Increased erythrocytic osmotic fragility in anemic domestic shorthair and purebred cats

Tritschler, Claudia

Abstract: Objectives: Increased osmotic fragility and splenomegaly were reported in anemic Abyssinian and Somali cats. Here we report about this condition in domestic shorthair cats and two other breeds. Methods: The osmotic fragility test was performed, known causes of anemia were excluded, the illness was followed, and medical records were reviewed. Results: Twelve cats were first found to be anemic between 0.5-9 years of age. Pallor, lethargy, inappetence, pica, weight loss and splenomegaly were commonly observed. A macrocytic and hypochromic anemia with variable regeneration was noted. Infectious disease screening, direct Coombs', and pyruvate kinase DNA mutation test results were negative. Freshly drawn blood did not appear hemolyzed but became progressively lyzed during storage. The sigmoid osmotic fragility curves were right shifted and cross-correction studies indicated a red cell effect. Most cats were treated with prednisolone and doxycycline. Five cats with recurrent or persistent anemia responded well to splenectomy. However, two had occasional recurrence of severe anemia. Finally, six cats were euthanized within 1 month and 7 years after initial presentation. Histopathology of 6 spleens revealed mainly congestion and extramedullary hematopoiesis. Conclusion: Similar to Abyssinians and Somalis, domestic shorthair cats and other breeds can also develop osmotic fragility with anemia and splenomegaly, which should be considered as a differential diagnosis in anemic cats. Ziele: Erhöhte osmotische Fragilität und Splenomegalie wurden bei anämischen Abessinier- und Somalikatzen beschrieben. Hier beschreiben wir diese Erkrankung bei Haus- und zwei weiteren Rassekatzen. Methoden: Die osmotische Fragilität wurde getestet, bekannte Anämieursachen ausgeschlossen, der Verlauf verfolgt und Krankheitsgeschichten überprüft. Resultate: Anämie wurde bei zwölf Katzen erstmals mit 0,5-9 Jahren diagnostiziert. Blässe, Lethargie, Appetitlosigkeit, Pica, Gewichtsverlust und Splenomegalie waren häufige Symptome. Charakteristika der Anämie waren Makrozytose, Hypochromie und variable Regeneration. Infektionskrankheiten, direkter Coombs und Pyruvatkinase-Mutations-Test fielen negativ aus. Frisches Blut erschien normal, hämolysierte aber während der Lagerung. Die sigmoidalen osmotischen Fragilitätskurven waren nach rechts verschoben und Kreutz-Korrektur-Studien deuteten auf ein Erythrozytenproblem. Die meisten Katzen wurden mit Prednisolon und Doxyzyclin behandelt. Fünf Katzen mit rezidivierender oder persistierender Anämie sprachen gut auf Splenektomie an. Zwei davon zeigten aber später Rezidive. Sechs Katzen wurden zwischen 1 Monat bis 7 Jahre nach der ersten Präsentation euthanasiert. Histopathologie von 6 Milzen zeigte Kongestion und extramedulläre Hämatopoese. Konklusion: Wie auch Abessinier und Somalis können Hauskatzen und andere Rassen osmotische Fragilität mit Anämie und Splenomegalie entwickeln, was als Differentialdiagnose berücksichtigt werden sollte.

Other titles: Erhöhte Erythrozytäre Osmotische Fragilität bei Anämischen Haus- und Rassekatzen

Originally published at:
Tritschler, Claudia. Increased erythrocytic osmotic fragility in anemic domestic shorthair and purebred cats. 2015, University of Zurich, Vetsuisse Faculty.

Klinik für Kleintiermedizin
der Vetsuisse-Fakultät Universität Zürich

Direktorin: Prof. Dr. Claudia Reusch

Increased Erythrocytic Osmotic Fragility in Anemic Domestic Shorthair and Purebred Cats

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Claudia Tritschler

Tierärztin
von Schaffhausen, Schaffhausen, Schweiz

genehmigt auf Antrag von

Prof. Dr. Urs Giger, Referent

Zürich 2015

Inhaltsverzeichnis

Summary

Vetsuisse-Fakultät Universität Zürich (2015)

Claudia Tritschler

Departement für Kleintiermedizin, Vetsuisse-Fakultät Universität Zürich
claudia.tritschler@gmx.ch

Increased Erythrocytic Osmotic Fragility in Anemic Domestic Shorthair and Purebred Cats

Objectives: Increased osmotic fragility and splenomegaly were reported in anemic Abyssinian and Somali cats. Here we report about this condition in domestic shorthair cats and two other breeds.
Methods: The osmotic fragility test was performed, known causes of anemia were excluded, the illness was followed, and medical records were reviewed.
Results: Twelve cats were first found to be anemic between 0.5-9 years of age. Pallor, lethargy, inappetence, pica, weight loss and splenomegaly were commonly observed. A macrocytic and hypochromic anemia with variable regeneration was noted. Infectious disease screening, direct Coombs˘, and pyruvate kinase DNA mutation test results were negative. Freshly drawn blood did not appear hemolyzed but became progressively lyzed during storage. The sigmoid osmotic fragility curves were right shifted and cross-correction studies indicated a red cell effect.
Most cats were treated with prednisolone and doxycycline. Five cats with recurrent or persistent anemia responded well to splenectomy. However, two had occasional recurrence of severe anemia. Finally, six cats were euthanized within 1 month and 7 years after initial presentation. Histopathology of 6 spleens revealed mainly congestion and extramedullary hematopoiesis.
Conclusion: Similar to Abyssinians and Somalis, domestic shorthair cats and other breeds can also develop osmotic fragility with anemia and splenomegaly, which should be considered as a differential diagnosis in anemic cats.

Key words: hemolytic anemia, in vitro lysis, erythrocyte membrane abnormality, splenomegaly, cross-correction

Zusammenfassung

Vetsuisse-Fakultät Universität Zürich (2015)

Claudia Tritschler

Departement für Kleintiermedizin, Vetsuisse-Fakultät Universität Zürich
claudia.tritschler@gmx.ch

Erhöhte Erythrozytäre Osmotische Fragilität bei Anämischen Haus- und Rassekatzen

Ziele: Erhöhte osmotische Fragilität und Splenomegalie wurden bei anämischen Abessinier- und Somalikatzen beschrieben. Hier beschreiben wir diese Erkrankung bei Haus- und zwei weiteren Rassekatzen.
Methoden: Die osmotische Fragilität wurde getestet, bekannte Anämieursachen ausgeschlossen, der Verlauf verfolgt und Krankheitsgeschichten überprüft.
Resultate: Anämie wurde bei zwölf Katzen erstmals mit 0,5-9 Jahren diagnostiziert. Blässe, Lethargie, Appetitlosigkeit, Pica, Gewichtsverlust und Splenomegalie waren häufige Symptome. Charakteristika der Anämie waren Makrozytose, Hypochromie und variable Regeneration. Infektionskrankheiten, direkter Coombs und Pyruvatkinase-Mutations-Test fielen negativ aus. Frisches Blut erschien normal, hämolysierte aber während der Lagerung. Die sigmoidalen osmotischen Fragilitätskurven waren nach rechts verschoben und Kreutz-Korrektur-Studien deuteten auf ein Erythrozytenproblem.
Die meisten Katzen wurden mit Prednisolon und Doxyzyclin behandelt. Fünf Katzen mit rezidivierender oder persistierender Anämie sprachen gut auf Splenektomie an. Zwei davon zeigten aber später Rezidive. Sechs Katzen wurden zwischen 1 Monat bis 7 Jahre nach der ersten Präsentation euthanasiert. Histopathologie von 6 Milzen zeigte Kongestion und extramedulläre Hämatopoese.
Konklusion: Wie auch Abessinier und Somalis können Hauskatzen und andere Rassen osmotische Fragilität mit Anämie und Splenomegalie entwickeln, was als Differentialdiagnose berücksichtigt werden sollte.

Schlüsselwörter: Hämolytische Anämie, In-vitro-Lyse, Erythrozytenmembran-Abnormalität, Splenomegalie, Kreuz-Korrektur

Introduction

The erythrocytic osmotic fragility (OF) test estimates the surface area to volume ratio of erythrocytes and is used to measure red blood cell (RBC) fragility in cases of chronic or recurring anemia.[1] Briefly, whole blood is incubated in isotonic to hypotonic sodium chloride (NaCl) solutions at 20°C. As water is drawn into RBCs during exposure to progressively hypotonic solutions, the RBCs swell, leak and then burst. The hemoglobin (Hb) released into the supernatant is assessed spectrophotometrically and is expressed as percentage of the completely lyzed blood in distilled water. The normal OF curve is sigmoid, and the mean osmotic fragility (MOF; NaCl concentration at which 50% of RBC lyzed) occurs around 0.5% NaCl in healthy animals. Generally, the OF is inversely related to the normal size of RBCs in animal species.[2]

In humans, an abnormal erythrocytic OF test result may suggest hereditary RBC membrane defects such as spherocytosis and stomatocytosis,[3] but is also observed with some acquired disorders such as renal failure, diabetes mellitus, and immune-mediated hemolytic anemia (IMHA).[1,3] Nowadays, the OF test is rarely conducted in human medicine and has been replaced by more specific tests, such as ektacytometry, protein electrophoresis, Coombs˘test, and DNA tests for hereditary RBC membrane defects.[3] Increased OF was also reported in dogs with hereditary spectrin deficiency,[4,5] hereditary stomatocytosis,[5-9] IMHA[10,11] and other anemias.[12]

Increased OF has also been documented in cats with IMHA,[13] and in Abyssinian and Somali cats, which may have a hereditary erythrocyte membrane defect.[14] In the latter, fragile RBCs were accompanied by chronic intermittent hemolytic, macrocytic-hypochromic and variably regenerative anemia, splenomegaly, hyperglobulinemia, lymphocytosis, hyperbilirubinemia, and increased serum hepatic enzyme activities. Here we report on increased erythrocytic OF in anemic domestic shorthair (DSH) cats

and other purebred cats with clinicopathological features similar to that previously reported in Somali and Abyssinian cats.

Material and Methods

Animals and Samples

Client-owned cats other than Abyssinians and Somalis that were diagnosed with chronic or intermittent anemia of unknown cause, had negative infectious disease screening and direct Coombs˘test results by the primary or referring veterinarian, and which revealed increased fragility in a requested erythrocytic OF test by the PennGen Laboratory (PennGen, University of Pennsylvania, School of Veterinary Medicine, Philadelphia, PA) between September 2009 and September 2014, were included. Fresh ethylenediaminetetraacetate (EDTA) anticoagulated blood samples (2-4 ml) were either collected at the Ryan Veterinary Hospital (University of Pennsylvania, Philadelphia, PA), or submitted by the referring veterinarian along with a sample from a healthy cat (frequently a donor cat that needed blood typing or other screening tests) by overnight shipping. Additional control blood samples were obtained from healthy cats from a research colony at the University of Pennsylvania. All samples were sent and kept refrigerated at 4°C and analyzed simultaneously the same and/or the following day. The study was performed according to the Guidelines for the Institutional Care and Use of Animals in Research.

Signalment and medical records including history, physical examination, clinicopathological information and therapeutic interventions taken at the first diagnosis of anemia were reviewed. Cats were followed after initial diagnosis of increased OF when possible.

Laboratory Tests

Routine laboratory tests utilized for this analysis, including complete blood count (CBC), reticulocyte count, packed cell volume (PCV), total protein (TP), microscopic blood smear evaluation, serum chemistry panel, and urinalysis, were performed at the same time or shortly before the first OF test. All of these tests were performed either by the primary clinic, their reference laboratories (IDEXX, Westbrook, ME or Antech, Irvine, CA) or the clinical laboratory of the Ryan Veterinary Hospital.

A direct Coombs˘test was performed at 37°C using regular commercial reagents (IgG, IgM, C3), by the reference laboratory utilized by the submitting clinic and follow up tests also by PennGen (Antiglobulin Feline Test, Alvedia, Limonest, France) at 20°C, which has not yet been independently validated. Similarly, feline blood typing[15] and pyruvate kinase (PK) deficiency DNA testing[15,16] was performed at PennGen.

All cats were tested for feline leukemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibodies by SNAP test (IDEXX) and hemoplasma PCR (*M. haemofelis, M haemominutum, M. turicensis*). Other infectious disease screening tests (NCSU, Vector Borne Disease Lab, Raleigh, NC) performed in some cats included antibody screening for *Bartonella* species using an immunofluorescence assay and/or Western blot (National Veterinary Laboratory, Franklin Lakes, NJ) and PCR testing for *Anaplasma, Babesia, Bartonella, Ehrlichia,* Hemoplasma*, Rickettsia*, and *Cytauxzoon* species.

Abdominal Imaging, Histopathology and Cytology

Abdominal imaging studies were evaluated when performed. Gross and histopathological or cytological findings of the spleen, liver, and bone marrow were reviewed, when necropsied, removed, biopsied and/or aspirated.

Erythrocytic In Vitro Lysis and Osmotic Fragility Test

The PCV, whole blood and plasma Hb concentrations were measured (B-Hemoglobin HemoCue, Ängelholm, Sweden)[17] to assess in vivo and in vitro lysis of samples at the time of receipt (freshly drawn or after shipping overnight chilled) and again the next day after storage at 4°C.

The OF test was performed as previously described by this laboratory[10,14] with minor modifications from the original assay.[18] Anticoagulated blood from patients and corresponding healthy control cats were processed simultaneously. Briefly, a series of phosphate buffered NaCl dilutions was prepared, containing concentrations of 0.85, 0.8, 0.75, 0.7, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.25 and 0% NaCl. Two milliliters of each solution were mixed well with 15 µl of whole blood in individual test tubes. After 30-45 minutes of incubation at 20°C, samples were centrifuged at 2000 g for 10 minutes. The optical densities of the supernatants were measured spectrophotometrically at 540 nm and the percentage of hemolysis of each tube was calculated. The MOF was determined from the obtained lysis curve.

Cross-Correction Testing

In order to differentiate between intrinsic (RBC) and extrinsic (plasma) issues, patient RBCs and plasma were simultaneously incubated separately with the reciprocal components from the healthy control cat. Briefly, after centrifuging ~0.5 ml EDTA blood at 1000 g for 3 minutes, the plasma was transferred into another tube, while the RBCs were suspended in 1 ml phosphate buffered saline (PBS) and then again centrifuged. Washing with PBS was repeated twice to remove all plasma.

An aliquot (~0.2 ml) of washed RBCs from the affected and its healthy control cat were then incubated at 4°C with an aliquot (0.2-0.3 ml) of plasma from the control cat

and from the affected cat, respectively. After 6 and 24 hours incubation, OF tests were performed with both sample combinations and the unwashed original blood samples at 20°C.

Analysis and Statistics

Following data acquisition by Excel 2013 (Microsoft Corporation, Redmond, WA) standard statistical analyses were applied (IBM SPSS Statistics 22, IBM, Armonk, NY). Data was tested for normal distribution with the Kolmogorov-Smirnow-test. Normally distributed parametric data was illustrated as mean ± standard deviation (m ± SD) and non-normally distributed data as median and range. The Wilcoxon Signed Rank test was performed to test the paired MOF results, from affected and control cats in OF testing and the cross-correction studies. Probability values of $p<0.05$ were considered statistically significant.

Results

Animals

In total, 12 cats (Table 1) including ten DSH cats, one Ocicat and one Siamese cat, all neutered and privately owned in different parts of the United States, exhibiting chronic or intermittent anemia and increased erythrocytic OF were included in this study. Nine DSH cats were adopted from shelters or found as stray cats, while the others (cat 2, 3 and 7) were obtained from breeders. All cats were kept indoors, fed a commercial cat food, and were never exposed to any known toxins according to the owners. Eight animals lived with at least one other cat in the same household, and one also with dogs. The cats˘coat colors varied, but four were tricolored, cream diluted tabby DSH cats from Southwestern Pennsylvania (Table 1): their estimated birth dates

ranged from 2006 to 2011. The Ocicat and one DSH cat (cat 11) had littermates and other relatives with anemia of unknown cause according to the owners, but those were not further studied.

Table 1 Clinical details about cats with increased OF including treatment, additional tests and clinical course

Cat	Sex	First anemia (age in years)	OF tested (age in years)	Additional infectious disease screen ¶	Doxy-cycline	Predni-solone	pRBC n events	Relapses post-medication	Spleen ultrasound findings	Splenectomy (age in years)	Relapses post-splenectomy	Histopathology spleen (S)/ Liver (L)	Current Age/Age of Death (years)
1 H,§	F	1.5	2.5	Vector borne disease panel	∓	∓	-	4 in 2 years	Mottled, nodules	4.5	0	S	6.75
2	M	2	2.75	Vector borne disease panel	∓	∓	-	2 in 0.66 year	Mottled	2.75	0	S/L	3.25
3*	M	4.25	4.5		∓	∓	1	No improvement	Not done	4.5	0	S	5.75
4 H,§	F	2.25	2.5	Vector borne disease panel	∓	∓	-	1 after 0.5 year	Normal echotexture	3	2 after 3 years within 0.5 year	S	6.75
5	M	2	5.25		∓	∓	1	2 in 3.25 years	Granular	5.25	2 after 2 years within 2 years	S	Euthanized 9
6 H,§	F	2	3		∓	∓	1	2 in 1.25 year	Mottled	No	-		3.5
7†	M	4	4		-	∓	-	None, doing well	Normal echotexture	No	-		4.75
8 H,§	F	7.5	7.5	Bartonella Western blot	-	∓	2	1 after 0.75 year	Nodules	No	-		Euthanized 8.5
9	F	0.5	0.6	Vector borne disease panel	∓	∓	1	No improvement	Not done	No	-		Euthanized 0.6
10§	F	9	9	Vector borne disease panel	∓	∓	6	No improvement	Mottled, nodules	No	-	S/L	Euthanized 9
11	F	0.5	0.65	Bartonella Western blot	∓	-	-	1 after 0.5 year	Not done	No	-	L	Euthanized 1
12	M	1.5	2	Vector borne disease panel	∓	∓	-	1 after 0.5 year	Mottled	No	-		Euthanized 2

* Ocicat, è Siamese, H dilute cream tabby cats, § Cases presented at UPenn, ¶ Vector borne disease panel: PCR for A*naplasma, Babesia, Bartonella, Ehrlichia,* Hemoplasma*, Rickettsia, Cytauxzoon* species and immunofluorescence and/or Western blot assay for *Bartonella* species

F = female; M = male; pRBC = packed RBC transfusion, (∓) = medication received or test performed.

Clinical Signs and Routine Test Results

Pallor (n = 11), lethargy (8) and splenomegaly (11) were the main clinical signs at presentation. Weight loss (5), inappetence (6) and pica (5) were additionally observed. Three of the dilute colored cats had increased body condition scores (BCS) ranging between 6-8 (normal BCS 5/9). One additional DSH cat (cat 2) with black fur also had an increased BCS of 8, but none had hyperlipidemia on presentation. First anemia was found between 0.5-9 years of age (median: 2.25 years).

All cats had a history of chronic or intermittent anemia before the OF test was performed (Table 1 and 2). They were found to be anemic with a wide range of hematocrits, which also fluctuated greatly over time. Common RBC characteristics at the initial time of OF testing were moderate to marked macrocytosis (9), mild hypochromasia (5), mild to marked anisocytosis (10; Figure 1), polychromasia (8), and reticulocytosis (7) [insert Figure 1]. If these characteristics were not noted initially, they were found at later examinations. Moreover, in seven cats, few to many normoblasts were seen on blood smear examination. Low or moderate numbers of Heinz bodies and Howell Jolly bodies were also found in four and three cats, respectively.

Total white blood cell (WBC) counts of 11 cats were in the reference interval (RI), except cat 12 (Table 1+2), which had a severe neutrophilia (39.6x10^3/µl, RI: 2.3-14.0 x10^3/µl) with normal cell morphology. Rare reactive lymphocytes were initially observed on blood smears of three cats, but were also seen in four additional cats at later examinations.

Table 2 Selected hematology and chemistry panel results from all 12 cats with increased OF at initial examination.

Parameter (Unit)	Mean ± SD or Median	Range	Reference interval
HCT (%)	20.3 ± 7.1	6.9 ¯ 26.0	31.7 - 48.0
RBC (x10^6/µl)	3.4 ± 1.4	1.0 - 5.5	6.6 - 11.2
Hb (g/dl)	6.31 ± 2.26	2.1 - 9.6	10.6 - 15.6
MCV (fl)	63.5 ± 12.3	43.0 - 79.0	36.7 - 53.7
MCHC (g/dl)	30.9 ± 2.6	27.9 - 38.3	30.1 - 35.6
Reticulocytes (x10^3/µl)	130	12 - 666	0 - 50
nRBC (/100 WBC)	3	0 - 126	0 - 1
WBC (x10^3/µl)	12.7	4.6 - 40.7	4.0 - 18.7
Serum bilirubin (mg/dl)	0.6	0.2 - 6.9	0.1 - 0.4

HCT = hematocrit; Hb = hemoglobin; MCV = mean corpuscular volume;

MCHC = mean corpuscular hemoglobin concentration;

nRBC = nucleated red blood cells; WBC = white blood cells

Urinalysis and serum chemistry results were normal, except five cats had mild and cat 12 marked hyperbilirubinemia. Cat 12 also had elevated serum liver enzyme activities: alanine aminotransferase (ALT): 401 U/L, RI: 33-152 U/L; aspartate aminotransferase (AST): 349 U/L, RI: 1-37 U/L.

Serum iron concentrations measured in cat 2 and 4 were normal. Direct Coombs˘ test and PK deficiency DNA test results were negative in all cats.

All animals were negative for FeLV, FIV and Hemoplasma infections. Additionally, six cats tested negative for the available entire vector borne feline infectious disease panel by PCR and serology and two cats were serologically negative for *Bartonella* species infections by Western blot (Table 1). Cat 4 was later found to be serologically strongly positive for *Bartonella vinsonii* (see below).

Abdominal Imaging, Histopathology and Cytology Findings

Abdominal ultrasound performed on nine cats showed mild to severe splenomegaly with varied changes and three (cat 5, 6, 8) also had mild hepatomegaly (Table 1).

Histopathological examination (Table 1) of splenic samples from splenectomy (5) and necropsy (1) revealed marked congestion (5), mild to moderate extramedullary hematopoiesis (5), mild hemosiderosis (3), mild scattered lymphoid follicles (1), and scattered clusters of macrophages (2). Hepatic samples from one wedge biopsy and two necropsies showed moderate extramedullary hematopoiesis (2), histiocytosis (1) and marked hemosiderosis (1). One bone marrow aspirate (cat 10) revealed mild erythroid hyperplasia and focal megakaryocytic hyperplasia, while the other seemed unremarkable (cat 5).

Erythrocytic In Vitro Lysis and Osmotic Fragility Test Results

While no to little hemolysis was observed at the time of blood collection, moderate to severe in vitro hemolysis (plasma Hb 0.8-5.5 g/dl) was observed in 8/10 EDTA blood samples evaluated after 6 and 24 hours (Figure 2) stored at 4°C. Simultaneously handled control blood samples revealed little to no hemolysis (plasma Hb ±0.3 g/dl) based upon visual examination of microhematocrit tubes and plasma Hb determinations [insert Figure 2.].

The OF curves of the anemic cats were moderately to severely right shifted (Figure 3), with MOF`s ranging from 0.63-0.87% NaCl at the time of initial testing, when compared to corresponding controls, for which the MOF`s ranged from 0.45-0.57% NaCl ($p=0.002$). The location and shape of the OF curve either stayed as initially

determined or the RBCs became even more fragile after 24 hours. In two cases (cat 3 and 10) only a small proportion of RBCs resisted storage at 4°C one day after blood collection, making OF testing nearly impossible [insert Figure 3.].

Cross-Correction Test Results

Plasma and washed RBC components of blood samples from three DSH cats (cats 4, 6, 11) were cross-corrected with components of their complementary control blood samples. In all three cases MOFs of RBCs from healthy control cats remained normal after incubation at 4°C with either their own plasma or plasma from the affected cats. In contrast, MOFs of RBCs from affected cats remained increased after incubation with either their own plasma or plasma from the control cats (Table 3). Thus, an intrinsic issue causing increased erythrocytic OF is suspected.

Table 3 Osmotic fragility of erythrocytes in in vitro cross-correction experiments. MOF˘s (%) of cross-correction test results from three cats expressed as median (range)

Incubation	**Patient RBCs**		**Control RBCs**	
(Hours)	**+ Patient Plasma**	**+ Control Plasma**	**+ Control Plasma**	**+ Patient Plasma**
0	0.65 (0.65-0.83)	-	0.54 (0.51-0.57)	-
6	0.65 (0.64-0.73)	0.62 (0.59-0.75)	0.55 (0.51-0.55)	0.52 (0.50-0.54)
24	0.66 (0.65-0.76)	0.67 (0.61-0.75)	0.55 (0.52-0.57)	0.53 (0.51-0.59)

Therapy and Follow-up Results

All cats were initially treated with doxycycline and/or immunosuppressive doses of prednisolone (1-2 mg/kg BID) presumptively for an infectious and/or immune process except for cat 8 (Table 1). Generally, when the PCV rose the prednisolone dose was

tapered stepwise every 2-4 weeks until withdrawn. Cats with persistent or recurring anemia according to follow-up routine laboratory results, were continually treated or retreated with prednisolone (1-2 mg/kg BID), splenectomized and/or euthanized. In critically ill cases of severe anemia, AB type compatible packed RBCs were transfused (Table 1). Few cats also received additional antibiotics, including amoxicillin/clavulanic acid (4), enrofloxacin (2), marbofloxacin (1), and azithromycin (1).

Of the five splenectomyzed cats, three recovered without relapse and recent follow up OF tests were normal in these cats. Cat 4 (Table 1; Figure 4) had an episode of recurrent anemia with abnormal OF 3 years after splenectomy and was found to be serologically positive for *B vinsonii*, but was negative by PCR test. Rapid improvement was observed after treatment with azithromycin and prednisolone. PCR test results for *Bartonella* species remained negative, and the serum antibody titer against *B vinsonii* declined drastically over 5 months to negative. However, this cat relapsed 6 months later with severe anemia (PCV 16%) and increased OF, but was completely negative for any infectious diseases and recovered well following prednisolone and azithromycin treatment (PCV 35% for past 8 months) [insert Figure 4.].

Cat 5 developed recurrent anemia of unknown cause 2 years after splenectomy, which appeared to respond well to intermittent prednisolone therapy for another 2 years, but the cat was then euthanized.

Special cases that did poorly and were not splenectomized are presented below:

Cat 8 was found to have macrocytic anemia on a routine exam and aside from routine blood work and the OF test, additional diagnostics and treatments were declined by the owner. However, 9 months later this cat developed severe anemia (PCV 9%). No cause was identified and medical emergency treatment appeared unsuccessful and the cat was euthanized.

Cat 11 was stable for 7 months with mild anemia (PCV 26%) before developing suddenly severe hyperbilirubinemia (from 20.1 to 25.5 mg/dl; PCV 21%) and mildly increased serum ALT activities (from 199 to 225 U/L) that appeared therapeutically unresponsive to routine emergency care. Necropsy revealed cholangiohepatitis with cholestasis, fibrosis and centrolobular atrophy.

Cat 12 showed intermittent recurring anemia over 6 months, then acutely developed non-regenerative anemia, neutrophilia, hypoproteinemia and also had elevated serum ALT activity (401 U/L) and was euthanized. No necropsy was performed.

Discussion

Anemia is a very common abnormality in cats in any clinical practice and can result from infectious, immune, toxic, metabolic and inherited causes.[19,20] Here we describe a unique condition of anemia with increased erythrocytic OF and splenomegaly in DSH and purebred cats similar to what this laboratory previously described in anemic Abyssinian and Somali cats.[5,14] Prevalence and underlying cause(s) still need to be determined.

A remarkably severe in vitro fragility of erythrocytes that is not typically observed in either feline anemia or other diseases was documented in these 12 anemic cats. While hemolysis was not observed in freshly collected blood, it became apparent and progressively increased within hours to days of regular storage in an EDTA tube at 4°C. This was readily evident by visual examination of the EDTA tube and/or after centrifuging a blood sample in a microhematocrit tube and without rewarming the blood. In fact some blood samples were nearly completely lyzed by the next day, whereas the simultaneously handled, shipped, and stored control samples from

healthy cats showed little to no hemolysis during the same time frame. Such in vitro hemolysis seems to be unique to these cats and the previously reported Abyssinian and Somali cats with increased OF.[5,14]

Concordantly, utilization of a classic OF test demonstrated that erythrocytes from these cats were markedly more fragile in hypotonic solutions than were RBCs from healthy control cats. In fact some erythrocytes started to lyze near physiological NaCl concentration. The OF curve was significantly shifted to the right, suggesting that all of the erythrocytes were similarly affected. They appeared to swell rapidly even in solutions utilized for automated hematology cell counters, resulting in increased macrocytic and lyzing artifacts observed during analysis. These observations are similar to those reported previously in Abyssinian and Somali cats.[14]

When examining the patients˘erythrocytes and plasma separately in in vitro cross-correction experiments from three anemic cats compared to controls, it became evident that the patients˘washed erythrocytes lyzed similarly in the presence of control and autologous plasma, while the controls˘erythrocytes remained stable in autologous and patient plasma. These limited data support an intrinsic RBC-related defect, and inherited or acquired erythrocyte abnormalities are suspected, but further studies are needed. Severely increased OF has been observed in Miniature and Middle Schnauzers, Alaskan Malamutes and Drentse Patrijshond with hereditary stomatocytosis, a disease assumed to be caused by a red cell membrane defect.[6-9] However, the cats in this report and also in the study of Abyssinian and Somali cats had few if any stomatocytes, although the observed high MCV was disproportional to the degree of reticulocytosis. Milder erythrocytic OF is also seen with IMHA in dogs[10-12] but has rarely been studied in cats with IMHA.[13] Despite extensive attempts to find an acquired cause, no immune, infectious (one exceptional cat was later found to have

a *Bartonella* infection as revealed by positive serology), or toxic cause was identified over extended follow up periods similar to the previously described Abyssinian and Somali cats.[14] And indeed increased erythrocytic osmotic fragility has been previously reported in a few cats with immune-mediated hemolytic anemia, hemoplasma infection, anemia of inflammatory disease and suspected hereditary poikilocytosis.[13,14,21-23] We did not find any support for these or other diseases in the cats of this report. We did not specifically prepare and examine the blood for cold antibodies and complement. Thus, we cannot entirely exclude cold agglutinin disease, cold hemolytic anemia, and paroxysmal cold hemoglobinuria as reported in human patients.[24] None of the cats had any evidence of massive pigmenturia or peripheral cold agglutinin disease. Furthermore, the lyzing observed in vitro at cold temperatures and increased OF seem unique to these cats.

Interestingly, four of the 12 affected stray cats from Southeastern Pennsylvania had a tricolored dilute hair coat, suggesting that perhaps they were related and, therefore, had a common hereditary red cell defect. While some cats including three of the tricolored cats with dilute hair coat were overweight, their plasma was not found to be lipemic during routine laboratory testing. Interestingly, lipemia has been associated with increased OF in different species, receiving a high fat diet and also in diabetic dogs.[25-29]

Despite the overwhelming in vitro evidence of erythrocytic fragility in the cats of this report, the in vivo evidence of hemolysis is indirect. These cats had intermittently severe anemia, but reached normal hematocrit values. There was never any evidence of blood loss, but some of the cats showed pica, which is a typical sign of blood loss with iron or other deficiencies. Based upon the lack of hemoglobinemia and hemoglobinuria, an extravascular hemolysis predominantly in the enlarged spleen

appears most likely. Hemolysis was suggested by a regenerative macrocytic hypochromic anemia with mild hyperbilirubinemia and reticulocytosis. The degree of reticulocytosis varied greatly, but was frequently less than what would be expected for the severity of the anemia, suggesting transient marrow issues hampering the regenerative response in these cats.

Most of the cats in this as well as the previous report on Abyssinian and Somali cats with increased OF[14] had moderate to severe splenomegaly. Diffuse splenomegaly in cats is often associated with marked extramedullary hematopoiesis and was indeed observed in Abyssinian and Somali cats with increased OF. Somewhat surprising, extramedullary hematopoiesis was not a major finding here. Splenomegaly can also occur in cats with hemoplasmosis, FeLV infection, and other infectious diseases[30,31] but tests and histopathology did not reveal any infectious agents. Similarly, despite an association of anemia with hemolymphatic neoplasias in cats[30,31] there was no evidence of cancer identified. Splenectomy in five cats with severe episodes of anemia seemed to reduce the degree of in vitro lysis and anemia for months to years suggesting that the spleen enhanced RBC fragility through an unknown mechanism, thereby accelerating RBC clearance. An improved osmotic fragility was also observed in cats with hemoplasma infection following splenectomy.[32]

Treatment of cats with immunosuppressive doses of prednisolone appeared to temporarily resolve or improve their anemia. As direct Coombs˘tests were all negative, prednisolone-mediated inhibition of RBC-specific autoantibodies is unlikely to account for the response. However, prednisolone may inhibit macrophage-mediated attack of altered RBCs and stabilize membranes.[20]

The ultimate reasons for euthanasia of six cats in this study were lack of response to medical treatment and progressive decline or development of hemolytic crisis,

severe icterus or recurring anemia. Interestingly, two cats with signs of hemolytic anemia signs also showed signs of severe primary hepatopathy and were euthanized. It is unclear at this time if the hepatopathy was related to OF in these two cats. No bilirubin calculi were found in any cats with increased OF, while in cats with PK deficiency it was occasionally observed.[33,34]

Conclusions

Anemic DSH and other purebred cats can develop severe OF and splenomegaly. The RBCs appeared to be extremely fragile in vitro and limited cross correction studies support an intrinsic RBC defect in promoting increased erythrocytic OF. Many of the observed clinicopathological and histopathological features resemble those previously reported in Abyssinian and Somali cats. No acquired, immune, infectious or toxic causes could be identified. Treatment with doxycycline, prednisolone, and blood transfusions may improve the clinical condition, but such responses appear transient. Splenectomy seems to be most effective for long-term improvement of anemia and OF, although a cure has not been achieved.

Funding

This study was supported in part by a grant from the National Institutes of Health # OD 010939.

Conflict of Interest Declaration

The authors are members of the nonprofit PennGen laboratory, which offers OF and other hematological testing.

Figures

Figure 1 Blood smears from two cats with increased OF showing anisocytosis (a, b) and polychromasia (a). Bar 6µm.

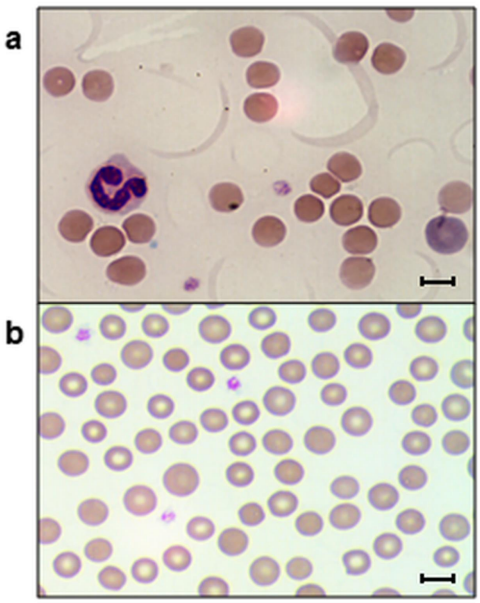


Figure 2 Microhematocrits from one cat at time of blood collection and 6 and 24 hours after blood collection stored at 4°C. PCV's decreased from 20% to 14% and 12%, respectively.

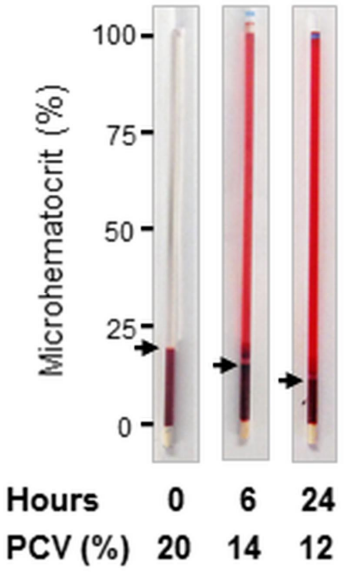


Figure 3 Osmotic fragility test results from an affected and control cat. An Osmotic fragility tube test result with the MOF is depicted with an arrow (a).

Right shifted curve of an affected cat with increased OF and a normal curve from the corresponding control cat (b). The curve of the control cat has a sigmoid shape, while the affected cat˘s curve is flattened and hemolysis is occurring in higher NaCl concentrations. Dashed lines indicate in both curves the MOFs at 50% hemolysis.

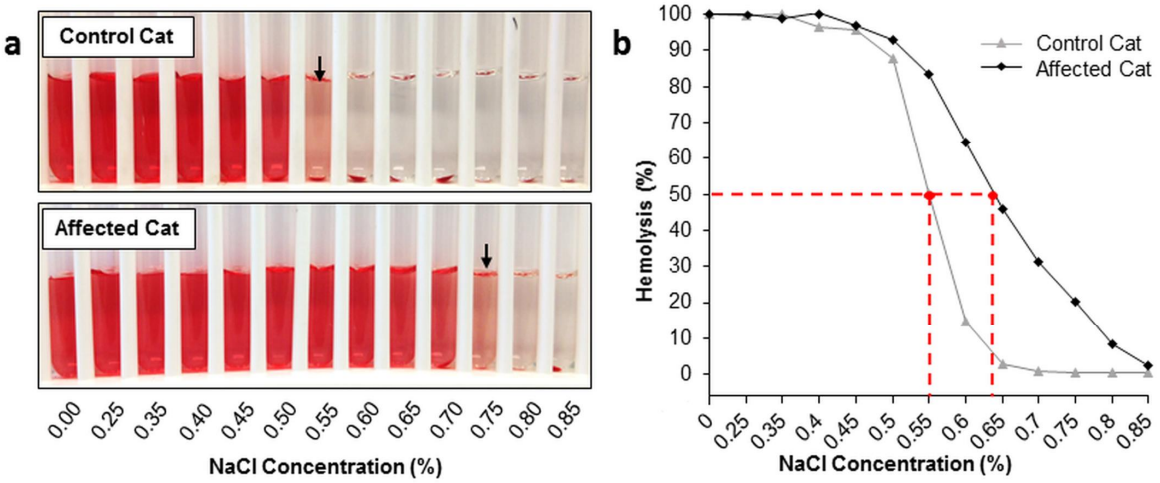
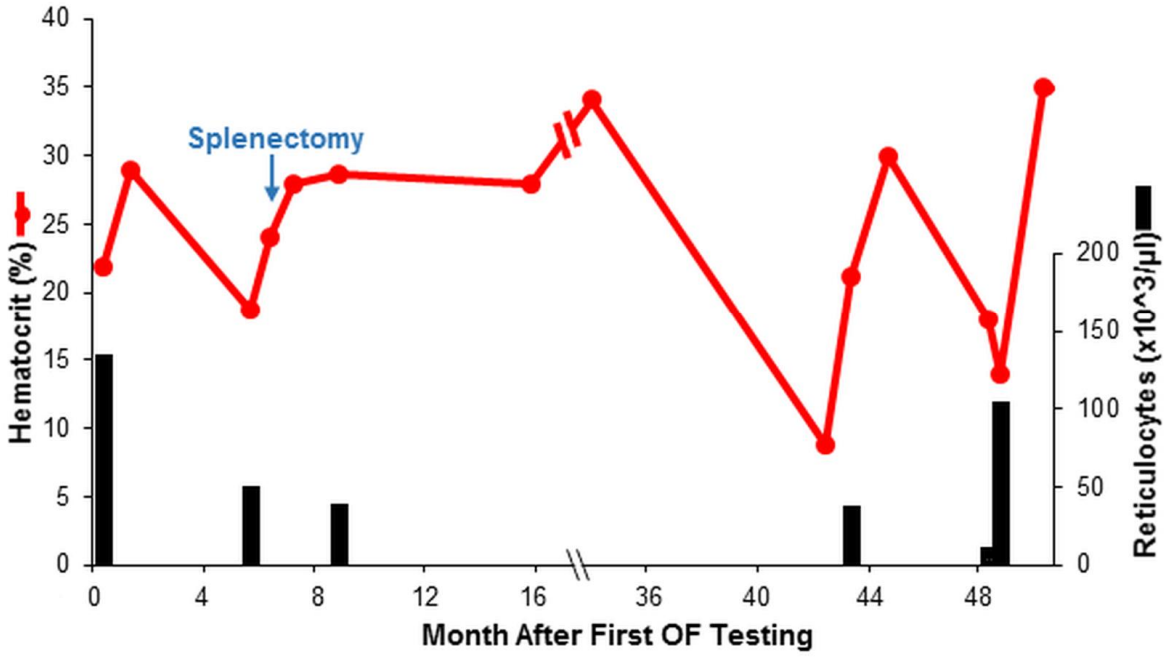


Figure 4 Clinical course in a cat with increased OF. Hematocrit and reticulocyte counts over a 4 year period following first OF testing.


Splenectomy
Hematocrit (%)
Reticulocytes (x10^3/µl)
Month After First OF Testing
0
4
8
12
16
36
40
44
48
0
5
10
15
20
25
30
35
40
0
50
100
150
200

References

1 Beutler E. **Osmotic Fragility**. In: Williams JW, Beutler E, Erslev AJ, et al (eds). *Hematology*. 4th ed. New York: McGraw Hill Publishing Co, 1990, pp 1726 - 1728.

2 Perk K, Hort I and Perri H. **The degree of swelling and osmotic resistance in hypotonic solutions of erythrocyes from various domestic animals**. *Refuah Vet* 1964; 20: 122.

3 King MJ and Zanella A. **Hereditary red cell membrane disorders and laboratory diagnostic testing**. *Int J Lab Hematol* 2013; 35: 237-243.

4 Slappendel RJ, van Zwieten R, van Leeuwen M, et al. **Hereditary spectrin deficiency in Golden Retriever dogs**. *J Vet Intern Med* 2005; 19: 187-192.

5 Giger U. **Hereditary Erythrocyte Disorders**. In: Bonagura JD and Twedt DC (eds) Kirk's Current Vetrinary Therapy [Evolve Recource]. 14 ed. Missouri: Saunders Elsevier, 2009.

6 Slappendel RJ, van der Gaag I, van Nes JJ, et al. **Familial stomatocytosis-hypertrophic gastritis (FSHG), a newly recognised disease in the dog (Drentse patrijshond)**. *Vet Q 1991*; 13: 30-40.

7 Pinkerton PH, Fletch SM, Brueckner PJ, et al. **Hereditary stomatocytosis with hemolytic anemia in the dog**. *Blood* 1974; 44: 557-567.

8 Giger U, Amador A and Meyers Wallen V, et al. **Stomatocytosis in miniature schnauzers** [abstract]. *American College of Veterinary Internal Medicine Proceedings* 1988: 754.

9 Bonfanti U, Comazzi S, Paltrinieri S, et al. **Stomatocytosis in 7 related Standard Schnauzers**. *Vet Clin Pathol* 2004; 33: 234-239.

10 Caviezel LL, Raj K and Giger U. **Comparison of 4 direct coombs' test methods with polyclonal antiglobulins in anemic and nonanemic dogs for in-clinic or laboratory use**. *J Vet Intern Med* 2014; 28: 583-591.

11 Piek CJ, Junius G, Dekker A, et al. **Idiopathic immune-mediated hemolytic anemia: treatment outcome and prognostic factors in 149 dogs**. *J Vet Intern Med* 2008; 22: 366-373.

12 Paltrinieri S, Comazzi S and Agnes F. **Haematological parameters and altered erythrocyte metabolism in anaemic dogs**. *J Comp Pathol* 2000; 122: 25-34.

13 Kohn B, Weingart C, Eckmann V, et al. **Primary immune-mediated hemolytic anemia in 19 cats: diagnosis, therapy, and outcome (1998-2004)**. *J Vet Intern Med* 2006; 20: 159-166.

14 Kohn B, Goldschmidt MH, Hohenhaus AE, et al. **Anemia, splenomegaly, and increased osmotic fragility of erythrocytes in Abyssinian and Somali cats**. *J Am Vet Med Assoc* 2000; 217: 1483-1491.

15 Seth M, Jackson KV and Giger U. **Comparison of five blood-typing methods for the feline AB blood group system**. *Am J Vet Res* 2011; 72: 203-209.

16 Giger U, Rajpurohit Y, Wang P, et al. **Molecular basis of erythrocyte pyruvate kinase (R-PK) deficiency in cats** [Abstract]. *Blood* 1997; 90 (Suppl): 5b.

17 Callan MB, Giger U, Oakley DA, et al. **Evaluation of an automated system for measurement in animals**. *Am J Vet Res* 1992; 53: 1760-1764.

18 Parpart AK, Lorenz PB, Parpart ER, et al. **The Osmotic Resistance (Fragility) of Human Red Cells**. *J Clin Invest* 1947; 26: 636-640.

19 Couto C. **Anemia**. In: Nelson RW and Couto CG (eds). Small Animal Internal Medicine. 5 ed. Missoure: Mosby Elsevier, 2014, pp 1201-1219.

20 Giger U. **Regenerative Anemias Caused by Blood Loss or Hemolysis**. In: Ettinger S and Feldman E (eds). Veterinary Internal Medicine. 6 ed. Missouri: Elsevier Saunders, 2005, pp 1886-1907.

21 Jain NC. **Osmotic fragility of erythrocytes of dogs and cats in health and in certain hematologic disorders**. *Cornell Vet* 1973; 63: 411-423.

22 Giger U, Reilly MP, Tasi A, et al. **Beta-thalassemia in a cat: A naturally-occuring disease model**. *Blood* 1994; 84: 364a.

23 Ottenjann M, Weingart C, Arndt G, et al. **Characterization of the anemia of inflammatory disease in cats with abscesses, pyothorax, or fat necrosis**. *J Vet Intern Med* 2006; 20: 1143-1150.

24 Gertz MA. **Management of cold haemolytic syndrome**. *Br.J.Haematol* 2007; 138: 422-429.

25 Abdelhalim MA and Moussa SA. **Biochemical changes of hemoglobin and osmotic fragility of red blood cells in high fat diet rabbits**. *Pak J Biol Sci* 2010; 13: 73-77.

26 Akahane K, Furuhama K and Onodera T. **Simultaneous occurrence of hypercholesterolemia and hemolytic anemia in rats fed cholesterol diet**. *Life Sci* 1986; 39: 499-505.

27 Behling-Kelly E and Collins-Cronkright R. **Increases in beta-lipoproteins in hyperlipidemic and dyslipidemic dogs are associated with increased erythrocyte osmotic fragility**. *Vet Clin Pathol* 2014; 43: 405-415.

28 Cooper RA, Leslie MH, Knight D, et al. **Red cell cholesterol enrichment and spur cell anemia in dogs fed a cholesterol-enriched atherogenic diet**. *J Lipid Res* 1980; 21: 1082-1089.

29 Sengupta A and Ghosh M. **Integrity of erythrocytes of hypercholesterolemic and normocholesterolemic rats during ingestion of different structured lipids**. *Eur J Nutr* 2011; 50: 411-419.

30 Couto CG. **Lymphadenopathy and Splenomegaly**. In: Nelson RW and Couto CG (eds). Small Animal Internal Medicine. 5 ed. Missouri: Mosby Elsevier, 2014, p.1264 - 1275.

31 Autran de Morais H and O'Brien RT. **Non-Neoplastic Diseases of the Spleen**. In: Ettinger SJ and Feldman EC (eds). Veterinary Internal Medicine. 6 ed. Missouri: Elsevier Saunders, 2005, pp 1944-1951.

32 Maede Y. **Studies on feline haemobartonellosis. V. Role of the spleen in cats infected with *Haemobartonella felis***. *Jpn J Vet Res* 1978; 40: 691-695.

33 Harvey AM, Holt PE, Barr FJ, et al. **Treatment and long-term follow-up of extrahepatic biliary obstruction with bilirubin cholelithiasis in a Somali cat with pyruvate kinase deficiency**. *J Feline Med Surg* 2007; 9: 424-431.

34 van Geffen C, Savary-Bataille K, Chiers K, et al. **Bilirubin cholelithiasis and haemosiderosis in an anaemic pyruvate kinase-deficient Somali cat**. *J Small Anim Pract* 2008; 49: 479-482.

Danksagung - Acknowledgement

I am using this opportunity to express my gratitude to everybody who supported me throughout the course of this dissertation project.

Foremost, I would like to offer my sincere appreciation and thanks to Professor Dr. Urs Giger for giving me this great opportunity to come to Philadelphia to work and write my dissertation at the Section of Medical Genetics at the School of Veterinary Medicine of the University of Pennsylvania. He was always available to provide valuable help and feedback for my project. Without his truly encouraging guidance, expertise, attentive supervision and professional advice this project would not have been as successful. I could not have imagined having a better or friendlier mentor and advisor for my doctoral thesis.

I place on record my sense of gratitude to my laboratory colleagues Karthik Raj, and Dr. Keijiro Mizukami for sharing their experience, supporting me and my experiments, and for all the helpful comments.

I am also truly grateful to the entire Medical Genetics and the Clinical Laboratory teams as well as all pathology residents at the Veterinary Hospital at the University of Pennsylvania for their assistance and encouragements,

Special thanks also go to all my new and old friends for their encouraging words and visits during my doctorate abroad.

Last but not least, I express my deepest gratitude to my beloved parents Markus and Nelly Tritschler for all of their sacrifices that they have made on my behalf and for their continuous support in helping me achieve all my professional goals. Finally, I would like to thank my partner from the bottom of my heart for giving me hope, encouragement and endless love throughout this challenging journey to complete and publish my thesis.

Curriculum Vitae

Persönliche Daten

Name, Vorname:	**Tritschler, Claudia**
Geburtsdatum:	02. Oktober 1985
Geburtsort:	Buenos Aires, Argentinien
Nationalität:	Schweiz
Heimatort:	Schaffhausen, Schaffhausen, Schweiz

Ausbildung und Anstellung

08.1992 ¯ 07.1999 **Gelbhausgarten,** Primarschule ¯ Schaffhausen

08.1999 ¯ 07.2001 **Bachschule,** Sekundarschule ¯ Schaffhausen

08.2001 ¯ 07.2005 **Kantonsschule Schaffhausen** ¯ Schaffhausen

- Schwerpunkt Chemie und Biologie
- Matura Typus Naturwissenschaften

09.2006 ¯ 11.2011 **Studium der Veterinärmedizin an der Vetsuisse-Fakultät, University Zürich,** Zürich, Schweiz

Abschlussprüfung vet. med. ¯ Staatsexamen Oktober 2011

Master of Science in Veterinärmedizin im Jahr 2011

- Schwerpunkt Pathobiologie
- Murdoch University Parasitology Department, Perth, WA, Australia
  Externship in Parasitologie, 01-03.2011

Bachelor of Science in Veterinärmedizin im Jahr 2009

01.2012 ¯ 10.2013 **Tierärztin, Gross- und Kleintiere**
Uehlinger Tierärzte-Team AG, Beringen, SH, Schweiz

02.2014 ¯ 04.2015	**Doktorandin, wissenschaftliche Mitarbeiterin** **Section of Medical Genetics, Veterinary Hospital** **University of Pennsylvania,** Philadelphia, PA, USA Anfertigung der Dissertationsarbeit unter Leitung von Prof. Dr. Urs Giger, Department für Kleintiermedizin, Universität Zürich, Schweiz
Ab 04.2015	**Assistenztierärztin, Kleintiere** **Kleintierklinik Breitensee Wien,** Wien, Österreich