male dog 2 (MHA). However, a nucleotide exchange (G →A) was identified in two healthy female dogs (FH, dogs 5 and 7) and in two healthy male dogs (MH, dogs 6 and 8). A G-A overlap (letter R) was found in a suspected female carrier dog (FC?, dog 4) and in a healthy female dog (FH, dog 9). The ellipses exhibit the single base change.*

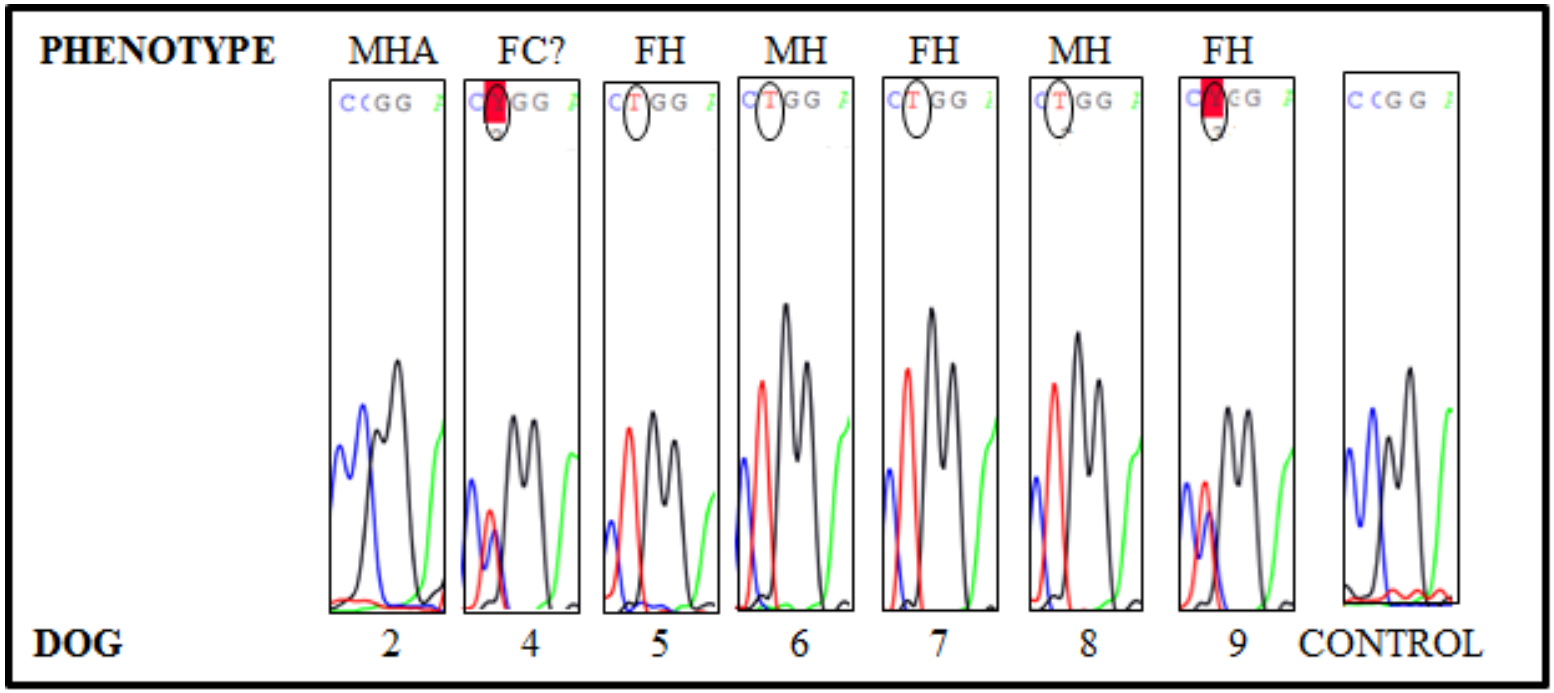

Figure 13: Single base change at nucleotide position 3608 in exon 14 (part 14-8) of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms represent the forward sequences of a part of exon 14, from nucleotide 3607 to 3611, from patients (dogs 2, 4-9) and from a control dog (CONTROL). The obtained sequences were compared with the published wild type. Exon 14 comparisons revealed no difference between the wild type and haemophilic male dog 2 (MHA). However, a nucleotide exchange (C →T) was identified in two healthy female dogs (FH, dogs 5 and 7) and in two healthy male dogs (MH, dogs 6 and 8). A C-T overlap (letter Y) was found in a suspected female carrier dog (FC?, dog 4) and in a healthy female dog (FH, dog 9). The ellipses exhibit the single base change.*

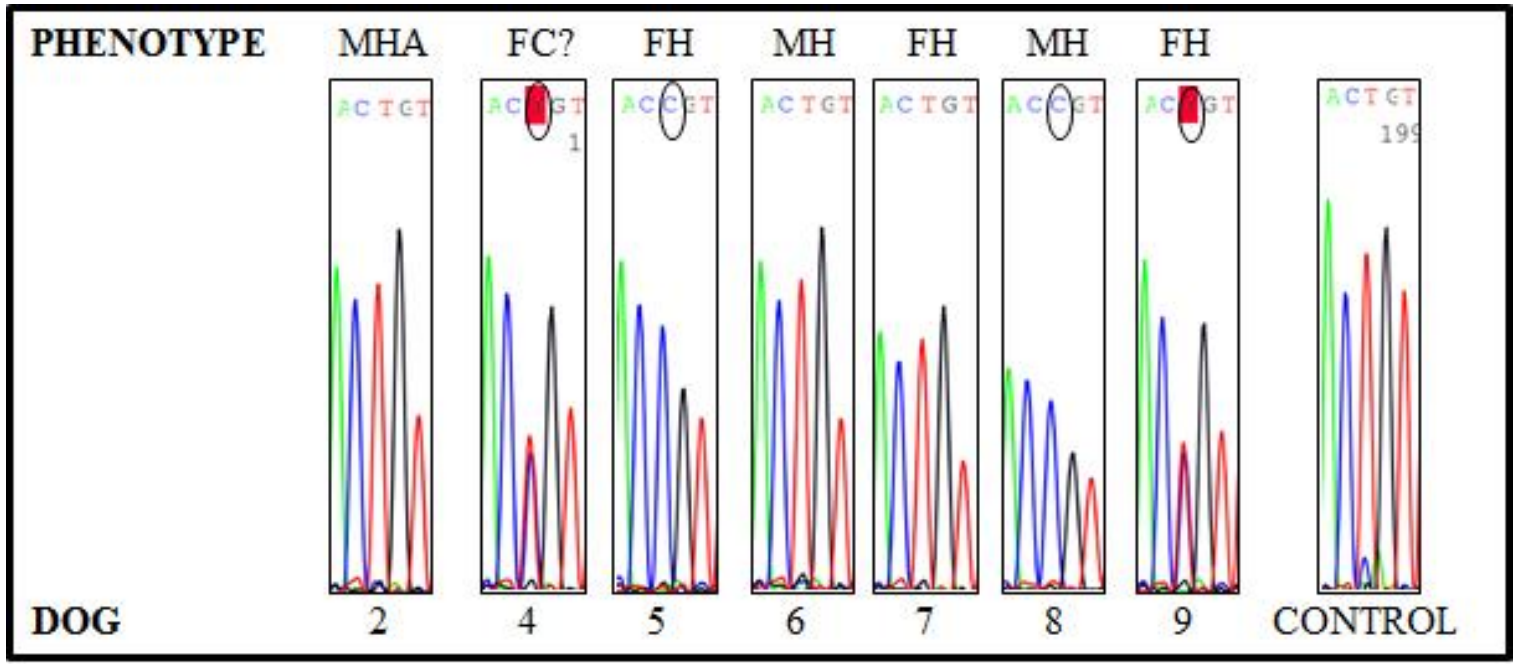

Figure 14: Single base change at position 2782 in exon 14 (part 14-4) of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms show the reverse sequences of a part of exon 14, from nucleotide 2780 to 2784, from dogs 2, 4-9 and from a control dog (CONTROL). Nucleotide sequences were obtained after the second amplification of exon 14 (part 14-4). These nucleotide sequences are identical to the obtained sequences during the first PCR reaction. No nucleotide change was observed in exon 14 (part 14-4) sequences from haemophilic male dog 2 (MHA) and two healthy dogs (dogs 6 and 7). A nucleotide exchange (T →C) was identified in a healthy female dog (FH, dog 5) and in a healthy male dog (MH, dog 8). A T-C overlap (letter Y) was found in a suspected female carrier dog (FC?, dog 4) and in a healthy female dog (FH, dog 9). The ellipses exhibit the single base change.*

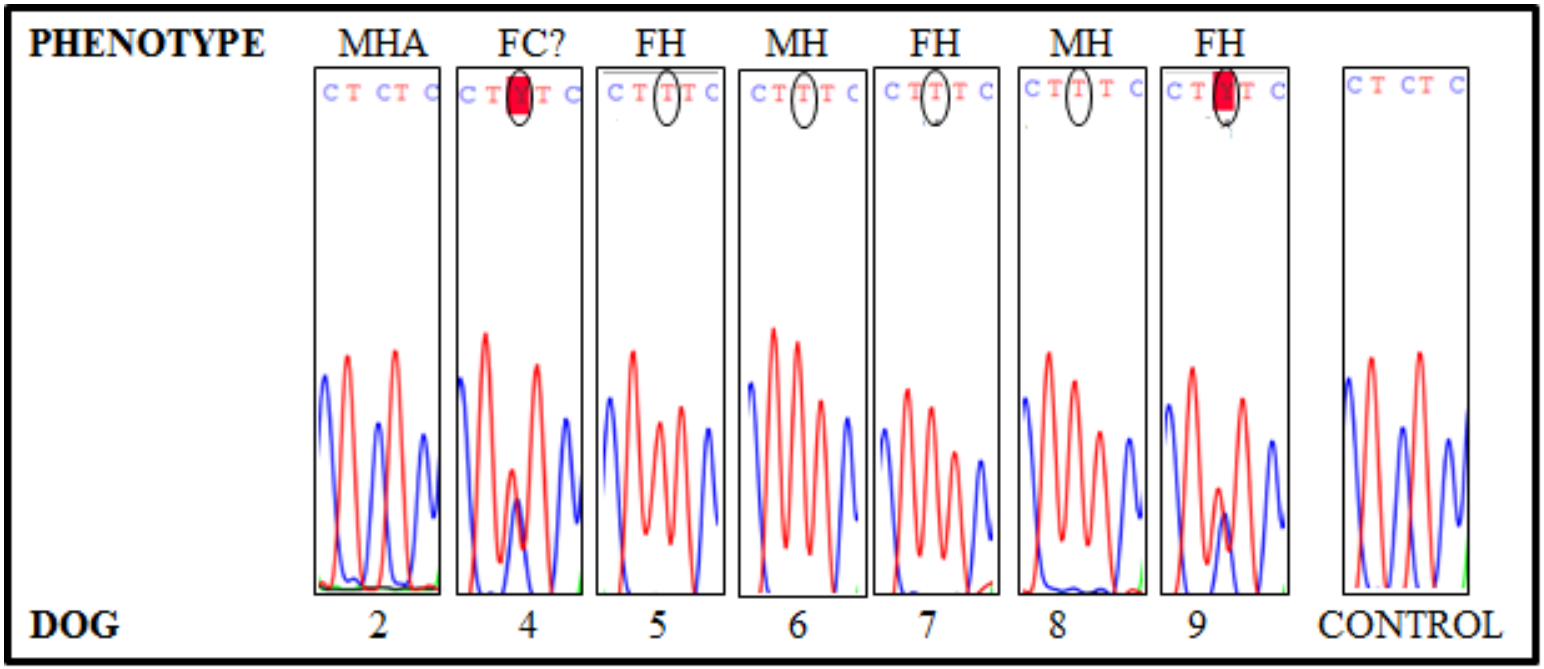

Figure 15: Single base change at position 2943 in exon 14 (part 14-4) of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms represent the reverse sequences of a part of exon 14, from nucleotide 2941 to 2945, from patients (dogs 2, 4-9) and from a control dog (CONTROL). Nucleotide sequences were obtained after the second amplification of exon 14 (part 14-4) from patients. These nucleotide sequences are identical to the obtained sequences during the first PCR reaction. No nucleotide change was observed in exon 14 (part 14-4) sequence from haemophilic male dog 2 (MHA). A nucleotide exchange (G →C) was identified in two healthy female dogs (FH, dogs 5 and 7) and in two healthy male dogs (MH, dogs 6 and 8). A T-C overlap (letter Y) was found in a suspected female carrier dog (FC?, dog 4) and in a healthy female dog (FH, dog 9). The ellipses exhibit the single base change.*

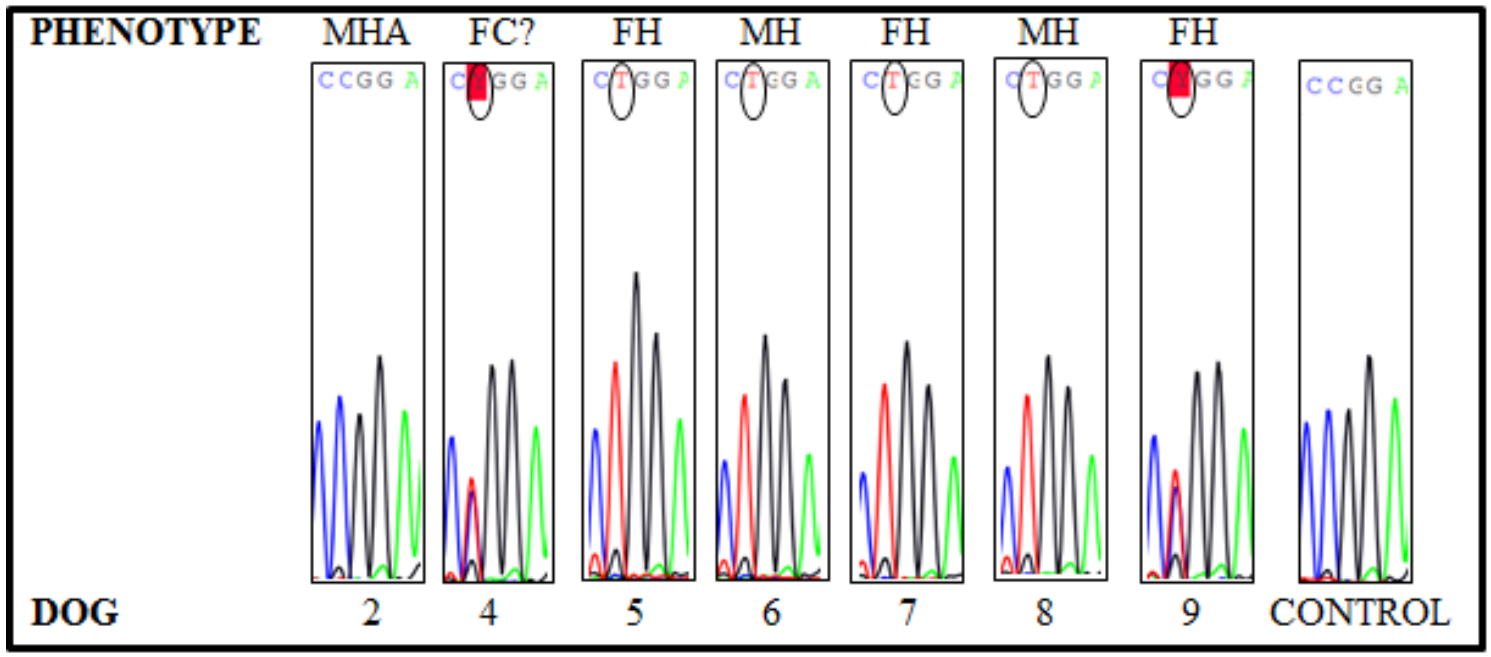

Figure 16: Single base change at nucleotide position 3608 in exon 14 (part 14-8) of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *The electropherograms represent the forward sequences of a part of exon 14, from nucleotide 3607 to 3611, from patients (dogs 2, 4-9) and from a control dog (CONTROL). Nucleotide sequences were obtained after the second amplification of exon 14 (part 14-8) from patients. Exon 14 (part 14-4) sequences from the patients were compared with the wild type and no nucleotide change was observed in haemophilic male dog 2 (MHA). A nucleotide exchange (C →T) was identified in two healthy female dogs (FH, dogs 5 and 7) and in two healthy male dogs (MH, dogs 6 and 8). A C-T overlap (letter Y) was found in a suspected female carrier dog (FC?, dog 4) and in a healthy female dog (FH, dog 9). The ellipses exhibit the single base change.*

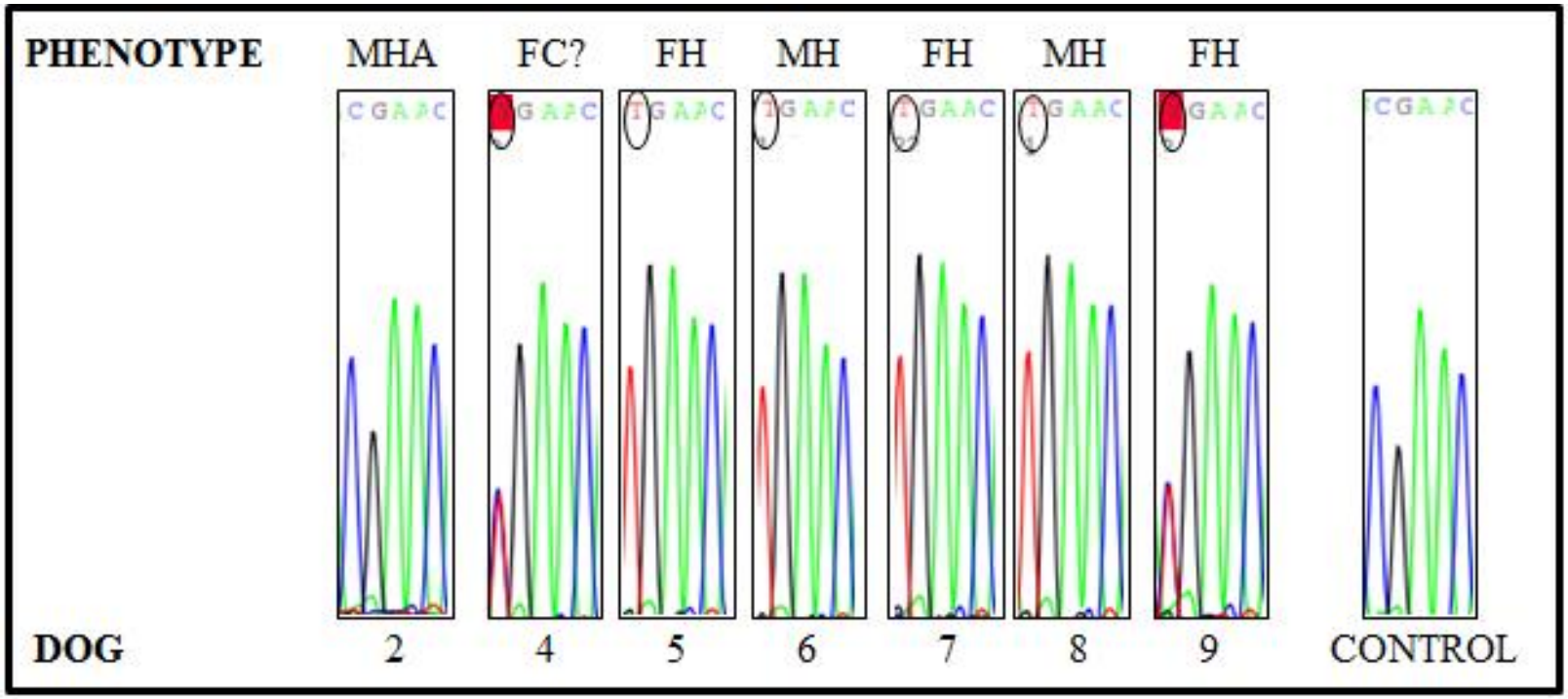

Figure 17: Single base change at position 5292 in exon 15 of the canine FVIII gene obtained from genomic DNA of related *Wire-Haired Dachshund*. *These electropherograms correspond to the forward sequences of a part of exon 15, from nucleotide 5292 to 5296, from patients (dogs 2, 4-9) and from a control dog (CONTROL). The obtained sequences were compared with the published wild type. No difference was observed between the wild type and haemophilic male dog 2 (MHA). However, a nucleotide exchange (C →T) was identified in two healthy female dogs (FH, dogs 5 and 7) and in two healthy male dogs (MH, dogs 6 and 8). A C-T overlap (letter Y) was found in a suspected female carrier dog (FC?, dog 4) and in a healthy female dog (FH, dog 9). The nucleotide change is indicated with an ellipse.*

4.2.5. Exon 14 (parts 14-4 and 14- 8) screening in ten random dog breeds

Nucleotide changes in exon 14 (parts 14-4 and 14-8) were also present in ten random dog breeds (Table 9, see p. 51). The exon 14 sequence of seven out of the ten dogs contained A at position 2782, and G in the other three dogs. C was observed at nucleotide position 3608 in the exon 14 sequence of six of them, T was identified in three of them, and two peaks (T and C) were identified at this position in one dog.

4.3. Male Great Dane with moderate haemophilia A

The coding region of the canine FVIII gene of the genomic DNA from dog 10 (Table 8, see p. 51) was compared with the respective sequences from the wild type canine FVIII cDNA (AF016234). All exon sequences and exon-intron boundaries were identical to those seen in the wild type canine FVIII cDNA, except for exon 21.

4.3.1. Single base change at position 6217 in exon 21 of the FVIII gene

In the haemophilic Great Dane, a nucleotide change c.6217T>C was observed in exon 21 of canine FVIII cDNA (Fig. 18, see p. 95). This abnormality was confirmed by a second amplification of exon 21.

4.3.2. Validation via a protein structure modelling analysis

The overlay of very similar models of the human wild type FVIII and the canine wild type FVIII, which were created by using the SwissModel programme, showed that the amino acid exchange (Trp^{2073}) was located in the C1 domain of the canine FVIII protein (Fig. 19, see p. 96).

Screening for active sites in the C domain of canine FVIII protein using 3D2Go server revealed the presence of two possible pockets (pockets 1 and 2) and two related active sites. The altered amino acid was observed close to pocket 1 (Fig. 20, see p. 97). A high number of hydrophobic amino acids were found in pocket 1 by using APBS software (Fig. 21, see p. 98). Evaluation of the ion conductance zone of pocket 1, using Mole programme, showed that the altered amino acid is located in the middle of the elaborated tunnel, causing an obstruction of the tunnel path (Fig. 22, see p. 99). Conversely, no obstruction was observed in the wild type

canine FVIII. Comparison of the tunnel size of the wild type FVIII with the defective canine FVIII confirmed an obstruction from 9 Å distance (Fig. 23, see p. 100).

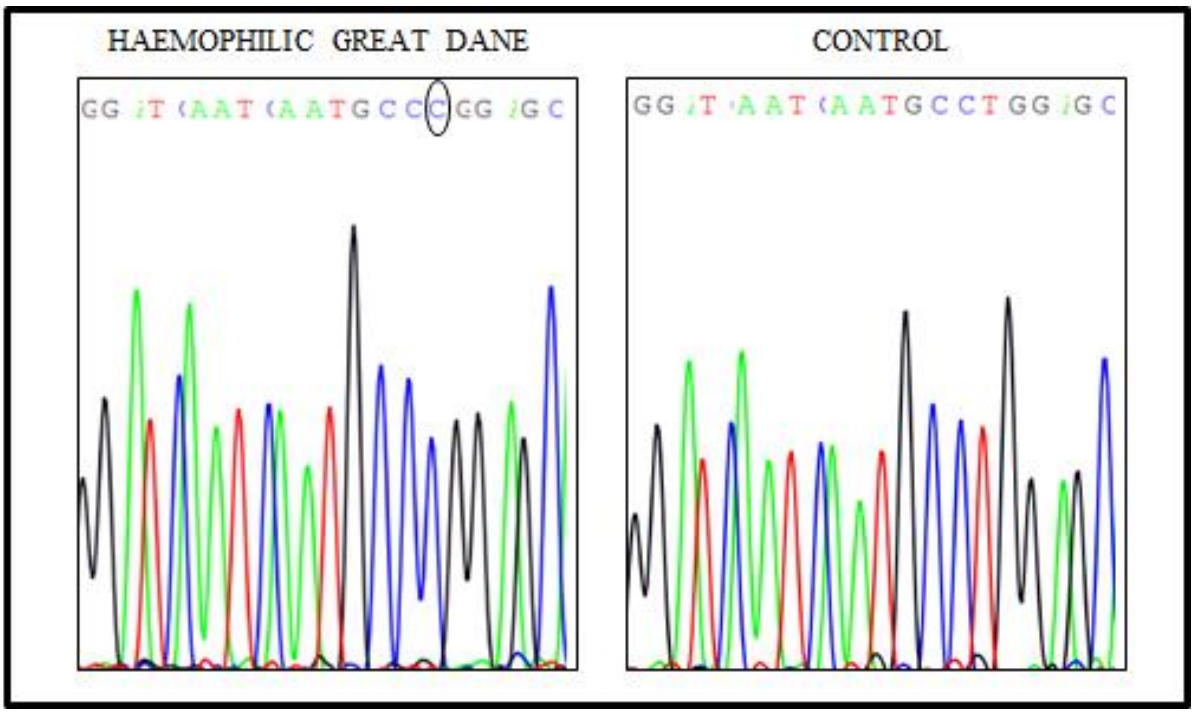

Figure 18: Nucleotide change at nucleotide position 6217 in exon 21 of the canine FVIII gene from genomic DNA of a Great Dane with haemophilia A. *The electropherograms show exon 21 sequence from the nucleotide 6202 to 6222. The left electropherogram was obtained from the genomic DNA of a male Great Dane with moderate haemophilia A and the right one from a healthy Spanish Mastiff dog, which was used as a control. The obtained sequences were compared with the published wild type. In this case, exon 21 sequence of the Great Dane shows T→C exchange at nucleotide position 6217 in comparison to the control dog and the wild type. The nucleotide change is identified with an ellipse.*

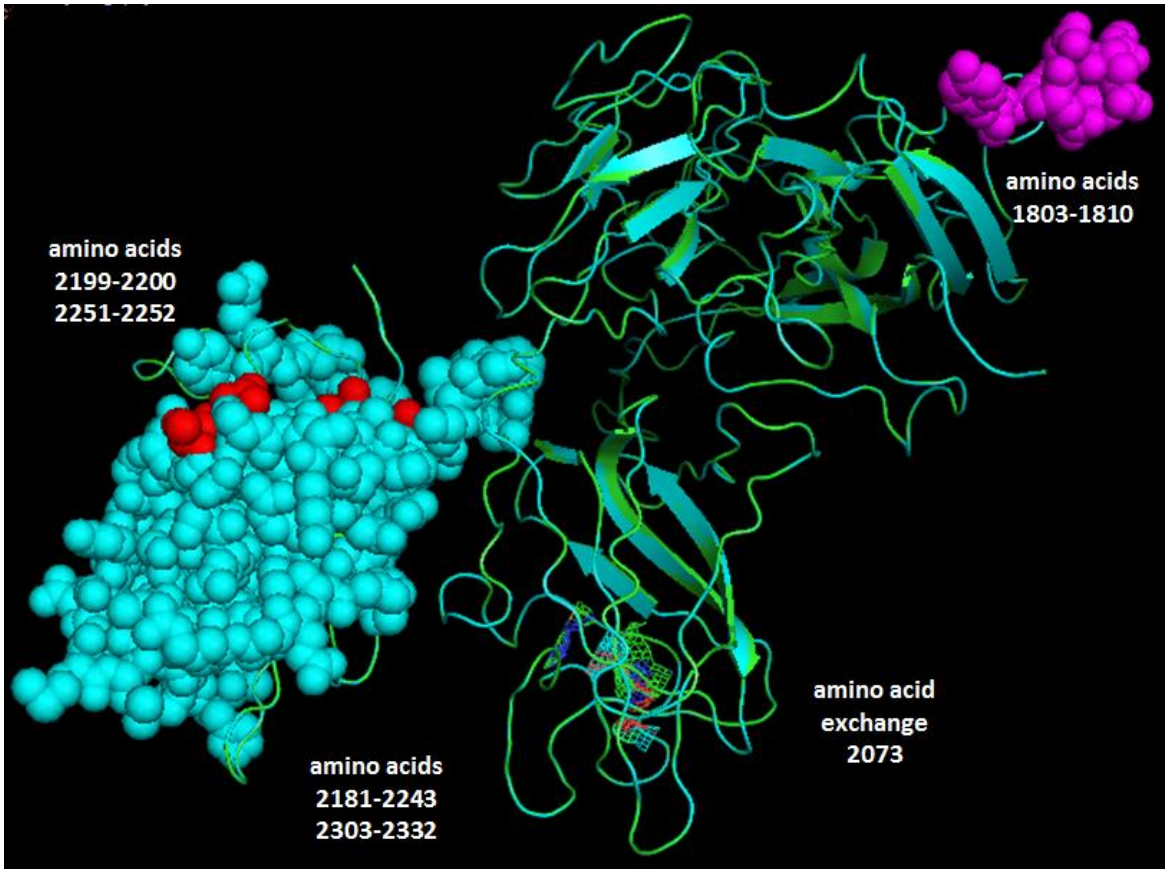

Figure 19: Protein model of canine FVIII from the haemophilic Great Dane by using 3D2Go programme. *The figure represents the overlay of A3, C1 and C2 domains of human FVIII (blue colour) and altered canine FVIII (green colour). The figure shows some known binding sites on human FVIII. Human FVIII binding site for FIXa (amino acids 1803-1810 in A3 domain) is marked by magenta spheres. Human FVIII binding sites for vWF (amino acids 2181-2243 and 2303-2332 in C2 domain) are marked by cyan spheres and Human FVIII binding sites for phospholipids (amino acids 2199-2200 and 2251-2252 in C2 domain) are marked by red spheres. The altered amino acid of canine FVIII from the Great Dane is symbolized as a multi-coloured mesh in C1 domain and is not close to the known human binding sites in A3 and C2 domains.*

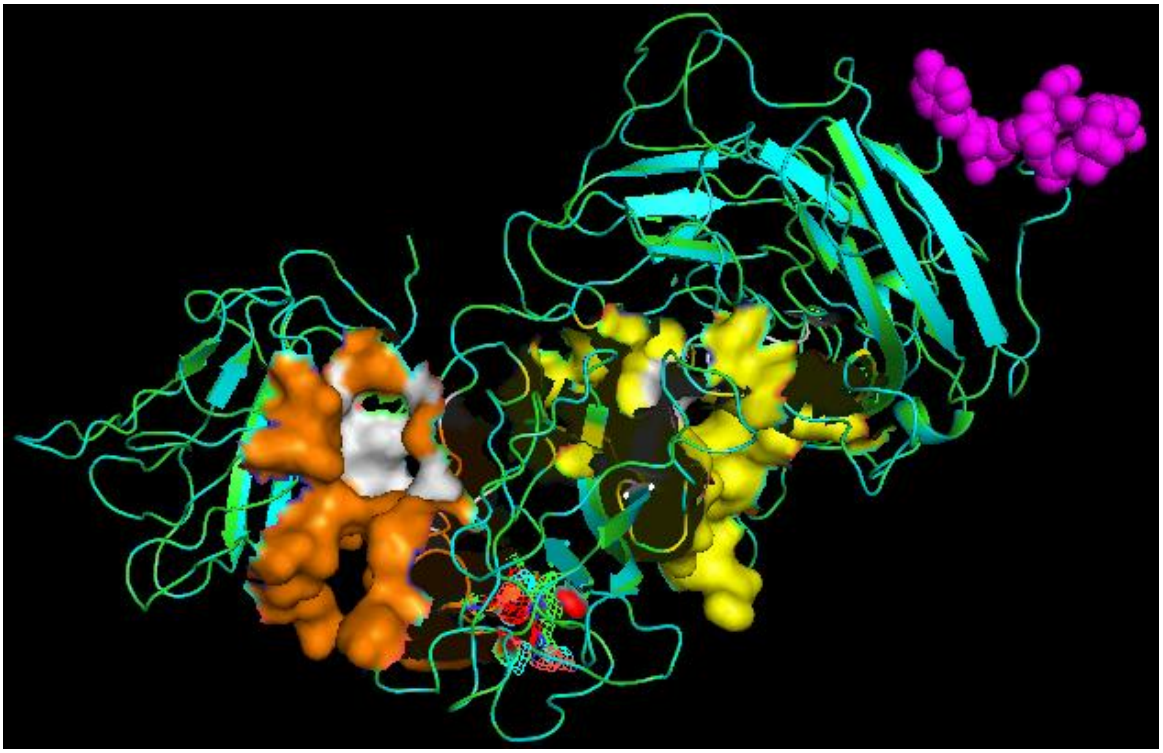

Figure 20: Protein model of canine FVIII from the haemophilic Great Dane, using 3D2Go programme. *The illustration shows an overlay of the human wild type FVIII (blue colour) and mutated canine FVIII (green colour), as well as two pockets (pockets 1 and 2) and active sites, which were identified in C domains of the canine FVIII protein. On the left side, the orange surface modelling indicates pocket 1 and the grey zone illustrates its active site. On the right side, the yellow surface modelling indicates pocket 2 and its active site is coloured in white. The mutation of tryptophan residue at position 2073 is symbolized as a multi-coloured mesh in the proximity of the pocket 1. The figure also shows the known human FIXa binding site (lilac colour), which is located at nucleotide position 1803-1818 in the A3 domain of FVIII.*

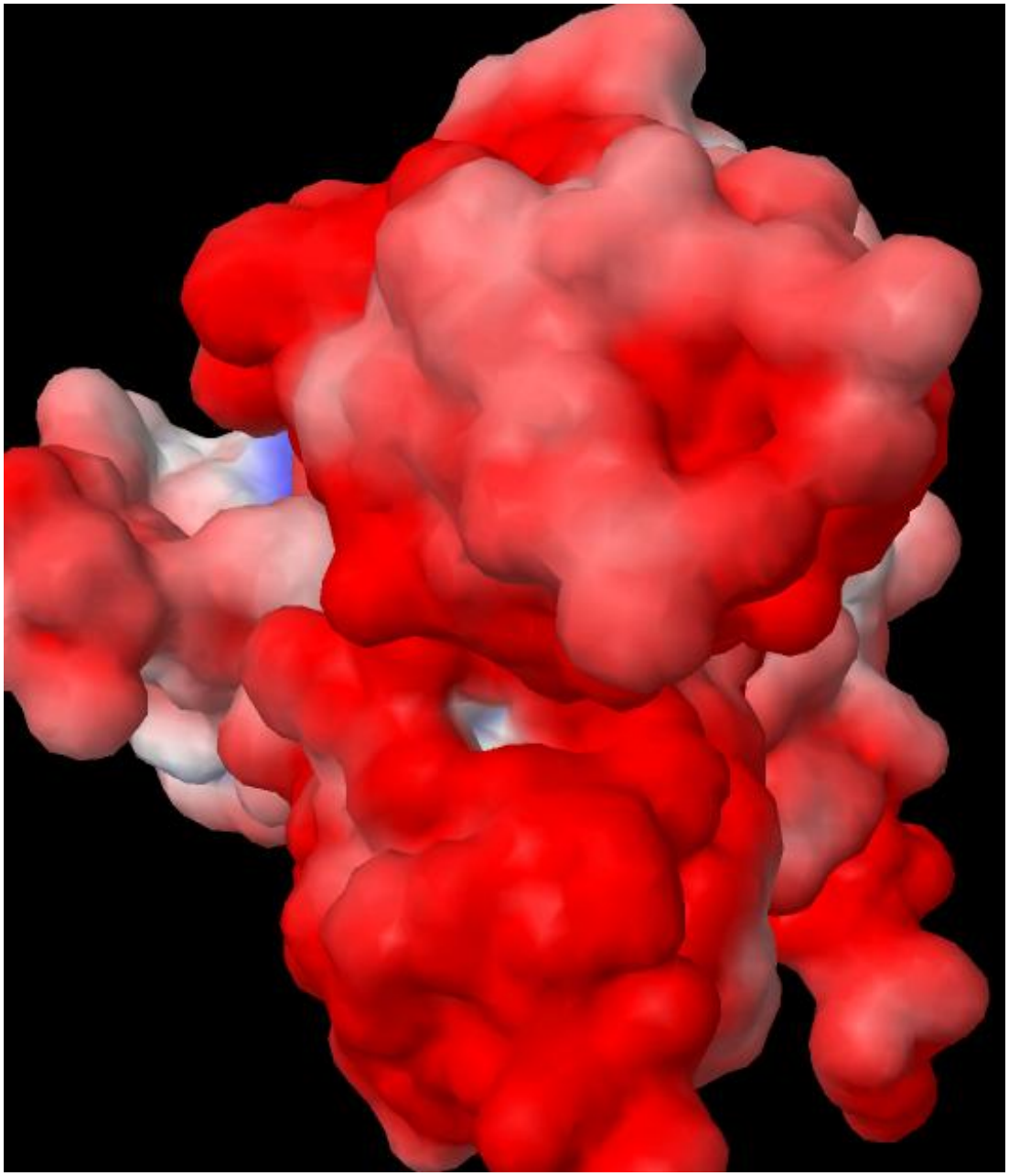

Figure 21: Protein model of canine FVIII from the haemophilic Great Dane, using APBS software. *The image shows the hydrophobicity of amino acid residues located close to identified pockets. The red colour becomes more intense as the amino acid becomes more hydrophobic. Pocket 1, which is located in the lower part of the image, shows a high intensity of the red colour; in other words, a great number of hydrophobic amino acids, in comparison to pocket 2 (upper part of the image), which shows a lower intensity of the colour red.*

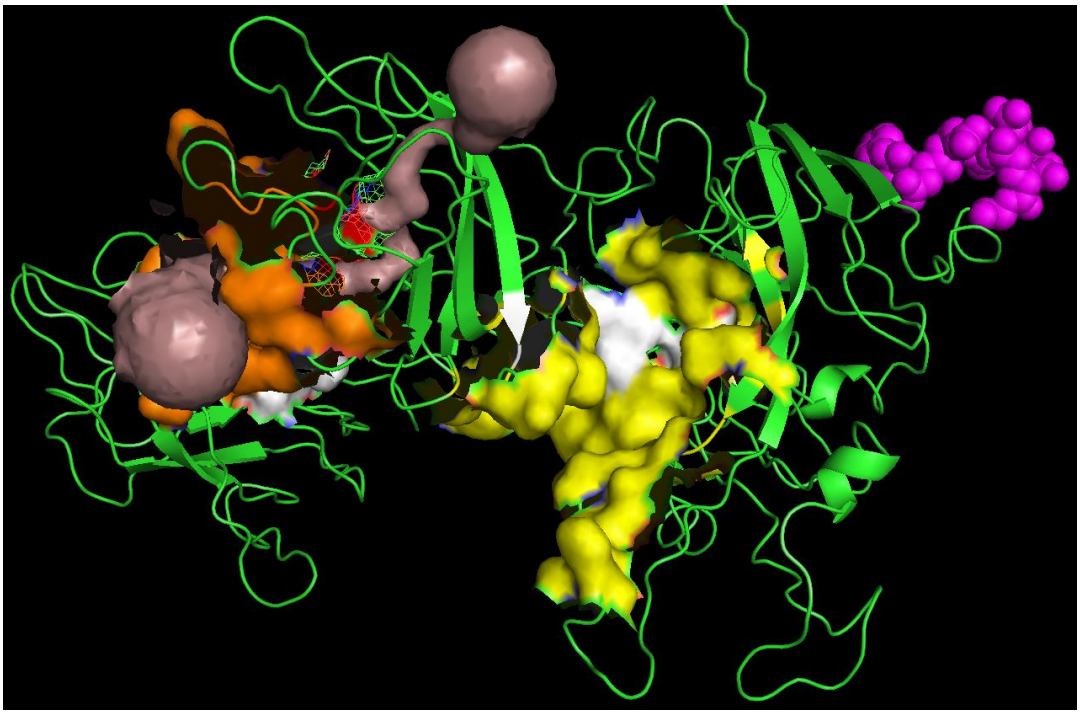

Figure 22: Evaluation of the ion conductance zone of pocket 1 in the canine FVIII molecule of a haemophilic Great Dane, using Mole programme. *The figure represents the canine FVIII protein modelling from the haemophilic Great Dane, where a tunnel through pocket 1 was elaborated to evaluate the ion conductance zone of this pocket. In the figure, the orange surface indicates pocket 1 and the path of the tunnel through this pocket, is marked by a grey mass. The altered amino acid (symbolized as a multi-coloured mesh) is located in the middle of the tunnel, causing an obstruction of the path. On the right side, the yellow surface modelling indicates pocket 2, and its active site is coloured in white. The figure also shows the human FIXa binding site (lilac colour) located in the A3 domain of FVIII.*

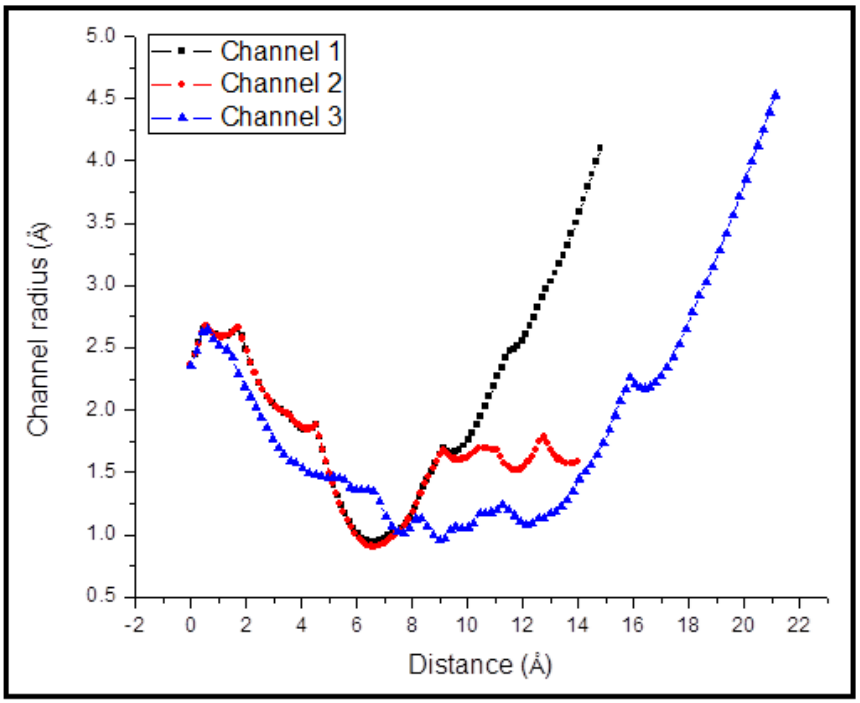

Figure 23: Graphic representation of the channel profiles. *The abscissa shows the length of the tunnel measured in Å and the ordinate represents the channel radius measured in Å. Channel 1 (black line) illustrates the obtained values in the tunnel of pocket 1 of the canine wild type FVIII. Channel 2 (red line) shows the obtained values in the tunnel of pocket 1 of mutated canine FVIII. Here, in comparison to the wild type, the radius shows an obstruction from 9 Å distance. Channel 3 (blue line) illustrates the obtained values in the tunnel of pocket 2 of the canine wild type FVIII.*

4.3.3. Exon 21 screening in genomic DNA of different dog breeds

Sequencing analysis of exon 21 sequences of the canine FVIII gene from ten healthy Great Danes (Table 9, see p. 51) did not show any differences to the wild type canine FVIII cDNA.

4.4. Results in Poodle mix with haemophilia A

Comparison of amplification products representing each exon of canine FVIII gene of genomic DNA from dog 11 (Table 8, see p. 51) were identical to the wild type FVIII cDNA (AF016234), except in exons 1, 14 and 15.

4.4.1. Single base changes in exon 1, 14 and 15 of the canine FVIII gene

Different single-base changes were observed in the canine FVIII gene of the haemophilic Poodle mix. Initially, an amino acid change, c.141C>T was detected in exon 1. After that, two single amino acid changes, c.2943G>A and c.3608C>T, were found in exon 14. Finally, a single-base exchange, c.5292C>T was identified in exon 15 (Fig. 24).

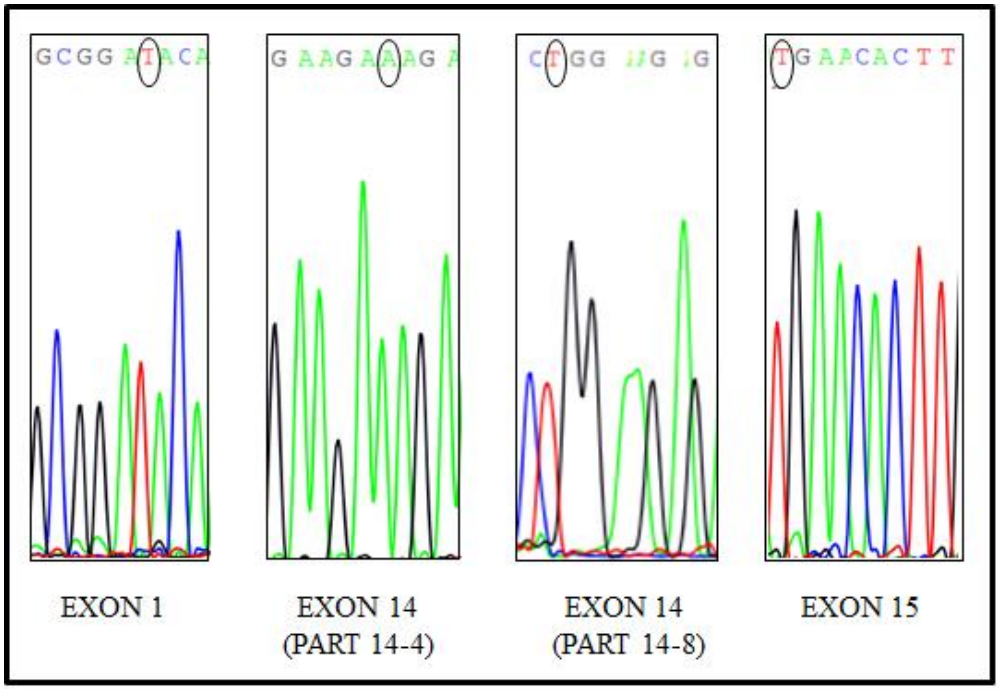

Figure 24: Nucleotide exchanges identified in the FVIII gene of genomic DNA from a Poodle mix with haemophilia A. *From left to right, the four electropherograms show a part of the nucleotide sequences of exon 1 (from nucleotide 136 to 144), exon 14 (from nucleotide 2938 to 2946 and from nucleotide 3607 to 3615) and exon 15 (from nucleotide 5292 to 5301) of canine FVIII gene from a Poodle mix. Sequence comparisons with the wild type canine FVIII cDNA revealed some nucleotide changes: c.141C>T in exon 1, c.2943G>A and c.3608C>T in exon 14 and c.5292C>T in exon 15, which are marked by an ellipse.*

4.4.2. Insertion in exon 14 of the canine FVIII gene

Differences in length of PCR products from exon 14 (part 14-6) were observed on agarose gel electrophoresis. When using DNA from the haemophilic Poodle mix, a band of approximately 300 bp was visualised, whereas an expected 250 bp fragment was observed using DNA of the control dog (Fig. 25, see p. 103).

Sequence analysis of the PCR product (forward sequence) of the haemophilic Poodle mix revealed an insertion after nucleotide 3216 in exon 14 of canine FVIII cDNA. The insertion is composed of a short sequence (TAAAG) and a poly-A tail (20-26 bp), followed by an overlapping sequence (Fig. 26, see p. 104). A poly-A tail followed by an overlapping sequence was also observed in the reverse sequence, however in this case, it was identified after nucleotide 3202 of the canine FVIII cDNA, due to the fact that the insertion is flanked by a repeated sequence of 15 bp, which corresponds to the nucleotide sequence 3202-3216 of exon 14 (Fig. 27, see p. 105).

Amplification reactions after using two new primer pairs, 14-4F+14-6R (A) and 14-6F+14-7R (B), produced an approximately 500 and 400 base pairs fragment, respectively. No marked differences in length were observed between the fragments from haemophilic Poodle mix and the control dog. Unlike the above, PCR reactions with the primer pair 14-6F and 14-6nR (C) produced fragments, which showed a small difference in size: approximately 200 base pairs from the genomic DNA from haemophilic Poodle mix and 170 base pairs from the control dog (Fig. 28, see p. 106). Sequence analyses of the obtained PCR products corroborate that exon 14 of canine FVIII gene from a haemophilic Poodle contained an insertion after nucleotide 3214 (Fig. 29 and 30, see pages 107-108).

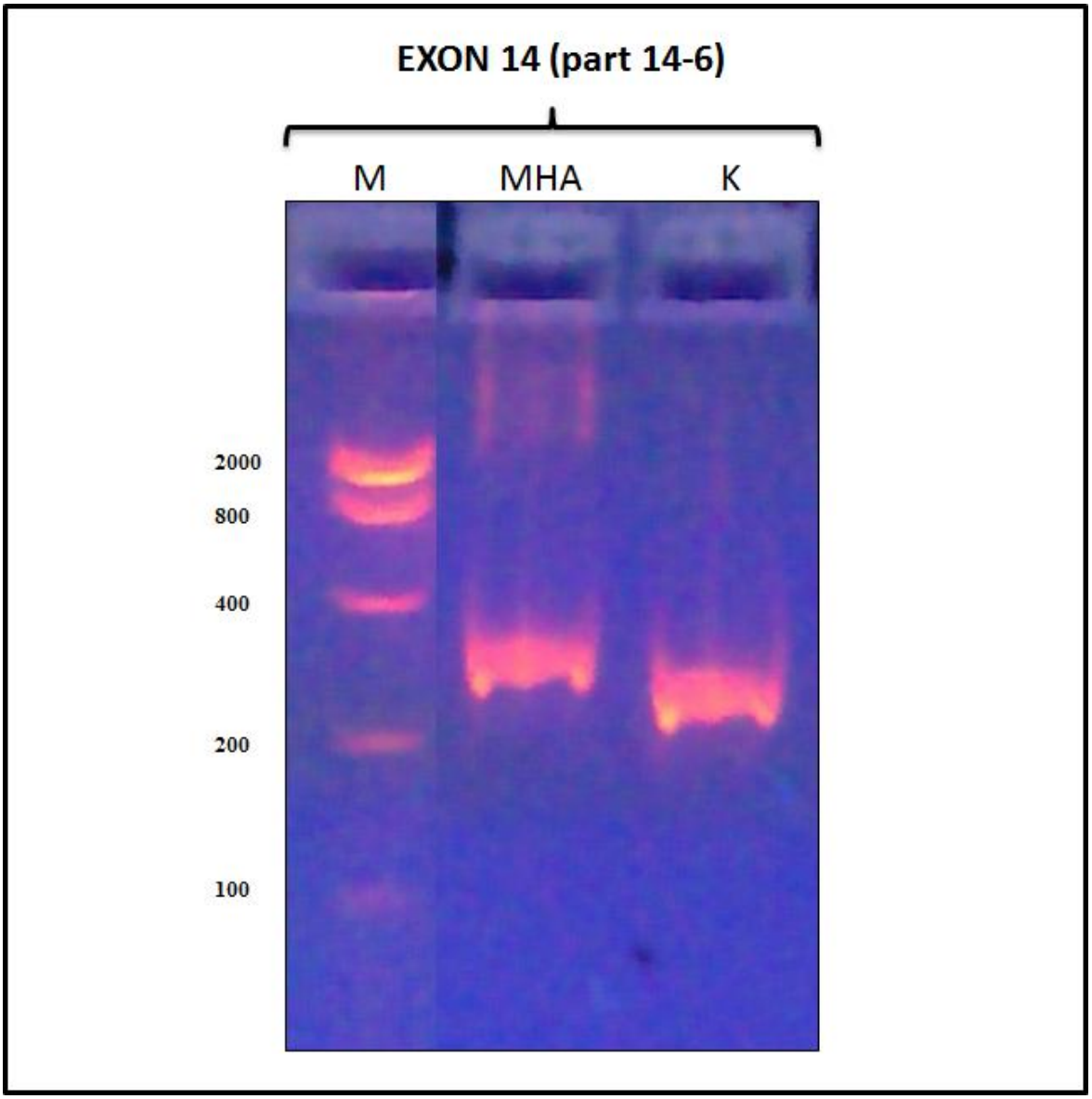

Figure 25: Agarose gel electrophoresis of exon 14 (part 14-6) of the canine FVIII gene from the haemophilic male Poodle mix. *The image illustrates the bands of PCR products from a haemophilic male Poodle mix (HMA) and a control Spanish Mastiff (K). The use of a marker (M) allowed the identification of the size of the bands. A band with the expected length (approximately 250 bp) was observed using DNA from a control dog. In HMA, amplification of exon 14 (part 14-6) yielded a band of approximately 300 bp, which is clearly longer that the obtained fragment from control dog.*

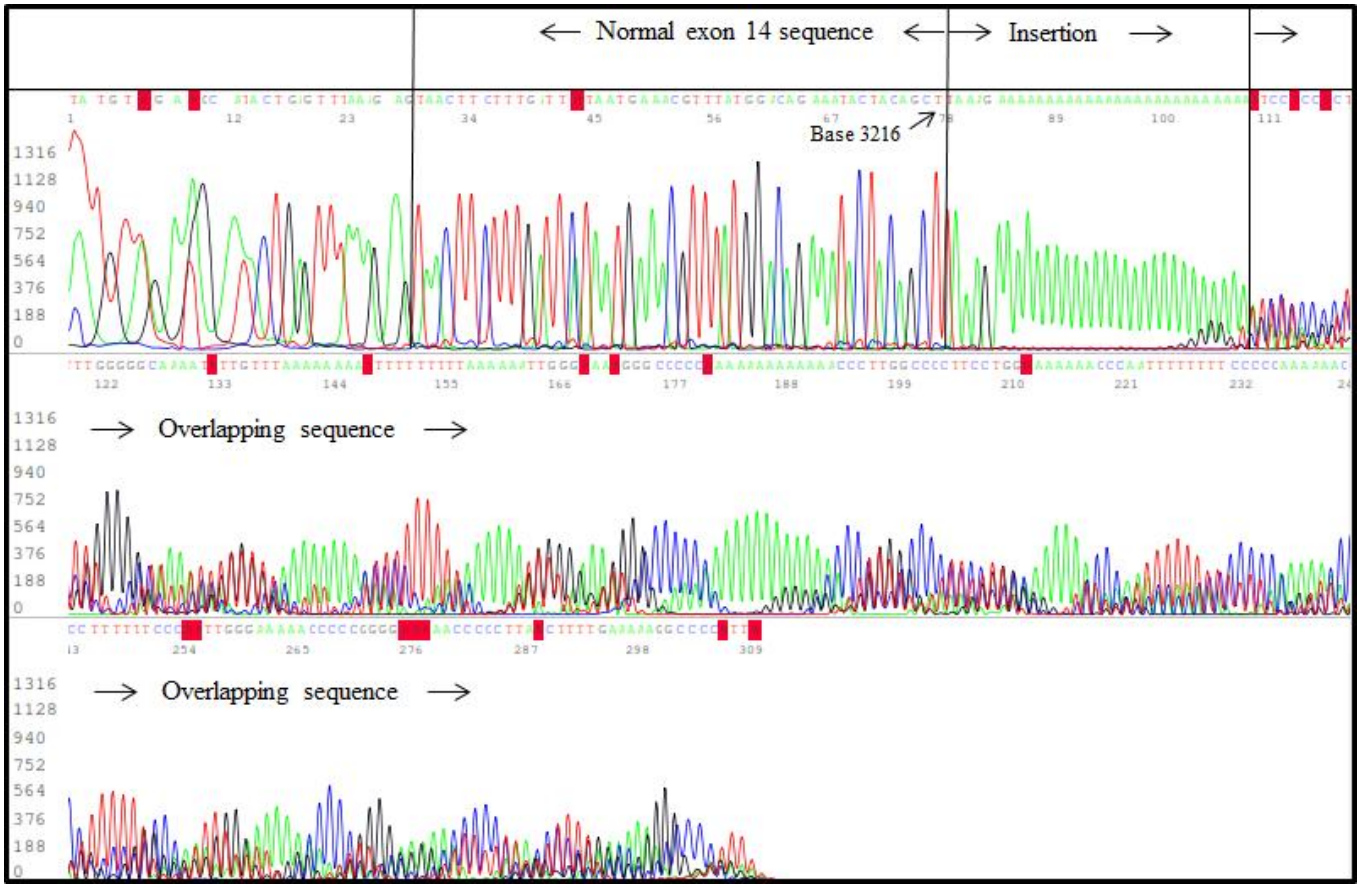

Figure 26: Forward sequencing electropherogram of a portion (part 14-6) of exon 14 of the canine FVIII gene obtained from genomic DNA of the haemophilic male Poodle mix. *PCR reaction was performed with the primer pair 14-6F and 14-6R. The image illustrates a part of the exon 14 sequence, from nucleotide 3167 to 3216, of the canine FVIII gene from haemophilic Poodle mix. This sequence is disrupted by an insertion after nucleotide 3216, which is followed by an overlapping sequence. The insertion is composed of a short sequence (TAAAG) and a poly-A tail (20-26 base pairs).*

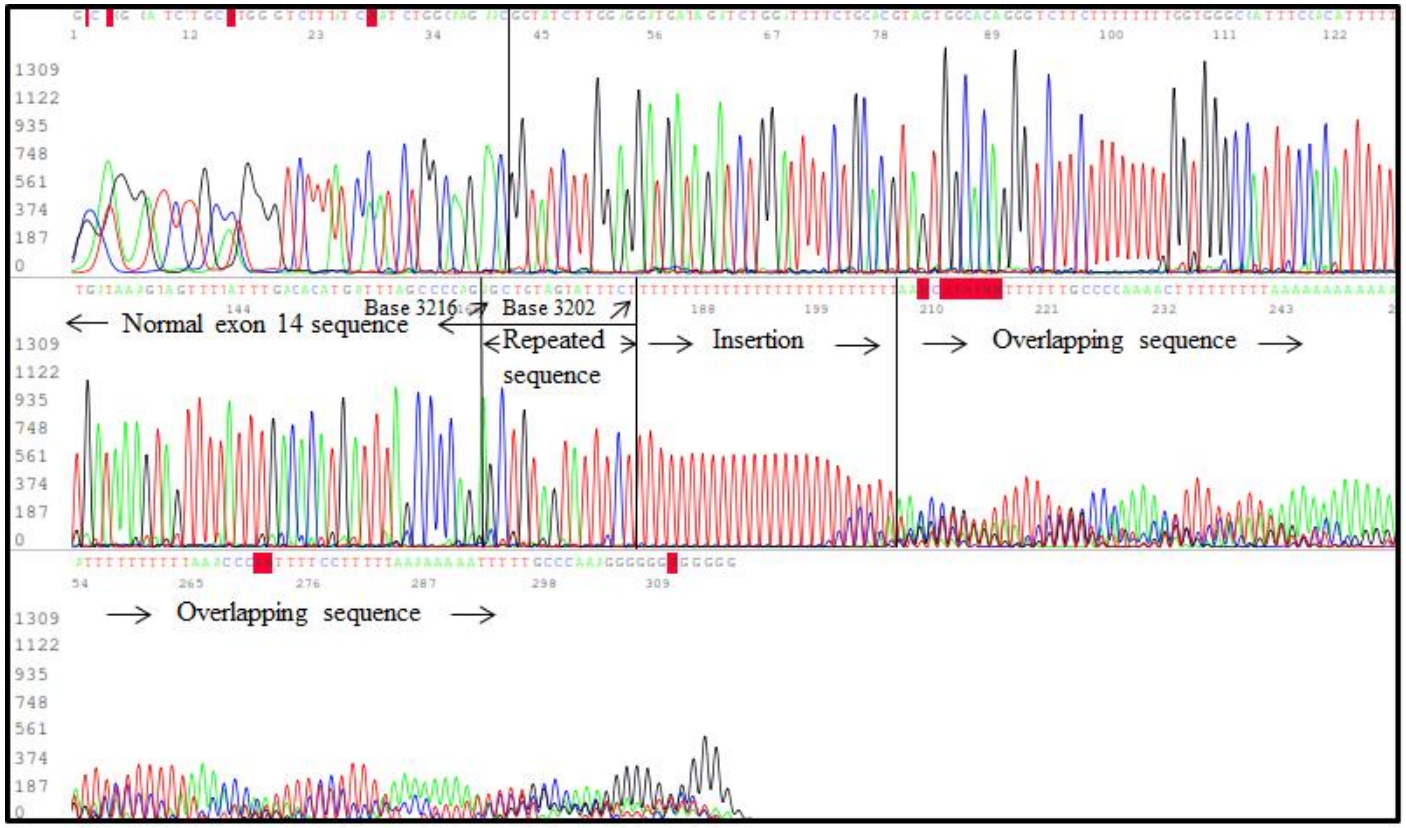

Figure 27: Reverse sequencing electropherogram of a portion (part 14-6) of exon 14 of the canine FVIII gene obtained from genomic DNA of the haemophilic Poodle mix. *PCR reaction was performed with the primer pair 14-6F and 14-6R. The electropherogram illustrates a part of the normal exon 14 sequence, from nucleotide 3341 to nucleotide 3217 of the canine FVIII gene from the haemophilic Poodle mix, which is followed by a repeated sequence of 15 bp (from nucleotide 3216 to 3202), a poly-A tail (20-25 base pairs) and an overlapping sequence.*

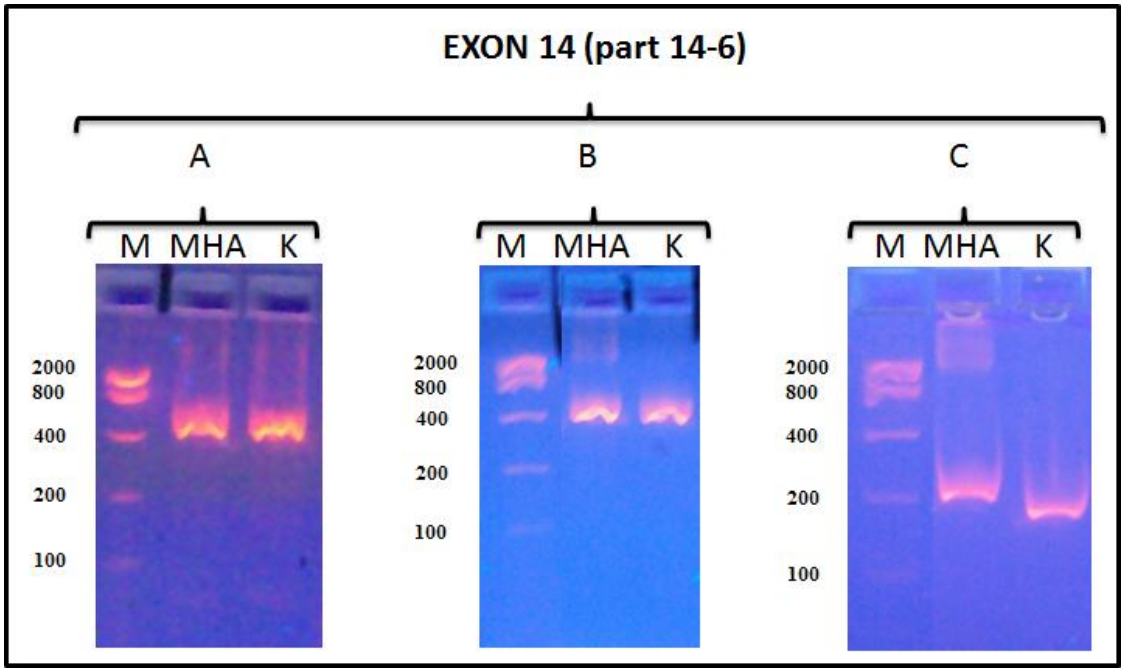

Figure 28: Agarose gel electrophoresis of exon 14 (part 14-6) of the canine FVIII gene from haemophilic male Poodle mix. *The image illustrates three PCR reactions (A, B and C), which were performed to amplify exon 14 (part 14-6) in genomic DNA from a haemophilic male Poodle (HMA) and a control Spanish Mastiff (K). The use of a marker (M) allowed the identification of the size of the bands. During PCR reaction A and B approximately 400base pair (bp) fragments were visualized, therefore no difference in length was observed between genomic DNA of HMA and K. The use of a new reverse primer located within part 14-6 of exon 14 (PCR reaction C) allowed the identification of a difference in length between both DNA samples. An approximately 200 bp fragment was visualized using DNA from HMA. This size differs from the obtained fragment using DNA of a control dog (approximately 170 bp).*

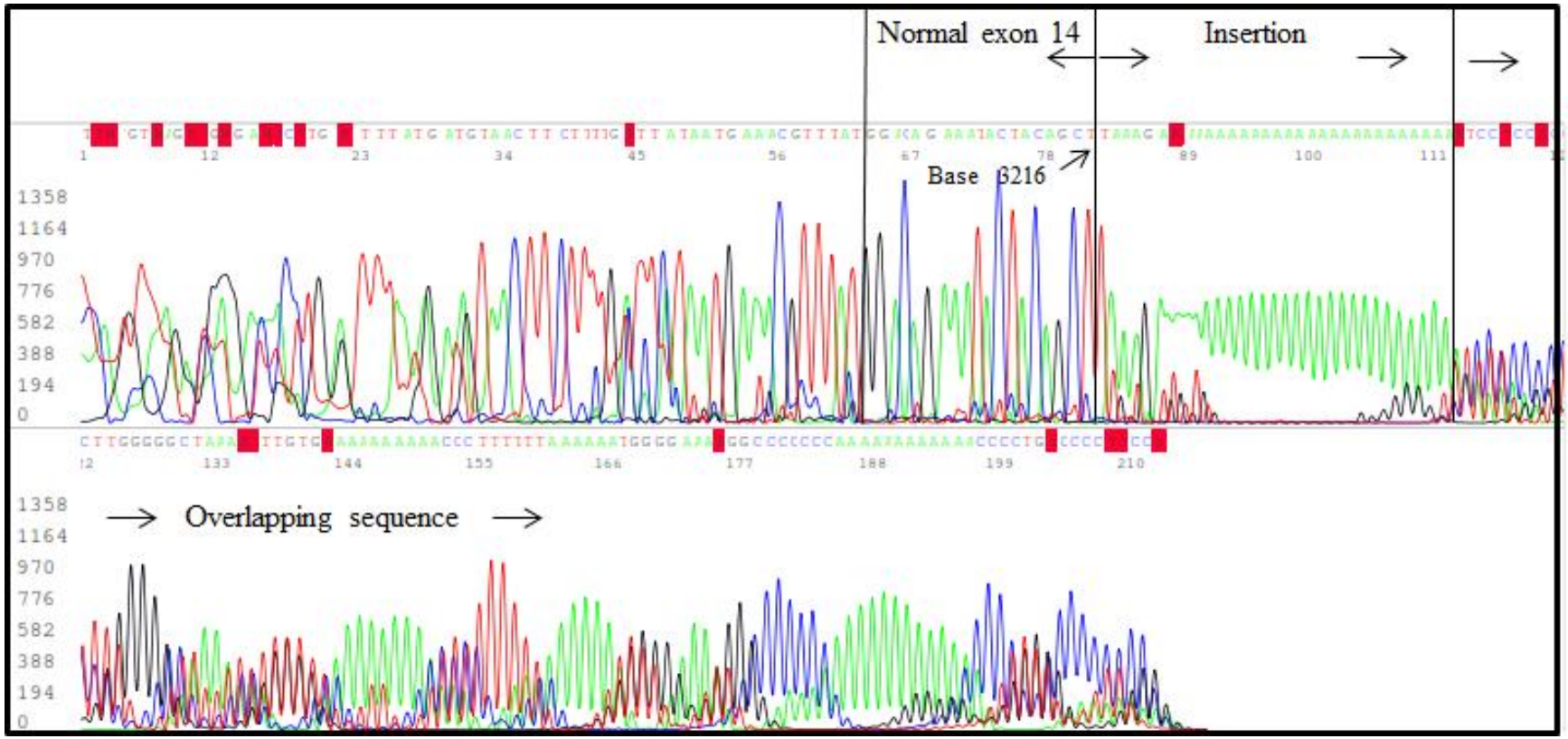

Figure 29: Forward sequencing electropherogram of a portion (part 14-6) of exon 14 of the canine FVIII gene obtained from genomic DNA of a haemophilic male Poodle mix. *PCR reaction (C) was performed with the primer pair 14-6F and 14-6nR. The image illustrates a part of the exon 14 sequence, from nucleotide 3198 to 3216 of the canine FVIII gene from the haemophilic Poodle mix, which is disrupted by an insertion after nucleotide 3216, followed by an overlapping sequence. The insertion is characterised by a short sequence (TAAAG) and a poly-A tail (20-26 base pairs).*

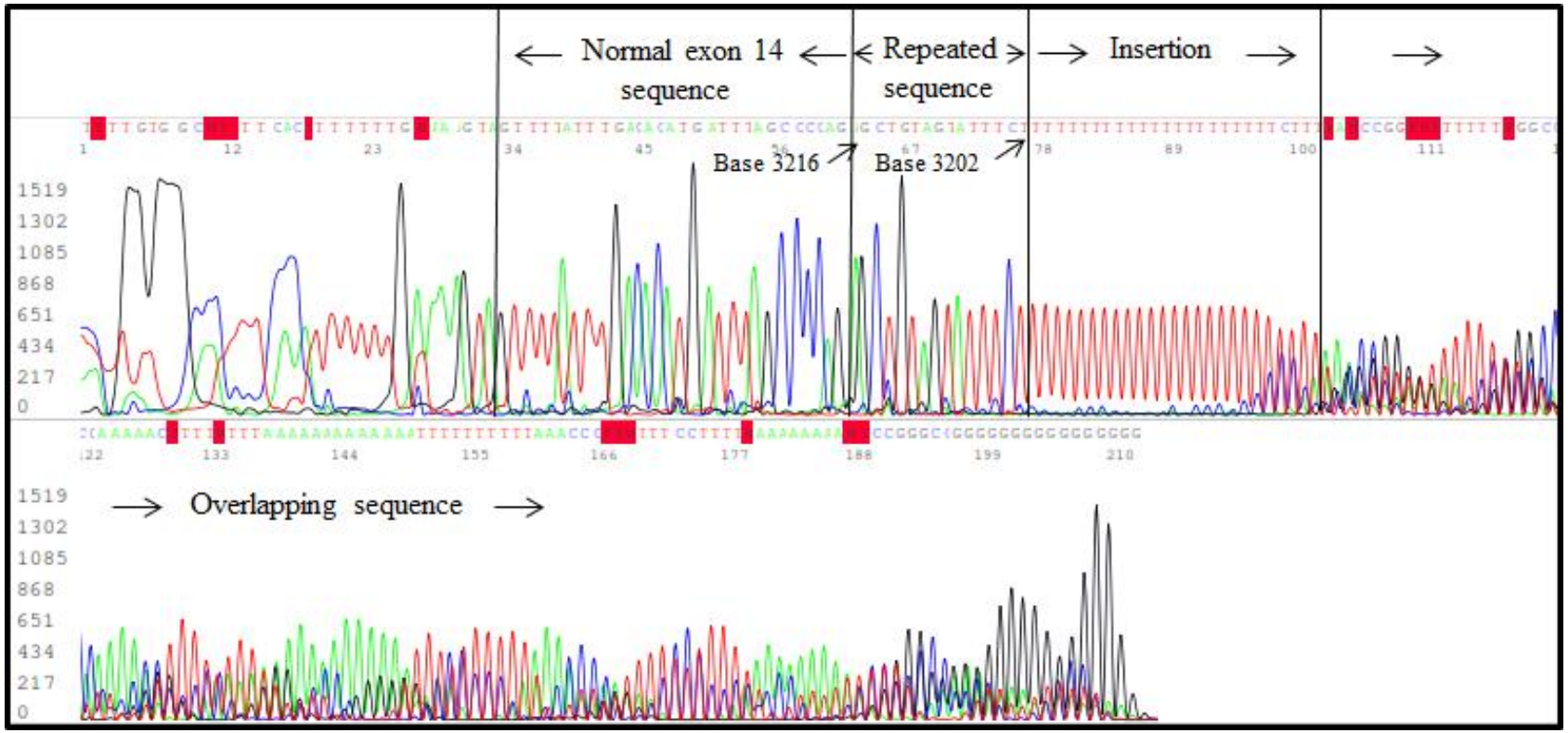

Figure 30: Reverse sequencing electropherogram of a portion (part 14-6) of exon 14 of the canine FVIII gene obtained from genomic DNA of a hamophilic male Poodle mix. *PCR reaction (C) was performed with the primer pair 14-6F and 14-6nR. The image illustrates a part of the normal exon 14 sequence, from nucleotide 3245 to nucleotide 3217 of the canine FVIII gene from the haemophilic Poodle mix. The sequence is followed by a 15 bases pairs duplication (from nucleotide 3216 to 3202), a poly-A tail (20-25 base pairs) and an overlapping sequence*.

5. DISCUSSION

5.1. Fila Brasileiro with severe form of haemophilia B

Results of the analysis of the coding region and exon intron boundaries of the canine FIX gene of a haemophilic male Fila Brasileiro revealed that a mutational event in exon 4 is responsible for the haemophilic phenotype in this dog. The analysis of intron sequences around exon 4 showed that the mutational event also involves the surrounding flanking regions, approximately 900 nucleotides upstream and at least 1000 nucleotides downstream of exon 4.

The exact type of mutation could not been identified due to the fact that the amplification of a long fragment, which involves 1418 bp of intron 3, exon 4 (114bp) and 4377 bp of intron 4 failed when using genomic DNA of patient and a control dog and two different polymerases.

The possibility of a poor quality of the DNA sample was taken into account but it was ruled out since most exons were correctly amplified from genomic DNA of the haemophilic Fila Brasileiro. Another plausible explanation for the failure of amplification of exon 4 from this patient could have been an error of the PCR technique. However, this possibility was excluded due to the fact that exon 4 amplification of the canine FIX gene of a control dog and three healthy Fila Brasileiro dogs yielded a PCR product of the expected size.

The failure of amplifications of exon 4 and surrounding regions from the patient suggested that a possible large deletion or an inversion involving exon 4, as well as part of intron 3 and 4, could be the likely cause for failure of amplification of exon 4 and intron sequences around it and, thereby, the causative mutation responsible for the haemophilic phenotype in the Fila Brasileiro. Between both possible types of genetic defects, a deletion involving exon 4 as well as part of intron 3 and 4 would be the likeliest causative mutation, because deletion is the most commonly identified mutation type of the canine FIX gene (approximately 50% of the known mutations) and no inversion has been identified to date (MISCHKE 2011C). Furthermore, all known deletions of the canine FIX gene have been reported in dogs with a severe form of haemophilia B (residual factor activity < 1 %), which was also present in the studied Fila Brasileiro.

For example, a 5 bp deletion followed by a c.777C>T was identified in exon 6 from Lhasa Apso dogs (MAUSER et al. 1996). A deletion in exon 8 was identified in a Labrador

Retriever and in a Airedale Terrier (SUGAHARA et al. 1996). A large deletion from exon 1 to exon 6 causes haemophilia B in Pit Bull Terrier mix dogs (GU et al. 1999). Finally, complete gene deletion was suspected as the causative mutation of haemophilia B in a Labrador Retriever due to the lack of any amplification product (BROOKS et al. 1997). In human beings, deletions are the second most common genetic defect of the FIX gene. Published mutations include an analogous deletion of the exon 4 and the flanking intron sequences in humans suffering from the severe form of haemophilia B (VIDAUD et al. 1986).

The last version of the human haemophilia B mutation database lists a total of 2891 patients[9] (last access: 03.06.12). In contrast to dogs, point mutations are the most common genetic defect in humans suffering from haemophilia B, and are present in approximately 90 % of those patients; 30 % of all these mutations are located in hypermutable sites (GC sites) (BOWEN 2002; PRUTHI 2005). Deletions are the second most common mutation. These include short deletions (less than 30 nucleotides), gross deletions and complete deletions[1]. As in dogs, insertions are the least common and are sometimes combined with other mutations, such as deletions (BOWEN 2002; PRUTHI 2005).

To the best of my knowledge, the suspected mutation in exon 4 is the first mutational event which has been described in this breed. Exon 4 encodes 35 amino acids of the EGF-like domain of the canine FIX gene (EVANS et al. 1989B). The role of this domain in dogs has not been described so far. In humans, this domain appears to bind to the A3 domain of FVIIIa, as well as to the FVIIa-TF complex (PERSSON et al. 2002). Formation of these complexes enhances activity of the catalytic domain of the human FIX and is, therefore, essential for the cleavage of FX and for the function of the whole blood clotting system (LENTING et al. 1996A, CELIE et al. 2002). This explains why large mutations in exon 4 leading to changes in the FVIII and or FVIIa-TF binding sites are associated with severely reduced activation of FIX and, consequently, FX causing hypocoagulability (VIDAUD et al. 1986). Amino acid alignments of FIX between different species have shown that the amino acid sequence of canine FIX is highly homologous to the human protein (EVANS et al. 1989, MAUSER et al. 1996), suggesting a similar protein structure and probably a similar severe functional defect.

9 http://www.kcl.ac.uk/ip/petergreen/intro.html

This is well in accordance with the severe phenotype of the studied dog, i.e. severe clinical signs and residual FIX activity < 1 %.

Further studies could be aimed at amplification of intron 4, followed by a long PCR reaction to amplify a region of the FIX spanning part of intron 3, exon 4 and part of intron 4 with the aim to determine the definite type of mutation. The forward primer for the PCR reaction should be designed to bind to intron 3 of the canine FIX gene and reverse primer to intron 4. It is necessary to design a specific PCR protocol, which should include a Taq DNA polymerase for amplification of long DNA fragments (DF-Taq DNA-Polymerase, Genaxxon bioscience, Germany). After the amplifications, PCR products should be separated by electrophoresis on 1 % agarose gel.

5.2. Wire-haired Dachshund with haemophilia A

Initially, sequence analysis of the canine FVIII gene of a suspected carrier (dog 4) and five healthy Wire-haired Dachshunds (dogs 5-9) identified five nucleotide changes in exons 1, 14 and 15 of the canine FVIII gene: c.141C>T in exon 1, c.2782A>G, c.2943G>A and c.3608C>T in exon 14 and c.5292C>T in exon 15. The presence of these nucleotide changes was additionally confirmed by using a new pair of primers. Based on the genetic code, three of these changes (c.141C>T, c.2943G>A, and c.5292C>T) do not result in an amino acid substitution during the translation. The c.141C>T changes a GAC into GAT at codon position 47 of the canine FVIII cDNA, but both triplet codons encode aspartic acid. The c.2943G>A changes a GAG into GAA at codon position 981 of the canine FVIII cDNA and both codons encode glutamic acid. The c.5292C>T changes an AAC into AAT at codon 1764 of the canine FVIII cDNA, but both codons encodes asparagine. Due to the fact that these three nucleotide substitutions do not change the amino sequence of FVIII protein, these nucleotide changes could be characterized as silent mutations or synonymous polymorphisms. This is also well in accordance with the fact that they were not identified in the haemophilic dog 2. Interestingly, nucleotide substitutions at the same three codon positions mentioned above were observed by HOUGH et al. (2002), while they investigated the causative mutation of haemophilia A in the dog colony at Queen's university (Kingston, Canada). In the cited study, nucleotide substitutions were identified in normal and haemophilic dogs, and consequently they were characterized as natural single nucleotide polymorphisms (SNP).

In contrast, the remaining two nucleotide changes, which were observed in the Dachshunds (c.2782A>G and c.3608C>T), theoretically result in amino acid changes. The first nucleotide exchange (c.2782A>G) leads to an AGT to GGT exchange, which during translation will result in an amino acid substitution of serine by glycine at residue 928 of the FVIII protein. The second nucleotide exchange (c.3608C>T) changes CCG into CTG, resulting in a substitution of proline by leucine at position 1203 of the FVIII protein. The identification of these nucleotide changes in exon 14 of some of ten random control dogs confirmed that both nucleotide exchanges are non-synonymous polymorphisms, which is also in accordance with the previous report by HOUGH et al. (2002).

The presence of five SNPs within the canine FVIII gene suggests that this gene is variable. Human FVIII gene has also been described as a variable gene, because polymorphisms have been detected (VIEL et al. 2007). Whether the identified SNPs produce an effect on the FVIII protein has not been investigated in dogs yet. In humans, a non-synonymous SNP at codon 1241 of the FVIII cDNA has been associated with variable FVIII activities (VIEL et al. 2007).

Sequence analysis of the canine FVIII gene from the haemophilic Wire-haired Dachshund (dog 2) did not reveal differences between the patient and the wild type canine FVIII cDNA (CAMERON et al. 1998), which suggested that the genetic defect responsible for haemophilia A in this dog does not lie within the coding region and exon-intron boundaries of the canine FVIII gene. Unfortunately, genomic analysis of the suspected carrier of the Wire-haired Dachshund (dog 3), which could have confirmed the described finding and which could have possibly contributed to identify the underlying defect of this dog family, was not possible due to the fact that the DNA sample was contaminated with human genomic DNA.

In dogs, only four causative mutations have been identified in the canine FVIII gene. Two of them were detected in the coding region using genomic DNA (MISCHKE et al. 2011A, MISCHKE et al. 2011B). In the other two cases, the coding region of the canine FVIII gene was normal and an aberrant transcript was identified (LOZIER et al. 2002, HOUGH et al. 2002). In the current genetic study, several hypotheses were taken into account to explain the unidentified mutation:

1. The haemophilic phenotype could result from intra-chromosomal recombination events that affect RNA processing. In the Chapel Hill haemophilic A dog colony, dogs show a normal coding region of the FVIII gene. However, the FVIII transcript contains an abnormal sequence (ch8) after exon 22 that also replaces the last four exons. Ch8 shows an 87 % identical homology to F8A, which is located in intron 22. Fluorescence in situ hybridization analysis also detected ch8 at the 5' end of the FVIII gene from normal dogs. This fact suggested that a homologous recombination event between these copies results in the intron 22 inversion (LOZIER et al. 2002). Another abnormal transcript was observed in the Queen's University (Kingston, Canada) dog colony. FVIII mRNA analysis revealed an abnormal sequence juxtaposed to exon 22, which was associated with a recombination event between intron 22 and other copies located in the canine genome that results in the intron 22 inversion and in the transcription of this novel sequence element (NSE). This NSE is identical to the one that was previously published by LOZIER et al. (2002), which indicates that this mutation can occur in unrelated haemophilic A families (HOUGH et al. 2002). In humans, intron 22 inversion has also been identified in 42 % of patients with severe haemophilia A (ANTONARAKIS et al. 1995). Based on these findings, intron 22 of the FVIII gene has been considered as an unstable DNA region (LOZIER et al. 2002). Intron 1 inversion also causes haemophilia A in humans, but it is less common than intron 22 inversion (ANDRIKOVICS et al. 2003). These data suggest that the haemophilic phenotype in the Wire-haired Dachshund can be the result of mutations that affect RNA processing. Unfortunately no RNA samples were available to analyse the transcript and to confirm or exclude this possibility.

2. Another possible explanation for the unidentified mutation is that mutations could be present in areas of the FVIII gene that were not analysed in the present study (e.g. promoter region, 5' UTR, 3' UTR, polyadenylation sites, intron sequences). This hypothesis is well in accordance with the results of a human study based on patients with severe (n=30) and mild to moderate (n=17) haemophilia A, respectively (HIGUCHI et al. 1991). In this study, PCR amplifications of the coding region, splice junctions, promoter region, 150 nucleotides from the 5' UTR, 300 nucleotides from the 3' UTR and polyadenylation site of the human FVIII gene were performed using genomic DNA. PCR products were analysed using gel electrophoresis, sequencing and Southern blot. Because underlying mutations were identified in only 16/30 patients with severe haemophilia A and 16/17 with mild to moderate disease,

HIGUCHI et al. (1991) concluded that about 50 % of the mutations responsible for severe haemophilia A in humans are not located in the coding region of the human FVIII gene. In addition, molecular changes in the promoter region of the human FVIII gene, where HIGUCHI et al. (1991) did not find mutations, have been identified by other authors in mildly affected human patients (BOGDANOVA et al. 2007, ZIMMERMANN et al. 2012). Finally, mutations in 5' UTRs and 3' UTRs have been associated with diseases in humans (CHATTERJEE et al. 2009). The international database of human haemophilia A lists large deletions involving exon 26 and part of the 3' UTR (last update: June 2012)[10] (KEMBALL-COOK et al. 1998).

Although the human studies described above suggest that mutations in these non-coding areas of the canine FVIII gene could be responsible for haemophilia A in the Wire-haired Dachshund family, in the present study, none of these non-coding areas could be investigated. In contrast to human beings, the promoter region of canine FVIII has not been reported and the canine 5' UTR has not been completely characterised so far. In addition, the canine 3' UTR, which has a very high G/C content (> 70 %) and the two polyadenylation sequences, which are located at the end of the 3'UTR were not analysed due to the risk of the primer dimer or mispriming formation, which would have impeded the amplification. These reasons probably explain why no examples of mutations in these non-coding areas of the canine FVIII gene have been reported so far.

Two further explanations were also considered, which are, however, less likely to be responsible for the inability to find the underlying mutation in the Wire-Haired Dachshund:

3. FVIII deficiency could be caused by an alteration of the FVIII binding site on the vWF, although the patient had a normal vWF concentration. A variant of vWD, where the FVIII binding site on the vWF is affected, has been described in humans as pseudohaemophilia or vWD type 2 Normandy (2N). In this vWD type, laboratory data (prolonged APTT, decreased FVIII activity as well as normal vWF:antigen [Ag] and vWF activity) initially led to a misdiagnosis as haemophilia A. However, measurements of the vWF capacity to bind FVIII made it possible to diagnose the disorder as vWD type 2N. Missense mutations in the coding region of the human vWF (exons 18-24), which encode amino acids residues 761 to 1074

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involved in the vWF binding site to FVIII, have been identified as the causative defect responsible for this disease (MAZURIER 1992, JACQUEMIN 2009). The relevance of this vWD subtype in dogs is unclear.

With respect to this possibility (primary change on the vWF protein), it has to be considered that the FVIII activity in vWD is not reduced to the same extent as the levels of vWF:Ag (DENIS et al. 1999, MATTOSO et al. 2010). Although individual studies of dogs suffering from vWD report low and/or variable FVIII activities ranging from 17 to 22 % (NICHOLS et al. 1993) or 35 to 61 % (JOHNSTONE 1986), which are explained with the inclusion of different vWD types (JOHNSTONE et al. 1981), most authors found FVIII concentrations within the normal range (JOHNSTONE et al. 1981, MATTOSO et al. 2010). FVIII activity in dogs suffering from vWD is not reduced in a similar extent as has been reported in humans suffering from vWD (TURECEK et al. 1997), which indicates that FVIII stabilisation in blood plasma by vWF appears to not to be as important in dogs as in humans (DENIS et al. 1999, BURGESS et al. 2009). Therefore, generally possible mutations in the FVIII binding site on the canine vWF, would probably lead to, if any, a much lower reduction of the FVIII activity. These facts make this hypothesis an unlikely cause for the reduction of FVIII in the Wire-haired Dachshund.

4. Sequence analysis of genomic DNA by PCR followed by electrophoresis could be a sub-optimal method. However, five known polymorphisms of the canine FVIII gene were identified in this dog family, and the mutation responsible for haemophilia A in the Great Dane and the Poodle mix was determined using genomic DNA samples, which gives an idea of its accuracy for the detection of mutations in genomic DNA. Moreover, methods based on sequence analysis of the coding region of the canine FVIII gene by PCR amplification followed by sequencing of the PCR products were also carried out on a German Shepherd (MISCHKE et al. 2011A) and Havanese dogs (MISCHKE et al. 2011B). In these cases the underlying mutation responsible for the haemophilic phenotype was identified.

In summary, further studies will be required to clarify which of the four hypothesis mentioned above is true and – finally – to identify the causative mutation of haemophilia A in this breed. These studies should include, in order of importance: analysis of RNA transcript, screening of the promoter region (when the respective sequence will be published), of the 5' UTR (when

the complete sequence will be published), of the 3' UTR, of polyandenylation sites (by using optimized PCR technique), and of intron sequences of the canine FVIII gene as well as analysis of the FVIII binding site on vWF.

5.3. Great Dane with haemophilia A

Results of this genetic study demonstrate with sufficient clarity that the identified c.6217T>C missense mutation in exon 21 is the causative mutation responsible for haemophilia A in this Great Dane. The mutation was confirmed by repeated amplifications of exon 21. Although no canine FVIII polymorphism has been documented at this position of the FVIII gene, the possibility that this nucleotide exchange represents a SNP was considered. However, the absence of nucleotide exchange at this position in the genomic DNA from ten healthy Great Danes ruled out the possibility that the detected mutation is a SNP. In dogs, only one point mutation has been reported in the FVIII gene so far. In this case, c.98G>A was identified in exon 1 of the canine FVIII gene from a German Shepherd with a severe form of haemophilia A. This nucleotide change obviously leads to a nonsense mutation that probably results in a very short truncated FVIII protein which is rapidly degraded (MISCHKE et al. 2011A). However, about 580 missense mutations are included in the international database of human haemophilia A, of which 11 have been identified in exon 21 of the FVIII gene (KEMBALL-COOK et al. 1998), which is also the location of the mutation in the Great Dane.

The effect of the identified missense mutation on transcription and translation could not be investigated due to the fact that no RNA sample was available. Theoretically, c.6217T>C leads to a codon exchange (TGG→CGG) during the transcription. This abnormal codon (CGG) results in a substitution of tryptophan ([Trp], Ph-NH-CH=C-CH_2-CH(NH_2)-COOH), a neutral and relatively nonpolar residue that contains an aromatic ring in the side chain, by arginine (HN=C(NH_2)-NH-(CH_2)$_3$-CH(NH_2)-COOH), a positively charged and polar residue that has an amino group in the side chain (TAYLOR 1986; BETTS et al. 2007). This amino acid exchange is located at amino acid position 2073 (Trp^{2073}) of the translated canine FVIII (2054 of the mature canine FVIII, or 2062 of the mature human FVIII).

Based on the sequence alignments of the mature FVIII protein from different species, which were published by CAMERON et al. (1998) and LIU et al. (2000), this missense mutation was localized in the C1 domain of the canine FVIII. In addition, the fact that C1 domain of

the mature human FVIII spans from Lys^{2020} (lysine) to Asn^{2172} (LENTING et al. 1998; GRAW et al. 2005), and that this domain shows 94 % (LIU et al. 2000) or 92 % (CAMERON et al. 1998) homology, at the amino acid level, with the canine C1 domain, suggested that the canine C1 domain could span from Lys^{2012} to Asn^{2164}. Furthermore, it was observed that this mutation affects one of the highly conserved amino acids; in other words, Trp^{2073} is in an analogous position in both species (human beings and dogs) as in other species such as mouse and pig (CAMERON et al. 1998) and beyond that conserved (identity of 96 %) in the discoidin domain family. This protein family is formed by eukaryotic and prokaryotic proteins with similar repeated C domains (BAUMGARTNER et al. 1998). Well in accordance with this finding in the Great Dane, the majority of the identified missense mutations in the C domains of the FVIII gene also affect conserved amino acids (GALE et al. 2000).

The structural and consequently functional relevance of the replacement of tryptophan by arginine is related to the great differences in charge, polarity and structure between these amino acids. Such amino acid replacements have been associated with possible protein disorders (BETTS et al. 2007). This may especially be the case, because the Trp^{2073} replacement affected a highly conserved protein residue, which can be regarded as vital amino acids for the structure and/or function of proteins because amino acid substitutions frequently result in loss of the protein function (POTEETE et al. 1992).

Results of elaborated protein models confirmed that the identified missense mutation in the C1 domain of the FVIII protein results in a change in the structure of the FVIII protein. This identified structural change is well in accordance with the known fact that missense mutations in C domains of the human FVIII protein result in disruptions of the FVIII structure and alterations of FVIII binding sites for other coagulation factors (BOWEN 2002; GALE et al. 2000). As far as the author knows, no binding site has been documented in the C1 domain of the canine FVIII so far. However, due to the high similarities between canine und human FVIII proteins, the known human binding sites could be located at similar positions in canine FVIII (CAMERON et al. 1998, MISCHKE 2011B).

Based on elaborated protein models, which were created in the present study, the altered amino acid was not close to known binding sites of human FVIII for FIX in the A3 domain (FAY 2005), for vWF in the C2 domain (HEALEY et al. 1998) and for phospholipids in the

C2 domain (BARROW et al. 2001; FAY 2005), which suggested that this amino acid did not directly participate in these bindings. In addition, elaborated protein models suggested that a possible active site is close to the altered amino acid. Unfortunately, the functional role of this active site could not be evaluated. Interestingly, human studies of the role of the FVIII C1 domain suggest that this region of the protein enhances the binding of the FVIII C2 domain for FIXa and FX (LÜ et al. 2011). Moreover, HSU et al. (2008) demonstrated that the FVIII C1 domain binds to platelets before and after platelet activation. Therefore, it can be hypothesized that the identified C-T missense mutation in exon 21 of the canine FVIII gene of the Great Dane alters the interaction of FVIII with FIXa, FX or platelets, i.e. the capacity to form a complex (known as intrinsic FXase complex) with FIXa and FX on the activated platelet surface. This can explain the significantly reduced FVIII activity of 4 %, which was detected in the Great Dane. With a factor VIII activity of 4 %, the degree of severity in the studied dog would be classified as "moderate form" based on human criteria (LIU et al. 2000). However, severe clinical signs present in the Great Dane are more suitable for a severe form, which is well in accordance with the phenomenon that haemophilic dogs have more severe clinical signs at a defined residual factor activity of haemophilic factors. This discrepancy can be mainly explained by the different life style of the two species (MISCHKE 2012).

5.4. Poodle mix dog with haemophilia A

Genetic analysis of the genomic DNA of a Poodle mix identified four single nucleotide changes in the coding region of the canine FVIII gene: c.141C>T in exon 1, c.2943G>A and c.3608C>T in exon 14, and c.5292C>T in exon 15. These substitutions were characterized as SNPs by HOUGH et al. (2002) and therefore, they are not responsible for haemophilia A in the Poodle mix.

Analysis of the exon 14 (part 14-6) sequence showed that an insertion in exon 14 is the underlying defect responsible for haemophilia A in the Poodle mix. This finding was confirmed by three PCR reactions, by using different primer pairs. The fact that the analysis of the reverse sequences identified this mutation after nucleotide 3202 instead of after nucleotide 3216 (observed after the analysis of forward sequences) revealed that this insertion also contains 15-bp duplication (from nucleotide 3202 to nucleotide 3216). Therefore, the

identified insertion in exon 14 is about 40-46 bp in length, and contains a short sequence (TAAAG), followed by a poly-A tail (20-26 bp), which is flanked by a DNA duplication (15-bp). During the interpretation of electropherograms, the exact length of the poly-A tail could not be calculated, because the poly-A tail was followed by an overlapping sequence. This overlapping sequence revealed that fragments with different lengths had been amplified during the PCR reaction. These fragments were probably created due to errors during the amplification of the identified long poly-A tail by the Taq DNA polymerase. Therefore, the size of the amplified poly A tail varied in length, from 20 to 26 nucleotides depending on the cycles of amplification. Amplification errors associated to mononucleotide repeats have been described previously in other studies (CLARKE et al. 2001; FAZEKAS et al. 2010).

The international data base of human haemophilia A lists 80 insertions as causative mutations responsible for mild to severe haemophilia A[2] (KEMBALL-COOK et al. 1998). These insertions range from several base pairs to retro-transposon (KAZAZIAN et al. 1988) or Alu elements (GANGULY et al. 2003). Approximately 50 % of these insertions are located in exon 14[2], but these mutations are not similar to the one which was detected in the Poodle mix. In contrast, only one insertion has been described in dogs so far. In this case, a 218 bp SINE insertion was found in exon 14 of the canine FVIII gene of Havanese dogs with haemophilia A. This insertion was identified after nucleotide 2675 of the FVIII cDNA (MISCHKE et al. 2011B). SINEs are eukaryotic transposable elements, 150-300 bp in length, which contribute to mammalian diversity but are also responsible for genetic diseases caused by insertional mutagenesis (WANG et al. 2005; WALTER-CONTE et al. 2011), as in the case of Havanese dogs. SINEs contain two promoter boxes (A and B), as well as a $(CT)_n$ region, followed by poly A/T with a polyadenylation signal (AATAAA), and these are flanked by target site duplications (8-15bp) (WALTER-CONTE et al. 2011). The insertion, which was identified in the haemophilic Poodle mix is also flanked by 15-bp duplications and contains a poly-A tail, which suggested retrotransposition of a transposable element. However, this possibility was ruled out due to the fact that the identified insertion is shorter than the described SINEs and lacks $(CT)_n$ region, as well as promoter boxes, which are necessary to enable RNA polymerase III to initiate the SINE amplification during the retro-transposition mechanism (WALTER-CONTE et al. 2011).

The effect of the identified insertion on the transcription and transduction could not be studied because no RNA sample was available. Analysis of the 40-46 bp insertion in exon 14 of the canine FVIII gene from the Poodle mix dog revealed a reading frame shift and a premature stop codon "TAA" at the beginning of the insertion, c.3217-3219. The presence of this stop codon probably results in a 1072 amino acids translation product, which is clearly shorter than the 2343 amino acids of the wild type canine FVIII. In addition, due to the fact that B, A3, C1 and C2 domains of the protein are encoded from exon 14 to exon 26 of the canine FVIII gene, and that the B domain is located at amino acid residues 687-1621 (CAMERON et al. 1998), the truncated protein of the Poodle mix would only contain A1, A2 and a part of the B domain (385 amino acids, from 687 to 1072), but would lack 59 % of amino acids, which are part of the B domain (from amino acid 1073 to 1621), as well as complete A3, C1 and C2 domains. The lack of these domains suggests that some FVIII interactions with other coagulant proteins would be altered. In dogs, the A3 domain of canine FVIII contains one binding site for vWF, which is localized at amino acid residues 1666 to 1676 (human residues 1649 to 1689 [FAY 2005]). This binding site lacks in the defect FVIII of the Poodle mix. In addition, one of the three thrombin binding sites (Arg^{1681}-Ser^{1682}; human residues 1689-1690 [FAY 2005]) also lacks, whereas the two remaining binding sites for thrombin (Arg^{366}-Ser^{367}; human residues 372-372 [FAY 2005]), and Arg^{734}-Ser^{735}, human residues 740-741 [FAY 2005]) are present in the truncated protein of the Poodle mix. In addition, although canine FIXa, FXa and phospholipid cleavage sites have not been published so far, theses binding sites appear to be located at similar positions to the human ones (CAMERON et al. 1998). The A3 domain of human FVIII contains a binding site for FIX at amino acid residues 1803-1810 (FAY 2005), which is lacking in the defect FVIII of the patient, but the remaining FIXa cleavage sites, which are located at position 489-493, 558-565 and 708-715 in the A2 domain (LENTING et al. 1996; GRAW et al. 2005), are present. Finally, due to the lack of the C2 domain in the altered FVIII molecule, very likely FXa and phospholipid binding sites are also lacking, because at least in humans, binding sites for these two components are located within the C2 domain (NOGAMI et al. 2002; FAY 2005). The lack of several binding sites with essential reaction partners suggests that the truncated FVIII molecule from the Poodle mix with haemophilia A probably has a low enzymatic activity, which explains the low detected FVIII activity of 5 % and severe clinical signs. As discussed for the case of the Great Dane, this is a

further example for the clinical observation, that haemophilic dogs have more severe clinical signs than haemophilic humans at a defined residual factor activity (MISCHKE 2012).

Alternatively, in the unlikely event that the identified insertion in exon 14 of the haemophilic Poodle mix could be transcribed, it would probably result in a FVIII molecule with an abnormal or even non-functional B domain. Human studies suggest that B domain plays an important role in the intracellular processing and/or secretion of FVIII protein (PRUTHI 2005), and during the FVIII activation (MCCONELL 2000).

5.5. Conclusion

The present genetic study shows that a mutational event, which involves exon 4 and surrounding intron sequences of the canine FIX gene, is responsible for haemophilia B in the studied Fila Brasileiro. Additionally, two different mutations, a c.6217T>C missense mutation in exon 21 and a 40-46 bp insertion in exon 14 of the canine FVIII gene, are responsible for haemophilia A in the examined Great Dane and Poodle mix, respectively. Furthermore, results of the analysis of the canine FVIII gene of the haemophilic Wire-Haired Dachshund reveal that the causative mutation responsible for the haemophilic phenotype in this dog is not located neither in the coding region of canine FVIII gene nor exon intron boundaries of the canine FVIII gene. To the knowledge of the author, these are the first known mutations in a haemophilic Fila Brasileiros, and in a Great Dane. Moreover, all observed mutations are novel mutations of the canine FIX or FVIII gene. The results, thereby, underline the fact that the haemophilic B or A phenotype in the canine population is caused by a diversity of mutations in the FIX and FVIII gene (BROOKS et al. 1997, GU et al. 1999; MISCHKE 2012). Mutational heterogeneity has been also confirmed in human haemophilia B and A (BOWEN 2002).

Unfortunately, the incidence of the identified mutations in the different investigated breeds could not be investigated due to the lack of a great number of samples collected from different individuals of these breed and especially dogs which are related to our patients. Development of specific genetic screening tests based on the defined mutations would be crucial to reliably identify possible carriers and to receive valuable data on the incidence of the defect in the studied breeds.

6. SUMMARY

Patricia Alcaraz Rodríguez

Molecular genetic analysis of haemophilia A and B in several dog breeds

The **objective** of this study was to identify the underlying genetic defect responsible for the haemophilic phenotype in different dogs.

Animals: The study included a Fila Brasileiro suffering from haemophilia B and three haemophilic A dogs: a Wire Haired Dachshund, a Great Dane, and a Poodle mix. The genetic study also included two related Wire-Haired Dachshund dogs, which were suspected to be carriers of haemophilia A, four related healthy Wire-Haired Dachshund dogs and further healthy control dogs.

Materials and methods: DNA was isolated from EDTA blood from all these dogs. In the haemophilic Fila Brasileiro, the coding region and exon intron boundaries of the canine FIX gene were amplified and sequenced by using polymerase chain reaction (PCR) and electrophoresis. The obtained nucleotide sequences of the FIX gene were compared with the wild type canine FIX mRNA. Furthermore, intron sequences around exon 4 were also analysed. Genomic DNA from three healthy Fila Brasileiros was used to validate the results.

In patients suffering from haemophilia A and related dogs, the coding region and exon intron boundaries of the canine FVIII gene were also analysed. The obtained nucleotide sequences were compared with the wild type canine FVIII mRNA. To confirm the results, specific exon screenings were carried out with new primer pairs. The results were validated with genomic DNA from ten random dog breeds and ten Great Danes. In addition, several protein structure modelling were carried out to evaluate the structural and functional relevance of the identified mutation in the Great Dane.

Results: The failure of the amplification of exon 4 and intron sequences around it from genomic DNA of the haemophilic Fila Brasileiro and not from the control dog, as well as the correct amplification of exon 4 from genomic DNA of three normal Fila Brasileiros, revealed that a mutational event involving exon 4 and approximately 900 nucleotides upstream and at

least 1000 nucleotides downstream of exon 4 is responsible for the haemophilic phenotype in this dog.

Sequence analysis of the coding region and exon intron boundaries of the canine FVIII gene of a suspected carrier (dog 4) and five healthy Wire-haired Dachshunds (dogs 5-9) revealed five single nucleotide polymorphisms (SNPs): a c.141C>T in exon 1, a c.2782A>G, a c.2943G>A and a c.3608C>T in exon 14, and c.5292C>T in exon 15. Three of these SNPs (c.141C>T, c.2943G>A, and c.5292C>T) were synonymous polymorphisms and the two remaining nucleotide changes (c.2782A>G and c.3608C>T), which were also identified in exon 14 of some of ten random control dogs, were characterised as non-synonymous polymorphisms. Interestingly, sequence analysis of the canine FVIII gene of the haemophilic Wire-haired Dachshund did not reveal differences between the patient and the wild type canine FVIII cDNA, which suggested that the genetic defect responsible for haemophilia A in this dog is not located in the coding region and exon intron boundaries of the canine FVIII gene. Unfortunately, analysis of the FVIII gene of the second suspected carrier, which could have contributed to identifying the underlying defect of this dog family, was not possible due to the fact that the DNA sample was contaminated with human genomic DNA.

The genetic defect which was responsible for haemophilia A in a Great Dane was identified as a c.6217T>C in exon 21 of canine FVIII gene, which leads to the replacement of tryptophan by arginine at amino acid position 2073 in the C1 domain of the canine FVIII protein. The relevance of this amino acid replacement is related to the great differences in charge, polarity and structure between these amino acids. This mutation was confirmed by the fact that screening of genomic DNA of ten healthy Great Danes did not show this missense mutation. In addition, elaborated protein models suggested that a possible active site is close to the altered amino acid, and confirmed a change in the structure of the truncated FVIII molecule. In contrast, no changes were observed in the FVIII molecule of healthy dogs.

Results of the elaborated genetic study in the Poodle mix demonstrated with sufficient clarity that a 40-46 bp insertion in exon 14 of canine FVIII gene was the causative mutation responsible for the haemophilic phenotype in this dog. The identified insertion contained a short sequence (TAAAG), followed by a poly-A tail (20-26 bp), and was flanked by a DNA duplication (15-bp). Analysis of the insertion revealed that a transcriptional frameshift and a

premature stop codon “TAA” was located at the beginning of the insertion, c.3217-3219, which probably results in a 1072 amino acids translation product. This truncated protein would probably lack 59 % of amino acids of the B domain, as well as complete A3, C1 and C2 domains. In addition, four known SNPs were identified: c.141C>T in exon 1, c.2943G>A and c.3608C>T in exon 14, as well as c.5292C>T in exon 15.

Conclusion: The results of the present study underline the fact that the molecular bases of canine haemophilia B and A are diverse in different breeds , which has also been confirmed in human haemophilia B and A. The development of specific genetic screening tests based on the identified mutations would enable the identification of possible carriers and receive valuable data on the incidence of the defect in the analysed breeds, which would be useful for the control of these hereditary disorders of haemostasis.

7. ZUSAMMENFASSUNG

Patricia Alcaraz Rodríguez

Molekulargenetische Analysen der Hämophilie A und B bei verschiedenen Hunderassen

Das **Ziel** dieser Studie war die Identifizierung des Gendefektes, der verantwortlich für die Hämophilie bei verschiedenen Hunderassen ist.

Tiere: Die Studie umfasste einen Fila Brasileiro mit Hämophilie B und drei weitere Hunde mit Hämophilie A: einen Rauhhaardackel, eine Deutsche Dogge und einen Pudel-Mischling. Zwei verwandte Rauhhaardackel mit Verdacht auf Hämophilie A und vier verwandte gesunde Rauhhaardackel sowie weitere gesunde Kontrolltiere.

Materialien und Methoden: Von allen diesen Hunden wurde die DNA aus EDTA-Blut isoliert. Im Falle des hämophilen Fila Brasileiro wurden die kodierende Region und Exon-Intron-Grenzen des caninen FIX-Gens mit Hilfe der Polymerase Kettenreaktion (PCR) und Elektrophorese amplifiziert und sequenziert. Die erhaltenen Nukleotidsequenzen des FIX-Gens wurden mit dem Wildtyp der caninen FIX mRNA verglichen. Weiterhin wurden Intronsequenzen vor und nach dem Exon 4 analysiert. Es wurde genomische DNA von drei gesunden Fila Brasileiro verwendet, um die Ergebnisse zu validieren.
Bei Patienten mit Hämophilie A und verwandten Hunden wurden die kodierende Regionen und Exon-Intron-Grenzen des caninen FVIII Gens analysiert. Die erhaltenen Nukleotidsequenzen wurden mit dem Wildtyp der caninen FVIII mRNA verglichen. Um die Ergebnisse zu bestätigen, wurden spezifische Exon-Screenings mit zusätzlichen Primerpaaren durchgeführt. Die Ergebnisse wurden mit genomischer DNA von zehn zufällig ausgewählten Hunderassen und zehn Doggen validiert. Darüber hinaus wurden Protein-Struktur-Modellierungen durchgeführt, um die strukturelle und funktionelle Relevanz der identifizierten Mutation in Hunden der Rasse Great Dane zu überprüfen.

Ergebnisse: Mittels PCR ließen sich Exon 4 und umgebende Intron-Sequenzen aus genomischer DNA der gesunden Fila Brasileiro erfolgreich amplifizieren. Die Amplifikation derselben Bereiche gelang nicht bei dem an Hämophilia B erkrankten Fila Brasileiro. Dies

Ergebnis lässt darauf schließen, dass eine Mutation im Exon 4 sowie ungefähr 900 Nukleotide „upstream“ und mindestens 1000 Nukleotide „downstream“ von Exon 4 für die Hämophilie B in diesem Hund verantwortlich ist.

Die Sequenzanalyse der kodierenden Regionen und Exon-Intron-Grenzen des caninen FVIII-Gens eines vermuteten Trägers der Hämophilie (Hund 4) sowie von fünf gesunden Rauhhaardackeln (Hunde 5–9) identifizierte fünf Einzel-Nukleotid-Polymorphismen („single nucleotide polymorphisms“, SNPs): c.141C>T, c.2782A>G, c.2943G>A, c.3608C>T und c.5292C>T. Drei SNPs (c.141C>T, c.2943G>A, und c.5292C>T) waren stumme Polymorphismen. Die anderen zwei Basenaustausche (c.2782A>G und c.3608C>T), die auch in Exon 14 von zehn zufällig ausgewählte Hunden identifiziert wurden, waren nicht-stumme Polymorphismen. Interessanterweise ergab die Sequenzanalyse des canine FVIII Gens des hämophilen Rauhhaardackels keinerlei Unterschiede im Vergleich zu gesunden Hunden und der caninen Wildtyp FVIII cDNA. Dies deutet darauf hin, dass sich der für Hämophilie A verantwortliche genetische Defekt bei diesem Hund weder in der kodierenden Region noch im Bereich dern Exon-Intron-Grenzen des caninen FVIII-Gens befindet. Leider war die DNA-Probe des zweiten verdächtigen Trägers mit humaner, genomischer DNA kontaminiert, so dass die Analyse der Mutation nicht möglich war.

Der für die Hämophilie A bei der Deutschen Dogge verantwortliche genetische Defekt wurde als c.6217T>C-Punktmutation im Exon 21 des caninen FVIII Gens identifiziert. Dieser Basenaustausch führt zum Ersatz von Tryptophan durch Arginin an der Aminosäureposition 2073 in der C1-Domäne des caninen FVIII-Proteins. Dieser Aminosäure-Austausch ist relevant für das FVIII-Molekül wegen der großen Unterschiede in Ladung, Polarität und Struktur zwischen diesen Aminosäuren. Als weiteren Hinweis auf die Kausalität dieser Mutation war das Ergebnis des Screenings der genomischen DNA von zehn gesunden Doggen zu werten. Keine der gesunden Doggen wies die beschriebene Mutation auf. Zur weiteren Untersuchung der Bedeutung dieser Mutation wurden Proteinmodelle erstellt. Analyse und Vergleich der Proteinstrukturen zeigten, dass sich in der Nähe der ausgetauschten Aminosäure ein mögliches aktives Zentrum befindet und die Struktur des FVIII-Moleküls vom Wildtyp abweicht.

Die Ergebnisse der genetischen Analyse des FVIII-Gens beim Pudel-Mischling ließen darauf schließen, dass eine 40-46 bp Insertion in Exon 14 des FVIII-Gens die ursächliche Mutation für den hämophilen Phänotyp bei diesem Hund war. Die identifizierte Insertion enthielt eine kurze Sequenz (TAAAG), gefolgt von einem Poly-A-Schwanz (20-26 bp), und wurde flankiert durch eine DNA-Duplizierung (15 bp). Die Analyse der Insertion zeigte, dass durch einen Frameshift ein vorzeitiges Stop-codon "TAA" zu Beginn der Insertion (c.3217-3219) entstand, das wahrscheinlich ein Translationsprodukt mit einer Länge von 1072 Aminosäuren ergibt. Diesem verkürzten Protein würden ca. 59 % der Aminosäuren der B-Domäne sowie die komplette A3-, C1- und C2-Domäne fehlen. Zusätzlich wurden vier bekannte SNPs identifiziert: c.141C>T, c.2943G>A, c.3608C >T und c.5292C>T.

Fazit: Die Ergebnisse der vorliegenden Studie bestätigen die Annahme, dass der caninen Hämophilie B und A bei verschiedenen Hunderassen unterschiedliche molekulare Veränderungen zugrunde liegen, wie auch bei der menschlichen Hämophilie B und A bestätigt wurde. Die Entwicklung spezifischer genetischer Screening-Tests, die auf den identifizierten Mutationen basieren, könnten mögliche Träger identifizieren. Diese Daten wären sehr hilfreich für die Kontrolle das Auftretens dieser Hämostasestörung beim Hund.

8. REFERENCES

AHMAD, S. S., R. RAWALA-SHEIKH u. P. N. WALSH (1989):
Comparative interactions of factor IX and factor IXa with human platelets.
J. Biol. Chem. 264, 6, 3244-3251

AHMAD, S. S., J. M. SCANDURA u. P. N. WALSH (2000):
Structural and functional characterization of platelet receptor-mediated factor VIII binding.
J. Biol. Chem. 275, 17, 13071-1381

AHMAD, S. S., F. S. LONDON u. P. N. WALSH (2003A):
The assembly of the factor X-activating complex on activated human platelets.
J. Thromb. Haemost. 1, 1, 48-59

AHMAD, S. S., F. S. LONDON u. P. N. WALSH (2003B):
Binding studies of the enzyme (factor IXa) with the cofactor (factor VIIIa) in the assembly of factor-X activating complex on the activated platelet surface.
J. Thromb. Haemost. 1, 11, 2348-2355

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS u. D. J. LIPMAN (1990):
Basic local alignment search tool.
J. Mol. Biol. 215, 3, 403-410

ANDRIKOVICS, H., I. KLEIN, A. BORS, L. NEMES, A. MAROSI, A. VARADI u. A. TORDAI (2003):

Analysis of large structural changes of factor VIII gene, involving intron 1 and 22, in severe haemophilia A.Haemotologica 88, 7, 778-784

ANTONARAKIS, S. E., J. P. ROSSITER, M. YOUNG, J. HORST, P. DE MOERLOOSE, S. S. SOMMER, R. P. KETTERLING, H. H. KAZAZIAN, C. NÉGRIER u. C.VINCIGUERRA (1995):
Factor VIII gene inversions in severe hemophilia A: results of an international consortium study.
Blood 86, 6, 2206-2212

ARCHER, R. K. u. R. S. T. BOWDEN (1959):
A case of true hemophilia in a Labrador dog.
Vet. Rec. 71, 560-561

ARNOLD, K., L. BORDOLI, J. KOPP u. T. SCHWEDE (2006):
The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling.
Bioinformatics 22, 2, 195-201

AXELROD, J. H., M. S. READ, K. M. BRINKHOUS u. I. M. VERMA (1990):
Phenotypic correction of FIX deficiency in skin fibroblasts of hemophilic dog.
Proc. Natl. Acad. Sci. 87, 13, 5173-5177

BAKER, N. A., D. SEPT, S. JOSEPH, M. J. HOLST u. J. A. MCCAMMON (2001):
Electrostatics of nanosystems: application to microtubules and the ribosome.
Proc. Natl. Acad. Sci. USA. 98, 18, 10037-10041

BARROW, R. T., J. F. HEALEY, M. G. JACQUEMIN, J. M. SAINT-REMY u. P. LOLLAR (2001):
Antigenicity of putative phospholipid membrane-binding residues in factor VIII.
Blood 97, 1, 169-174

BARTLETT, J. M. S. u. D. STIRLING (2003):
A Short History of the Polymerase Chain Reaction
in: J. M. S. BARTLET u. D. STIRLING: PCR protocols.
Humana Press Inc., Totowa, USA, 3-6

BAUMGARTNER, S., K. HOFMANN, R. CHIQUET-EHRISMANN u. P. BUCHER (1998):
The discoidin domain family revisited: new members from prokaryotes and a homology-based fold prediction.
Protein Sci. 7, 7, 1626-1631

BENN, D. M., P. A. GENTRY u. I. B. JOHNSTONE (1978):
Classic hemophilia (hemophilia A) in a family of collies.
Can. Vet. J. 19, 8, 221-225

BERTINA, R. M., R. CUPERS u. A. WIJNGAARDEN (1984):
Factor IXa protects activated factor VIII against inactivation by activated protein C.
Biochem. Biophys. Res. Commun. 125, 1, 177-183

BETTS, M. J. u. R. B. RUSSELL (2007):
Amino acid properties and consequences of substitutions
In: M. R. BARNES u. I. C. GRAY: Bioinformatics for Geneticists.
John Wiley & Sons, West Sussex, England, 311-342

BLOSTEIN, M. D., B. C. FURIE, I. RAJOTTE u. B. FURIE (2003):
The Gla domain of factor IXa binds to factor VIIIa in the tenase complex.
J. Biol. Chem. 278, 33, 31297-31302

BOGDANOVA, N., A. MARKOFF, R. EISERT, C. WERMES, H. POLLMANN, A. TODOROVA, M. CHLYSTUN, U. NOWAK-GÖTTL u. L. HORST (2007):
Spectrum of molecular defects and mutation detection rate in patients with mild and moderate hemophilia A.
Hum. Mutat. 28, 1, 54-60

BOS, M. H. u. R. M. CAMIRE (2010):
Blood coagulation factors V and VIII: Molecular Mechanisms of Procofactor Activation.
J. Coagul. Disord. 2, 2, 19-27

BOUDREAUX, M. K., J. L. CATALFAMO u. M. KLOK (2007):
Calcium-diacylglycerol guanine nucleotide exchange factor I gene mutations associated with loss of function in canine platelets.
Transl. Res. 150, 2, 81-92

BOUDREAUX, M. K. u. J. L. CATALFAMO (2001):
Molecular and genetic basis for thrombasthenic thrombopathia in otterhounds.
Am. J. Vet. Res. 62, 11, 1797-1804

BOUDREAUX, M. K. u. M. MARTIN (2011):
P2Y12 receptor gene mutation associated with postoperative haemorrhage in a Greater Swiss Mountain dog.
Vet. Clin. Pathol. 40, 2, 202-206

BOWEN, D. J. (2002):
Haemophilia A and haemophilia B: molecular insights.
Mol. Path. 55, 1-18

BRINKHOUS, K. M., P. D. DAVIS, J. B. GRAHAM u. W. J. DODDS (1973):
Expression and linkage of genes for X-linked hemophilias A and B in the dog.
Blood 41, 4, 577-585

BROOKS, M. B., W. GU u. K. RAY (1997):
Complete deletion of factor IX gene and inhibition of factor IX activity in a labrador retriever with hemophilia B.
J. Am. Vet. Med. Assoc. 211, 11, 1418-1421

BROOKS, M. (1999):
A Review of Canine Inherited Bleeding. Disorders: Biochemical and Molecular. Strategies for Disease characterization and Carrier Detection.
J. Hered. 90, 112-118

BROOKS, M. B., W. GU, J. L. BARNAS, J. RAY u. K. RAY (2003):
A Line 1 insertion in the Factor IX gene segregates with mild hemophilia B in dogs.
Mamm. Genome. 14, 11, 788-795

BROOKS, M. B., J. L. BARNAS, J. FREMONT u. J. RAY (2005):
Cosegregation of a factor VIII microsatellite marker with mild hemophilia A in Golden Retriever dogs.
J. Vet. Intern. Med. 19, 2, 205-210

BROOKS, M. B., R. MACNGUYEN, R. HALL, R. GUPTA u. J. G. BOOTH (2008):
Indirect carrier detection of canine haemophilia A using factor VIII microsatellite markers.
Anim. Genet. 39, 3, 278-283

BROOKS, M., K. ETTER, J. CATALFAMO, A. BRISBIN, C. BUSTAMANTE u. J. MEZEY (2010):
A genome-wide linkage scan in German shepherd dogs localizes canine platelet procoagulant deficiency (Scott syndrome) to canine chromosome 27.
Gene 450, 1-2, 70-75

BURGESS, H. J., J. P. WOODS, A. C. G. ABRAMS-OGG u. R. D.WOOD (2009):
Evaluation of laboratory methods to improve characterization of dogs with von Willebrand disease.
Can. J. Vet. Res. 73, 4, 252–259

BUTLER, J. M. (2011):
Capillary electrophoresis: Principles and Instrumentation
in: J. M. BUTLER: Advance topics in forensic DNA typing: Methodology.
Elsevier, London, England, 141-166

CALLAN, M. B., J. S. BENNETT, D. K. PHILLIPS, M. E. HASKINS, J. E. HAYDEN, J. G. ANDERSON u. U. GIGER (1995):
Inherited platelet delta-storage pool disease in dogs causing severe bleeding: an animal model for a specific ADP deficiency.
J. Thromb. Haemost. 74, 3, 949-953

CALLAN, M. B., M. N. ALJAMALI, P. MARGARITIS, M. E. GRIOT-WENK, E. S. POLLAK, P. WERNER, U. GIGER u. K. A. HIGH (2006):
A novel missense mutation responsible for factor VII deficiency in research Beagle colonies.
J. Thromb. Haemost. 4, 12, 2616-2622

CAMERON, C., C. NOTLEY, S. HOYLE, L. MCGLYNN, C. HOUGH, S. KAMISSUE, A. GILES u. D. LILLICRAP (1998):
The canine factor VIII cDNA and 5' flanking sequence.
J. Thromb. Haemost. 79, 2, 317-322

CAMIRE, R. M. u. M. H. A. BOS (2009):
The molecular basis of factor V and VIII procofactor activation.
J. Thromb. Haemost. 7, 12, 1951–1961

CAMPBELL, K. L., C. E. GREENE u. W. J. DODDS (1983):
Factor IX deficiency (hemophilia B) in a Scottish terrier.
J. Am. Vet. Med. Assoc. 182, 2, 170-171

CELIE, P. H., G. VAN STEMPVOORT, C. FRIBOURG, L. J. SCHURGERS, P. J. LENTING u. K. MERTENS (2002):
The connecting segment between both epidermal growth factor-like domains in blood coagulation factor IX contributes to stimulation by factor VIIIa and its isolated A2 domain.
J. Biol. Chem. 277, 23, 20214-20220

CESARMAN-MAUS, G. u. K. A. HAJJAR (2005):
Molecular mechanisms of fibrinolysis.
Br. J. Haematol. 129, 3, 307–321

CHATTERJEE, S. u. J. K. PAL (2009):
Role of 5'- and 3'-untranslated regions of mRNAs in human diseases.
Biol. Cell. 101, 5, 251-262

CHOI, J. S., J. S. KIM, C. O. JOE, S. KIM, K. S. HA u. Y. M. PARK (1999):
Improved cycle sequencing of GC-rich DNA template.
Exp. Mol. Med. 31, 1, 20-24

CLARKE, L. A., C. S. REBELO, J. GONÇALVES, M. G. BOAVIDA u. P. JORDAN (2001):
PCR amplification introduces errors into mononucleotide and dinucleotide repeat sequences.
Mol Pathol. 54, 5, 351-353

COOK, A. K., L. L. WERNER, S. L. O'NEILL, M. BROOKS u. B. F. FELDMAN (1993):
Factor X deficiency in a Jack Russell terrier.
Vet. Clin. Pathol. 22, 3, 68-71

CRUZ, M. A., R. I. HANDIN u. R. J. WISE (1993):
The interaction of the von Willebrand factor-A1 domain with platelet glycoprotein Ib/IX. The role of glycosylation and disulfide bonding in a monomeric recombinant A1 domain protein.
J. Biol. Chem. 268, 28, 21238-21245

CRUZ, M. A., J. CHEN, J. L. WHITELOCK, L. D. MORALES u. J. A. LOPEZ (2005):
The platelet glycoprotein Ib-von Willebrand factor interaction activates the collagen receptor alpha2beta1 to bind collagen: activation-dependent conformational change of the alpha2-I domain.
Blood 105, 5, 1986-1991

DAVIS, B., M. TOIVIO-KINNUCAN, S. SCHULLER u. M. K. BOUDREAUX (2008):
Mutation in beta1-tubulin correlates with macrothrombocytopenia in Cavalier King Charles Spaniels.
J. Vet. Intern. Med. 22, 3, 540-545

DE LUCA, M., D. A. FACEY, E. J. FAVALORO, M. S. HERTZBERG, J. C. WHISSTOCK, T. MCNALLY, R. K. ANDREWS u. M. C. BERNDT (2000):
Structure and function of the von Willebrand factor A1 domain: analysis with monoclonal antibodies reveals distinct binding sites involved in recognition of the platelet membrane glycoprotein Ib-IX-V complex and ristocetin-dependent activation.
Blood 95, 1, 164-172

DENIS, C. V. u. D. D. WAGNER (1999):
Insights from von Willebrand disease animal models.
Cell. Mol. Life. Sci. 56, 11-12, 977-990

DO, H., J. F. HEALEY, E. K. WALLER u. P. LOLLAR (1999):
Expression of factor VIII by murine liver sinusoidal endothelial cells.
J. Biol. Chem. 274, 28, 19587-19592

DUNNING, M. D., G. F. AVERIS, H. PATTINSON, M. TARGETT, S. CADE u. M. E. HERRTAGE (2009):
Haemophilia A (factor VIII deficiency) in a litter of Weimaraners.
J. Small Anim. Pract. 50, 7, 357-359

DUTRA, A. S., E. MIGNOT u. J. M. PUCK (1996):
Gene localization and syntenic mapping by FISH in the dog.
Cytogenet. Cell. Genet. 74, 1-2, 113-117

DUTTON, C. M., C. PAYNTON u. S. S. SOMMER (1993):
General method for amplifying regions of very high G+C content.
Nucleic. Acids Res. 21, 12, 2953-2954

EATON, D., H. RODRIGUEZ u. G. VEHAR (1986):
Proteolytic processing of human FVIII. Correlation of specific cleavages by thrombin FXa and activated protein C with activation and inactivation of Factor VIII coagulant activity.
Biochemistry 25, 2, 505–512

ELDER, B., D. LAKICH u. J. GITSCHIER (1993):
Sequence of the murine factor VIII cDNA.
Genomics 16, 2, 374-379

ESMON, C.T. u. P. LOLLAR (1996):
Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and factor VIII.
J. Biol. Chem. 271, 23, 13882-13887

EVANS, J. P., K. M. BRINKHOUS, G. D. BRAYER, H. M. REISNER u. K. A. HIGH (1989A):
Canine hemophilia B resulting from a point mutation with unusual consequences.
Proc. Natl. Acad. Sci. 86, 24, 10095–10099

EVANS, J. P., H. H. WATZKE, J. L. WARE, D. W. STAFFORD u. K. A. HIGH (1989B):
Molecular cloning of a cDNA encoding canine factor IX.
Blood 74, 1, 207-212

FAZEKAS, A., R. STEEVES u. S. NEWMASTER (2010):
Improving sequencing quality from PCR products containing long mononucleotide repeats.
Biotechniques 48,4, 277-285

FAUSTINO, N. A. u. T. A. COOPER (2003):
Pre-mRNA splicing and human disease.
Genes Dev. 17, 4, 419-437

FAY, P. J., T. M. SMUDZIN u. F. J. WALKER (1991):
Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. Identification of cleavage sites and correlation of proteolysis with cofactor activity.
J. Biol. Chem. 266, 30, 20139-20145

FAY, P. J., M. MASTRI, M. E. KOSZELAK u. H. WAKABAYASHI (2001):
Cleavage of factor VIII heavy chain is required for the functional interaction of a2 subunit with factor IXa.
J. Biol. Chem. 276, 15, 12434–12439

FAY, P. J. (2004):
Activation of factor VIII and mechanisms of cofactor action.
Blood Rev. 18, 1, 1-15

FAY, P. J. (2005):
Factor VIII Structure and Function.
Int. J. Hematol. 83, 2, 103-108

FAY, P. J. u. P. V. JENKINS (2005):
Mutating factor VIII: lessons from structure to function.
Blood Rev. 19, 1, 15-27

FELDMAN, D. G., M. B. BROOKS u. W. J. DODDS (1995):
Hemophilia B (factor IX deficiency) in a family of German shepherd dogs.
J. Am. Vet. Med. Assoc. 206, 12, 1901-1905

FIGUEIREDO, M. S. u. G. G. BROWNLEE (1995):
Cis-acting elements and transcription factors involved in the promoter activity of the human factor VIII gene.
J. Biol. Chem. 270, 20, 11828-11838

FUJIKAWA, K., M. E. LEGAZ, H. KATO u. E. W. DAVIE (1974):
The mechanism of activation of bovine factor IX (Christmas factor) by bovine factor XIa (activated plasma thromboplastin antecedent).
Biochemistry 13, 22, 4508-4516

GAILANI, D. (2000):
Activation of factor IX by factor XIa.
Trends. Cardiovasc. Med. 10, 5, 198-204

GAILANI, D., D. HO, M. F. SUN, Q. CHENG u. P. N. WALSH (2001):
Model for a factor IX activation complex on blood platelets: dimeric conformation of factor XIa is essential.
Blood 97, 10, 3117-3122

GALE, A. J., J. L. PELLEQUER, E. D. GETZOFF u. J. H. GRIFFIN (2000):
Structural basis for haemophilia A caused by mutations in the C domains of blood coagulation factor VIII.
Thromb. Haemost. 83, 1, 78-85

GALE, A. J., T. J. CRAMER, D. ROZENSHTEYN u. J. R. CRUZ (2008):
Detailed Mechanisms of the Inactivation of Factor VIIIa by Activated Protein C in the Presence of Its Cofactors, Protein S and Factor V*.
J. Biol. Chem. 283, 24, 16355–16362

GALE, A. J. (2011):
Current Understanding of Hemostasis.
Toxicol. Pathol. 39, 1, 273-280

GANGULY, A., T. DUNBAR, P. CHEN, L. GODMILOW u. T. GANGULY (2003):
Exon skipping caused by an intronic insertion of a young Alu Yb9 element leads to severe hemophilia A.
Hum. Genet. 113, 4, 348-352

GENTRY, P. A., I. B. JOHNSTONE u. S. E. SANFORD (1977):
Diagnosis of classic hemophilia (hemophilia A) in a standard poodle.
Can. Vet. J. 18, 3, 79-81

GIANNELLI, F., P. M. GREEN, S. S. SOMMER, M. POON, M. LUDWIG, R. SCHWAAB, P. H. REITSMA, M. GOOSSENS, A. YOSHIOKA, M. S. FIGUEIREDO u. G. G. BROWNLEE (1998):
Haemophilia B: database of point mutations and short additions and deletions--eighth edition.
Nucleic. Acids Res. 26, 1, 265-268

GILES, A. R., S. TINLIN, H. HOOGENDOORN, P. GREENWOOD u. R. GREENWOOD (1984):
Development of factor VIII:C antibodies in dogs with hemophilia A (factor VIII:C deficiency).
Blood 63, 2, 451-456

GITSCHIER, J., W. I. WOOD, T. M. GORALKA, K. L. WION, E. Y. CHEN, D. H. EATON, G. A. VEHAR, D. J. CAPON u. R. M. LAWN (1984):
Characterization of the human factor VIII gene.
Nature 312, 326-330

GOLDEN, J. G., A. R. BANKNIEDER u. M. E. BRUESTLE (1980):
Hemophilia in a Great Pyrenees.
Mod. Vet. Pract. 61, 8, 671-674

GOREE, M., J. L. CATALFAMO, S. ABER u. M. K. BOUDREAUX (2005):
Characterization of the mutations causing hemophilia B in 2 domestic cats.
J. Vet. Intern. Med. 19, 2, 200-204

GRAW, J., H. H. BRACKMANN, J. OLDENBURG, R. SCHNEPPENHEIM, M. SPANNAGL u. R. SCHWAAB (2005):
Haemophilia A: from mutation analysis to new therapies.
Nat. Rev. Genet. 6, 6, 488-501

GU, W., M. BROOKS, J. CATALFAMO, J. RAY u. K. RAY (1999):
Two distinct mutations cause severe hemophilia B in two unrelated canine pedigrees.
J. Thromb. Haemost. 82, 4, 1270-1275

GUILLIATT, A. M. (2002):
Agarose and polyacrylamide Gel Electrophoresis
in: B. D. M. THEOPHILUS u. R. RAPLEY: PCR Mutation Detection Protocols.
Humana Press Inc., New Jersey, USA, 1-12

HAMER, R. J., J. A. KOEDAM, N. H. BEESER-VISSER, R. M. BERTINA, J. A. VAN MOURIK u. J. J. SIXMA (1987):
Factor VIII binds to von Willebrand factor via its Mr-80,000 light chain.
Eur. J. Biochem. 166, 1, 37–43

HANDFORD, P. A., M. MAYHEW, M. BARON, P. R. WINSHIP, I. D. CAMPBELL u. G. G. BROWNLEE (1991):
Key residues involved in calcium-binding motifs in EGF-like domains.
Nature 351, 6322, 164-167

HEALEY, J. F., I. M. LUBIN u. P. LOLLAR (1996):
The cDNA and derived amino acid sequence of porcine factor VIII.
Blood 88, 11, 4209-4214

HEALEY, J. F., R. T. BARROW, H. M. TAMIM, I. M. LUBIN, M. SHIMA, D. SCANDELLA u. P. LOLLAR (1998):
Residues Glu2181-Val2243 contain a major determinant of the inhibitory epitope in the C2 domain of human factor VIII.
Blood 92, 10, 3701-3709

HEALY, P. J., C. A. SEWELL, T. EXNER, A. G. MORTON u. B. S. ADAMS (1984):
Haemophilia in Hereford cattle: factor VIII deficiency.
Aust. Vet. J. 61, 4, 132-133

HEIN, H. (1986):
Haemophilia A in German shepherd dogs.
Vet. Rec. 118, 22, 619

HELLING, R. B., H. M. GOODMAN u. H. W. BOYER (1974):
Analysis of endonuclease R-EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis.
J. Virol. 14, 5, 1235-1244

HENNINGER, R. W. (1988):
Hemophilia A in two related quarter horse colts.
J. Am. Vet. Med. Assoc. 193, 1, 91-94

HIGUCHI, M., H. H. KAZAZIAN, L. KASCH, T. C. WARREN, M. J. MCGINNISS, J. A. PHILLIPS, C. KASPER, R. JANCO u. S. E. ANTONARAKIS (1991):
Molecular characterization of severe hemophilia A suggests that about half the mutations are not within the coding regions and splice junctions of the factor VIII gene.
Proc. Natl. Acad. Sci. USA. 88, 16, 7405–7409

HILL, B. L., R. D. ZENOBLE u. W. J. DODDS (1982):
Prothrombin deficiency in a cocker spaniel.
J. Am. Vet. Med. Assoc. 181, 3, 262-263

HILL-EUBANKS, D. C., C. G. PARKER u. P. LOLLAR (1989):
Differential proteolytic activation of factor VIII-von Willebrand factor complex by thrombin.
Proc. Natl. Acad. Sci. USA. 86, 17, 6508-6512

HSU, T. C., K. P. PRATT u. A. R. THOMPSON (2008):
The factor VIII C1 domain contributes to platelet binding.
Blood 111, 1, 200-208

HOLLESTELLE, M. J., T. THINNES, K. CRAIN, A. STIKO, J. K. KRUIJT, T. J. VAN BERKEL, D. J. LOSKUTOFF u. J. A. VAN MOURIK (2001):
Tissue distribution of factor VIII gene expression in vivo - a closer look.
J. Thromb. Haemost. 86, 3, 855-861

HOLLESTELLE, M. J., P. P. POYCK, J. M. HOLLESTELLE, H. A. MARSMAN, J. A. MOURIK u. T. M. GULIK (2005):
Extra-hepatic factor VIII expression in porcine fulminant hepatic failure.
J. Thromb. Haemost. 3, 10, 2274-2280

HOUGH, C., S. KAMISUE, C. CAMERON, C. NOTLEY, S. TINLIN, A. GILES u. D. LILLICRAP (2002):
Aberrant splicing and premature termination of transcription of the FVIII gene as a cause of severe canine hemophilia A: similarities with the intron 22 inversion mutation in human hemophilia.
J. Thromb. Haemost. 87, 4, 659-665

HYNDMAN, D. L. u. M. MITSUHASHI (2003):
PCR primer design
in: J. M. S. BARTLETT u. D. STIRLING: PCR protocols.
Humana Press Inc., Totowa, USA, 81-88

IAZBIK, P. M., R. M. SILVA u. S. A. MARQUEZ (1997):
Hemophilia A in a Yorkshire Terrier family.
Av. Cienc. Vet. 12, 1, 57-60

JACQUEMIN, M., A. BENHIDA, K. PEERLINCK, B. DESQUEPER, L. VANDER ELST, R. LAVEND'HOMME, R. D'OIRON, R. SCHWAAB, M. BAKKUS, K. THIELEMANS, J. G. GILLES, J. VERMYLEN u. J. M. SAINT-REMY (2000):
A human antibody directed to the factor VIII C1 domain inhibits factor VIII cofactor activity and binding to von Willebrand factor.
Blood 95, 1, 156-163

JACQUEMIN, M., A. NEYRINCK, M. I. HERMANNS, R. LAVEND'HOMME, F. REGA, J. M. SAINT-REMY, K. PEERLINCK, D. VAN RAEMDONCK u. C. J. KIRKPATRICK (2006):
FVIII production by human lung microvascular endothelial cells.
Blood. 108, 2, 515-517

JACQUEMIN, M. (2009):
Factor VIII-von Willebrand factor binding defects in autosomal recessive von Willebrand disease type Normandy and in mild hemophilia A. New insights into factor VIII-von Willebrand factor interactions.
Acta Haematol. 121, 2-3, 102-105

JENSEN, M. A., M. FUKUSHIMA u. R. W. DAVIS (2010):
DMSO and betaine greatly improve amplification of GC-rich constructs in de novo synthesis.
PLoS One 5, 6, 1-5

JOHNSON, G. S., M. A. TURRENTINE u. K. H. KRAUS (1988):
Canine von Willebrand's disease. A heterogeneous group of bleeding disorders.
Vet. Clin. North Am. Small Anim. Pract. 18, 1, 195-229.

JOHNSTONE, I. B. u. S. CRANE (1981):
Von Willebrand's Disease in Two Families of Doberman Pinschers.
Can. Vet. J. 22, 8, 239–243

JOHNSTONE, I. B. u. A. M. NORRIS (1984):
A moderately severe expression of classical hemophilia in a family of german shepherd dogs.
Can. Vet. J. 25, 5, 191-194

JOHNSTONE, I. B. (1986):
Canine Von Willebrand's Disease: A Common Inherited Bleeding Disorder in Doberman Pinscher Dogs.
Can. Vet. J. 27, 9, 315–318

JOHNSTONE, I. B., J. C. MORTON u. D. G. ALLEN (1987):
Factor VIII Deficiency in a Cat.
Can. Vet. J. 28, 10, 671-673

JOSEPH, S. A., M. B. BROOKS, P. J. COCCARI u. S. C. RIBACK (1996):
Hemophilia A in a German shorthaired pointer: clinical presentations and diagnosis.
J. Am. Anim. Hosp. Assoc. 32, 1, 25-28

KAAE, J. A., M. B. CALLAN u. M. B. BROOKS (2007):
Hereditary factor VII deficiency in the Alaskan Klee Kai dog.
J. Vet. Intern. Med. 21, 5, 976-981

KAMMERMANN, B., J. GMÜR u. H. STÜNZI (1971):
Afibrinogenemia in dogs.
Zentbl. Vetmed. A. 18, 3, 192-205

KASTHURI, R. S., S. L. GLOVER, J. BOLES u. N. MACKMAN (2010):
Tissue factor and tissue factor pathway inhibitor as key regulators of global hemostasis: measurement of their levels in coagulation assays.
Semin. Thromb. Hemost. 36, 7, 764-771

KAZAZIAN, H. H., C. WONG, H. YOUSSOUFIAN, A. F. SCOTT, D. G. PHILLIPS u. S. E. ANTONARAKIS (1988):
Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man.
Nature 332, 6160, 164-166

KEMBALL-COOK, G., E. G. TUDDENHAM u. A. I. WACEY (1998):
The factor VIII Structure and Mutation Resource Site: HAMSTeRS version 4.
Nucleic. Acids Res. 26, 1, 216-219

KENT, W. J. (2002):
BLAT - the BLAST-like alignment tool.
Genome Res. 12, 4, 656-664

KIEFER, F., K. ARNOLD, M. KÜNZLI, L. BORDOLI u.T. SCHWEDE (2009):
The SWISS-MODEL Repository and associated resources.
Nucleic. Acids Res. 37, 387-392

KIRCHHOFER, D. u. Y. NEMERSON (1996):
Initiation of blood coagulation: the tissue factor/factor VIIa complex.
Curr. Opin. Biotechnol. 7, 386–391

KNOWLER, C., U. GIGER, W. J. DODDS u. M. BROOKS (1994):
Factor XI deficiency in Kerry Blue Terriers.
J. Am. Vet. Med. Assoc. 205, 11, 1557-1561

KOBAYASHI, E. u. M. MIYATA (1995):
Allotransplantation of whole spleen in patients with hepatic malignant tumors or hemophilia A.
Arch. Surg. 130, 6, 676-677

KOEDAM, J. A., J. C. MEIJERS, J. J. SIXMA u. B. N. BOUMA (1988):
Inactivation of human factor VIII by activated protein C: cofactor activity of protein S and protective effect of von Willebrand factor.
J. Clin. Invest. 82, 4, 1236-1243

KOEDAM, J. A., R. J. HAMER, N. H. BEESER-VISSER, B. N. BOUMA u. J. J. SIXMA (1990):
The effect of von Willebrand factor on activation of factor VIII by factor Xa.
Eur. J. Biochem. 189, 2, 229-234

KRAMER, J. W., P. J. VENTA, S. R. KLEIN, Y. CAO, W. D. SCHALL u. V. YUZBASIYAN-GURKAN (2004):
A von Willebrand's factor genomic nucleotide variant and polymerase chain reaction diagnostic test associated with inheritable type-2 von Willebrand's disease in a line of german shorthaired pointer dogs.
Vet. Pathol. 41, 3, 221-228

KUMAR, S. u. C. W. FULLER (2007):
Advances in Dye-Nucleotide Conjugate Chemistry for DNA sequencing
in: K.R. MITCHELSON: New high throughput technologies for DNA sequencing and genomics 2.
Elsevier, Amsterdam, The Netherlands, 119-146

LACOBELLI, N. (2008):
Variant Canine Coagulation Factor IX as a Novel Approach to the Treatment of Hemophilia B.
PennScience 6, 2, 27-32

LACROIX-DESMAZES, S., A. M. NAVARRETE, S. ANDRE, J. BAYRY, S. V. KAVERI u. S. DASGUPTA (2008):
Dynamics of factor VIII interactions determine its immunologic fate in hemophilia A.
Blood 112, 2, 240-249

LANE, D. A., H. PHILIPPOU u. J. A. HUNTINGTON (2005):
Directing thrombin.
Blood 106, 8, 2605-2612

LAPAN, K. A. u. P. J. FAY (1997):
Localization of a factor X interactive site in the A1 subunit of factor VIIIa.
J. Biol. Chem. 272, 4, 2082-2088

LAPAN, K. A. u. P. J. FAY (1998):
Interaction of the A1 subunit of factor VIIIa and the serine protease domain of factor X identified by zero-length cross-linking.
J. Thromb. Haemost. 80, 3, 418-422

LAWSON, J. H. u. K. G. MANN (1991):
Cooperative activation of human factor IX by the human extrinsic pathway of blood coagulation.
J. Biol. Chem. 266, 17, 11317-11327

LENTING, P. J., J. A. VAN MOURIK u. K. MERTENS (1998):
The Life Cycle of Coagulation Factor VIII in View of Its Structure and Function.
Blood 92, 11, 3983-3996

LENTING, P. J., J. W. VAN DE LOO, M. J. DONATH, J. A. VAN MOURIK u. K. MERTENS (1996A):
The sequence Glu1811-Lys1818 of human blood coagulation factor VIII comprises a binding site for activated factor IX.
J. Biol. Chem. 271, 4, 1935-1940

LENTING, P. J., O. D CHRISTOPHE , H. MAAT ,D. J REES u. K. MERTENS (1996B):
Ca2+ binding to the first epidermal growth factor-like domain of human blood coagulation factor IX promotes enzyme activity and factor VIII light chain binding.
J. Biol. Chem. 271, 41, 25332-25337

LEVINSON, B., S. KENWRICK, D. LAKICH, G. HAMMONDS u. J. GITSCHIER (1990):
A transcribed gene in an intron of the human factor VIII gene.
Genomics 7, 1, 1-11

LEVINSON, B., S. KENWRICK, P. GAMEL, K. FISHER u. J. GITSCHIER (1992A):
Evidence for a third transcript from the human factor VIII gene.
Genomics 14, 3, 585-589

LEVINSON, B., J. R. BERMINGHAM, A. METZENBERG, S. KENWRICK, V. CHAPMAN u. J. GITSCHIER (1992B):
Sequence of the human factor VIII-associated gene is conserved in mouse.
Genomics 13, 3, 862-865

LEWIS, J. H., J. A. SPERO u. U. HASIBA (1983):
A hemophiliac dog colony: genetic studies and coagulation findings in hemophiliac and normal dogs.
Comp. Biochem. Physiol. A. 75, 2, 147-151

LEYTE, A., M. P. VERBEET, T. BRODNIEWICZ-PROBA, J. A. VAN MOURIK u. K. MERTENS (1989):
The interaction between human blood-coagulation factor VIII and von Willebrand factor. Characterization of a high-affinity binding site on factor VIII.
Biochem. J. 257, 3, 679–683

LINDQUIST, P. A., K. FUJIKAWA u. E. W. DAVIE (1978):
Activation of bovine factor IX (Christmas factor) by factor XIa (activated plasma thromboplastin antecedent) and a protease from Russell's viper venom.
J. Biol. Chem. 253, 6, 1902-1909

LIPSCOMB, D. L., C. BOURNE u. M. K. BOUDREAUX (2000):
Two genetic defects in alphaIIb are associated with type I Glanzmann's thrombasthenia in a Great Pyrenees dog: a 14-base insertion in exon 13 and a splicing defect of intron 13.
Vet. Pathol. 37, 6, 581-588

LITTLEWOOD, J. D., S. A. BEVAN u. M. J. CORKE (1991):
Haemophilia A (classic haemophilia, factor VIII deficiency) in a Thoroughbred colt foal.
Equine Vet. J. 23, 1, 70-72

LIU, M. L., B. W. SHEN, S. NAKAYA, K. P. PRATT, K. FUJIKAWA, E. W. DAVIE, B. L. STODDARD u. A. R. THOMPSON (2000):
Hemophilic factor VIII C1- and C2-domain missense mutations and their modeling to the 1.5-angstrom human C2-domain crystal structure.
Blood 96, 3, 979-987

LOOHUIS, M., P. A. J. LEEGWATER u. B. A. VAN OOST (2004):
Annotation of four missense mutations in the von Willebrand gene in German Wirehair pointers with type 2 von Willebrand's disease.
The 2nd International Conference: Advances in Canine and Feline Genomics, Utrecht, 14-16.10.2004

LÖWENBERG, E. C., J. C. MEIJERS u. M. LEVI (2010):
Platelet-vessel wall interaction in health and disease.
Neth. J. Med. 68, 6, 242-251

LOZIER, J. N., A. DUTRA, E. PAK, N. ZHOU, Z. ZHENG, T. C. NICHOLS, D. A. BELLINGER, M. READ u. R. A. MORGAN (2002):
The Chapel Hill hemophilia A dog colony exhibits a factor VIII gene inversion.
Proc. Natl. Acad. Sci. USA. 99, 20, 12991-12996

LOZIER, J. (2006):
Factor VIII biosynthesis. New inspirations?.
Blood 108, 2, 414-415

LU, G., G. J. JR. BROZE u. S. KRISHNASWAMY (2004):
Formation of factors IXa and Xa by the extrinsic pathway: differential regulation by tissue factor pathway inhibitor and antithrombin III.
J. Biol. Chem. 279, 17, 17241-17249

LÜ, J., S. W. PIPE, H. MIAO , M. JACQUEMIN u. G.E. GILBERT (2011):
A membrane-interactive surface on the factor VIII C1 domain cooperates with the C2 domain for cofactor function.
Blood 117, 11, 3181-3189

MA, Y. Q., J. QIN u. E. F. PLOW (2007):
Platelet integrin alpha(IIb)beta(3): activation mechanisms.
J. Thromb. Haemost. 5, 7, 1345-1352

MACPHERSON, R., J. SCHERER, M. L. ROSS u. P. A. GENTRY (1999):
Factor VII deficiency in a mixed breed dog.
Can. Vet. J. 40, 7, 503-505

MAGGIO-PRICE, L. u. W. J. DODDS (1993):
Factor IX deficiency (hemophilia B) in a family of British shorthair cats.
J. Am. Vet. Med. Assoc. 203, 12, 1702-1704

MANN, K. G., M. E. NESHEIM, W. R. CHURCH, P. HALEY u. S. KRISHNASWAMY (1990):
Surface-dependent reactions of the vitamin K-dependent enzyme complexes.
Blood 76, 1, 1–16

MANN, K. G., S. BUTENAS u. K. BRUMMEL (2003):
The Dynamics of Thrombin Formation.
Arterioscler. Thromb. Vasc. Biol. 23, 1, 17-25

MANNUCCI, P. M. u. E. G. TUDDENHAM (2001):
The hemophilia from royal genes to gene therapy.
N. Engl. J. Med. 344, 23, 1773-1779

MANSELL, P. D., B. W. PARRY u. P. J. VAN ORSOUW (1990):
Haemophilia A in a family of miniature poodles.
Aust. Vet. J. 67, 11, 420-421

MATHUR, A., D. ZHONG, A. K. SABHARWAL, K. J. SMITH u. S. P. BAJAJ (1997):
Interaction of factor IXa with factor VIIIa. Effects of protease domain Ca2+ binding site, proteolysis in the autolysis loop, phospholipid, and factor X.
J. Biol. Chem. 272, 37, 23418-23426

MATTOSO, C. R., R. K. TAKAHIRA, S. L. BEIER, J. P. ARAÚJO u. J. E. CORRENTE (2010):
Prevalence of von Willebrand disease in dogs from Sao Paulo State, Brazil.
J. Vet. Diagn. Invest. 22, 1, 55-60

MAUSER, A. E., J. WHITLARK, K. M. WHITNEY u. C. D. LOTHROP JR (1996):
A deletion mutation causes hemophilia B in Lhasa Apso dogs.
Blood 88, 9, 3451-3455

MAZURIER, C. (1992):
Von Willebrand disease masquerading as haemophilia A.
J. Thromb. Haemost. 67, 4, 391-396

MCCONELL, M. F. (2000):
Overview of haemostasis
in: M. J. DAY, A. MACKING u. J. LITTLEWOOD: Manual of canine and feline haematology and transfusion medicine.
Br. Small Anim. Vet. Assoc., Gloucester, England, 165-171

MILLS, J. N., R. H. LABUC u. M. J. LAWLEY (1997):
Factor VII deficiency in an Alaskan malamute.
Aust. Vet. J. 75, 5, 320-322

MISCHKE, R. (2000):
Activated partial thromboplastin time as a screening test of minor or moderate coagulation factor deficiencies for canine plasma: sensitivity of different commercial reagents.
J. Vet. Diagn. Invest. 12, 5, 433-437

MISCHKE, R., C.WILHELM, A. CZWALINNA, M. VARVENNE, K. NARTEN u. M. VON DEPKA (2011A):
Canine haemophilia A caused by a mutation leading to a stop codon.
Vet. Rec. 169, 19, 496b

MISCHKE, R., A. KEHL, P. KUEHNLEIN, M. JAHN, R. ERTL, D. KLEIN, A. CECIL, T. DANDEKAR u. E. MUELLER (2011B):
Genetic analysis of haemophilic Havanese dogs.
Conference Proceedings of the 13th Conference of the ESVCP/ECVCP 2011, Dublin, 31.08.-03.09.2011 c.S.30

MISCHKE, R., P. KÜHNLEIN, A. KEHL, I. LANGBEIN-DETSCH, F. STEUDLE, A. SCHMID, T. DANDEKAR, A. CZWALINNA u. E. MÜLLER (2011C):
G244E in the canine factor IX gene leads to severe haemophilia B in Rhodesian Ridgebacks.
Vet. J. 187, 1, 113-118

MISCHKE, R. (2012):
Hämophilia A und B beim Hund.
Tierärzl. Prax. kleintiere 40, K, 44-53

MONROE, D. M. u. N. S. KEY (2007):
The tissue factor-factor VIIa complex: procoagulant activity, regulation, and multitasking.
J. Thromb. Haemost. 5, 6, 1097-1105

MULLIS, K., F. FALOONA, S. SCHARF, R. SAIKI, G. HORN u. H. ERLICH (1986):
Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction.
Cold. Spring Harb. Symp. Quant. Biol. 51, 1, 263-273

MULLIS, K. B. (1990):
The unusual origin of the polymerase chain reaction.
Sci. Am. 262, 4, 56-61, 64-65

MYLES, T., T. H. YUN u. L. L. LEUNG (2002):
Structural requirements for the activation of human factor VIII by thrombin.
Blood 100, 8, 2820-2826

NAKATA, M., M. SAKAI u. T. SAKAI (2006):
Hemophilia B in a crossbred Maltese dog.
J. Vet. Med. Sci. 68, 11, 1223-1224

NAYLOR, J. A., D. BUCK, P. GREEN, H. WILLIAMSON, D. BENTLEY u. F. GIANNELLI (1995):
Investigation of the factor VIII intron 22 repeated region (int22h) and the associated inversion junctions.
Hum. Mol. Genet. 4, 7, 1217-1224

NEUENSCHWANDER, S. u. V. PLISKA (1994):
Factor VIII in blood plasma of haemophilic sheep: analysis of clotting time-plasma dilution curves.
Haemostasis 24, 1, 27-35

NEWELL, J. L. u. P. J. FAY (2007):
Proteolysis at Arg740 facilitates subsequent bond cleavages during thrombin-catalyzed activation of factor VIII.
J. Biol. Chem. 282, 35, 25367-25375

NICHOLS, T. C., D. A. BELLINGER, R. L. REDDICK, S. V. SMITH, G. G. KOCH, K. DAVIS, J. SIGMAN, K. M. BRINKHOUS, T. R. GRIGGS u. M. S. READ (1993):
The roles of von Willebrand factor and factor VIII in arterial thrombosis: studies in canine von Willebrand disease and hemophilia A.
Blood 81, 10, 2644-2651

NICHOLS, T. C., D. A. BELLINGER, E. P. MERRICKS, R. A. RAYMER, M. T. KLOOS, N. DEFRIESS, M. V. RAGNI u. T. R. GRIGGS (2010):
Porcine and canine von Willebrand factor and von Willebrand disease: hemostasis, thrombosis, and atherosclerosis studies.
Thrombosis (Doi: 10.1155/2010/461238)

NIESWANDT, B., C. BRAKEBUSCH, W. BERGMEIER, V. SCHULTE, D. BOUVARD, R. MOKHTARI-NEJAD, T. LINDHOUT, J. W. HEEMSKERK, H. ZIRNGIBL u. R. FASSLER (2001):
Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen.
EMBO J. 20, 9, 2120–2130

NITSCHE, A. (2007):
Oligonucleotide design for in house Real-time PCR Applications in Microbiology
in: I.M. MACKAY: Real time PCR in microbiology: from diagnosis to characterization.
Caister Academic Press, Norfolk, USA, 41-69

NOGAMI, K., M. SHIMA, K. NISHIYA, K. HOSOKAWA, E. L. SAENKO, J. C. GIDDINGS, I. TANAKA u. A. YOSHIOKA (2002):
Anticoagulant effects of a synthetic peptide containing residues Thr-2253-Gln-2270 within factor VIII C2 domain that selectively inhibits factor Xa-catalysed factor VIII activation.
Br. J. Haematol. 116, 4, 868-874

O'BRIEN, D. P., D. JOHNSON, P. BYFIELD u. E. G. TUDDENHAM (1992):
Inactivation of factor VIII by factor IXa.
Biochemistry 31, 10, 2805-2812

OHKUBO, Y. Z., J. H. MORRISSEY u. E. TAJKHORSHID (2010):
Dynamical view of membrane binding and complex formation of human factor VIIa and tissue factor.
J. Thromb. Haemost. 8, 5, 1044-1053

OSTERUD, B. u. S. I. RAPAPORT (1977):
Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation.
Proc. Natl. Acad. Sci. USA. 74, 12, 5260-5264

OTTO, C. M., W. J. DODDS u. C. E. GREENE (1991):
Factor XII and partial prekallikrein deficiencies in a dog with recurrent gastrointestinal hemorrhage.
J. Am. Vet. Med. Assoc. 198, 1, 129-131

PARK, D. (2007):
Genomic DNA isolation from different Biological materials
in: E. HILARIO u. J. MACKAY: Protocols for nucleic acid analysis by nonradioactive probes.
Human Press Inc., New Jersey, USA, 3-13

PEDERSEN, H. D., J. HÄGGSTROM, L. H. OLSEN, K. CHRISTENSEN, A. SELIN, M. L. BURMEISTER u. H. LARSEN (2002):
Idiopathic asymptomatic thrombocytopenia in Cavalier King Charles Spaniels is an autosomal recessive trait.
J. Vet. Intern Med. 16, 2, 169-173

PERSSON, K. E., B. O. VILLOUTREIX, A. M. THÄMLITZ, K. E., KNOBE u. J. STENFLO (2002):
The N-terminal epidermal growth factor-like domain of coagulation factor IX. Probing its functions in the activation of factor IX and factor X with a monoclonal antibody.
J. Biol. Chem. 277, 38, 35616-35624

PETERS, M. F. u. C. A. ROSS (2001):
Isolation of a 40-kDa Huntingtin-associated protein.
J. Biol. Chem. 276, 5, 3188-3194

PETERSON, M. E. u. W. J. DODDS (1979):
Factor IX deficiency in an Alaskan Malamute.
J. Am. Vet. Med. Assoc. 174, 12, 1326-1327

PETŘEK, M., P. KOŠINOVÁ, J. KOČA u. M. OTYEPKA (2007):
MOLE: A Voronoi Diagram-Based Explorer of Molecular Channels, Pores, and Tunnels.
Structure 15, 11, 1357-1363

PIKE, R. N., A. M. BUCKLE, B. F. LE BONNIEC u. F. C. CHURCH (2005):
Control of the coagulation system by serpins. Getting by with a little help from glycosaminoglycans.
FEBS J. 272, 19, 4842–4851

PITTMAN, D. D. u. R. J. KAUFMAN (1988):
Proteolytic requirements for thrombin activation of anti-hemophilic factor (factor VIII).
Proc. Natl. Acad. Sci. USA. 85, 8, 2429-2433

POLLER, L., J. M. THOMSON, C. H. SEAR u. W. THOMAS (1971):
Identification of a congenital defect of factor VII in a colony of beagle dogs: the clinical use of the plasma.
J. Clin. Pathol. 24, 7, 626-632

POTEETE, A. R., D. RENNELL u. S. E. BOUVIER (1992):
Functional significance of conserved amino acid residues.
Proteins 13, 1, 38-40

PRUTHI, R. K. (2005):
Hemophilia: a practical approach to genetic testing.
Mayo Clin. Proc. 80, 11, 1485-1499

RANDOLPH, J. F., S. A. CENTER u. W. J. DODDS (1986):
Factor XII deficiency and von Willebrand's disease in a family of miniature poodle dogs.
Cornell Vet. 76, 1, 3-10

RAU, J. C., L. M. BEAULIEU, J. A. HUNTINGTON u. F. C. CHURCH (2007):
Serpins in thrombosis, hemostasis and fibrinolysis.
J. Thromb. Haemost. 5, 1, 102-115

REES, D. J., I. M. JONES, P. A. HANDFORD, S. J. WALTER, M. P. ESNOUF, K. J. SMITH u. G. G. BROWNLEE (1988):
The role of beta-hydroxyaspartate and adjacent carboxylate residues in the first EGF domain of human factor IX.
EMBO J. 7, 7, 2053–2061

REGAN, L. M., B. J. LAMPHEAR, C. F. HUGGINS, F. J. WALKER u. P. J. FAY (1994):
Factor IXa protects factor VIIIa from activated protein C. Factor IXa inhibits activated protein C-catalyzed cleavage of factor VIIIa at Arg562.
J. Biol. Chem. 269, 13, 9445-9452

RIEGER, M., H. P. SCHWARZ, P. L. TURECEK, F. DORNER, J. A. VAN MOURIK u. C. MANNHALTER (1998):
Identification of mutations in the canine von Willebrand factor gene associated with type III von Willebrand disease.
J. Thromb. Haemost. 80, 2, 332-337

ROWSELL, H. C., H. G. DOWNIE, J. F. MUSTARD, J. E. LEESON u. J. A. ARCHIBALD (1960):
A disorder resembling hemophilia B (Christmas disease) in dogs.
J. Am. Vet. Med. Assoc. 137, 247-250

ROZEN, S. u. H. J. SKALETSKY (2000):
Primer3 on the WWW for general users and for biologist programmers
in: S: KRAWETZ u. S. MISENER: Bioinformatics Methods and Protocols: Methods in Molecular Biology.
Humana Press, Totowa, USA, 365-386

RUMBAUT, R. E. u. P. THIAGARAJAN (2010):
Platelet-Vessel Wall Interactions
in N. GRANGER u. J. P. GRANGER: Hemostasis and Thrombosis.
Morgan & Claypool life science. Mississippi, USA, 1-45

RYCHLIK, W., W. J. SPENCER u. R. E. RHOADS (1990):
Optimization of the annealing temperature for DNA amplification in vitro.
Nucleic. Acids Res. 18, 21, 6409-6412

SADLER, J. E. (1998):
Biochemistry and genetics of von Willebrand factor.
Annu. Rev. Biochem. 67, 1, 395-424

SAENKO, E. L. u. D. SCANDELLA (1997):
The acidic region of the factor VIII light chain and the C2 domain together form the high affinity binding site for von willebrand factor.
J. Biol. Chem. 272, 29, 18007-18014

SAENKO, E. L., M. SHIMA u. A. G. SARAFANOV (1999):
Role of activation of the coagulation factor VIII in interaction with vWf, phospholipid, and functioning within the factor Xase complex.
Trends. Cardiovasc. Med. 9, 7, 185–192

SAIKI, R., D. GELFAND, S. STOFFEL, S. SCHARF, R. HIGUCHI, G. HORN, K. MULLIS u. H. ERLICH (1988):
Science 239, 4839 487-491

SANGER, F., S. NICKLEN u. A. R. COULSON (1977):
DNA sequencing with chain-terminating inhibitors.
Proc. Natl. Acad. Sci. USA. 74, 12, 5463-5467

SARGAN, D. (2011):
Inherited diseases in dogs, "http://www.vet.cam.ac.uk/idid/results.php", SARGAN, D., 2011

SARRATT, K. L., H. CHEN, M. M. ZUTTER, S. A. SANTORO, D. A. HAMMER u. M. L. KAHN (2005):
GPVI and alpha2beta1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow.
Blood 106, 4, 1268-1277

SAVAGE, B., E. SALDÍVAR u. Z. M. RUGGERI (1996):
Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor.
Cell 84, 2, 289-297

SCANDURA, J. M., S. S. AHMAD u. P. N. WALSH (1996):
A binding site expressed on the surface of activated human platelets is shared by factor X and prothrombin.
Biochemistry 35, 27, 8890-8902

SHEN, B. W., P. C. SPIEGEL, C. H. CHANG, J. W. HUH, J. S. LEE, J. KIM, Y. H. KIM u. B. L. STODDARD (2008):
The tertiary structure and domain organization of coagulation factor VIII.
Blood 111, 3, 1240-1247

SHERDING, R. G. u. S. P. DIBARTOLA (1980):
Hemophilia B (factor IX deficiency) in an Old English Sheepdog.
J. Am. Vet. Med. Assoc. 176, 2, 141-142

SLAPPENDEL, R. J. (1975):
Hemophilia A and hemophilia B in a family of French bulldogs.
Tijdschr. Diergeneeskd. 100, 20, 1075-1088

SMITH, J. W., T. K. DAY u. A. MACKIN (2005):
Diagnosing Bleeding Disorders.
Compend. Contin. Educ. Veterinarian Pract. Vet. 27, 11, 828-839, 842-843

SMITH, L. M., J. Z. SANDERS, R. J. KAISER, P. HUGHES, C. DODD, C. R. CONNELL, C. HEINER, S. B. KENT u. L. E. HOOD (1986):
Fluorescence detection in automated DNA sequence analysis.
Nature 321, 6071, 674-679

SMITH, S. B. u. D. GAILANI (2008):
Update on the physiology and pathology of factor IX activation by factor XIa.
Expert Rev. Hematol. 1, 1, 87–98

SPURLING, N. W., L. K. BURTON, R. PEACOCK u. T. PILLING (1972):
Hereditary factor-VII deficiency in the beagle.
Br. J. Haematol. 23, 1, 59-67

STIRLING, D. (2003):
Sequencing
in: J. M. S. BARTLETT u. D. STIRLING: PCR protocols.
Humana Press Inc. Totowa, USA, 337-340

STOKOL, T. (2005):
Disorders of hemostasis
in: E. VILLIERS u. L. BLACKWOOD: Manual of canine and feline Clinical Pathology.
Br. Small Anim. Vet., Gloucester, England, 83-98

SUGAHARA, Y., J. CATALFAMO, M. BROOKS, E. HITOMI, S. P. BAJAJ u. K. KURACHI (1996):
Isolation and characterization of canine factor IX.
J. Thromb. Haemost. 75, 3, 450-455

TATSUMI, K., K. OHASHI, M. KATAOKA, C. TATENO, M. SHIBATA, H. NAKA, M. SHIMA, M. HISANAGA, H. KANEHIRO, T. OKANO, K. YOSHIZATO, Y. NAKAJIMA u. A. YOSHIOKA (2008):
Successful in vivo propagation of factor IX-producing hepatocytes in mice: potential for cell-based therapy in haemophilia B.
J. Thromb. Haemost. 99, 5, 883-891

TAYLOR, W. R. (1986):
The classification of amino acid conservation.
J. Theor. Biol. 119, 2, 205–218

TCHERNEVA, E. u. U. GIGER (2007):
Molecular base of coagulation factor XI deficiency in Kerry Blue Terrier.
Bulg. J. Vet. Med. 10, 4, 247-255

TURECEK, P. L., H. GRITSCH, L. PICHLER, W. AUER, B. FISCHER, A. MITTERER, W. MUNDT, U. SCHLOKAT, F. DORNER, H. J. BRINKMAN, J. A. VAN MOURIK u. H. P. SCHWARZ (1997):
In vivo characterization of recombinant von Willebrand factor in dogs with von Willebrand disease.
Blood 90, 9, 3555-3567

VALLEIX, S., J. C. JEANNY, S. ELSEVIER, R. L. JOSHI, P. FAYET, D. BUCCHINI u. M. DELPECH (1999):
Expression of human F8B, a gene nested within the coagulation factor VIII gene, produces multiple eye defects and developmental alterations in chimeric and transgenic mice.
Hum. Mol. Genet. 8, 7, 1291-1301

VAN DIEIJEN, D., G. TANG, J. ROSING u. H. C. HEMKER (1981):
The role of phospholipid and factor VIIIa in the activation of bovine factor X.
J. Biol. Chem. 256, 7, 3433-3442

VARGA-SZABO, D., I. PLEINES u. B. NIESWANDT (2008):
Cell Adhesion Mechanisms in Plateltets.
Arterioscler. Thromb. Vasc. Biol. 28, 3, 403-412

VELTKAMP, J. J., E. ASFAOU, K. VAN DE TORREN, J. A. VAN DER DOES, N. H. VAN TILBURG u. E. K. PAUWELS (1974):
Extrahepatic factor VIII synthesis. Lung transplants in hemophilic dogs.
Transplantation 18, 1, 56-62

VENTA, P. J., G. J. BREWER, V. YUZBASIYAN-GURKAN u. W. D. SCHALL (2000A):
DNA encoding canine von Willebrand factor and methods of use, http://www.patentgenius.com/patent/6074832.html, VENTA, P. J., G. J. BREWER, V. YUZBASIYAN-GURKAN, W. D. 2000.

VENTA, P. J., J. LI, V. YUZBASIYAN-GURKAN, G. J. BREWER u. W. D. SCHALL (2000B):
Mutation causing von Willebrand's disease in Scottish Terriers.
J. Vet. Intern. Med.14, 1, 10-19

VERLANDER, J. W., N. T. GORMAN u. W. J. DODDS (1984):
Factor IX deficiency (hemophilia B) in a litter of Labrador retrievers.
J. Am. Vet. Med. Assoc. 185, 1, 83-84

VIDAUD, M., C. CHABRET, C. GAZENGEL, L. GRUNEBAUM, J. P. CAZENAVE u. M. GOOSSENS (1986):
A de novo intragenic deletion of the potential EGF domain of the factor IX gene in a family with severe hemophilia B.
Blood 68, 4, 961-963

VIEL, K. R., D. K. MACHIAH, D. M. WARREN, M. KHACHIDZE, A. BUIL, K. FERNSTROM, J. C. SOUTO, J. M. PERALTA, T. SMITH, J. BLANGERO, S. PORTER, S. T. WARREN, J. FONTCUBERTA, J. M. SORIA, W. D. FLANDERS, L. ALMASY u. T. E. HOWARD (2007):
A sequence variation scan of the coagulation factor VIII (FVIII) structural gene and associations with plasma FVIII activity levels.
Blood 109, 9, 3713-3724

WALKER, F. J., S. I. CHAVIN u. P. J. FAY (1987):
Inactivation of factor VIII by activated protein C and protein S.
Arch. Biochem. Biophys. 252, 1, 322-328

WALLACE, R. B., J. SHAFFER, R. F. MURPHY, J. BONNER, T. HIROSE u. K. ITAKURA (1979):
Hybridization of synthetic oligodeoxyribonucleotides to phi chi 174 DNA: the effect of single base pair mismatch.
Nucleic Acids Res. 6, 11, 3543-3557

WALTERS-CONTE, K. B., D. L. JOHNSON, M. W. ALLARD u. J. PECON-SLATTERY (2011):
Carnivore-specific SINEs (Can-SINEs): distribution, evolution, and genomic impact.
J. Hered. 102, 1, S2-S10

WANG, W. u. E. F. KIRKNESS (2005):
Short interspersed elements (SINEs) are a major source of canine genomic diversity.
Genome Res. 15, 12, 1798-1808

WEBSTER, W. P., C. F. ZUKOSKI, P. HUTCHIN, R. L. REDDICK, S. R. MANDEL u. G. D. PENICK (1971):
Plasma factor VIII synthesis and control as revealed by canine organ transplantation.
Am. J. Physiol. 220, 5, 1147-1154

WION, K. L., D. KELLY, J. A. SUMMERFIELD, E. G. TUDDENHAM u. R. M. LAWN (1985):
Distribution of factor VIII mRNA and antigen in human liver and other tissues.
Nature 317, 6039, 726-729

WU, D. Y., L. UGOZZOLI, B. K. PAL, J. QIAN u. R. B. WALLACE (1991):
The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction.
DNA Cell Biol. 10, 3, 233-238

ZIMMERMANN, M. A., D. MEIER, J. OLDENBURG, C. R. MÜLLER u. S. ROST (2012):
Identification and characterization of mutations in the promoter region of the factor VIII gene.
J. Thromb. Haemost. 2, 314-317

9. PUBLICATIONS

Poster session:

P. Alcaraz, A. Kehl, P. Kuehnlein, A. Cecil, T. Dandekar, E. Mueller, R. Mischke (2012): Canine haemophilia A caused by a point mutation in a Great Dane. 58 th Congress of the German Society for Small Animal Medicine in Dusseldorf, Germany. 18-21 October 2012.

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