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Concentration of Serum Amyloid A (SAA) in European Brown Hare (*Lepus europaeus*)

Diplomarbeit

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1 Introduction

1.1 History and Definition of Amyloidosis

Amyloidosis is a general term for several protein misfolding and aggregational diseases (Benson et al. 2018). Protein misfolding disorders come in all different shapes and forms. They can be described as e.g. genetic or acquired, localized or systemic, intracellular or extracellular (Buxbaum 2009). Inappropriate aggregation of proteins is usually controlled by a complex cellular quality control mechanism but under certain circumstances some proteins are able to aggregate into insoluble highly ordered structures(Aguzzi and O'Connor 2010).

Although amyloidoses are considered to be a very heterogeneous group of diseases they have some common features.

The Nomenclature Committee of the International Society of Amyloidosis has given following definition for amyloid: “Amyloid is an extracellular deposition of protein fibrils with characteristic appearance in electron microscope, typical X-ray diffraction pattern, and affinity for Congo red with concomitant green birefringence” (Benson et al. 2018).

In the mid-19th century Virchow(1884) found amorphous pathological deposits in the human central nervous system and later in other organs, with positive iodine staining reaction, like starch or wood in plant material. Believing the deposits to be of carbohydrate origin he introduced the term amyloid into human medicine. The term is derived from the Greek word *amylon* which means starch. Only a few years later, in 1859, the proteinaceous nature of amyloid was proven (Friedreich and Kekulé 1859).

The macroscopic appearance of amyloidosis is not specific for amyloid. The fact that amyloidoses are a very heterogeneous group of diseases plays an important role in the distribution and appearance of lesions. Deposits are either limited to a single organ or tissue or affect more than one site. Affected organs may be enlarged, moderately firm and abnormally discolored or may not even show any distinct gross lesions at all (Snyder 2007).

Microscopic findings of the deposition may correspond to the gross lesions, but sometimes deposits are only seen at microscopic examination. Amyloid should be differentiated from other similar appearing extracellular deposits such as collagen and fibrin (Snyder 2007).

Light microscopically amyloid appears as homogeneous masses. When looked at under polarized light amyloid shows birefringence. This effect is increased after staining with Congo

red. When looked at under the light microscope after staining, amyloid turns orange to red and shows the characteristic apple green birefringence under polarized light (Ménsua et al. 2003, Snyder 2007). Other staining methods can be used to reveal the presence of amyloid in tissue but Congo red staining remains the golden standard until today (Ménsua et al. 2003).

Congo red staining is not chemically specific for amyloid, but rather for its conformational arrangement into so-called beta-pleated sheets. This cross-beta structure was revealed through X-ray diffraction analysis (Eanes and Glenner 1968).The diffraction pattern can be used to make a distinction between parallel beta-sheet and cross-beta structure of proteins.

Amyloid fibrils were discovered by Cohen and Calkins(1959) with the help of an electron microscope. The electron microscopic examination revealed fibrils about 10nm wide and undefined length (Cohen and Calkins 1959).

1.2 Classification

It is important to define the context of usage when defining the term amyloid (Fändrich 2007). “The problem of nomenclature is that at present the medical and biophysical scientific communities are using different definitions of "amyloid". The designation amyloid comes from the medical field but has been adopted by biochemists and biophysicists and is now generally used for all cross β-sheet fibrils.”(Benson et al. 2018). For example, in vitro generated “amyloid fibrils” can derive from all different kind of proteins, even from those that have no amyloidotic potential in vivo (Fändrich 2007). In comparison to in vivo fibrils, the in vitro generated amyloid-like fibrils lack other minor components, which are part of amyloid deposit (Benson et al. 2018).

For a long time amyloidoses were divided into two large groups. Systemic amyloidoses, where the deposits may be present in many organs and tissues, and localized amyloidoses (Merlini and Westermark 2004). The modern classification is, in the first place based on the precursor proteins that form the amyloid fibrils (Falk et al. 1997), and are thereafter further characterized as systemic and/or localized.

1.2.1 Precursor Proteins

The first ever described amyloid protein was protein AA. The first A stands for amyloid fibril protein, which is the same for all types of amyloid proteins (Benson et al. 2018), and the second A simply stands for the first letter in the alphabet (Benditt and Eriksen 1971).The second type discovered was protein AL. Originally it was designated protein B to follow fit the nomenclature

of the first protein (Benditt and Eriksen 1971). From the second International Symposium on Amyloidosis on, which was held in Helsinki in 1974, a committee was formed to discuss the nomenclature of amyloid and amyloidosis and the basics for the designation were postulated in their proceedings (Husby et al. 1991). Once every two years the International Society of Amyloidosis (ISA) nomenclature committee gathers to discuss the introduction of new protein misfolding diseases into the group of amyloidoses.

The guideline is, that protein A is followed by a suffix that is an abbreviation of the precursor protein name. This designation should also be used for the associated amyloid disease (Benson et al. 2018).

According to the current state of knowledge 46 different proteins, of which 36 are of human nature (Table 1) and at least ten are of other vertebrate nature (Table 2), have been identified (Benson et al. 2018).

Table 1: Amyloid fibril proteins and their precursors in humans (Benson et al. 2018)

Fibril protein	Precursor protein	Systemic and/or localized	Acquired or hereditary	Target organs
AL	Immunoglobulin light chain	S, L	A, H	All organs, usually except CNS
AH	Immunoglobulin heavy chain	S, L	A	All organs except CNS
AA	(Apo) Serum amyloid A	S	A	All organs except CNS
ATTR	Transthyretin, wild type	S	A	Heart mainly in males, Lung, Ligaments, Tenosynovium
	Transthyretin, variants	S	H	PNS, ANS, heart, eye, leptomen.
Aβ2M	β2-Microglobulin, wild type	S	A	Musculoskeletal System
	β2-Microglobulin, variant	S	H	ANS
AApoAI	Apolipoprotein A I, variants	S	H	Heart, liver, kidney, PNS, testis, larynx (C terminal variants), skin (C terminal variants)
AApoAII	Apolipoprotein A II, variants	S	H	Kidney
AApoAIV	Apolipoprotein A IV, wild type	S	A	Kidney medulla and systemic
AApoCII	Apolipoprotein C II, variants	S	H	Kidney
AApoCIII	Apolipoprotein C III, variants	S	H	Kidney
AGel	Gelsolin, variants	S	H	PNS, cornea
ALys	Lysozyme, variants	S	H	Kidney
ALECT2	Leukocyte Chemotactic Factor-2	S	A	Kidney, primarily
AFib	Fibrinogen α,variants	S	H	Kidney, primarily
ACys	Cystatin C, variants	S	H	PNS, skin
ABri	ABriPP, variants	S	H	CNS
ADan	ADanPP, variants	L	H	CNS
Aβ	Aβ protein precursor, wild type	L	A	CNS

	Aβ protein precursor, variant	L	H	CNS
AαSyn	α-Synuclein	L	A	CNS
ATau	Tau	L	A	CNS
APrP	Prion protein, wild type	L	A	CJD, fatal insomnia
	Prion protein variants	L	H	CJD, GSS syndrome, fatal insomnia
ACal	Prion protein variant	S	H	PNS
	(Pro)calcitonin	L	A	C-cell thyroid tumors
AIAPP	Islet amyloid polypeptide**	L	A	Islets of Langerhans, insulinomas
AANF	Atrial natriuretic factor	L	A	Cardiac atria
APro	Prolactin	L	A	Pituitary prolactinomas, aging pituitary
AIns	Insulin	L	A	Iatrogenic, local injection
ASPC	Lung surfactant protein	L	A	Lung
AGal7	Galectin 7	L	A	Skin
ACor	Corneodesmosin	L	A	Cornified epithelia, hair follicles
AMed	Lactadherin	L	A	Senile aortic media
AKer	Kerato-epithelin	L	A	Cornea, hereditary
ALac	Lactoferrin	L	A	Cornea
AOAAP	Odontogenic ameloblastassociated protein	L	A	Odontogenic tumors
ASem1	Semenogelin 1	L	A	Vesicula seminalis
AEnf	Enfurvitide	L	A	Iatrogenic
ACatK	Cathepsin K	L	A	Iatrogenic Tumor associated

Table 2: Amyloid fibril proteins and their precursors in animals (Benson et al. 2018)

Fibril protein	Precursor protein	Systemic and/or localized	Affected organs or syndrome	Species
AL	Immunoglobulin Light Chain	S,L	Plasmacytoma	Cat, Horse
AA	(Apo) Serum Amyloid A	S	Chronic Inflammation or Infections	Many mammalian and avian species: Mouse, Cat, Cow, Dog, Duck, Guinea pig, etc.
AApoAI	Apolipoprotein AI	S	Age-related	Dog
AApoAII	Apolipoprotein AII	S	Age-related	Mouse
ATTR	Transthyretin	S	Age-related	Vervet monkey
AFib	Fibrinogen Aa	S	Spleen, Liver	Stone marten
Aβ	Aβ precursor protein	L	Age-related	Dog, Sheep, Wolverine
AIAPP	Islet Amyloid Polypeptide	L	Islets of Langerhans, Insulinoma	Apes, Cat, Racoon
AIns	Insulin	L	Islets of Langerhans	Octodon degus
ACas	A-S2C casein	L	Mammary gland	Cow

The entity of the precursors is diverse and unrelated. However apolipoproteins and polypeptide hormones seem to be over-represented amongst amyloid fibril proteins (Merlini and Westermark 2004). In spite of this variety, all of them produce amyloid fibrils with the common cross beta structure (Falk et al. 1997). Usually the amyloid fibrils are made up of only one type of protein precursor (Benson et al. 2018) and each one is associated with a specific clinical syndrome (Woldemeskel 2012).

In most instances, the precursor is a regular plasma protein which is occurring at an abnormally high concentration, e.g. serum amyloid A (SAA) (Merlini and Westermark 2004). While many amyloid forming proteins are known to have a notable proportion of beta-sheet structure, the preexistence in the precursor is not a requirement for amyloid formation (Johnson et al. 1996). Somehow an alpha-helix to beta-sheet conversion must take place in this case (Merlini and Westermark 2004). Johnson et al. (1997) proposed that for example incomplete degradation of proteins could lead to more amyloidogenic segments because of conformational changes, or because of a proportional shift in the beta sheet content in shorter peptide sequences.

Other precursors represent amyloidogenic mutants of regular plasma protein, like in some hereditary forms of amyloidosis (Gruys 2004). Here the secondary structure already contains beta sheets in a higher content.

1.3 AA Amyloidosis

Amyloid A protein (AA) amyloidosis is also known as secondary or inflammation associated amyloidosis. It occurs in species with persistently high plasma SAA concentrations over long time, caused by chronic inflammations, infections and neoplasia (Blancas-Mejía and Ramirez-Alvarado 2013).

In veterinary medicine AA amyloidosis is the most common encountered form of amyloidoses, with a characteristic deposition pattern predominantly in the central organs, such as the kidneys, liver, spleen, enteric mucosa and arterial walls, but in fact can be found in any organ except the brain (Blancas-Mejía and Ramirez-Alvarado 2013, Gruys 2004). Its nature is progressive and ultimately leads to organ failure followed by death (Gillmore et al. 2001). Systemic AA amyloidosis occurs sporadically in most species, since not all individuals with long-standing inflammations generate secondary amyloidosis. It is still not clear why only a minority of individuals with chronically elevated SAA blood levels develop AA amyloidosis (Johan et al. 1998).

A study showed that anti-inflammatory therapy which decreased SAA concentrations to physiological reference ranges, could lead to regression of amyloid in tissue and even to recovery of organ function of affected organs (Gillmore et al. 2001).

1.3.1 Amyloid Fibril

Although other components are present in the deposit, the amyloid fibril, made up by the precursor protein, is the main component of the amyloid substance (Woldemeskel 2012). The fibrils are non-branching and insoluble with a diameter of about 7.5−10 nm, irrespective of the type of amyloid, are of indefinite length (Cohen 1966, Cohen and Calkins 1959) and have a hydrophobic core (Li et al. 1999). Many of the main characteristics of amyloid depend on the molecular organization of the amyloid fibril proteins (Merlini and Westermark 2004).

The fibril itself is a polymeric structure. The protein monomers are held together by hydrogen bonds and fold to a very stable secondary beta sheet structure (Benson et al. 2018). Multiple b-sheets interact via side-chains (Serpell 2014). The beta sheets are perpendicularly arranged to the fibril axis and form long and thin protofilaments (Glenner 1980). In comparison to this, for example beta keratin is arranged in parallel beta sheets, where the beta sheets are ordered in the same direction as the fibrilar axis (Fändrich 2007). Two or more of these protofilaments, depending on the precursor protein, intertwine to form the actual amyloid fibril (Glenner 1980).

1.3.2 SAA

Serum amyloid A (SAA) is a family of~12 kDa proteins (104–112 amino acids) and is a sensitive acute phase reactant (Steel and Whitehead 1994) and an apolipoprotein of high-density lipoprotein (HDL) (Gruys et al. 2005). In 1985 it was proven that SAA is the actual precursor protein for AA amyloidosis (Husebekk et al. 1985).

The acute phase reaction (APR) is the first inflammatory response of the body and part of the innate immunity. During the acute phase reaction a cascade of pro-inflammatory cytokines is put in motion (Uhlar and Whitehead 1999) which results in systemic effects like fever and leukocytosis and further causes a reaction in the release of the so called acute phase proteins (APP) (Gabay and Kushner 1999). In case of an APR the concentration in plasma of some of these proteins decreases (negative APP) and of some increase (positive APP) (Ceron et al. 2005). Together with C-reactive protein (CPR) and others, SAA is one of the major positive APPs (Steel and Whitehead 1994).

Various infections, inflammation, trauma, immunologic reactions and cancer can activate the APR and therefore lead to an increase of SAA under the regulation of interleukin (IL)-1, IL-6 and tumor necrosis factor (Gaillard et al. 2018, Uhlar and Whitehead 1999). In human medicine rheumatoid arthritis, tuberculosis, familial Mediterranean fever and metastatic cancers are mentioned frequently as cause of AA amyloidosis (Blancas-Mejía and Ramirez-Alvarado 2013).
The degree of reaction to these insults vary in range from 10 to even 1000-fold increase of SAA during severe bacterial infections or flare-ups of chronic processes (De Buck et al. 2016).

The main source of SAA is the liver (Takahashi et al. 1985) but additional extra-hepatic local expression was described for different species at various sites (Upragarin et al. 2005).

After expression and release into the blood stream, most circulating SAA can be found in association with plasma HDL, and therefore it is considered to be an apolipoprotein of HDL (apoSAA) (Benditt and Eriksen 1977). During inflammatory conditions the proportion of apoSAA is increased by replacing apolipoprotein 1 and apolipoprotein 2, which are usually the main components in combination with HDL (Malle et al. 1993).

In physiological conditions SAA is removed from the circulation by hepatocytes within 12-24 hours. During an acute phase reaction the clearance is reduced by about 30 % (Gressner and Arndt 2019).

1.3.2.1 SAA Isoforms

SAA proteins are highly conserved through evolution (Gursky 2020). The only species known to be an exception are rats, due to incomplete gene sequences no functioning SAA exists in this species (Yu et al. 2000). The comparison of sequences of various species revealed strictly conserved regions within the SAA genes, which are believed to apply to all other mammalian species (Tamamoto et al. 2008).

Up to five SAA genes and corresponding protein isotypes are known in different species (Rygg et al. 1993).
In mice, two acute phase isoforms of SAA exist, murine SAA1 (mSAA1) and mSAA2 are both acute phase reactants, but protein AA only seems to be deriving from mSAA2 (Hoffman et al. 1984). Murine SAA3 is expressed in extrahepatic tissues by different cell types, mainly by macrophages and adipocytes, as a local response to injury and inflammation (Meek et al. 1992, Tannock et al. 2017). In humans for example, three isotypes of SAA are known. Human SAA1

(hSAA1) and hSAA2 are acute phase isotypes and therefore upregulated in the presence of inflammatory cytokines, whereas hSAA4 is produced consistently (De Buck et al. 2016).

Besides for mice, specific isoforms linked to the formation of AA amyloidosis were described for mink, hamsters, horses, cows and others (Gruys et al. 2005).

1.3.2.2 SAA Structure

Up to now four SAA structures resolved by X-ray crystallography have been published. Two of human SAA1 (hSAA1) (Lu et al. 2014) and two of murine SAA3 (mSAA3) (Derebe et al. 2014, Hu et al. 2019). For this measurements SAA is brought to a crystalline structure. This structure is made up by the protein monomers which assemble as dimers, trimers or hexamers for hSAA1 (Lu et al. 2014), while mSAA3 showed small differences and is only arranged in tetramers (Derebe et al. 2014). But regardless of the composition of the crystal structure, all of them showed a similar SAA monomer fold, therefore an evolutionary conservation of this fold is suggested (Hu et al. 2019).

The hSAA1 monomer structure as determined by Lu et al. (2014) is made up of four alpha helices arranged in a Y-shape. Most helical structures can be found from helix 1 (h1) to h3 in lipid bound and lipid-free state (Das and Gursky 2015). H1 and h3 make up the lipid binding site, with h3 being partially unstructured in the non-bound state but shifting to a helical structure when associated with lipids (Tanaka et al. 2017). Therefore HDL binding to this site prevents amyloid formation by blocking the amyloidogenic residues in h1-h3 (Das and Gursky 2015). The remaining SAA residues (70-104), the C-term, lack a specific secondary structure and, flexible wrapped around the alpha helix bundle, they seem to be maintaining the stability of the SAA structure (Das and Gursky 2015, Lu et al. 2014). A proteolytic cleavage of the C-term could thus lead to an aggregation prone SAA (Lu et al. 2014).

1.3.2.3 SAA Functions

As reviewed by Gursky (2020), multiple, even conflicting, functions have been reported for SAA so far, which seem to be dependent on the isoform, lipidation status, site of synthesis and other factors, such as the usage of recombinant hSAA1, in which certain amino acid sequences differ from regular hSAA1 (Gursky 2020, Sack 2018). To give an overview of its functions, SAA takes part in modulation of the specific and the innate immunity as well as in the lipid transport during inflammation (Zhang et al. 2019).

Non SAA-HDL has antioxidant and anti-inflammatory functions and plays a major role in the cholesterol efflux, where the cholesterol is transported either to the liver and excreted via the bile, or to the steroidogenic organs and used for the production of steroid hormones (Barter et al. 2004).
By reversible binding HDL (Tannock et al. 2017, Wilson et al. 2018), SAA is able to activate several HDL receptors that are responsible to bind modified lipoproteins (Kisilevsky and Manley 2012). By doing so, it can retain cellular cholesterol from the efflux for cell repair (Kisilevsky and Manley 2012) and impairs anti-inflammatory functions of HDL (Han et al. 2016). However, SAA is also capable of self-assembling into temporary SAA only lipoproteins (Gursky 2020). These seem to be able to encapsulate membrane lipids and incorporate them into nanoparticles and therefore play a role in the removal of cell membrane debris at the site of affection (Jayaraman et al. 2018).

Besides the hepatic expression of SAA, it is also secreted locally, especially by macrophages at sites of inflammation, where it stimulates cytokine production and attracts immune cells (De Buck et al. 2016).

Further functions of SAA amongst others are: the binding and transport of retinol, which regulates innate intestinal immunity (Derebe et al. 2014), activation of toll-like receptors for immune response (Ye and Sun 2015), binding and opsonising of gram-negative bacteria (Hari-Dass et al. 2005, Zheng et al. 2020) as well as antiviral activity against hepatitis-C virus (Lavie et al. 2006).

The different functions of SAA are routed in its ability to bind a multitude of ligands (Gursky 2020). Without going into too much detail, those can include cell receptors involved in host defence or lipid metabolism, lipids, small lipophilic molecules, basal membrane proteins, plasma proteins, bacterial outer membrane proteins, anion and cations as reviewed by Sack (2018) as well as Frame and Gursky (2016). Frame and Gursky (2016) proposed that this ability to bind this variety of ligands comes from a flexible conformation of SAA. Proteins with binding promiscuity have, according to Gursky (2020), disordered secondary or tertiary structures in the non-bound state, but as soon as they bind to a ligand they optimize their fold for individual interactions. This seems to be the case for SAA as well. Murine SAA1 is largely unfolded in solution at physiological pH and temperature when unlipidated, but as soon as it engages lipid binding the alpha helical proportion increases in dependence of nature and quantity of lipid (Jayaraman et al. 2015).

1.3.3 Amyloid formation

Protein AA is the N-terminal cleavage product of SAA (Johan et al. 1998). The formation of AA amyloidosis is a biphasic process with a long predeposition phase, the so called preamyloid or lag phase (Kisilevsky 1999). This phase involves the accumulation and aggregation of precursor proteins and it can take several days or up to years (Cui et al. 2002). Fibrillation, in terms of thermodynamics and kinetics, is a very unfavorable process, therefore a long lag phase is expected (Obici and Merlini 2012).

The second phase, the amyloid phase, takes place after an initial nucleation event, this seems to be the case for all amyloid types (Gajdusek 1994). At one point high amounts of precursor proteins lead to the formation of a nidus or seed onto which the elongation of the amyloid fibril takes place (Kisilevsky 1999). The rate of addition to this preformed seed fibril exceeds the formation of new fibrils through a nucleation event and therefore the aggregation is accelerated (Buell et al. 2014).

“It is now clear that its pathogenesis is multifactorial and influenced by many variables. These include the primary structure of the precursor protein, an acute phase response, the presence of non-fibril proteins (e.g. AP, apo E, GAGs and proteoglycans), receptors, lipid metabolism and proteases” (Röcken and Shakespeare 2002).

An involvement of cells from the reticuloendothelial system was postulated from early on (Smetana 1927). Especially different macrophage proteases were held accountable for taking part in the cleavage process of SAA to AA protein, most probably by cathepsin B (Claus et al. 2017, Kluve-Beckerman et al. 2002). It was proven that macrophages can internalise SAA (Kluve-Beckerman et al. 2001, Kluve-Beckerman et al. 2002) and amyloid fibrils were detected in macrophages in histological samples of amyloidogenic mice (Shirahama and Cohen 1975, Takahashi et al. 1989).

Today the lysosomal origin of AA amyloidosis has been solidified. After a series of in vitro investigations, Meinhardt (2017) concluded following steps in the pathogenic pathway of secondary AA amyloidosis. First, the internalisation of SAA in macrophages and monocytes leads to aggregation of SAA in lysosomes. This aggregation is followed by disintegration of the lysosomes and ultimately leads to cell death (Claus et al. 2017, Meinhardt 2017). Intracellular processed fibrils are released into the extracellular matrix and growth of the SAA derived amyloid fibrils takes place. Furthermore, Jayaraman et al. (2017) revealed that at about pH 4.3

mSAA1 forms stable proteolysis-resistant oligomers, that are able to disrupt lipid bilayers and shift to a lipid-induced beta-sheet conformation.

1.3.4 Minor components

Next to the main fibril protein other small non-fibril proteins are present in amyloid deposits. These small proteins are called minor components. Most of those components are normally circulating plasma proteins or a derivate of those (Real de Asúa et al. 2014). They seem to be closely associated with the fibril (Benson et al. 2018). The importance of all the different components and their part in the pathogenesis of amyloidosis has not been fully enlightened up to now. Particularly serum amyloid P-component (SAP), glygosaminoglycans (GAG), in this especially heparan sulfate proteoglycan (HSPG), and apolipoprotein E (ApoE) seem to be the most constant components in the different types of amyloid and more or less ubiquitous for the different animal species (Buxbaum 2009, Merlini and Westermark 2004). Heparan sulphate plays a key role in amyloid formation, as it dissociates SAA from HDL and therefore enables an incorporation into the cell (Noborn et al. 2012). In a transgenic murine study, a lack of heparan sulphate, leads to AA amyloidosis resistant mice(Li et al. 2005).

The role of SAP within amyloidosis is the stabilization of amyloid deposits (Real de Asúa et al. 2014). “The induction of reactive amyloidosis is retarded in mice with targeted deletion of the SAP gene” but not prevented by it (Botto et al. 1997). However, Miyazaki et al. (2020) showed that SAP may not be preset in feline amyloid deposits.

Apo E was detected in amyloid deposits in different animals including mice (Miyahara et al. 2018) and cats (Miyazaki et al. 2020). Therefore Miyazaki et al. (2020) proposed that apoE might be universal for all animal species. It has been shown to influence AA amyloidogenesis in experiments with knockout mice, where it accelerates the formation and deposition of AA amyloid but has no effect on the deposition pattern, and furthermore a lack of apoE does not prevent the formation of AA amyloid (Kindy and Rader 1998).

1.3.5 Induction, Seeding and Transmission

SAA protein synthesis is increased in vivo during the acute phase reaction in response to various challenges. In susceptible mouse strains and other animals, AA amyloidosis can be experimentally induced by repeatedly using inflammatory stimuli that cause a major increase in the SAA blood levels, like endotoxins, silver nitrate ($AgNO_3$), casein etc. (Uhlar and

Whitehead 1999). After a lag-phase of a few weeks AA amyloidosis develops in those animals. These inflammation inducible AA models were one of the earliest models and are widely used since the application is quite simple (Buxbaum 2009). Another used model for studying human AA amyloidosis uses transgenic mice, which express human IL-6 spontaneously, so the reintroduction of the inflammatory stimulus is unnecessary (Wall et al. 2008).

A transmissible principle, similar to that of prion diseases, has been discussed for AA amyloidosis (Lundmark et al. 2002).

The fact that the lag-phase can be shortened in experimentally induced murine AA amyloidosis by introduction of amyloid laden tissue from other mice was known since the 1960s. Since the exact mechanism or cause for this event was not clear at that time, it was called amyloid enhancing factor (AEF) (Axelrad et al. 1982). Lundmark et al. (2002) demonstrated that AEF is the AA amyloid fibril or a fragment of it itself. By using AEF from mice the lag time for the development of amyloidosis could be shortened from weeks to days in recipient mice (Glenner 1980). AEF is efficient at very high dilutions and doses as little as 15 pg protein (Lundmark et al. 2002) and can be received by injections or over the oral route (Glenner 1980). However, AEF is only able to trigger a AA amyloidosis in susceptible individuals, which means without sustained high SAA values, no AA amyloidosis will develop when using AEF alone (Cui et al. 2002).

As reviewed by Westermark et al. (2018) intraspecies transmission has been described for many animal species, for example hamsters, mink, domestic hen, and cheetah, to mention only a few.

Cui et al. (2002) demonstrated that murine AA amyloidosis was also accelerated not only by semi-purified amyloid fibril originated from mice but also by bovine AA fibrils and human AI fibrils. Further interspecies transmissibility was experimentally shown by Horiuchi et al. (2008) with Sore Hock affected rabbits and Liu et al. (2007), who showed that transmission of avian AA amyloidosis is not restricted to bird species, when swan derived AA fibrils were able to trigger murine AA amyloidosis in an experiment.

In addition to this, beta-sheet rich fibrils like silk or bacterial curli, as well as nanotechnically engineered beta-sheet rich material (Westermark and Westermark 2009) and other (synthetic) generated fibrils, with hight beta-sheet contents (Lundmark et al. 2005) can have amyloid-enhancing impact.

Up to date no non-experimental animal-to-human or *vice versa* transmission has been reported (Gursky 2020). However, if the conditions in a recipient are right, such an event could not be ruled out. In fact non-experimental horizontal transmission could be an answer to the question, why only a subset of individuals with longstanding inflammations develop AA amyloidosis.

For humans three possible routes were postulated by Westermark and Westermark (2009). First a transmission over blood transfusion could be possible, similar to TSE pathologies (Llewelyn et al. 2004). Sponarova et al. (2008) showed that spreading of AA amyloidosis throughout the body is likely to happen by seeding with preformed fibrils trough blood circulation. They showed that monocytes contain AEF and could act as a transport vessel and were able to induce AA amyloidosis in other susceptible recipients. In addition to this (Tasaki et al. 2010) postulated that they discovered cell-free circulating AEF in murine blood and plasma.

Second, by organ transplants (Westermark and Westermark 2008) and third through heterologous seeding by ingestion of food containing amyloid fibrils (Westermark and Westermark 2009). In animals heterologous seeding over feeding as well as horizontal transmission over faeces, as described for the cheetah (Zhang et al. 2008a) may be the most relevant possible transmission routes.

In several mammalian and bird species, especially in water fowl, AA amyloidosis is relatively common (Guo et al. 1996). Commercially available pate de foie gras was proven to contain amyloid fibrils and was able to accelerate the formation of AA amyloid fibrils in a mouse model (Solomon et al. 2007). Cooking of the foie gras merely led to a reduced AEF activity. Omoto et al. (2007) came to the same conclusion, AEF activity decreases after heat treatment and suggested, similar to prion diseases, autoclaving or chemical decontamination is necessary to fully eliminate AEF activity. Apart from this not only foie gras but also meat derived from other animals, for example sheep (Ménsua et al. 2003) and cattle (Tojo et al. 2005), may be a dietary source for seeding material.

1.3.6 Animals

In veterinary medicine, AA amyloidosis is the most frequent encountered form of amyloidoses in mammals and birds. In most species extracellular fibrils are deposited in liver and or kidneys as well as other organs, where they can lead to failure of them (Terio et al. 2008). The main mechanism of organ failure is induced by mass effect which results in a inhibition of blood

support (Merlini and Westermark 2004) but also some degree of toxic effect, as described for beta amyloid fibrils (Schubert et al. 1995), has been proposed for oligomeric pre-stages of AA protein.

AA amyloidosis has been described in many different domestic, captive and free-living species (reviewed by (Woldemeskel 2012). Although, most of the time sporadically occurring as a consequence of severe and longstanding inflammations, neoplasia and in few species idiopathic, some animal species seem to be particularly prone to the formation of AA amyloid deposits (Woldemeskel 2012).

In water fowl AA amyloidosis is quite common (Guo et al. 1996) and is often associated with chronic inflammatory conditions such as bumblefoot (Shinsuke et al. 2008).

A familial form of amyloidosis is known in Siamese and Abyssinian cats as well as Shar Pei dogs. In those breeds AA amyloidosis can develop in the absence of chronic inflammations. All of them have different specific amino acid sequence variations that result in more amyloidogenic forms of SAA and different deposition patterns of AA fibrils (Boyce et al. 1984, Johnson et al. 1995, Van der Linde-Sipman et al. 1997). While in Abyssinian cats and Shar Pei dogs a renal form of AA amyloidosis prevails, a deposition of AA fibrils in the liver is the most common finding in Siamese cats and other oriental breeds (Van der Linde-Sipman et al. 1997).

Considering non-domestic animal species, the cheetah is one to mention in connection with AA amyloidosis. In captive cheetahs the development of AA amyloidosis is often associated with chronic lympho-plasmatic gastritis (Johnson et al. 1997, Papendick et al. 1997). Furthermore, a mutation in the SAA promoter region, like in humans with rheumatoid arthritis, leads to exaggerated SAA transcription under inflammatory status (Zhang et al. 2008b). A combination of chronic stress with above mentioned risk factors make captive cheetahs particularly vulnerable for a development of AA amyloidosis (Terio et al. 2004). Chronic stress due to captivity, breeding or other causes may also be a risk factor for the development of AA amyloidosis in other animal species.

Genetic risk factors for AA amyloidosis have also been identified for humans and other animal species, like Dorcas gazelles and black footed cats and brown layer hens (reviewed by (Woldemeskel 2012). In brown layer-hen a deposition of amyloid fibrils into the joints are frequently encountered, while in white layer-chicken this deposition pattern is not seen at all (Murakami et al. 2014, Ovelgönne et al. 2001).

1.4 Research Objectives and Hypothesis

One animal order, in which secondary AA amyloidosis has rarely been described, is the order of Lagomorphs.

„The order Lagomorphs comprises about 90 living species, divided in 2 families: the pikas (Family *Ochotonidae*), and the rabbits, hares, and jackrabbits (Family *Leporidae*)", of which a quarter is listed as threatened (Fontanesi et al. 2016).

In central Europe one of the most common free ranging species is the European brown hare (*Lepus europaeus*) with a natural distribution from Great Britain and western Europe through to western Siberia and south western Asia (Hacklander and Schai-Braun 2019). It has been introduced as a game species in several other countries (Hacklander and Schai-Braun 2019).

"The European hare is the smallest mammalian species in Europe dwelling above ground or without shelter throughout the year" (Schai-Braun et al. 2015). Their juveniles, the leverets, are left behind indepressions in the ground and females only come by to feed the juveniles once or twice a day. " Hares are born fully furred and praecocial, are generally larger" and tend to be more solitary living than the European rabbit (*Oryctolagus cuniculus*) (Graham 2015).

All lagomorphs are herbivores. European hares are most frequently feeding on arable crops, due to their abundance but are selectively feeding on different types of weeds when available (Reichlin et al. 2006). In order to optimise the nutritional intake from plant material lagomorphs are hindgut fermenters and practice caecotrophy (Graham 2015).

Although listed as "least concerned" in the red list of the International Union for Conservation of Nature (Hacklander and Schai-Braun 2019) over the last 60 years a significant decline in their population has been noticed locally, leading to specific red listings as "near threatened" and "threatened" in countries like Austria, Switzerland, Germany and Norway (Reichlin et al. 2006). While climate change, predation and specific diseases might play a role in their population decline, intensification of agriculture was found to be the main cause for this phenomenon (Smith et al. 2005).

Most information on the lagomorph immune system has been gained from studies with the European rabbit (Pinheiro et al. 2016). This species is, next to the mouse, one of the most frequently used animals in biomedical research (Fontanesi et al. 2016).

Horiuchi et al. (2008) and Murakami et al. (2011) reported on the transmission of AA amyloidosis in so called sore hocks affected rabbits, where bovine AA fibrils were able to induce AA amyloidosis in rabbits.

Rabbit SAA is like the human SAA made up from 104 amino acids, and has the same conserved regions, as described in other animal species (Liepnieks et al. 1991).

However, while studies with rabbits might give us some insight, one must not forget that the European hare and the European rabbit are two different species. Only a few reports on pathological findings of AA amyloidosis in hares exist (Geisel and Linke 1988).

Furthermore, knowledge gained from studying a species under controlled laboratory conditions, cannot be simply translated onto a free-ranging species (Abolins et al. 2017). “In wild populations, individuals are regularly exposed to a wide range of pathogens. In this context, organisms must elicit and regulate effective immune responses to protect their health while avoiding immunopathology”, while in laboratory conditions the exposure to infections is strictly limited and the species are mostly inbred lines, that enable very controlled and specific studies (Pedersen and Babayan 2011). Semi-wild study species can be a compromise to get an insight into a wild population. Therefore in this study semi wild European hare were used as study objects.

In European brown hares, particularly the occurrence of AA amyloidosis has not been given much attention so far. But with hares being an important prey and a major game species (Fontanesi et al. 2016) and the possibility of horizontal transmission by meat and other animal product consumption (Solomon et al. 2007, Tojo et al. 2005) some awareness should be paid to that phenomenon.

“Understanding of disease prevalence, epidemiology and pathogenesis is a vital part of effective management of any population of animals, domestic or wild” (Papendick et al. 1997).

Posautz et al. (2016) were able to detect several cases of AA amyloidosis in free-ranging European brown hare, originating from parts of Austria and northern Germany. Interestingly incidence of AA amyloidosis varied considerably for the different populations.

In humans and several animal species, SAA is considered to be an inflammatory and diagnostic marker for different pathogens and pathologic conditions, as SAA is rapidly increasing after inflammatory stimulation (Sasaki et al. 2003, Witkowska-Piłaszewicz et al.

2019, Zhang et al. 2019). In order to find out if SAA could also be of use in diagnostics for AA amyloidosis in hares, the SAA values in the blood of European brown hares were looked at.

Therefore the serum levels of SAA were measured from 30 hares which have been treated in different ways during an attempt to experimentally induce systemic AA amyloidosis.

The hypothesis is that the treatment with AEF and silver nitrate will lead to an increase of SAA in the blood of hares.

Furthermore, although SAA might play a role in the development of systemic AA amyloidosis, the serum protein levels are assumed not to be an accurate indicator of disease status.

2 Material & Method

2.1 Study Layout

2.1.1 Hares

The study was performed on semi-wild European brown hares (*Lepus europaeus*), which were bred and raised at the Research Institute of Wildlife Ecology in Vienna (FIWI).

The Blood samples of these brown hares were collected during a previous experiment at the FIWI [GZ 68.205/0111-WF/V/3b/2016; ETK-28/04/2016]. Thirty hares were chosen for the experiment and divided into three groups, each consisting of ten individuals. It was attempted to have comparable groups regarding sex and age.

Besides group-specific treatment, all hares were kept under the same conditions and received the same fodder. Treatment and groups will be explained further below. Afterwards an ELISA was performed to measure the SAA concentrations in the blood of those hares.

Since, after the ELISA performance a few testing spots remained unused, the decision was made to look at additional blood samples from eight free ranging European brown hares with AA amyloidosis, which are not connected to the hares from the experiment.

The study was planned as previously described by Lundmark et al. (2002).

2.1.2 Blood sampling

Blood samples of each animal were taken four times during the 96 days of the experiment (Table 4). Blood was taken by a veterinarian from the jugular vein. The hares were mildly sedated and restrained by a second person. Serum sampling tubes were used. The serum was centrifuged immediately after collection and separated straight away and frozen at -80°C for further use.

2.1.3 Groups

The hares received different treatment in order to group affiliation. The general treatment plan is shown in Table 3. Ten animals were handled as control group, and further 20 received amyloid inducing measures over different routes and can be read about in detail hereinafter.

2.1.3.1 Group A

Group A served as the control group for this experiment. As such, no special treatments were implemented on this group (Table 3). Drinking water was provided *ad libitum.* Hares were kept under the same conditions in singularly occupied wooden boxes.

2.1.3.2 Group B

Amyloid enhancing factor (AEF) was added to the drinking water to promote the development of systemic amyloidosis (Table 3). The AEF was obtained using the protocol by Lundmark et al. (2002). In brief, AEF was obtained as followed: Spleen samples from AA amyloidosis affected hares were homogenised in 0.15 M NaCl and then centrifuged. The sediment was again homogenised with NaCl and centrifuged. This procedure was repeated four times with NaCl and successively two times with distilled water. The water was decanted from the tube and the pellet, rich in amyloid, was extracted. The presence of amyloidotic material was checked by making a smear, staining with Congo red and looking for the typical green birefringence under polarized light, as well as by western blot. The pellet was finally watered down to a concentration of 1 mg/ml of AEF within their drinking water and presented to the hares *ad libitum*.

2.1.3.3 Group C

This group, like group B, was supplied with the same amount of AEF within their drinking water. In addition, hares in this group received subcutaneous injections of 0.5 ml silver nitrate($AgNO_3$) (1 %, s. c., Silvernitrate, Sigma-Aldrich, St.Louis, Missouri, United States of America) at three times during the experiment to trigger an inflammatory reaction. $AgNO_3$ is proven to induce the development of systemic AA amyloidosis in mice e .g. (Axelrad et al. 1982) by elevating SAA levels and is frequently used in amyloidosis induction experiments (Gruys and Snel 1994). Silver nitrate was administered at different intervals before the blood collection, to control its influence on the SAA blood levels.

Actual timetable for management of blood collections and silver nitrate administrations, starting from day zero of the experiment, is shown in Table 4.

Table 3 general treatment plan for hares of group A, B and C

Group	Number of hares	Treatment		
		Blood collection	AEF p.o.	$AgNO^3$ s.c.
A	10	+	-	-
B	10	+	+	-
C	10	+	+	+

Table 4: Timetable of blood collections of group A, B and C and of administration of silver nitrate in group C in days starting from day zero

Day	0	23	47	48	77	85	96
Treatment	Blood collection	AgNO3 injection	AgNO3 injection	Blood collection	AgNO3 injection	Blood collection	Blood collection

2.1.3.4 free-ranging hare (Group D)

Besides the blood samples from these 30 hares, eight more blood samples from free-ranging European brown hares, which were sampled during annual hunts, were analysed. Blood samples were taken as soon as possible after death, latest 45minutes post mortem. Following an extensive pathological screening those hares showed signs of AA amyloidosis. The infection was confirmed through pathohistology, Congo red staining and Immunohistochemistry (IHC). Hares of this group were not part of the original experiment but could add interesting information of the SAA blood levels of actually diseased hares. Serum samples from these hares were also stored at -80 C.

2.2 ELISA

In order to measure/determine the concentration of SAA in the serum of the brown hares, a multispecies SAA ELISA was performed. The used ELISA kit is the PHASE™ range from Tridelta Development Ltd. It is a solid phase sandwich ELISA. In total, 128 samples in duplicates were analysed using four plates 96 wells plates.

For incubation and photometric analysis, a Thermo Fisher Scientific™ Multiskan™ GO Microplate Spectrophotometer was used in combination with the SkanIt™ Software Version 3.2 for Microplate Readers from Thermo Fisher Scientific™.

2.2.1 Principles of Sandwich-ELISA

Enzyme-linked Immunosorbent Assay (ELISA) is an immunological method to determine the presence of proteins, viruses, hormones, toxins, etc., i.e., it detects an antigen, with the help of a specific antibody, in different biological samples such as body fluids or tissues. Sandwich ELISAs use two different antibodies (a primary and a secondary antibody) to do so.

A solid phase, mostly a microtiter plate, is coated with the primary specific antibody. The sample, which contains antigen, together with a secondary specific enzyme-linked antibody are administered into the plate and are incubated for a specific time. Present antigen will be captured between the two specific antibodies. In the following step, non-bound material is washed out, and in a next step, substrate is added to trigger a colour reaction caused by the enzyme activity. The yielded colour is in direct proportion to the captured antigen. The concentration of antigen can be determined by photometric measurement and application of the Lambert-Beer law (Gressner and Arndt 2019). A series of concentrations of the standard antigen (or calibrator) are also measured photometrically and a calibration plot is set up which can then be used to determine the concentration of the antigen is the biological samples.

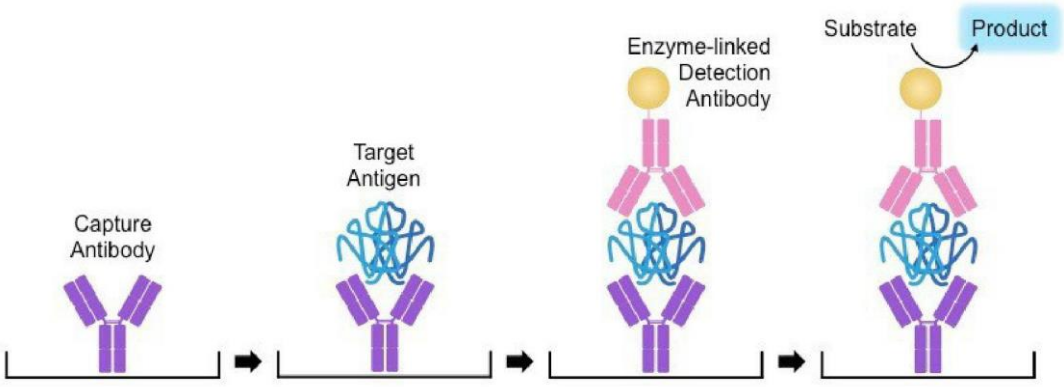

Figure 1: principle of sandwich ELISAhttps://ib.bioninja.com.au/_Media/elisa_med.jpeg (ELISA | BioNinja, accessed Apr 15, 2021)

2.2.2 Assay Preparation

The ELISA kit contained all necessary assay reagents. The sample/calibrator diluent as well as the wash buffer were provided in concentrated form and the required quantity had to be prepared according to the manual, by diluting them with distilled water.

The desired serum samples were taken out from the -80°C freezer and slowly thawed on ice. A small aliquot, depending on the dilution degree, was taken from the sampling tubes and diluted with sample diluent. The remaining serum was immediately refrozen at -80°C.

Figure 2 remaining thawed serum sample of hare A1

For the first test run, serum samples were diluted 500-fold, as was advised by the manufacturer of the kit. Thereafter 50, 100 and 200-fold dilutions were tried out.

The top calibrator was diluted with 1 ml of the prepared calibrator diluent, and was then further diluted 1:2 respectively to obtain the following four SAA calibrators. The sixth calibrator represented the zero calibrator and only contained calibrator diluent.

2.2.3 Assay Procedure

All the following steps were carried out as instructed by the manual. In short:

The number of 8-well strips needed for the experimental run was determined and attached to the microplate frame. Remaining unused wells were put back into the bag and stored in the refrigerator.

First step was pipetting 50 µl of ready-to-use Anti-SAA conjugate, which is the secondary antibody linked to Horseradish Peroxidase (HRP). Thereafter, 50 µl of the prepared calibrators or the samples, each in duplicates, were added. Whenever a new plate was used, a calibration curve was performed. If the remaining plate was used within 24 hours no new calibration was performed to economise space on the plates.

The plate was then put into the microplate reader to incubate at 37°C for one hour.

After the incubation, the content of the wells was drained and the plate was washed by pipetting 400 µl of the prepared wash buffer into each well four times successively. After the last wash, the plate was tapped dry on absorbent tissue paper to get rid of all remaining wash buffer.

100 µl of ready-to-use 3,3',5,5'-tetramethylbenzidine substrate (TMB) was then administered to all the wells. If SAA is present, TMB solution yields a blue colour. The intensity of the blue colour is in direct proportion to the concentration of SAA in the samples. The microplate was covered and left in a drawer to incubate for another 15 minutes at room temperature.

The last step before reading the microplate was to put another 100 µl of stop reagent, also ready-to-use, into all of the wells. By adding the stop reagent, the blue colour in the wells turned into yellow, which were then read at 450 nm by the use of a microplate reader.

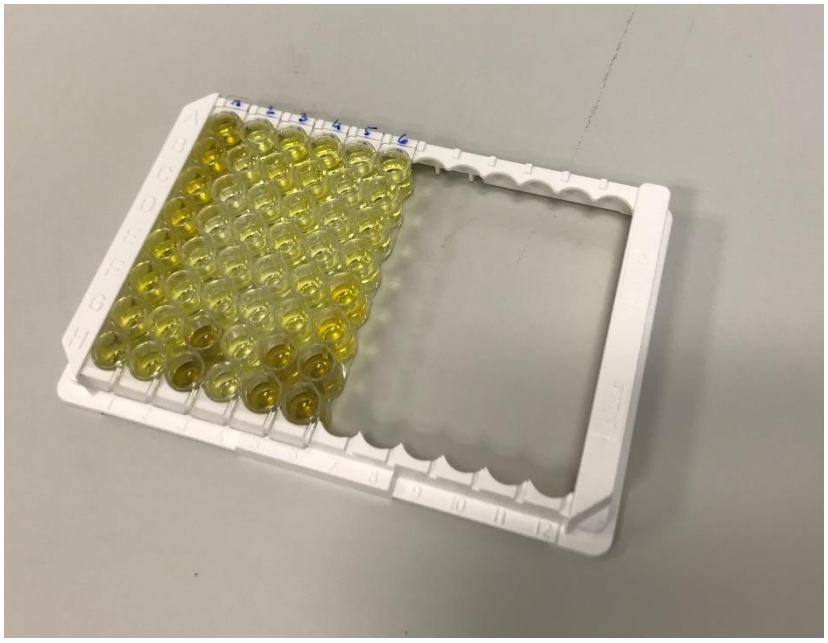

Figure 3. Microtiter plate after stop reagent was added; it is now ready to use in the microplate reader. The intensity of the yellow colour is in direct proportion to the SAA concentration.

2.2.4 Interpretation of Absorption Results

First, the mean absorbance for each sample and calibrators was calculated.

Then, a calibration curve was created by plotting the absorbances of the calibrators/standards against the concentrations of the standards given by the manual. Using Excel software, a best-fit curve was drawn through the standard/calibrator data points in order to create the calibration curve.

The SAA concentrations in the diluted experimental sample could then be determined from the calibration curve (using the equation of the best-fit curve). To obtain the SAA concentration in the original non-diluted sample, the concentrations had to be multiplied by the dilution factor.

2.3 Statistics

The sampling consisted of 30 individuals in three treatment groups each measured four times during the experiment. In total 120 samples from those hares were analysed in duplicates. Additionally eight samples of wild ranging European brown hares with confirmed Amyloidosis were analysed in duplicates as well. Yielding 256 measured SAA concentrations in total. Statistical analysis was carried out using the program *R (*Version 3.6.2).

2.3.1 Laboratory study

To determine differences in concentrations of SAA due to treatment between the groups A, B and C, the data was analysed by using a generalized additive model (or GAM). The use of GAMs allows to unravel both linear and non-linear relationships between the response variable and the explanatory variable. The mathematical expression of GAMs is conceptualised in Equation 1 for a single response variable (*Y*).

$$g^{-1}[E(Y|\boldsymbol{X})] = \beta_0 + \sum_{j=1}^{k} f_j(X_j) + \varepsilon$$

Equation 1

With g^{-1} the inverse link function, Y the response variable, $E(Y|\boldsymbol{X})$ the expected distribution of Y conditional to the set of predictors ($\boldsymbol{X} = [X_1, X_2, \dots, X_k]^T$), X_j the jth predictor (out of k predictors), β_0 the intercept, f_j the smoothed function related to the predictor X_j and ε the remaining error.

In this regard, it is useful to note that GAM is variation of a generalized linear model (GLM), in which the explanatory variable is smoothed by smoothing functions. The linear combination of these functions is linked to the response variable, making GAMs able to deal with non-linear, non-monotonic relationships between the explanatory and response variables (Guisan *et al.*, 2002). More information on GLMs and related extensions (e.g. generalised additive models (GAMs), generalised linear mixed models (GLMMs), generalised additive mixed models (GAMMs)) can be found in Zuur *et al.* (2009).

To develop the GAM, the following steps were taken:

1. The response variable (SAA) was log(x+1)-transformed
2. A group-specific intercept was considered appropriate (thus, fixed effect)
3. The response variable (SAA) varies with time, being group-specific (thus, smoothed fixed effect)
4. Individual hares can introduce random variability (thus, random effect)
5. Sex of the hares can introduce random variability (thus, random effect)

As such, the following input was used in R (package *mgcv*):

```
m = gam(log(Value+1)~ Group +
                        s(Day,by = Group,k = 4) +
```

```
s(Hare_id,bs="re")+
s(sex,bs="re"),data = dat)
```

With *Value* the concentration of SAA in the original blood sample, *Group* the three different treatments (group A, B and C), *Day* the sampling day, *Hare_id* the identity of the hare, *repeat.* the sample duplicate and *dat* the overall dataset.

Considering the setup of this study, the following results are expected under the null hypothesis (H_0*: There is no difference between the three groups*):

1. There is no significant difference in intercept between the three groups
2. A similar *edf* (*empirical degrees of freedom*) and significance score for the smoother, regardless of the group

Under the alternative hypothesis (H_a*: There is a difference between the three groups*), a difference in (1) intercept or (2) smoother is expected.

2.3.2 Field data

To compare group A, B and C to the fourth group D, which were the hares with infested amyloidosis, only the blood samples from the final blood collection (day 96) were compared to the samples of group D.
For this group comparison the Kruskal-Wallis test or H-test was performed (Kruskal and Wallis 1952).

The Kruskal-Wallis test is a non-parametric method and can be seen as the extension of the Mann-Whitney U test, which can only compare two groups. It is also called one-way analysis of variance (ANOVA) on ranks. In contrast to ANOVA, which can only be performed on normally distributed parametric data, a normal distribution of the residuals is not required (Weaver et al. 2017).

Following steps have to be made to be able to perform the H-test (Universität Zürich 2021):

1. All groups will be ranked concerning one response variable and overlooking all group affiliation, from 1-*N*.
2. After that, the ranking will be transferred into the different groups

3. If the same value appears more than once, so called tied ranks, the medians of those ranks will be assigned to the tied ranks instead and the H statistic will be corrected.
4. The sum of the ranks of each group will then be calculated and can be used for the calculation of the H-test, which reads as follows:

$$H = (N-1)\frac{\sum_{i=1}^{g} \boldsymbol{n_i}\left(\bar{\boldsymbol{r}}_{\boldsymbol{j.}} - \bar{\boldsymbol{r}}\right)^2}{\sum_{i=1}^{g}\sum_{j=1}^{n_j}(\boldsymbol{r_{ij}} - \bar{\boldsymbol{r}})^2}$$

Equation 2

$$H = \frac{12}{N(N+1)}\sum_{i=1}^{k}\frac{R_j^2}{n_i} - 3(N+1)$$

Equation 3 H-test without tied ranks

R_j= sum of the ranks, N= sample size, n_i= group size, k= number of groups

Degree of freedome df= k-1

5. In the end, to test the null hypothesis, H will be compared to a critical value H_c, which is dependent on the significance level and the degree of freedom and can be looked at in corresponding tables or softwares.

The following R notation has been used for the Kruskal-wallis test:

```
kruskal.test(Value~Group,data = df.dat)
```

This test will be able to tell if there is a statistical significant difference between the groups, yet cannot determine which and how many groups differ from each other. When performing the Kruskal-Wallis test following null hypothesis can be formulated:

H_0: There is no significant difference between the groups

Consecutive alternative hypothesis can be expected, H_a: There is at least one group differing from the other groups.

After a significant difference between groups has been observed, a Dunn's test, which is a post-hoc test for pairwise comparison of groups, has to be performed, to identify one or more differing groups (Glen 2017). More detailed information about the Dunn's test can be found in Dunn (1961).

For the Dunn's test with automated Benjamini-Hochberg correction for multiple testing, following input was used on R (package dunn.test):

```
dunn.test(df.dat$Value,g = df.dat$Group,method = 'bh')
```

3 Results

3.1 Group A

Since up to date no reference values for SAA in hares exist, an experiment specific reference range has been set up. All measured values from group A were considered into the estimated reference range. The boxplot (Figure 5) indicated at least three outliers, therefor it was chosen to define the maximum working reference value as mean plus two times the standard deviation, and rounded this value up to 25 mg/L. Hence, the specific reference range for this work extends from 0 mg/L to 25 mg/L.

SAA concentrations in Group A ranged from 0.5 mg/L to 35.57 mg/L (mean ± SD = 6.09 ± 9.21 mg/L; N = 40). No trend over time could be observed. In view of the reference range, SAA values higher than 25 mg/L can be considered as raised. Three raised values have been observed in group A. Hares A2 as well as A6 had higher SAA blood concentration during the first and A10 had raised SAA levels during the last blood sampling.

Table 5 Group A hares: individual hares and their SAA blood levels from multispecies ELISA in mg/L for all four blood takings

Group	hare	Day0 [mg/L]	Day47 [mg/L]	Day85 [mg/L]	Day96 [mg/L]
A	1	2.76	0.81	0.65	0.41
	2	33.87	7.41	5.65	10.56
	3	1.83	18.72	5.50	6.58
	4	0.77	1.04	0.35	0.58
	5	0.53	3.38	2.20	1.39
	6	30.85	3.75	7.96	8.49
	7	2.24	4.05	4.05	23.02
	8	1.77	3.66	1.49	4.08
	9	0.87	3.50	0.75	0.41
	10	0.04	1.79	0.11	35.57

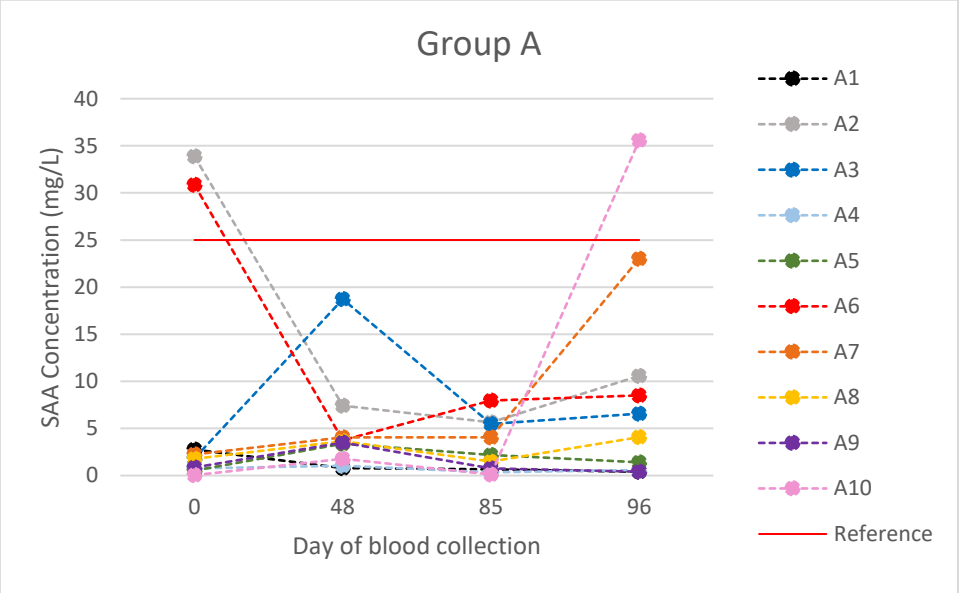

Figure 4 individual hares from group A (untreated) and the trend of their SAA values over the course of all blood takings

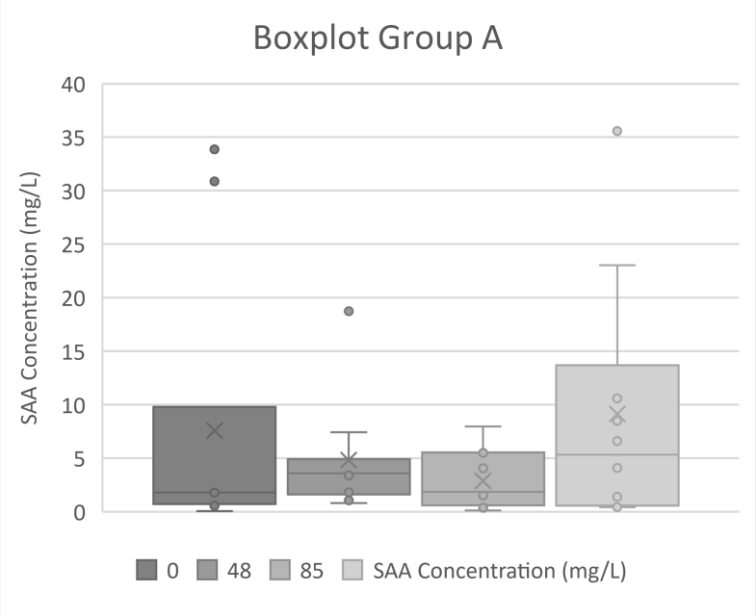

Figure 5 Boxplot of SAA concentrations in the blood of hares from four different timepoints, group A (untreated)

3.2 Group B

Measured SAA blood levels in group B ranged from 0.22 mg/L to 35.39 mg/L (mean ± SD = 6.36 ± 8.68 mg/L; N = 40). In this group, four SAA values were outside the reference range. Hare B5 had a SAA value higher than the maximum reference during the first and second sampling. Hare B2 as well as hare B4 had raised SAA values during the second blood collection.

Table 6 Group B hares: individual hares and their SAA blood levels from multispecies ELISA in mg/L for all four blood takings

Group	hare	Day0 [mg/L]	Day47 [mg/L]	Day85 [mg/L]	Day96 [mg/L]
B	1	0.68	0.44	0.83	0.22
	2	4.87	35.39	1.47	5.92
	3	1.30	4.29	4.74	0.71
	4	3.27	34.37	2.92	3.60
	5	27.35	25.20	13.39	7.07
	6	4.21	2.05	4.85	3.30
	7	1.52	6.37	3.90	1.57
	8	6.60	5.77	4.76	5.38
	9	3.49	6.82	3.13	3.19
	10	6.50	0.22	1.58	1.03

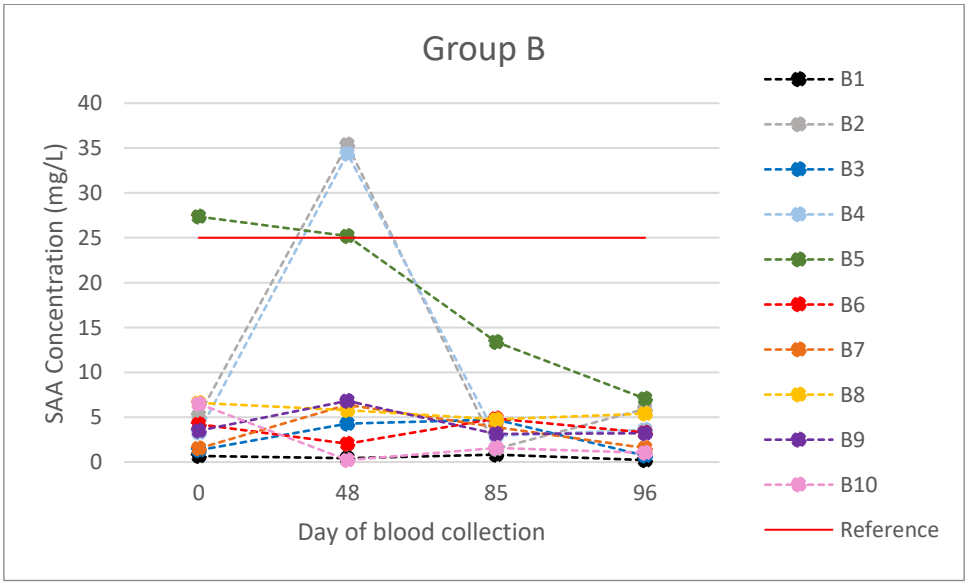

Figure 6 individual hares from group B (AEF) and the trend of their SAA values over the course of all four blood takings

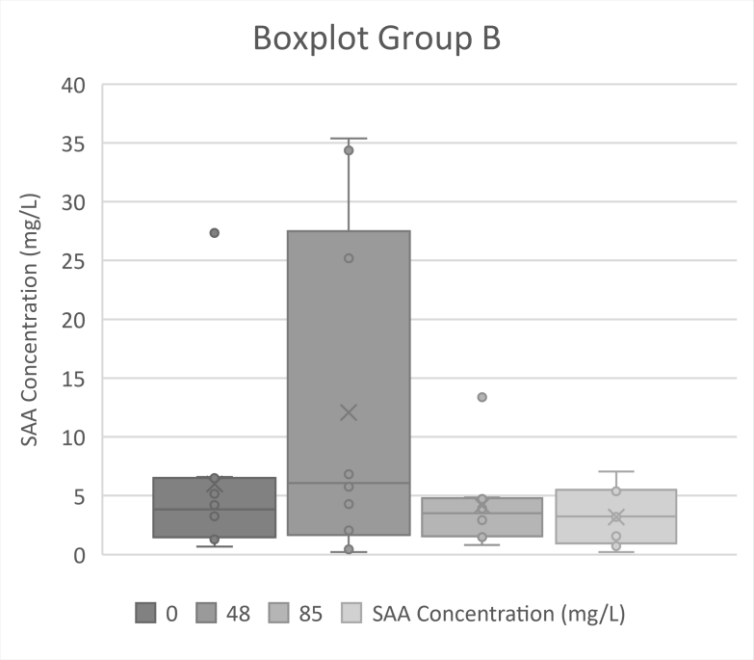

Figure 7 Boxplot of SAA Concentrations in the blood of hares from four different timepoints, group B (AEF)

3.3 Group C

In group C the SAA blood concentrations from all four time points ranged from 0.33 mg/L to 70.12 mg/L (mean ± SD = 15.78 ± 19.10 mg/L; N = 40) (Table 7). When comparing to the setup reference value, following samples can be described as raised. Hare C5 had elevated SAA blood levels at the beginning of the experiment. At day 47 of the experiment, which was one day after the $AgNO_3$ injection, all hares except for C6 had SAA concentrations above the reference range, and all, except hare C5, had their peaking concentration during this blood collection. Only one hare (C4) showed increased levels of SAA during the last blood collection. Two hares stood out to the rest of the group. Hare C4, as already mentioned above, had raised SAA values throughout all blood collections. Hare C6 had relatively low SAA concentrations throughout all four blood collections. However C6 still had its peak concentration at day 47 of the experiment.

Table 7 Group C hares : individual hares and their SAA blood levels from multispecies ELISA in mg/L for all four blood takings

Group	hare	Day0 [mg/L]	Day48 [mg/L]	Day85 [mg/L]	Day96 [mg/L]
C	1	2.06	33.55	14.39	8.25
	2	2.61	29.36	2.39	1.58
	3	1.20	68.78	19.44	2.39
	4	23.28	70.12	14.89	34.25
	5	52.97	35.03	3.11	2.03
	6	0.49	6.73	0.37	0.33
	7	0.53	37.65	2.54	0.90
	8	16.46	36.81	6.70	6.43
	9	3.26	36.55	10.84	4.57
	10	1.23	34.94	0.97	1.23
mean		**10.41**	**38.95**	**7.57**	**6.20**

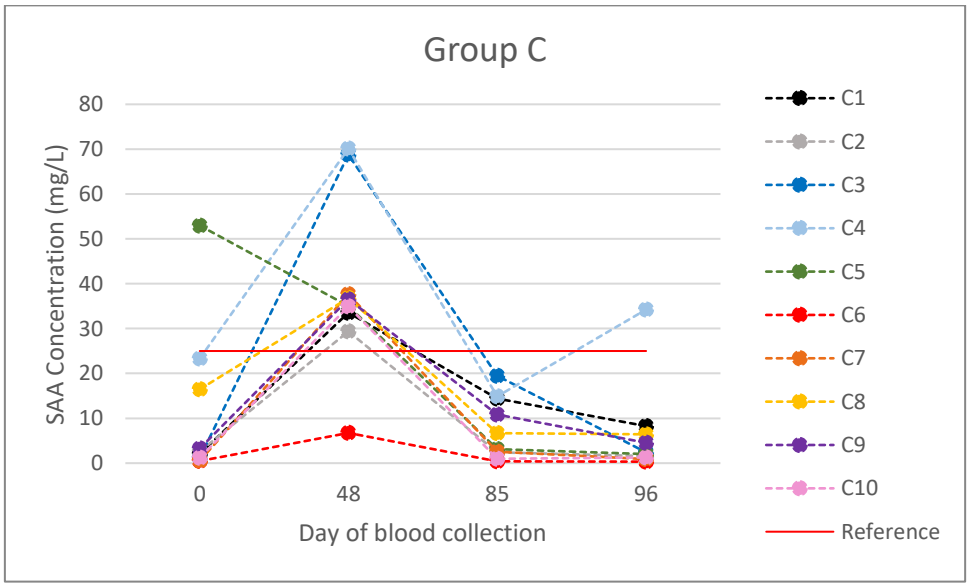

Figure 8 individual hares from group C (AEF + $AgNO_3$) and the trend of their SAA values over the course of all four blood takings

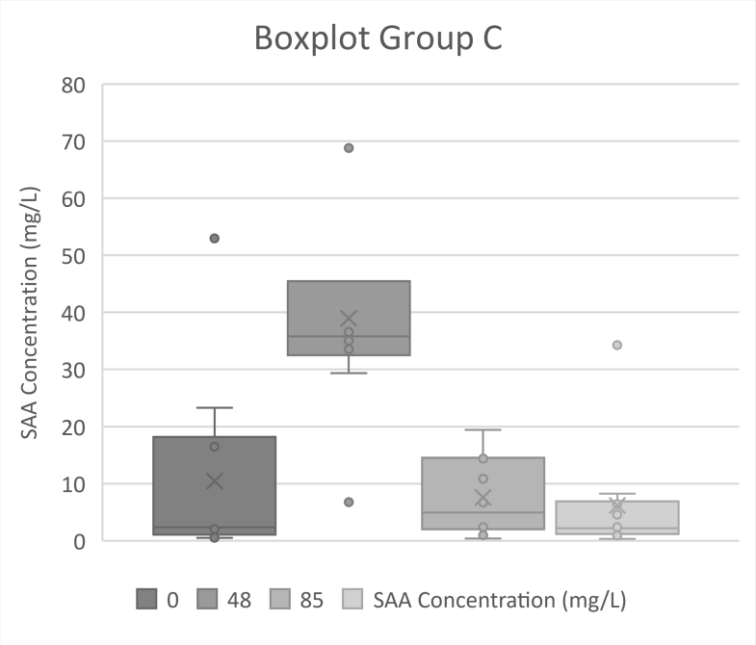

Figure 9 Boxplot of SAA Concentrations in the blood of hares from four different timepoints, treatment Group C (AEF and $AgNO_3$)

3.4 Comparison of treatments

The treatment-specific intercepts showed to differ significantly from 0, with similar values for Group A and Group B. The intercept for Group C was significantly higher than the baseline intercept (Table 8). The applied smoothing yields an almost horizontal line (*edf* ≈ 1) for groups A and B, which is visible in Figure 10. For Group C a significant smoothing occurs that differs from a simple linear approach (Table 9).

The identified random effects (*Hare_id* and *Sex*) were both not significant (*p*> 0.05; Table 9).

Table 8: Treatment-specific intercepts. All intercepts differ significantly from 0, with Group C showing the highest specific intercept (and being significantly different from Group A and Group B).

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	3.38014	0.09709	34.813	<2e-16	***
GroupB	0.16607	0.14177	1.171	0.2439	
GroupC	0.44366	0.17249	2.572	0.0114	*

Table 9: Treatment-specific intercepts. All intercepts differ significantly from 0, with Group C showing the highest specific intercept (and being significantly different from Group A and Group B).

	edf	Ref.df	F	p-value	
s(Day):GroupA	1.00000	1.000	0.267	0.606045	
s(Day):GroupB	1.05531	1.108	0.554	0.437419	
s(Day):GroupC	2.19423	2.496	7.508	0.000266	***
s(Ind2)	0.09423	1.000	0.104	0.295576	
s(Sex)	0.01237	1.000	0.013	0.316178	

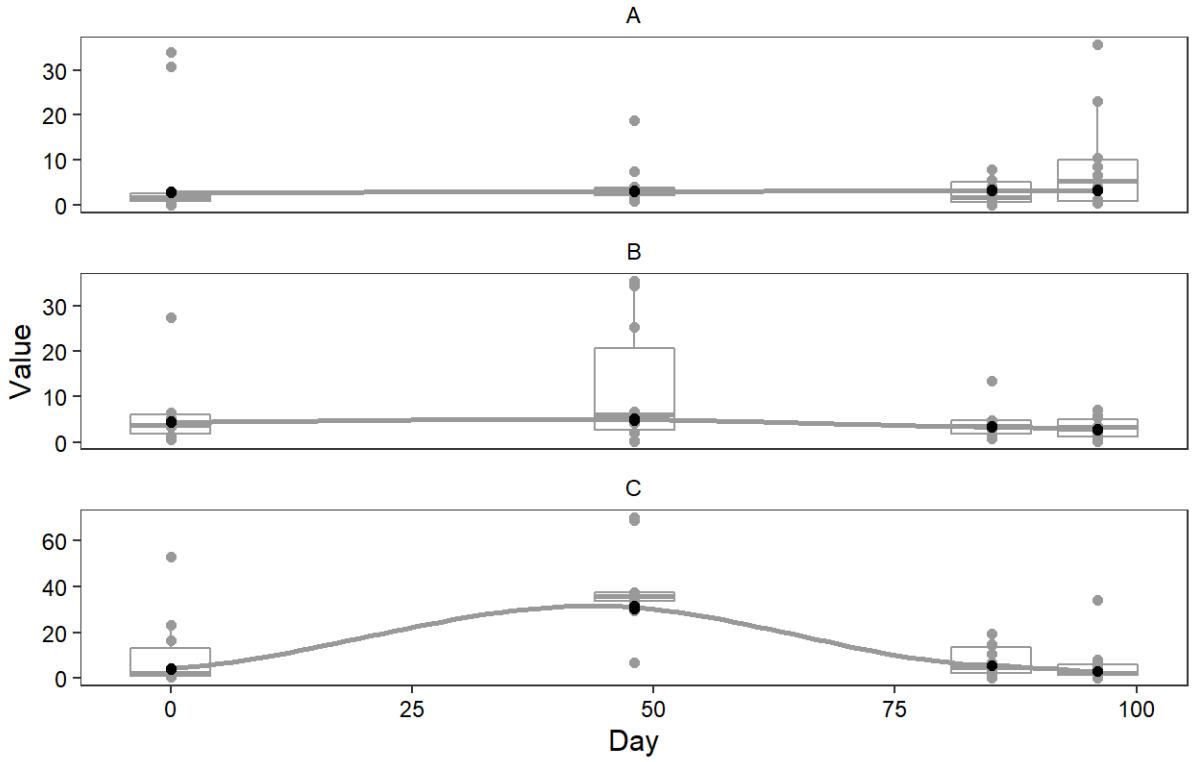

Figure 10: Treatment-specific responses. Observations (boxplots) versus Predictions (line). The predictions are based on a GAMM with time and group as fixed effects, while the individual hare and the sex were random effects.

Analysis of the residuals showed the absence of remaining patterns in function of the fitted values and the QQ-plot provided a satisfactory result (Figure 11). No perfect normal distribution of the residuals was obtained, as the majority of the residuals was slightly higher than zero. This pattern is also visible in the effect-specific analyses, showing a generally acceptable distribution of residuals around zero for the different groups and days. For Day 55 most residuals seem to be higher than zero (Figure 11). The explanation for this pattern is twofold: (1) within Group B several observations showed very high values, yet they hardly impacted the smoother and (2) within Group C the predictions from the model are lower than most

observations, caused by the limitations of the selected hyperparameters for the smoother (Figure 10).

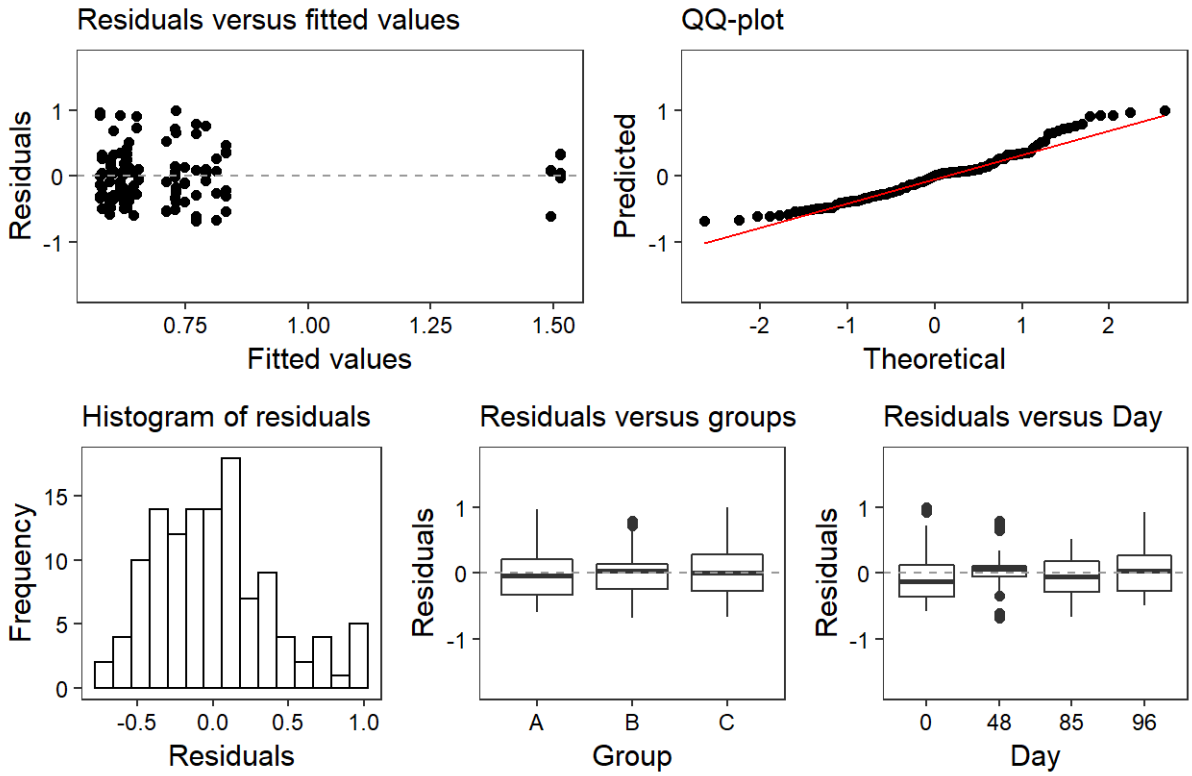

Figure 11: Analysis of the residuals resulting from the GAMM depicted in Figure 10. Residuals showed a random distribution around zero in function of the fitted values. Also effect-specific distributions of the residuals (second row) showed to be relatively well distributed around zero. Only for 'Day 55' most residuals were higher than zero, which is linked to the few high values in Group B and the underprediction of Group C (see also Figure 10).

3.5 Free-ranging hares (Group D)

This group has no connection with the three previous groups. All eight brown hares were free ranging wild hares. All of them had shown AA amyloidosis in pathohistological examinations and were therefore chosen for additional SAA quantification.

Measured SAA concentrations in group D ranged from 11.75 mg/L to 34.88 mg/L (mean ± SD = 24.68 ± 8.86 mg/L; N = 8). Four hares (D3, D4, D7 and D8) had values outside the set up reference range.

Table 10 SAA concentrations in the blood of free ranging brown hares affected by AA amyloidosis in mg/L

Group	hare	SAA [mg/L]
D	1	11.75
	2	22.95
	3	34.67
	4	30.64
	5	14.41
	6	19.99
	7	34.88
	8	28.16

3.6 Comparison with hares from the experiment under controlled conditions

A significant difference can be observed with the three groups of hares from previous experiment (Figure 12), which was confirmed by a Kruskal-Wallis and post-hoc Dunn test with Benjamini-Hochberg correction (Table 11). Group D showed significantly higher levels of SAA compared with the other three groups, which showed similar levels.

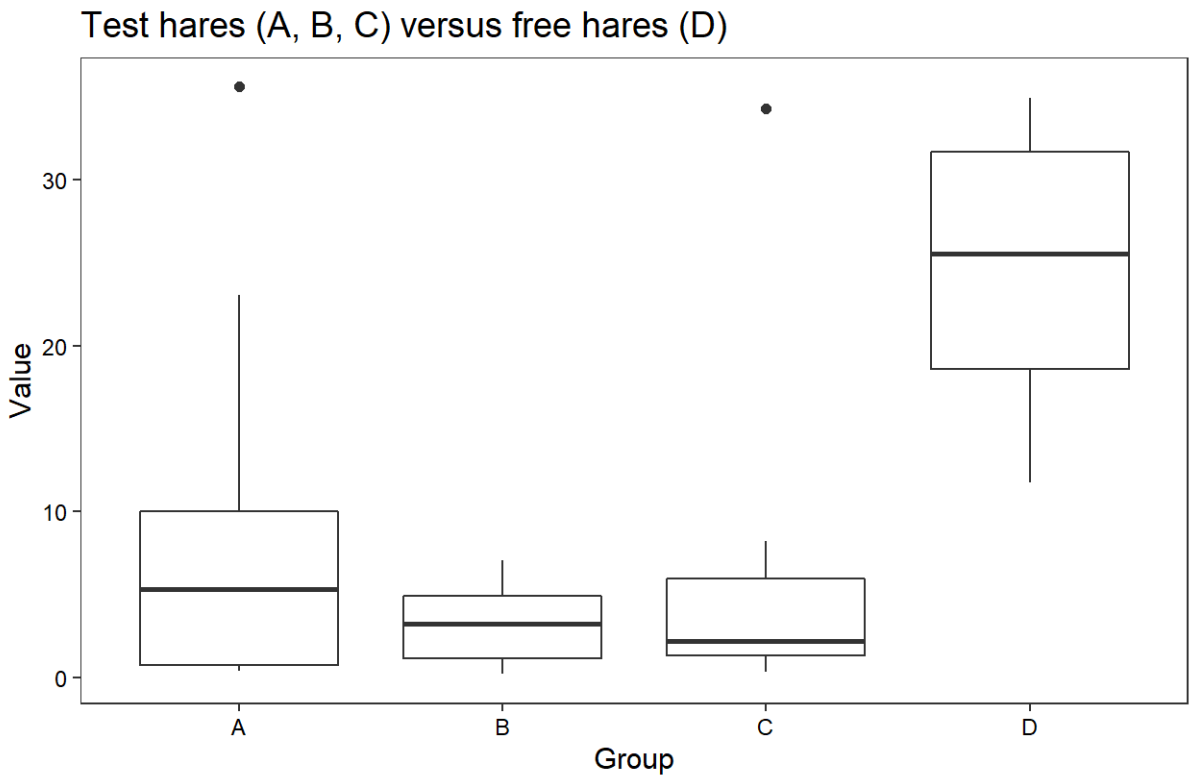

Figure 12: Observations of free hares (Group D) in contrast to measurements in hares under semi-controlled conditions (Group A, B and C; see previous section). The data suggest a clear difference between group D and the other groups.

Table 11: Results from the post-hoc Dunn test with Benjamini-Hochberg correction indicating the differences between the groups of hares. Group D showed to be significantly different from Group A, Group B and Group C.

Col Mean-| Row Mean	A	B	C
B	0.945691		
	0.2582		
C	0.563390	-0.382300	
	0.3439	0.3511	
D	-2.589452	-3.481058	-3.120621
	0.0096*	0.0015*	0.0027*

4 Discussion

“Domestic and wild lagomorphs, especially rabbits and hares, are important from an economic, ecological and public health point of view. Both rabbits and hares are susceptible to a wide variety of pathological disorders, so that the knowledge of the different risk factors, causes of death or disease and prevalence rates is relevant from a health, economic and welfare perspective” (Espinosa et al. 2020)

The aim of the study was to contribute to this knowledge. AA Amyloidosis and its development in free-ranging European brown hare has rarely been reported. During health screening of different hare populations in Austria and Germany, highly varying incidences of AA amyloidosis were noted between these populations (Posautz et al. 2016). Questions about transmissibility and the role of SAA in the development of AA amyloidosis in European brown hare have arisen.

With this study, we give a first reference point of SAA values in European brown hares. Due to the small sample size, the reference value is of course expected to be a rough assessment of the real status. International recommendations are to consider at least 120 reference values into the estimation of reference values (Geffré et al. 2009). In order to create proper reference values, the blood and health status of more hares should be considered and further research in this direction is needed. While the diagnostic potential of SAA is known for cats, dogs and horses, it is used as an inflammatory and a diagnostic marker for several diseases in these animals (Christensen et al. 2012, Eckersall and Bell 2010, Zhang et al. 2019) the same cannot be stated for hares. In search for hare SAA levels, experimental SAA comparison values have been found for a few rabbit species (Argente et al. 2014, Cray et al. 2013, Lennox et al. 2020). Since hares and rabbits are not the same species (they differ immensely in their lifestyle and upbringing of the juveniles), those reference values cannot be compared.

Furthermore the study gave a first insight into how SAA in brown hares reacts to inflammatory stimulation with s.c. injection of silver nitrate, which acts as a potent inflammatory agent, and how it responds to oral administration of AEF.

It is expected that the three treatment groups resulted in different responses. The generalised additive mixed model (GAMM) that links the measured value (response value) with a temporal component and a specific treatment approached the observed data relatively well (Figure 11). Still, only 23% of the deviance was explained by the model, which is linked to the high variability in obtained values on each specific time point (i. e. range of the boxplots).

The identified random effects (*Hare_id* and *Sex*) attributed to the explained deviance of the model but were not significant ($p > 0.05$; Table 9). This is in accordance to observations of Yamada et al. (1989). Both random effects were retained in the final model, but are not discussed further in order to provide a stronger focus on the fixed effects and the differences between the selected treatments.

Our study showed no evidence that SAA blood levels are influenced by oral intake of AEF in brown hares. The SAA levels in hares from group B, which continuously received AEF orally, did not differ from hares from group A, the control group. The treatment-specific intercepts showed similar values for Group A and Group B (Table 8).The similarity in intercept between Group A and Group B is not surprising when considering the applied smoothing, which indicates that for groups A and B an almost horizontal line is obtained ($edf \approx 1$) visible in Figure 10.

Possible causes for SAA being unresponsive to oral AEF administering in Brown hares could be, that the concentration of AEF received orally was not high enough, that AEF might not be effective over the oral route in this animal, or that AEF does not influence SAA blood levels at all in this species, regardless the dose and the way of application.

While no data for hares are available yet, the introduction of AA amyloidosis orally by AEF or other amyloid containing material is proven for some animal species, as cheetah, white hen and mouse (Cui et al. 2002, Murakami et al. 2013, Zhang et al. 2008a). AEF is proven to be effective at very minimal doses, in fact for intravenously administered AEF the biological effect does not seem to be dose dependent (Lundmark et al. 2002), and works by shortening the lag phase and promoting the development of AA amyloidosis (Liu et al. 2007). Raised SAA blood levels are usually described in combination with inflammatory stimulation. Therefore it is not surprising that the SAA values from group B, where hares had access to AEF within their drinking water, did not diverge from those in group A. While the finding of this study seems to be in accordance with most amyloid introduction studies (Lundmark et al. 2005), Brissette et al. (1989) postulated that in mice circulating SAA rapidly increased after intravenous AEF injections.

High levels of SAA over a long period of time are a prerequisite for the development of AA amyloidosis (Obici et al. 2009). Even then, only a subset of animals develop amyloid deposits. Transmission of AA amyloidosis in brown hare by ingestion of AEF or preformed amyloid fibrils,

without additional underlying inflammatory conditions, is therefore very unlikely to occur in field conditions.

Next to other inflammatory stimulants, silver nitrate ($AgNO_3$) is used in AA Amyloidosis introduction experiments and studies to investigate the mechanism involved (Brissette et al. 1989, Glojnaric et al. 2007). Hares from group C received s. c. injections with $AgNO_3$ and were exposed to AEF through their drinking water.

The SAA values in group C were significantly higher than measured values in group A and B (Table 8). While in group A two SAA values out of 40 measurements were raised (3/40) as well as in group B two out of 40 (4/40), in group C it were twelve out of 40 (11/40). Most of this raised SAA concentrations can be seen during the second blood collection (Day 47), which took place 24 hours after the first s.c. injection of silver nitrate and shows the inflammatory potential of $AgNO_3$. Eight out of ten hares in group C had elevated SAA concentrations during the second blood taking. While hare C2 was just under the reference maximum, C8 has a distinctly lower SAA level than the other hares from this group and furthermore had very low detected levels of SAA throughout the experiment.

During or after administering inflammatory stimulation, for example silver nitrate, LPS or casein, 100 to 1000 times increases of SAA levels in different animal species are reported (De Buck et al. 2016, Jensen and Whitehead 1998, Sponarova et al. 2008). SAA levels in this experiment reacted after $AgNO_3$ injections but changes to the former described extent, based on our experimental reference value, were not observed. Considering the mean SAA level in the control group was 6.09 mg/L (mean of the reference value equals 4.58 mg/L) and the mean value measured after the second blood collection in group C was 38.95 mg/L (the highest measured SAA concentration was 70.12 mg/L), the increasing factor is around six. Even when comparing the mean SAA level of the reference value with the highest SAA level measured, only a factor of 15 times is reached. This stands in contrast to factors of 100 or even 1000 mentioned for humans or other animal species after infections or experimental stimulation (De Buck et al. 2016, Jensen and Whitehead 1998, Sponarova et al. 2008).

One considerable uncertainty, which must be mentioned in this context, is that no further testing on the fit of the multispecies ELISA was performed. The control of the chosen immunoassay was left out, due to the restricted space on the microtiter plates. The following propositions are based on accurate fit of ELISA and hare samples in general and the high sample concentrations specifically.

What follows could be reasons for the moderate increase in SAA blood concentrations reached in this experiment:

a) Compared to the reference values of SAA in other animal species, the set up reference value is quite broad. Other animals have lower base levels of SAA (Moritz 2014) with reference values slightly differing from sources to source and by method. For humans for example roughly under 10 mg/L is a widely spread standard (Gressner and Arndt 2019).
b) A missed concentration peak, for mice peaks of 20 h (Brissette et al. 1989) to 24 h (Glojnaric et al. 2007) can be found, and or relatively quick clearance of SAA from circulation, could be possible. SAA half-life span is only around 90 minutes in mice (Tape and Kisilevsky 1990).
c) SAA might not be a major acute phase reactant in the European brown hare and therefore peaking levels cannot be expected to rise that high. SAA is described a major APP for most animal species, except the chicken and Nonhuman primates (Cray 2012), therefore this is not expected to be the case for hares.
d) The hares used in this experiment were semi-wild bred hares, and hence were not bred and raised in sterile and controlled laboratory conditions. The immune system of wild animals is used to a wide range of stressors (Abolins et al. 2017). Maybe higher concentrations of $AgNO_3$ or other more potent inflammatory stimulants would be necessary to reach severe increases in SAA levels in hares.Rygg et al. (1993) reported, though not significant, SAA mRNA levels in rabbits vary with different inflammatory stimulants.

In addition to the SAA level measurement, the hares were dissected by Posautz et al. (2017, unpublished). While none of the animals from group B developed amyloidosis, higher levels of SAA were detected a few times. Some elevated levels could be explained by pathological findings. In other hares, in spite of pathological lesions, the SAA levels remained within the reference range. In that case, the severeness of these findings may not have been sufficient enough to cast systematic immune reactions, and remained locally restricted, as described by Yamada (1999).

Hares from group C received in total three injections of silver nitrate to boost the development of AA amyloidosis. $AgNO_3$ is proven to induce the development of systemic AA amyloidosis in mice e. g. (Axelrad et al. 1982) by elevating SAA levels and is frequently used in amyloidosis induction experiments (Gruys and Snel 1994). AA amyloid deposits were only found in a single

hare from group C. Interestingly, hare C4 is the only one, that was not bred and born within the institution, but came there as a young foundling.

As Posautz et al. (2016) described varying occurrence of amyloidosis in different populations, transmission of this condition, or locally increased incidence of other diseases, which could lead to higher incidence of secondary amyloidosis, as well as genetic influences might play a role in this irregular distribution among populations. Furthermore (Posautz et al. 2022) postulated that the composition of intestinal microbiome might influence the development of AA amyloidosis. Various enterobacteria are known to form extracellular proteinaceous fibers, the so called curli (Barnhart and Chapman 2006) and are known to have amyloid-fibril like properties, e. g. accelerate AA amyloid formation in induction experiments (Lundmark et al. 2005). It is also reported that animals infected with *Staphylococcus aureus*, like sore hock affected rabbits (Horiuchi et al. 2008, Murakami et al. 2011) and waterfowl affected by bumblefoot (Shinsuke et al. 2008) seem to be more prone to the development of AA amyloidosis.

Furthermore different studies suggest, that SAA levels are not a sufficient amyloidosis marker. Murakami et al. (2011) as well as Ludlage et al. (2005) reported, that they could not find significant differences in levels of SAA in both research species affected and not affected by AA amyloidosis. However, Murakami et al. (2011) reported, though differences were not significant, AA Amyloidosis affected hares showed the highest mean SAA levels. Since in the course of this experiment only one hare developed amyloidosis it was hard to draw conclusions from it.

For this reason, we brought in another set of blood samples. Those samples originate from hares of different populations, collectively called group D, and were gathered during routine pathology. In histopathology these hares were found to be affected by AA amyloidosis and therefore picked for SAA ELISA analysis in retrospective. It is tricky to compare blood collected from wild free ranging hares and the blood of semi-wild hares under controlled test conditions nonetheless it still might be interesting to have data from amyloidosis affected hares. Therefore the comparison of group D with the other groups must be looked at with caution.

The samples of group D were compared to the last blood samples of group A, B and C each, to have similar conditions. A significant difference between group D and the other groups was detected, which was not surprising when we look at the SAA values. The free ranging hares showed higher levels of SAA in their blood, which corroborates with the observation of being

affected by amyloidosis (while the hares of this experiment, except for one, were not showing signs of this illness). The mean SAA level of group D (24.68 mg/L) is higher than the means of each group A, B and C (average = 6.09; 6.36; 15.78 mg/L). Aside from this, SAA levels in group D were not majorly high, compared to the reference range, four out of eight SAA levels (25 %) were above reference and the lowest measured SAA concentration in group D was 11.75 mg/L. The SAA levels in group D might hint to the fact, that the chosen reference value is too generous.

When compared to the SAA concentrations in group C, which were provoked by $AgNO_3$ injection, the mean concentration in group C at the second time point is higher than the mean concentration in group D. This might suggest similar conclusions as Murakami et al. (2011) and Ludlage et al. (2005) gained from their studies. The development of AA Amyloidosis requires a certain high level of SAA over a long time, but those levels do not necessarily exceed or differ from SAA concentrations during infections and inflammations.

Looking at hare C4, which was the only hare to develop AA amyloidosis, the SAA blood levels fit right into the range of levels measured in group D.

Considering this we postulate that SAA is not a sufficient AA Amyloidosis marker in the European brown hare, but still might be of diagnostic interest for detecting inflammatory and infectious disorders.

Conclusion

SAA functions in brown hare are not elucidated, but one can speculate that, since SAA structure is very conserved throughout mammalian and avian species (Gursky 2020), functions might be similar to murine and human SAA isoforms.

To gain more insight into SAA function and reaction in brown hares a similar setup with different treatment groups and SAA measurements in shorter intervals from the first injection on could be considered. Different inflammatory stimuli and varying doses could be used, to see if SAA in brown hares can be considered a major acute phase reactant or if the induction of SAA would call for more potent inflammatory stimulation.

While “no animal-to-human or human-to-human transmission has been reported to date” (Gursky 2020) transmission of AA Amyloidosis has been proven in experimental (Cui et al. 2002, Liu et al. 2007, Lundmark et al. 2002, Murakami et al. 2013) and non-experimental (Shinsuke et al. 2008, Zhang et al. 2008a) settings for different species. Details about the precise mechanism of oral transmission remain unclear, but similarities with the ingestion and

absorption of prions in the intestinal tract, where the payers plaques play an important part, are suspected (Westermark and Westermark 2009). Despite the fact of a barrier for cross-species transmission (Cui et al. 2002), the risk of transmission trough ingestion of AA fibril containing foodstuff (Solomon et al. 2007, Yamada et al. 2006) should not be ignored.

Abstract

To date, little attention has been given to AA Amyloidosis and its development in free-ranging European brown hare (*Lepus europaeus*). During health screenings of different hare populations in Austria and Germany, highly varying incidences of AA amyloidosis were noted among populations. Questions about transmissibility and the role of SAA in the development of AA amyloidosis in European brown hare have arisen.

In humans and several animal species, SAA is a frequently used inflammatory and diagnostic marker for different pathogens and pathologic conditions. As part of the acute phase reaction, SAA is rapidly increasing after inflammatory stimulation.

With the help of a commercially available multispecies ELISA, SAA has been detected in the blood of 30 European brown hares, which have been treated in different ways during a first attempt to experimentally induce systemic AA amyloidosis. Group A was the control group, in Group B hares received amyloid enhancing factor (AEF) p.o., while group C received AEF p.o. as well as s.c. injections of silver nitrate. Four blood samples of each hare were taken within the 96 days of the experiment. Furthermore, blood samples of eight free-ranging hares, which were shot during annual hunts and showed pathological signs of AA amyloidosis (validated by IHC), were included in the thesis and measured by ELISA.

As a first, an experimental working reference value for SAA was set up and gives first impressions on the scale of SAA concentrations in hares, which ranges from 0 to 25 mg/L. While SAA blood levels were not influenced by oral intake of AEF in the experiment, a p.o. transmission through ingestion of AEF cannot be dismissed in general. The diagnostic usefulness for the detection of AA amyloidosis in hares could not be proven. Increased SAA serum levels were observed following injections with silver nitrate, and hint at the potential of SAA as an inflammatory marker similarly to other animal species. This study should encourage further research.

Zusammenfassung

Das Vorkommen und die Entstehung von AA Amyloidose im europäischen Feldhasen (*Lepus europaeus*) hat bis heute nur wenig Aufmerksamkeit erhalten. In Österreich und Deutschland wurden, im Rahmen von Gesundheitsuntersuchungen, unterschiedliche Inzidenzen von AA Amyloidose zwischen den verschiedenen Hasenpopulationen festgestellt. Fragen über eine mögliche Krankheitsübertragung aber auch über die spezifische Entwicklung von AA Amyloidose im Feldhasen wurden aufgebracht.

SAA ist im humanmedizinischen Bereich, aber auch bei unterschiedlichen Tierarten, ein bereits häufig genutzter diagnostischer Marker für verschiedene Krankheiten und Erreger. Als Teil der Akuten Phase-Reaktion steigt SAA schnell im Blut bei entzündlichem Geschehen.

Mit Hilfe eines kommerziell erhältlichen Multispezies ELISA wurde SAA im Serum von 30 Hasen gemessen. Die Tiere waren Teil eines erstmaligen Amyloidose-Induktion Experiments beim Feldhasen und erhielten im Zuge dessen unterschiedliche Behandlungen. Es ergaben sich drei Gruppen für das Experiment. Gruppe A, die Kontrollgruppe, Gruppe B bekam Amyloid Enhancing Factor (AEF) p.o. und Gruppe C bekam zusätzlich zu AEF p.o., Silbernitrat-Injektionen s.c. verabreicht. Innerhalb der 96 Tage Laufzeit wurden jedem Hasen zu vier unterschiedlichen Zeitpunkten Blutproben entnommen. Zusätzlich wurden acht Blutproben von freilebenden Feldhasen mit demselben ELISA analysiert und in die Diplomarbeit inkludiert. Diese Feldhasen wurden bei der alljährlichen Jagd geschossen und zeigten in weiteren Untersuchungen Anzeichen von AA Amyloidose (bestätigt durch IHC).

Zum ersten Mal konnte durch diese Arbeit die Größenordnung von SAA im Blut des Feldhasen dargestellt werden und ein experiment-spezifischer Referenzbereich von 0 bis 25 mg/L wurde erstellt. Die, im Serum gemessenen, SAA Werte wurden durch die Ingestion von AEF im Experiment nicht beeinflusst, dennoch kann eine p.o. Übertragung von AA Amyloidose durch orale Aufnahme von AEF nicht ausgeschlossen werden. Ein Nutzen in der Diagnosefindung für AA Amyloidose konnte nicht festgestellt werden. Erhöhte SAA Werte wurden nach den Silbernitratinjektionen beobachtet und deuten auf das Potential als diagnostischer Entzündungsmarker, wie bereits bei anderen Tierarten genutzt, hin.
Diese Arbeit sollte als Anregung für weitere Forschung dienen.

List of abbreviations

AA	Amyloid A protein
AEF	amyloid enhancing factor
$AgNO_3$	silver nitrate
apoE	apolipoprotein E
APP	acute phase protein
APR	acute phase reaction
GAM	generalised additive model
GAMM	generalised additive mixed model
GLM	generalised linear model
HDL	high density lipoprotein
hSAA	human serum Amyloid A
mSAA	murine serum Amyloid A
SAA	serum Amyloid A
SAP	serum Amyloid P component

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