Phospholipids in equine summer eczema and its therapy

Raija Hallamaa

Academic dissertation

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Auditorium XII of Helsinki University, Unioninkatu 34, Helsinki, on 29th September, 2017, at 12 noon.

Helsinki 2017
To the memory of Thomas Tallberg
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Summer eczema is one of the most common diseases that causes discomfort and impairs the quality of life of horses worldwide. This recurrent, insect hypersensitivity-linked allergic pruritus affects horses typically during the summer months, when horses are predisposed to insect bites. Although horses of various breeds may be affected, this disorder has been demonstrated to be more common among some horse breeds than others. The lack of a feasible treatment has made equine summer eczema a challenge for veterinary medicine.

The main aims of this study were to examine serum phospholipids and their use in the therapy of summer eczema. The hypotheses were that the profiles of the major serum phospholipids differ between affected and healthy horses and these phospholipids are concentrated in the autologous serum preparations applied in therapy depending on the clinical status of the horse. Other aims were to delineate clinical features of summer eczema among Finnhorses and the other breeds affected in Finland.

The efficacy of the autoserum preparation in therapy was investigated in 28 horses in a randomized, placebo-controlled and double-blinded study, and the usefulness of this treatment was also evaluated according to long-term information collected from the owners of the 343 horses treated with this therapy over 12 years. Serum phospholipids and their changes after autoserum therapy were analysed in 10 horses with summer eczema and 10 matched healthy controls by liquid chromatography coupled with a triple-quadrupole mass spectrometry. Content of phospholipids in autoserum preparations made from the sera of 10 affected and 6 healthy horses were analysed by electrospray ionization mass spectrometry. The autoserum preparation was made from the horse’s own serum by serial washings to separate water-soluble molecules from water-insoluble lipids. Finally, the extracted lipids were mixed with ethanol and absorbed in sugar granules for oral administration.

Horses in the placebo group showed significant aggravation in their clinical signs compared with horses treated with autoserum therapy at the same time (P=0.0329). According to long-term data from the owners, 70% of the horses treated with an autoserum preparation benefited from this therapy (95% CI 0.64-0.75, P=0.0001) and 16% did not, and 14% of the owners did not provide a clear opinion. No harmful side effects related to this therapy were observed over the 12-year period.

Horses with summer eczema displayed significantly lower concentrations of phosphatidylcholine (P<0.0001) and sphingomyelin (P=0.0115) in their sera than healthy horses. After a 4-week autoserum therapy, no significant difference in the concentrations of these phospholipids between affected horses and their matched controls could be demonstrated. The change in clinical signs correlated significantly with the alterations in sphingomyelin concentrations (P=0.0047). Of the specific molecular species, sphingomyelin 15:0 showed a significant association with the change in clinical status (P=0.0268).

Analysis of autoserum preparations revealed that these preparations contained major serum phospholipids, however, in significantly differing concentrations between horses with summer eczema and healthy controls. Affected horses showed more abundant concentrations of phosphatidylcholine (P=0.042) and sphingomyelin (P=0.0017) than healthy horses. In addition, concentrations of these phospholipids correlated significantly with the clinical status of the horse (both P values <0.001).

Finnhorses formed the largest group of horses enrolled in this study. Most Finnhorses had become affected with summer eczema before the age of 5 years and showed moderate clinical signs. Features of the disease were mainly uniform between Finnhorses and the other affected breeds. Severity of the signs was not related to age at onset. No significant correlation existed between duration and severity of the disease.

This study showed that an autoserum preparation containing serum phospholipids was a favourable method to treat equine summer eczema. Horses with summer eczema displayed significant differentiations in their serum phospholipid profiles and these alterations seemed to change according to the clinical status of the horse. Lipids as signalling molecules may become targets of intense research also in veterinary medicine, and applications of this autoserum therapy for other allergic manifestations of horses should be explored.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:


The copyright holders of these original publications kindly gave their permission to reproduce them in this thesis. In addition, some unpublished material is presented.
# Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>ApoM</td>
<td>apolipoprotein M</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>ELA</td>
<td>equine leucocyte antigen</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
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<tr>
<td>EV</td>
<td>extracellular vesicle</td>
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<tr>
<td>FOXP3</td>
<td>forkhead box protein 3</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GWAS</td>
<td>genome-wide association study</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>HL</td>
<td>hepatic lipase</td>
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<tr>
<td>IBH</td>
<td>insect bite hypersensitivity</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<tr>
<td>LCAT</td>
<td>lecithin-cholesterol acyltransferase</td>
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<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LIR</td>
<td>leucocyte immunoglobulin-like receptor</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>LTP</td>
<td>lipid-transfer protein</td>
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<tr>
<td>lysoPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>lysoPC</td>
<td>lysophosphatidylcholine</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MR1 protein</td>
<td>MHC class I-related protein</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
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<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PAR2</td>
<td>protease-activated receptor-2</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>PI</td>
<td>phosphatidylinositol</td>
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<tr>
<td>PL</td>
<td>phospholipid</td>
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<tr>
<td>PLTP</td>
<td>phospholipid transfer protein</td>
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<td>PS</td>
<td>phosphatidylycerine</td>
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<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
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<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
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<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
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<tr>
<td>SRM</td>
<td>selective reaction monitoring</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>Th1 cell</td>
<td>T helper 1 cell</td>
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<td>Th2 cell</td>
<td>T helper 2 cell</td>
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<tr>
<td>Treg cell</td>
<td>regulatory T cell</td>
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<tr>
<td>TRP</td>
<td>transient receptor potential</td>
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<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
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<td>VLDL</td>
<td>very low-density lipoprotein</td>
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1. INTRODUCTION

Equine summer eczema is the most common allergic skin disease in horses (Barbet 1992, Scott & Miller 2003), manifesting recurrently during the summer months, when animals are exposed to biting insects, especially species of *Culicoides* (Halldorsdottir et al. 1989, Barbet 1992). Therefore, this disease is also known as insect hypersensitivity or insect bite hypersensitivity (Kurotaki et al. 1994, van Grevenhof et al. 2007), although it was firstly named Queensland itch according to the place where its relation to these midges was initially described and introduced (Riek 1953, Wilson 2014). Currently, equine summer eczema has been documented worldwide in locations where *Culicoides* exist. Iceland is the only country in which summer eczema does not afflict horses due to absence of these insects (Broström et al. 1987). However, equine summer eczema is commonly found in Icelandic horses exported from Iceland (Halldorsdottir et al. 1989, Björnsdotir et al. 2006), and therefore, this breed has been a target for various investigations related to this disorder. Besides Icelandic horses, summer eczema affects horses of multiple breeds, especially many native horse breeds (Kurotaki et al. 1994, van Grevenhof et al. 2007, Velie et al. 2016). Although the disease has been recognized in Finnhorses for decades, its features have not thus far been thoroughly delineated in this breed.

Pruritus is the main clinical sign, usually beginning at the mane and tail (Halldorsdottir & Larsen 1991, Björnsdottir et al. 2006). Local alopecia, self-excoriation and secondary skin infections follow depending on the intensity of scratching, and horses with severe itch may have large lesions over the body. Treatment is challenging and no effective therapy exists. Therefore, the main aim is to minimize contacts with biting insects by stabling horses during the time when insects are active or by using insecticides or special protective blankets. Antihistamines, glucocorticoids and diverse skin ointments have been used to relieve clinical signs (Barbet 1992, Scott & Miller 2003). However, all available treatments have limitations concerning their efficiency, feasibility, side effects or cost. Euthanasia is not an unusual alternative in horses with severe signs of summer eczema. In addition, this disease causes great financial loss to owners since the summer seasons are the best time for riding and other outdoor activities with horses. Equine summer eczema is one of the diseases most commonly impairing the quality of life of horses.

This study originated from the well-known difficulties encountered in the therapy of equine summer eczema. The main purpose was to develop and apply a new treatment for this harmful disease and to evaluate the efficacy of this therapy according to a randomized, placebo-controlled double-blinded study and a long-standing follow-up. In this therapy, a horse’s own serum was specifically prepared and used for oral administration. This autoserum therapy is based on the hypothesis that certain serum phospholipids could be involved as signalling molecules in the pathogenesis of equine summer eczema, and these lipid molecules could also be key players in the treatment. Upon specific processing of serum, these phospholipid molecules may be concentrated in autoserum preparations.

Research on lipids has been activated during the past two decades. Advanced methods to analyse and monitor lipids have created pivotal possibilities to unravel the multitude of lipid molecules involved in various metabolic reactions. At the same time, lipid profiling has become an important tool to delineate relevant fingerprints typical of distinct human diseases. However, lipids and their associations with various disorders have thus far been poorly investigated in horses. The development of mass spectrometry combined with modern computer systems has enabled serum phospholipids, even when present at minute levels, to be analysed. Here, lipids were analysed both in the serum and in the autoserum preparations of horses with summer eczema. In addition, changes in serum lipid profiles were assessed according to alterations in the clinical status of horses.

The aims of this study were to examine clinical features of summer eczema in native Finnhorses, to assess efficacy of autoserum therapy and to delineate phospholipids in the serum and autoserum preparations and to evaluate their association with the clinical course of summer eczema.
Features of equine summer eczema

Equine summer eczema, also known as insect hypersensitivity or insect bite hypersensitivity (IBH) especially in the scientific literature, is the most common allergic skin disease of the horse (Barbet 1992, Scott & Miller 2003). It is found worldwide, however, regional differences exist (Rick 1953, Broström et al. 1987, Steinman, et al. 2003a, van Grevenhof et al. 2007). Although records of this disorder have been found dating back over one hundred years, its relation to biting insects was not suggested until 1953 by Rick in Australia. Later, several studies focused particularly on antibodies against *Culicoides* species have supported this aetiology (Halldorsdottir et al. 1989, Hellberg et al. 2006, Wagner et al. 2006, Langner et al. 2009, Meulenbroeks et al. 2013). The genus of *Culicoides* comprises over one thousand species found worldwide (Huldén et al. 2008). Iceland is the only country in which these insects have not been observed (Broström et al. 1987). Different species of IBH-linked *Culicoides* prevail in different parts of the world, *C. obsoletus*, *nubeculosus* and *sonorensis* being the main ones (Langner et al. 2008, van der Meide et al. 2013).

In Finland, this pruritic disorder has been a well-recognized problem in the Finnhorse, a native Finnish coldblood breed that has been officially bred over 100 years. Summer eczema has been known as “jouhikutka” or “harja-ja häntäkutka” in the early Finnish veterinary literature. It was supposed to be associated with the neglected cleaning of the mane and tail, and later, with unspecified hypersensitivity reactions (Westermarck 1949, Ora 1963). In Finland, 25 species of *Culicoides* have been recorded (Huldén & Huldén 2014), and of these, particularly *Culicoides obsoletus* has spread throughout the country (Huldén et al. 2008). The major species affecting horses have not yet been determined.

Although horses worldwide are exposed to *Culicoides*, not all horses develop this allergic dermatitis. However, there is a clear tendency for some horse breeds to be more affected than others. Usually these horses represent native or local breeds or populations, e.g. Friesian horses and Shetland ponies in the Netherlands (van Grevenhof et al. 2007, Schurink et al. 2009) and Exmoor ponies in the United Kingdom (Velie et al. 2016). Icelandic horses imported from Iceland are particularly prone to this allergy (Halldorsdottir & Larsen 1991). Over the decades, the hereditary component of sensitivity to summer eczema has been researched and special interest has been targeted to equine leucocyte antigens (ELAs) encoded by genes in chromosome 20 (Halldorsdottir et al. 1991, Lazary et al. 1994, Andersson et al. 2012) and to genetic regions on several other chromosomes (Schurink et al. 2012, 2013, Shrestha et al. 2015, Velie et al. 2016). Recent genome-wide association studies (GWASs) have revealed that most of these regions are linked to immune defence reactions (Andersson et al. 2012, Schurink et al. 2013, Shrestha et al. 2015). However, no clear and purely hereditary-based background to the outbreak of this disease has emerged. Therefore, it is commonly thought that equine summer eczema is a multifactor disease resulting from a harmful combination of hereditary susceptibility and environmental influence (Steinman, et al. 2003a, van Grevenhof et al. 2007, Andersson et al. 2012, Velie et al. 2016), as in humans with atopic dermatitis (Elias & Wakefield 2011, Novak & Leung 2011, Rutkowski et al. 2014).

The main clinical sign of summer eczema is pruritus (Scott & Miller 2003). Severity of secondary skin lesions depends on the intensity of scratching and these lesions are typically found in the mane and tail (Halldorsdottir & Larsen 1991), but in severely affected horses also on large areas all over the body (Figure 1). Due to an allergic reaction, a variable amount of swelling is usually found in the mane, particularly with more advanced disease. Horses show clinical signs recurrently during the summer season when insects are active, and these periods vary between different countries. *Culicoides* have their breeding season when the mean 24-h temperature is 10°C or above (Halldorsdottir & Larsen 1991, Barbet 1992), and in Finland this period, known as thermal summer, usually extends from May to September in Turku, or from June to August in Sodankylä, the more northern part of the country (Finnish Meteorological Institute 2016).
Diagnosis of equine summer eczema is based on clinical examination and typical, seasonally recurring signs (Broström et al. 1987, Barbet 1992, Scott & Miller 2003, Hellberg et al. 2006, van Grevenhof et al. 2007, Olsén et al. 2011). Serological assays for identification of specific allergens have not shown uniform results with intradermal testing and are thus regarded as unreliable (Morgan et al. 2007, Langner et al. 2008). Horses with allergic dermatitis usually display positive reactions to various antigens both in skin tests (Jose-Cunilleras et al. 2001) and in serological assays (Frey et al. 2008) and may also show non-specific inflammatory skin reactions at the site of administration (Langner et al. 2008). Neither total serum IgE (Wagner et al. 2003) nor allergen-specific IgE analysis (Frey et al. 2008) with enzyme-linked immunosorbent assay (ELISA) have proven useful for diagnosis since no significant differences have been found between healthy and affected horses with these methods. Moreover, concentrations of free serum IgE do not correlate with the severity of clinical signs, although higher levels have been detected from the sera of affected horses (Meulenbroeks et al. 2013). Langner et al. (2008) demonstrated that tests based on the histamine release from basophils or mast cells are diagnostically more reliable than serological assays. However, current techniques with the use of recombinant Culicoides antigens produced in E. coli or baculovirus expression systems, instead of crude extracts, have provided new and more accurate diagnostic tools to distinguish healthy and affected horses (Langner et al. 2009, van der Meide et al. 2013). The diversity of Culicoides species and the cross-reactivity between these antigens (Langner et al. 2009, van der Meide et al. 2013) render execution of these studies somewhat challenging. The main differential diagnoses of summer eczema are parasitic skin diseases, dermatophytosis and other allergic disorders (Barbet 1992, Scott & Miller 2003).

Treatment of this allergy is difficult. The main principle is to avoid contact with biting insects. However, total isolation is impossible to achieve since the summer months are otherwise the best time for outdoor activities with horses. Special blankets, insecticides, glucocorticoids, antihistamines and various kinds of skin ointments are the most commonly used treatments (Barbet 1992, Scott & Miller 2003, Björnsdottir et al. 2006, Schaffartzik et al. 2012, Wilson 2014). Unfortunately, none of the available therapies have been uniformly beneficial, and all have limitations relating to efficacy, feasibility, side effects or cost. Special blankets have been designed for protection from bites, and their use in combination with overnight stabling has been demonstrated to be a significantly more favourable measure than either a blanket or overnight stabling alone (Olsén et al. 2011). Insecticides have been used for decades and their disadvantage is a rather short evaporation time (Barbet 1992). Medical treatment has been based on the use of glucocorticoids.
and antihistamines. However, both of these medications have disadvantages; glucocorticoids due to detrimental side effects (Barbet 1992, Foster et al. 1998, Schaffartzik et al. 2012) and antihistamines due to low bioavailability after oral administration (Barbet 1992, Foster et al. 1998, Dirikolu et al. 2008, Olssén et al. 2008, Kuroda et al. 2013) and poor efficacy relative to placebo treatment (Olssén et al. 2011). Immunotherapy has been used occasionally, but without marked success (Barbet 1992, Ginel et al. 2014), although Anderson et al. (1996) did describe strongly positive outcomes in six of the 10 severely affected horses that were subcutaneously treated with whole-body Culicoides extracts combined with a mycobacterial cell wall adjuvant. Besides these crude whole-body preparations (Barbet 1992, Anderson et al. 1996), commercial Culicoides extracts that are currently available, have been used. Ginel et al. (2014) conducted a placebo-controlled study by using these commercial Culicoides antigens combined with other environmental allergens adjusted according to horses’ positive reactions to the commercial ELISA, but they observed no significant benefit from this therapy. Preventive immunization schedules with recombinant antigens have been recently introduced in both humans (Valenta et al. 2012) and horses (Jonsdottir et al. 2015). The idea of this approach for horses is to immunize Icelandic horses already in Iceland before export, thereby probably managing to avoid the outbreak of summer eczema. This process is in its pilot phase (Jonsdottir et al. 2015).

**Immune responses in allergic reactions of the skin and in equine summer eczema**

The skin is an important organ for sensing various environmental antigens and together with the gastrointestinal tract represents the first sentinel against foreign intruders and substances (Scott & Miller 2003, Chinthrajah et al. 2016). Cells assembling in allergic reactions are situated in both the epidermis and the dermis (Scott & Miller 2003, Kendall & Nicolaou 2013), and immune cells are also recruited from the circulation when necessary (Galli et al. 2008). In the epidermis, Langerhans cells (LCs) are the main cell group participating in immune responses (Ginhoux & Merad 2010). Keratinocytes collaborate by producing a variety of cytokines and lipid mediators (Scott & Miller 2003, Kawakami et al. 2009, Kendall & Nicolaou 2013). LCs are classified as a subgroup of dendritic cells (DCs) (Clausen & Kel 2010, Ginhoux & Merad 2010, Otsuka & Kabashima 2015), and despite many similarities with classical DCs, LCs have features that distinguish them from DCs, which predominate in the dermis (Ginhoux & Merad 2010, Haniffa et al. 2015). For example, proliferation and differentiation of these cells follow different routes (Ginhoux & Merad 2010, Haniffa et al. 2015). However, both of these cell types are known as antigen-presenting cells (APCs) (Clausen & Kel 2010, Ginhoux & Merad 2010). LCs recognize antigens in the epidermis and transport these captured molecules to regional lymph nodes for presentation to naive T cells (Clausen & Kel 2010, Dubrac et al. 2010). It has been demonstrated that migrated APCs are detected in the draining lymph nodes 18 hours after antigen challenge (Sokol et al. 2008). Both LCs (Clausen & Kel 2010, Ginhoux & Merad 2010) and DCs (Steinman et al. 2003b) express major histocompatibility complex (MHC) class II molecules, through which protein antigens are presented. Depending on the amount of antigen, either LCs or DCs will be activated for presentation, LCs being more sensitive against low antigen doses (Bacci et al. 1997). There is evidence that DCs regulate the balance of immune responses, especially deleting extraneous T cells and/or promoting the expansion of regulatory T (Treg) cells, after antigen presentation has been accomplished (Steinman et al. 2003b, Chinthrajah et al. 2016). In addition to antigen-presenting MHC molecules, APCs express CD1 (cluster of differentiation) molecules that are specialized in presenting lipid antigens (Jayawardena-Wolf & Bendelec 2001, Leslie et al. 2008, De Libero & Mori 2010, Salio et al. 2010, Girardi & Zajonc 2012, Adams 2014), and distinct MHC class I-related (MR)1 proteins, which in turn are associated with small antigen molecules for T cell presentation (Layre et al. 2014, Pierce et al. 2014, Birkinshaw et al. 2015). Lipid and glycolipid antigens are firstly sorted by extra- and intracellular lipid-transfer proteins, LTPs (Mori & De Libero 2008), and depending on, for example the length and unsaturation degree of acyl chains these lipid antigens are presented via different types of CD1 molecules on the plasma membrane of APCs (Jayawardena-Wolf & Bendelec 2001, De Libero & Mori 2010, Layre et al. 2014, Birkinshaw et al. 2015). These
targets for lipids were not described until the late 1980s by Porcelli et al. (Porcelli et al. 1989, De Libero & Mori 2010), and in recent years comprehension of CD1 molecules and their function has grown enormously (Jayawardena-Wolf & Bendelec 2001, De Libero & Mori 2010, Salio et al. 2010, Jyonouchi et al. 2011, Birkirkshaw et al. 2015).

In addition to APCs, mast cells have an important role in dermal immune defences, especially in allergic responses (Olivera & Rivera 2005, Silveira e Souza et al. 2011). Mast cells are stimulated when allergens bind to IgE, leading to crosslinking of FcεRI receptors, the high-affinity receptors for IgE on the plasma membrane of mast cells (Kawakami & Galli 2002, Price et al. 2008, Galli et al. 2011). This crosslinking of captured FcεRI receptors induces a degranulation of mast cells, followed by leakage of histamine and other inflammatory mediators, including prostaglandins and leukotrienes (Boyce 2007, Metcalfe et al. 2016). These substances are released within minutes after FcεRI activation (Gould et al. 2003, Kulinski et al. 2015) and are responsible for the clinical signs, such as oedema, erythema and pruritus, associated with an allergic reaction (Galli et al. 2008). Sphingosine-1-phosphate (S1P) is an important lipid mediator of mast cells that is produced later after FcεRI aggregation (Kulinski et al. 2015). This bioactive sphingolipid derivative acts in an autocrine and paracrine fashion not only by regulating mast cells (Price et al. 2008, Olivera & Rivera 2011) but also by controlling migration of APCs to lymph nodes (Reines et al. 2009) and promoting the egress of lymphocytes from lymphoid organs (Cyster & Schwab 2012, Maceyka & Spiegel 2014). In an allergy-focused milieu, S1P modifies T-cell functions, especially towards responses of T helper 2 (Th2) cells (Olivera & Rivera 2011), but also has a role in the maturation and behaviour of Treg cells (Cyster & Schwab 2012).

After antigen presentation by APCs and release of mediators by mast cells and the other inflammatory cells involved, T cells are induced towards a Th2 phenotype, and these T cells in turn stimulate B lymphocytes to produce more allergen-specific IgE, leading to further crosslinking with IgE-loaded FcεRI receptors and the antigen on mast cells (Kawakami & Galli 2002, Galli et al. 2008). Simultaneously, other leucocytes, such as basophils and eosinophiles, are recruited from the circulation at the site of inflammation (Galli et al. 2008), and, additionally, basophils from the circulation migrate to the draining lymph nodes, where they promote Th2 skewing after allergen challenge (Sokol et al. 2008). The role of basophils has long been ignored (Falcone et al. 2006, Min & Paul 2008); however, these cells have recently been demonstrated to participate in various pivotal phases during allergic responses (Mukai et al. 2005, Falcone et al. 2006, Schneider et al. 2010). Besides mast cells, basophils express FcεRI receptors, but also leucocyte immunoglobulin-like receptors (LIRs) through which basophils are either activated (LIR7) or inhibited (LIR3) to release histamine, cysteinyl leukotrienes and interleukin (IL)-4 (Sloane et al. 2004). Thus, being stimulated via LIRs, basophils are able to act independently from IgE-mediated crosslinking with FcεRI receptors (Sloane et al. 2004). Moreover, LIR3 is able to link with LIR7 and FcεRI, resulting in inhibition of these receptors and their related mediators (Sloane et al. 2004). Taken together, populations of both innate and adaptive immune cells are implicated in the later stages of allergic reactions (Galli et al. 2008, Schneider et al. 2010).

Of the cytokines assembling in allergic responses, IL-4 and IL-13 are the most important for promoting inflammation (Akdis et al. 2005, Galli et al. 2008, Matsuoka et al. 2013), while IL-10, transforming growth factor (TGF)-β (Akdis et al. 2005, Sakaguchi et al. 2009, Schmetterer et al. 2012, Chinthrajah et al. 2016) and IL-2 are associated with suppression, IL-2 actually being involved in the differentiation of Treg cells (Sakaguchi et al. 2009, Josefowicz et al. 2012). Matsuoka et al. (2013) have recently introduced an additional inhibitory cytokine, IL-27, which is produced by DCs and targeted for Th2 suppression. Of the promoting cytokines, IL-4 is particularly linked to Th2 skewing (Falcone et al. 2006, Sokol et al. 2008), likewise thymic stromal lymphopoietin (TSLP) produced by basophils (Sokol et al. 2008, Otsuka & Kabashima 2015). Various cell types assembling in allergic inflammations are able to secrete these substances depending on the status of responses (Galli et al. 2008, Schmetterer et al. 2012). Studies on cultured peripheral blood mononuclear cells (PBMCs) from horses with and without summer eczema have shown that the concomitant expressions of IL-10 and TGF-β down-regulate IL-4-producing cells and these cells are more pronounced in cultures of horses with summer eczema (Hamza et al. 2008).

These cells are differentiated from the other T cell types according to their CD molecules and an expression of the transcription factor, forkhead box protein 3 (FOXP3) (Sakaguchi et al. 2009, Schmetterer et al. 2012). The phenotype of CD4+CD25+FOXP3+CD127− is currently considered to represent an exact definition for naturally occurring Treg cells (Schmetterer et al. 2012). Treg cells produce IL-10 and TGF-β (Akdis et al. 2005), and these cytokines in turn prevent activation of LCs and DCs (Sakaguchi et al. 2009, Schmetterer et al. 2012), which is essential for the promotion of Th2 responses and sustenance of allergic disorders (Clausen & Kel 2010, Dubrac et al. 2010). Additionally, Treg cells express various cell-surface molecules that promote their suppressive actions by, for example, abrogating effector T cells from interactions with APCs (Sakaguchi et al. 2009, Schmetterer et al. 2012).

Normally, the immune responses occurring after contact with various allergens are self-limiting without noxious consequences (Gould et al. 2003, Wilson 2014) and are controlled by interactions between APCs, mast cells and Treg cells, leading to tolerance against innocuous antigens (Steinman, et al. 2003b, Price et al. 2008, Reines et al. 2009, Sakaguchi et al. 2009, Chinthrajah et al. 2016). Responses to bites of insects are usually local and transient and are classified as type I or immediate hypersensitivity reactions, resulting mainly from mast cell activation (Kurotaki et al. 1994, Scott & Miller 2003, Wagner et al. 2006, Wilson 2014). However, in horses with summer eczema these responses are prolonged as horses are exposed to allergens (Wilson 2014), leading to high IgE levels, continuous antigen presentation to T cells with subsequent Th2-cell polarizations (Hellberg et al. 2006, Heimann et al. 2011, Meulenbroeks et al. 2015) and finally a possible switch towards a delayed, type IV hypersensitivity state (Kurotaki et al. 1994, Scott & Miller 2003, Schaffartzik et al. 2012). Actually, both of these hypersensitivity types seem to exist simultaneously (Meulenbroeks et al. 2015).

It has been demonstrated recently that in affected horses the balance between Th2 and Treg cells is disturbed, and this asymmetry may be pivotal in the pathogenesis of summer eczema (Heimann et al. 2011, Hamza et al. 2012), as has been observed also in allergic humans (Akdis et al. 2005). Although healthy horses and horses with summer eczema have not shown differences in the amounts of Treg cells in the circulation (Hamza et al. 2012) or in skin biopsies (Meulenbroeks et al. 2013), affected horses are incapable of elevating the number of Treg cells after antigen stimulation, while healthy horses develop a significant increase of these cells (Hamza et al. 2012). This was suggested to be the result of the excessive IL-4 secretion detected in eczema horses (Hamza et al. 2012). A more recent study has demonstrated that also T helper 1(Th1)-cell polarization seems to prevent IBH since healthy horses respond more commonly with Th1 skewing to allergen challenge (Meulenbroeks et al. 2015).

Kurotaki et al. (1994) distinguished three stages in the progression of histopathological changes related to insect hypersensitivity. The initial phase comprises epidermal intercellular oedema, an increase in the number of LCs and perivascular infiltrations of eosinophils and mast cells. In the more advanced stage, the number of mast cells and eosinophils increase further, simultaneously with infiltrating T cells and DCs in the dermo-epidermal junction, and finally, when clinical signs start to regress along with cooling weather conditions, hyperkeratosis and epidermal hyperplasia present (Kurotaki et al. 1994). Van der Haegen et al. (2001) described similar acute-phase changes with an abundance of eosinophils, mast cells and lymphocytes, and Meulenbroeks et al. (2015) demonstrated that these reactions were more pronounced in affected horses, even in wintertime when IBH-affected horses were subjected to the intradermal challenge of Culicoides antigens.

Atopic dermatitis of humans has many features in common with equine summer eczema. Intense pruritus, excessive IgE-mediated sensitization and Th2 polarization are typical findings of both disorders (Jose-Cunilleras et al. 2001, Dubrac et al. 2010, Elias & Wakefield 2011, Heimann et al. 2011, Novak & Leung 2011, Schaffartzik et al. 2012). In addition, their histopathological pictures have many similarities (Kurotaki et al. 1994, Kawakami et al. 2009). In humans, disturbed epidermal barrier function due to inherited defects of filaggrin protein is one of the main predisposing factors making the skin more sensitive to external intruders (Proksch et al. 2003, Elias & Wakefield 2011, Novak & Leung 2011). In horses, this has not yet been investigated (Schaffartzik et al. 2012).
Mechanisms of pruritus

Pruritus is the main clinical manifestation of equine summer eczema. Therefore, it is important to understand the mechanisms underlying itch. Pruritus that originates purely from the skin and affects only the skin is categorized as pruritoceptive itch, as opposed to neurogenic, psychogenic and neuropathic itch, the initial causes of which are unrelated to skin disorders (Patel & Dong 2011, Garibyan et al. 2013). Pruritus is transmitted via sensory nerves that protrude their fibres into the epidermis, while the bodies of these cells are situated in the dorsal root ganglia in the vicinity of the spinal cord (Steinhoff et al. 2006, Garibyan et al. 2013). The endings of these unmyelinated C-fibres express receptors and ion channels that convey itch signals following interactions with pruritogens, the mediators of pruritus (Garibyan et al. 2013). In the next phase, nerve impulses are conducted with spinal neurons and finally received in the brain (Steinhoff et al. 2006, Garibyan et al. 2013). Debate exists as to whether itch and pain stimuli possess the same nerve routes or whether there are specific neurons for each impulse (Patel & Dong 2011, Garibyan et al. 2013). However, current understanding supports the theory that both types of neurons exist, and itch-sensitizing neurons are a subset of pain neurons that are activated when the stimulus does not reach the threshold of the pain stimulus (Oude Elferink et al. 2011, Patel & Dong 2011, Garibyan et al. 2013).

Various cells and pruritogens are able to communicate with nerve endings and to contribute to the sense of pruritus (Steinhoff et al. 2006). Histamine is the main cause of itching by stimulating cutaneous sensory nerves (Gould et al. 2003, Ohsawa & Hirasawa 2014), although mast cell tryptase is also suggested to have pruritogenic effects (Kawakami et al. 2009). In addition, substance P (SP) of mast cells and IL-31 of Th2 cells are mediators of pruritus (Garibyan et al. 2013). Tryptase has been detected also in mast cells from horses with IBH (van der Haegen et al. 2001). All of these pruritogens have their own targets on the nerve endings, e.g. G-protein coupled receptors (GPCR) H1 and H4 for histamine (Garibyan et al. 2013, Ohsawa & Hirasawa 2014), protease-activated receptor (PAR)-2 for tryptase, neurokinin receptors for SP and IL-31 receptors for IL-31 (Steinhoff et al. 2006, Patel & Dong 2011, Garibyan et al. 2013). The exact mode for IL-31 action is thus far obscure since IL-31 has receptors both on the sensory nerves and in the plasma membrane of keratinocytes (Steinhoff et al. 2006). In addition to specific receptors, stimuli to nerve cells are mediated via the ion channels of the transient receptor potential (TRP) family (Garibyan et al. 2013). When the receptors or ion channels are activated, sensory nerve endings release neuromediators, transferring itch signals to the spinal cord and the brain (Steinhoff et al. 2006).

Keratinocytes seem to be additional key players in the circuit of pruritus. These cells are in a close contact with sensory nerve endings and express receptors and ion channels involved in conduction of itch stimulus (Steinhoff et al. 2006, Garibyan et al. 2013). Moreover, keratinocytes are able to release both enhancing and suppressing mediators (Steinhoff et al. 2006). Keratinocytes are currently the focus of intense research (Garibyan et al. 2013). Taken together, various cell types and their mediators are involved in the mechanism of pruritus. Therefore, therapies based only on the use of antihistamines have usually shown limited effects on both horses and humans with allergic skin diseases (Olsén et al. 2011, Garibyan et al. 2013).

General aspects of phospholipids and their roles in allergy

Lipids and phospholipids

Lipids are a diverse group of molecules that act as the main structural components of cellular membranes (van Meer 2011, Holthuis & Menon 2014). Lipids also participate in various intra- and extracellular reactions, including inflammation, apoptosis, cell signalling and a plethora of hormonal and enzymatic activities (Subbaiah & Liu 1996, van Meer 2005, Fadeel & Xue 2009, Blom et al. 2015). The defining feature of lipids is their insolubility in water, an ability that enables them to form bilayer membranes (Nelson & Cox 2008, Holthuis & Menon 2014) and serve as a cornerstone for the existence of uni- and multicellular organisms. To date, biological lipids are divided into eight main groups, including fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides, of which glycerophospholipids and
sphingolipids are also known as phospholipids (PLs) (Nelson & Cox 2008, Bublin et al. 2014). The cellular lipidome—the total set of lipid molecules in the specific cell types—comprises more than 1000 distinct lipids, and in the plasma, there are thousands of lipid species (Quehenberger et al. 2010, van Meer & de Kroon 2011). Of the content in human plasma, lipids represent the majority of biological molecules (Quehenberger & Dennis 2011).

PLs are composed of a polar head group, including a phosphate group and its substitute, two acyl chains and a glycerol backbone in glycerophospholipids or one acyl chain and a sphingosine backbone in sphingolipids (Nelson & Cox 2008). The polar head group defines the class of PLs and based on their acyl chain composition, i.e. the degree of unsaturation and the length of acyl chains, PLs are distinguished into different molecular species (Nelson & Cox 2008). The nomenclature of species is based on the total number of carbon atoms and double bonds in acyl chains (Nelson & Cox 2008), i.e. PC 36:2 has 36 carbon atoms with 2 double bonds in acyl chains. The main PL classes found in mammalian cells and circulation are phosphatidylethanolamine (PE), phosphatidyserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA) and sphingomyelin (SM) (van Meer 2005, Quehenberger & Dennis 2011). PLs constitute the second largest group of lipids in human plasma; only sterol lipids, in major part as cholesterol existing in esterified or non-esterified forms (Nelson & Cox 2008), are found more abundantly (Quehenberger & Dennis 2011). Of the PLs, PC and SM are the most abundant in horse serum, while PE and PI have been detected at minor levels (Fuchs et al. 2009).

**Phospholipids as structural components of membranes and circulating lipoproteins**

PLs are enriched in the plasma membrane, where they form a bilayer barrier between extra- and intracellular spaces (Kierszenbaum 2002). In this bilayer, lipids are settled so that their hydrophobic acyl chains are towards the inner side of this bilayer, while the polar head groups are at the outer side (Kierszenbaum 2002, Holthuis & Menon 2014). As a result, the plasma membrane has two leaflets, outer and inner, and these leaflets have their own specific lipid compositions (Kierszenbaum 2002, van Meer 2011). This lipid asymmetry is crucial for the transmembrane trafficking of the cell (Fadeel & Xue 2009, van Meer 2011). PC and SM are the major PLs in the outer leaflet, whereas PS, PE, PI and PA prefer the inner leaflet (Kierszenbaum 2002, Nelson & Cox 2008, Silveira e Souza et al. 2011). In addition to the plasma membrane, PLs are enriched in intracellular membranes, such as endoplasmic reticulum, where their compositions and amounts depend on the organelles and activities involved (Holthuis & Menon 2014).

In the mammalian circulation, lipids are carried mainly as a part of lipoprotein particles (Figure 2) (Shepherd 1991, Watson et al. 1993), whereas free fatty acids are bound to albumin (Watson et al. 1993). Lipoprotein particles (Figure 2) consist of surface-amphipathic apolipoproteins and specific compositions of lipids, including cholesterol, cholesteryl esters, triglycerides and PLs (Watson et al. 1991, Watson et al. 1993). Four classes of plasma lipoproteins have been identified in horses (Shepherd 1991, Watson et al. 1993). High-density lipoprotein (HDL) is the major class in horses, accounting about 60% of the total lipoprotein mass, and with low-density lipoprotein (LDL) they are the main carriers of cholesterol and PLs (Watson et al. 1991). In contrast to HDL or LDL, very low-density lipoproteins (VLDLs) and chylomicrons are triglyceride-rich lipoproteins, mostly involved in energy metabolism (Watson et al. 1991, 1993). Chylomicrons carry dietary lipids supplied from the intestine, whereas VLDL transports endogenous triglycerides derived from the liver (Watson et al. 1993). Equine VLDL contains both small and large molecular apolipoproteins (Watson et al. 1993). In addition to lipoproteins, blood contains lipid molecules that are bound in vesicular structures such as extracellular vesicles (EVs); however, their lipid content is low in comparison with that in lipoproteins (Caby et al. 2005, Bobrie et al. 2011, Chaput & Théry 2011, Raposo & Stoorvogel 2013).

Phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) regulate trafficking between circulating lipoproteins (Guyard-Dangremont et al. 1998, Zannis et al. 2015). Unlike humans, horses have not shown CETP activity (Watson et al. 1993, Guyard-Dangremont et al. 1998). In addition to PLTP, equine lipid homeostasis is controlled by lecithin-cholesterol acyltransferase (LCAT) and lipolytic enzymes such as lipoprotein lipase (LPL) and hepatic lipase (HL) (Watson et al. 1993). Breed, gender or a postprandial stage has shown no relation to the activity of LCAT, LPL or HL in adult horses (Watson et al. 1993).
In intracellular spaces, lipids are transported via both vesicular structures and specific LTPs (Mori & De Libero 2008, Wong et al. 2017). LTPs organize intracellular trafficking of PLs by extracting lipid molecules from membranes and by transporting them between organelles (Holthuis & Menon 2014, Wong et al. 2017). Some LTPs are specialised for particular lipids, while others carry various types of lipid molecules (Wong et al. 2017).

Figure 2. Simplified model of lipid transport between the intestinal lumen, circulation and tissues (modified from Shepherd 1991, Watson et al. 1991, Zannis et al. 2015). Chylomicrons derived from dietary lipids are postprandially hydrolysed by lipoprotein lipase (LPL) in the circulation. Generated free fatty acids are directed to adipose tissue for storage or to muscles to be used for energy production, while chylomicron remnants are cleared by the liver. Very low-density lipoproteins (VLDLs) are synthesized in the liver and in the circulation, they are converted to low-density lipoproteins (LDLs) in a process catalysed by LPL and hepatic lipase (HL). High-density lipoproteins (HDLs), which are mainly synthesized in the liver, recycle cholesterol from peripheral tissues back to the liver where extra cholesterol is secreted in the bile. This is known as the reverse cholesterol transport (RCT) process. Lipoproteins are the principal lipid carriers in the circulation. The major lipids carried in lipoprotein particles are cholesteryl esters, free cholesterol, triglycerides and various phospholipids. Apolipoproteins are major structural components on the particle surface and due to their amphipathic nature they are responsible for solubilisation of lipids. A detailed structure of the lipoprotein particle is presented.

Phospholipids in allergic inflammation
Membrane PLs are a prime source of arachidonic acid (AA), the precursor for both various pro- and anti-inflammatory lipid mediators such as eicosanoids, endocannabinoids and lipoxins (Boyce 2007, Bannenberg & Serhan 2010, Serhan & Petasis 2011, Kendall & Nicolaou 2013, Demetz et al. 2014). In inflammatory conditions, these bioactive lipids act in a paracrine or autocrine fashion, usually at nanomolar concentrations (Boyce 2007, Bannenberg & Serhan 2010, Stables & Gilroy 2011). In inflammation and its resolution, lipid mediators co-operate iteratively to restore homeostasis (Serhan & Petasis 2011, Stables & Gilroy 2011, Fanning & Boyce 2013).

Of the major PLs, PC is usually profiled as a structural lipid and a source for production of other lipids, whereas SM has essential functions related to epidermal barrier metabolism and sig-
SMs are important precursors of S1P, the pivotal lipid mediator produced by mast cells in allergic responses (Price et al. 2008, Kulinski et al. 2015). After activation of FceRI receptors, S1P is generated by sphingosine kinases in mast cells and released for the engagement with G-protein coupled S1P receptors on the membrane of the parent cells and nearby cells (Olivera & Rivera 2005, Price et al. 2008, Kolter 2011, Hanson et al. 2012, Maceyka & Spiegel 2014, Kulinski et al. 2015). Five types of S1P receptors, numbered from S1P1 to S1P5, have been identified (Reines et al. 2009, Chun et al. 2010). These receptors comprise differing numbers of amino acids and their engagements result in distinct influences (Chun et al. 2010). For example, the binding site on the S1P1 receptors is highly sensitive to hydrophobicity of the ligand (Hanson et al. 2012). The concentration gradient of S1P between blood and tissues is continuously maintained since decreased blood levels subject one to lymphopenia (Price et al. 2008, Kulinski et al. 2015). Erythrocytes are the main source of blood S1P (Price et al. 2008, Cyster & Schwab 2012). In the circulation, S1P is bound to albumin and apolipoprotein M (apoM) in HDL, apoM being the main and specific carrier for S1P (Christoffersen et al. 2011). The ability of S1P to affect APC and lymphocyte trafficking has created new therapeutic options for use of S1P receptors (Reines et al. 2009, Cyster & Schwab 2012), particularly in the treatment of autoimmune disorders (Maceyka & Spiegel 2014).

Since lipids participate in various metabolic reactions at different steps in mammalian biology, these molecules are involved in a wide range of disorders. As already mentioned, PL derivatives are key players in inflammation and its resolution, and therefore, disturbances in their metabolism are associated especially with immune-mediated afflictions, of which allergies and autoimmune responses are the most common (Kolter 2011, Jovanovic et al. 2013, Korematsu et al. 2014, Lis-Swiety et al. 2014, Maceyka & Spiegel 2014). In skin allergies, particularly in atopic disorders, PL derivatives have roles as both structural components of skin and regulators of allergic responses. Ceramides are the main components of the epidermal lipid barrier and active metabolites derived from SM (Proksch et al. 2003, Kolter 2011, Olivera & Rivera 2011, Maceyka & Spiegel 2014). Production of ceramides and SM is linked, since ceramides can be synthesized from SM and vice versa (Kolter 2011, Maceyka & Spiegel 2014). Ceramides protect skin from excess water loss (Proksch et al. 2003, Kolter 2011) and ceramides detected in the epidermis have typically long acyl chains (Kolter 2011). SM is a precursor for two of the seven types of ceramides identified in the skin (Proksch et al. 2003). In atopic disorders, an altered SM metabolism leads to disturbed production of ceramides, resulting in defects in barrier integrity (Proksch et al. 2003). In the atopic skin, the amount of ceramide has been demonstrated to be lower than in the healthy skin (Proksch et al. 2003). Apart from the skin, ceramides have an important role in the endothelium by responding to various inflammatory signals (Maceyka & Spiegel 2014). Intriguingly, SM seems to be involved in allergic reactions also as a part of allergens, especially in dairy products, where they promote the secretion of Th2 skewing cytokines (Jyonouchi et al. 2011).

3. AIMS OF THE STUDY

There were two major aims in the present thesis. The first aim was to examine serum PLs and their therapeutic use in horses with summer eczema. The preliminary hypotheses were that profiles of the major serum PLs differ between affected and healthy horses and these PLs typical of the horse are present in autologous serum preparations used in therapy of the disease. The second major aim was to delineate clinical features of summer eczema in Finnhorses. Specific aims were as follows:

1. To delineate characteristics of summer eczema among Finnhorses and other breeds affected in Finland (I).

2. To evaluate feasibility of autoserum therapy based on the results of the placebo-controlled double-blind study (II) and long-term information collected for this treatment (III).

3. To analyse PL compositions both in autologous serum preparations (IV) and in sera of horses with summer eczema and healthy controls and to assess whether these lipid profiles change after therapy (V).
4. MATERIALS AND METHODS

Horses

Altogether 363 horses with clinical signs of summer eczema and 16 healthy controls were included in this work comprising a series of studies performed in 1997-2014. Features of the disease were delineated in 275 horses between 1997 and 2007 (Study I), whereas studies on autoserum therapy comprised 343 horses and were carried out in 1997-2008 (Studies II and III). Of these 343 horses, 28 participated in the randomized, placebo-controlled and double-blinded study performed in 1997-1998 (Study II), and additionally, Study II included 39 horses treated with autoserum preparation but not placebo controlled. Besides the 67 horses participating in Study II, there were 276 horses in Study III that had not participated in the earlier studies. Taken together, Study III comprised 343 horses and this study was carried out to obtain long-term clinical experience of autoserum therapy. Serum PLs were analysed from autoserum preparations of 10 affected and 6 healthy horses in 2012 (Study IV). Differences in serum PLs and changes after therapy were examined between the 10 horses with summer eczema and their 10 matched healthy controls in 2014 (Study V).

Horses that had taken part in the first clinical study on autoserum therapy (II) were recruited through active advertising in the main horse magazines in Finland, while subjects in the other studies (I, III-V) consisted of horses whose owners or local veterinarians had contacted the author due to summer eczema over the years. The diagnosis was based on a clinical examination performed by the author or by local veterinarians. All horses with typical clinical signs of summer eczema during thermal summer (mean 24-h temperature 10ºC or above) were included. Horses with pruritus before or after this summer season were excluded. Furthermore, horses that had been simultaneously medicated with antihistamines or glucocorticoids were excluded, while all other treatments presented in detail later (Table 3) were permitted. The additional inclusion criterion for eczema horses in Study V was that a matched control horse could be obtained from the same stable where the affected horse was kept.

Healthy horses without a history of summer eczema were used as controls in Studies IV and V. In Study IV, all controls were Finnhorses and they were collected from stables that simultaneously housed horses suffering from summer eczema, while Study V comprised matched healthy controls that lived on the same farms and were fed with a similar fodder as their affected counterparts.

The studies on therapy were approved by the Animal Care and Use Committee of the University of Helsinki (25.04.1997), and the use of healthy horses in Study V was approved by the Regional State Administrative Agency of Southern Finland (ESAVI/1016/04.10.07/2014). Owners’ signed informed consent for inclusion was obtained.

Data of horses

Information about the horses was acquired by questionnaire (Appendix I) and supplemented with interviews when necessary. For all horses, data were collected on breed, gender, age and clinical signs. Additionally, age at onset, duration of disease and previous treatments were recorded. Clinical signs were categorized by the author as mild, moderate or severe depending on the size of skin lesions (Figure 3). Signs were regarded as mild if pruritus was the only clinical sign. Horses with pruritus and mild skin lesions in the mane, tail and/or body were classified as having moderate signs, while horses with pruritus and large skin lesions were graded as having severe signs. Owners’ opinions about aggravating factors were also requested.

Information about the autoserum therapy was collected yearly in 1997-2008, and the questionnaires were sent to owners in late autumn of the year that therapy had been started (II, III). The mode of the questionnaire (Appendix II) was similar over the study period. However, it was not possible to acquire full data for all horses in Studies I and III, and thus, the total numbers of horses vary for the individual variables presented in the tables.
Figure 3. Clinical signs of summer eczema were classified as mild (a, b), moderate (c, d) or severe (e, f) depending on the severity of skin lesions in the mane, tail and body (I-V).
**Autoserum preparation and therapy**

Horses in Studies II, III and V were treated with an autologous serum preparation that was introduced in Study II. A blood sample was collected into a 10 ml plain serum tube when a horse had shown typical clinical signs of summer eczema for at least 2 weeks. The sample was kept at room temperature and sheltered from sunshine until harvesting. Serum was harvested without using a centrifuge when blood cells were clotted and clear serum was visible. A sample of 0.05-0.1 ml was taken from the superficial layer of the serum. This aliquot was washed twice by shaking with sterile water (1:100), and the same volume was again taken from the superficial layer of the dilution. After the second washing, the same volume was taken again from the superficial layer and the final dilution was made in 40-48% ethanol. Ten drops of this solution were absorbed into 20 g of ordinary sugar granules. These granules were stored in plastic or glass bottles at room temperature and sheltered from sunshine. The administered dose consisted of 10-20 sugar granules and it was given orally once a day for 2 weeks, followed by a 1-week pause, after which horses were treated again for one week. After this basic period, treatment was repeated as 1-week courses whenever horses showed clinical signs (II, III).

In Study II, 28 of the total of 67 horses participated in the randomized, placebo-controlled and double-blinded part of the study, and these horses were randomly divided into placebo and autoserum therapy groups. Horses in the placebo group were treated with a preparation that was made otherwise similarly, except that no serum was added.

The efficacy of the autoserum therapy in the randomized, placebo-controlled and double-blinded part of Study II was evaluated after a 4-week treatment by the author according to the same scale that was used to grade the clinical signs. The treatment was regarded as beneficial when clinical signs became milder on this scale. After this 4-week study period, all horses in the placebo group were treated also with autoserum preparation.

Results of the horses without placebo controlling and blinding in Studies II and III were based on owners’ opinions of the therapy acquired by questionnaires and interviews. Their opinions were expressed as none, little or considerable benefit. If the owner could not express clear opinion, the answer was recorded as no opinion. In addition, information about the time needed for possible alleviation of clinical signs, frequency of medication and any related side effects was acquired. Owners whose horses participated in the original placebo-controlled study (II) were interviewed 5 years later. Information regarding the use of autoserum preparation and their horses’ clinical condition over the 5-year period was collected (III).

Although Study V did not focus on the clinical efficacy of autoserum therapy per se, the same scale used in Study II was applied in Study V to assess changes in clinical signs after therapy.

**Blood samplings for lipid analyses**

PL contents were analysed from both autologous serum preparations (IV) and horses’ sera (V). In Study IV, autoserum preparations were made as introduced earlier in this text, however, the amount of serum used was 0.065 ml in all samples and the final dilution was made in 48% ethanol. Lipid analysis of autoserum preparations was performed using these final dilutions, and they were stored at room temperature until analysed. Sera from healthy controls were collected at the same time as affected horses showed clinical signs and were prepared and stored similarly.

The first blood samples for serum PL analyses (V) were drawn when eczema horses had shown typical clinical signs of summer eczema for at least 2 weeks, and the second samples when the eczema horses had been on autoserum therapy for 4 weeks. Blood samples were taken using 10 ml plain vacuum tubes that were filled fully and kept at room temperature for at least 3 hours before harvesting. Sera were harvested without using centrifugation. Of the serum, 0.065 ml was taken from the superficial layer of the serum and added to 4.2 ml of 48% ethanol and stored at room temperature until analysed. Samples were collected from the matched controls similarly and simultaneously and also handled in the same manner.
Phospholipid analyses

PLs were analysed from both autologous serum preparations and sera of affected and healthy horses. Lipid analyses were performed in the laboratory of Docent Pentti Somerharju, Department of Biochemistry and Developmental Biology, Faculty of Medicine, Helsinki University. Autoserum preparations (IV) were analysed by electrospray ionization mass spectrometry (ESI-MS) with direct injection into the mass spectrometer, while liquid chromatography mass spectrometry (LC-MS) with selective reaction monitoring (SRM) was used to detect lipids in the serum (V). The main PL classes analysed were PC, PE, PS, PI, PA and SM.

Lipid analysis of autoserum preparation: The samples in 48% ethanol were dried under a N2 stream and the residue was reconstituted in 500 µl of chloroform/methanol (1:2) and stored at -20°C. Half of each sample was taken for preliminary mass spectrometry analysis to ascertain the constitution of the lipids in it, and unlabelled external standards were added to the remainder in order to quantify the amount of lipids. After addition of aqueous NH4OH (4%, i.e. 1% of NH4OH), the sample was infused at 6 µl/min to a Micromass Quattro Micro triple-quadrupole mass spectrometer (Waters, Milford, MA, USA) (Hermansson et al. 2005). Specific scans in both positive and negative ion modes for the detection of individual PL classes based on their head groups and species based on their acyl chain compositions were performed. Spectra were acquired for 5 min over a mass range of m/z (mass-to-charge ratio) 400-950 at a frequency of 4 scans/min. Lipid standards, di-22:1 PC, lysophosphatidylcholine (lysoPC) 20:0 and di-18:1 PA were each added to all samples to quantify the concentrations of the lipid species in these classes. The spectra obtained were exported to Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and the detected lipids were quantified by using LIMSA software (University of Helsinki, Helsinki, Finland).

Lipid analysis of serum: All samples stored in 48% ethanol were subjected to Folch’s method (Folch et al. 1957) for lipid extraction, dried under a N2 stream, reconstituted in 500 µl of chloroform/methanol (1:2) and further spiked with the following labelled standards (Avanti Polar Lipids, Alabaster, AL, USA) corresponding to each head group: D9(di-44:2) and D9(di-40:2) for PC, D4(di-40:2) and D4(di-20:0) for PE, D3(di-40:2) and D3(di-44:2) for PS, D6(di-36:2) and D6(di-28:0) for PI and finally unlabelled 25:0-SM. LC-MS with SRM was used for the analyses. Waters ACQUITY Ultra Performance LC system (Waters, Milford, MA, USA) equipped with a Waters ACQUITY BEH C18 column (1.0 × 100 mm) was used to separate the molecular species using gradient elution. Solvent A was acetonitrile/H2O (60:40) with 10 mM ammonium formate and 1% NH4OH, while solvent B was isopropanol/acetonitrile (90:10) containing 10 mM ammonium formate and 1% NH4OH. The flow rate was 0.13 ml/min and the column temperature 60°C. Solvent B was set to 40% at injection and increased linearly to 100% in 14 min, remained at this value for 3 min, decreased back to 40% in 1 min and then remained there until the end of the gradient at 20 min. The eluent was directed to the ESI source of Waters Quattro Premier triple-quadrupole mass spectrometer (Waters, Milford, MA, USA) operated in the positive ion mode. For SRM transitions, proton adducts of the PC, PE, PS and PI species were selected as the precursors, while the product ion was either the head group (PC, SM, PI) or the diacylglycerol fragment (PE, PS). For quantification purposes, the SRM chromatograms were integrated and the relative concentrations of the individual molecular species were calculated using QuanLynx software (Waters, Milford, MA, USA).
**Statistical analyses**

Characteristics of summer eczema (I) were evaluated by Fisher’s exact test for qualitative and the t test for quantitative variables. One-way analysis of variance was used to assess the relation between the age of onset or duration of disease and the severity of clinical signs.

Results of autoserum therapy were analysed by Fisher’s exact test in the placebo-controlled study (II). Binomial test was used to compare proportions of the horses with or without benefit from this treatment (II, III). Changes in clinical signs before the therapy and after a 5-year follow-up (III) were evaluated by the exact McNemar’s test in the horses that participated in the original placebo controlled study (II).

Differences between PL concentrations detected from autoserum preparations of healthy and eczema horses were assessed by using the Mann-Whitney test due to non-parametric distribution of the data verified by the Shapiro-Wilk test, and the correlation between PL concentrations and clinical signs by using Spearman’s correlation test (IV). The Friedman test was used for pairwise comparisons of PL concentrations in sera between the matched groups and over time (V). The relationship between the change in PL concentrations and clinical signs after therapy was evaluated by Spearman’s correlation test (V).

Analyses were performed using statistical software StatsDirect (StatsDirect Ltd., Sale, Cheshire, UK) and PASW Statistics 18.0 [(SPSS) IBM Corporation, Armonk, NY, USA]. Two-sided P values <0.05 were considered significant.
5. RESULTS

Characteristics of summer eczema

Of the 275 horses in Study I, 139 were Finnhorses, 71 Icelandic horses, 45 ponies and 12 other breeds, while breed was unspecified in 8 horses. All Finnhorses were born in Finland, as were 11 of the Icelandic horses, whereas 56 horses were imported from Iceland. The origin of 4 Icelandic horses remained unknown. Detailed information on affected horses and their disease is presented in Table 1.

Table 1. Characteristics of summer eczema in 275 horses in Finland between 1997 and 2007 with detailed information on four breeds most afflicted by summer eczema (I).

<table>
<thead>
<tr>
<th>Horses</th>
<th>Gender (%)</th>
<th>Age at onset mean±sd (years)</th>
<th>Clinical signs (%)</th>
<th>Duration of disease mean±sd (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>♂</td>
<td>♀</td>
<td>mild</td>
<td>moderate</td>
</tr>
<tr>
<td>All horses n=275</td>
<td>48</td>
<td>52</td>
<td>6.1±4.1</td>
<td>9</td>
</tr>
<tr>
<td>Finnhorses n=107</td>
<td>49</td>
<td>51</td>
<td>4.6±3.6</td>
<td>14</td>
</tr>
<tr>
<td>Icelandic horses n=60</td>
<td>46</td>
<td>54</td>
<td>9.0±2.9</td>
<td>3</td>
</tr>
<tr>
<td>imported n=49 born in Finland n=11</td>
<td>45</td>
<td>55</td>
<td>9.6±2.0</td>
<td>4</td>
</tr>
<tr>
<td>New Forest ponies n=15</td>
<td>53</td>
<td>47</td>
<td>4.5±3.6</td>
<td>0</td>
</tr>
<tr>
<td>Shetland ponies n=15</td>
<td>69</td>
<td>31</td>
<td>3.8±3.1</td>
<td>0</td>
</tr>
</tbody>
</table>

Male and female horses were equally affected, and no significant differences existed between their clinical symptoms. Most of the horses showed moderate clinical signs (Table 1). However, New Forest ponies (n=15) and imported Icelandic horses suffered significantly more often from severe clinical signs than Finnhorses (P=0.0082 and P=0.0183, respectively), while no significant difference was found between Finnhorses and Icelandic horses born in Finland (Table 1). Severity of clinical signs was not associated with age at onset (Table 2). This was uniformly observed in both all horses and Finnhorses. Neither was there any significant relation between the duration of the disease and the severity of summer eczema (Table 2).
Table 2. Age at onset and duration of summer eczema in 207 horses with different types of clinical signs (I).

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>mild</th>
<th>moderate</th>
<th>severe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at onset</strong> (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All horses (n=207)</td>
<td>5.4±3.9</td>
<td>6.2±4.0</td>
<td>6.3±4.5</td>
</tr>
<tr>
<td>Finnhorses (n=107)</td>
<td>4.7±3.6</td>
<td>4.6±3.6</td>
<td>4.1±4.2</td>
</tr>
<tr>
<td><strong>Duration of disease</strong> (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All horses (n=207)</td>
<td>2.6±1.8</td>
<td>3.5±2.5</td>
<td>2.8±1.8</td>
</tr>
</tbody>
</table>

When the horses entered Study I, 81% had shown clinical signs in two consecutive summers or more. Most of the horses showed clinical signs from May to October. Yet, the time of onset varied; some horses had signs already in the spring, others not until autumn. Of the owners, 241 answered the question dealing with aggravating factors. Most (64%) regarded biting insects as one of the aggravating factors. In addition, grass fodder was recorded by 19% and sunlight by 12%, while 22% of owners were unable to specify particular factor.

**Autoserum therapy**

The 28 horses participating in the placebo-controlled and double-blinded study had suffered from summer eczema for 4.3±2.5 years (mean±sd, range 1-10). Of these horses, 6 (21%) had been affected with mild, 14 (50%) with moderate and 8 (29%) with severe clinical signs during the years before this study. Of the horses enrolled without placebo-controlling but treated with autoserum preparation in 1997-2008, 10% had shown mild, 76% moderate and 14% severe clinical signs before enrollment (III).

Most of the horses had been treated with various methods presented in Table 3. Special blankets designed for eczema horses were not in common use when Study II started, while many of the horses enrolled in the later study (III) used these coveralls (Table 3). In 8 horses, the beginning of blanket use and autoserum therapy coincided.

Table 3. Most common treatments used in horses with equine summer eczema before treatment with autoserum. Horses in a placebo-controlled part of Study II are indicated as A and the other horses as B. Horses participating in Study III, but not in Study II are indicated as C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Study II horses enrolled in 1997-98, A</th>
<th>Study II horses enrolled in 1997-98, B</th>
<th>Study III horses enrolled in 1999-08, C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blanket</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>103 (49%)</td>
<td>103 (38%)</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>10 (34%)</td>
<td>11 (33%)</td>
<td>34 (16%)</td>
<td>55 (20%)</td>
</tr>
<tr>
<td>Antihistamines</td>
<td>5 (18%)</td>
<td>2 (6%)</td>
<td>10 (5%)</td>
<td>17 (6%)</td>
</tr>
<tr>
<td>Ointments</td>
<td>17 (61%)</td>
<td>24 (73%)</td>
<td>133 (63%)</td>
<td>174 (64%)</td>
</tr>
<tr>
<td>Insecticides</td>
<td>8 (29%)</td>
<td>10 (30%)</td>
<td>60 (28%)</td>
<td>78 (29%)</td>
</tr>
<tr>
<td>No treatments</td>
<td>1 (4%)</td>
<td>1 (3%)</td>
<td>21 (10%)</td>
<td>23 (8%)</td>
</tr>
</tbody>
</table>
The overall evaluation of this autoserum treatment was based on three components: a placebo-controlled study (II), long-term information on clinical experience acquired from questionnaires by owners (III) and a 5-year follow-up of horses (III) originally participating in the placebo-controlled study (II). Results of these studies are provided in Table 4.

Table 4. Clinical efficacy of autoserum therapy (II, III).

<table>
<thead>
<tr>
<th>Placebo-controlled study, n=28</th>
<th>not aggravated</th>
<th>(milder, same)</th>
<th>aggravated</th>
</tr>
</thead>
<tbody>
<tr>
<td>autoserum group, n=14</td>
<td>13</td>
<td>(8, 5)</td>
<td>1</td>
</tr>
<tr>
<td>placebo group, n=14</td>
<td>7</td>
<td>(5, 2)</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Owners’ opinion of therapy, n=300</th>
<th>benefit</th>
<th>no benefit</th>
<th>no opinion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>209 (70%)</td>
<td>48 (16%)</td>
<td>42 (14%)</td>
</tr>
</tbody>
</table>

Clinical signs after a 4-week treatment

Clinical signs

<table>
<thead>
<tr>
<th>5-year follow-up, n=26*</th>
<th>mild</th>
<th>moderate or severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>before therapy</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>5 years after</td>
<td>12**</td>
<td>14***</td>
</tr>
</tbody>
</table>

* 2 of the original 28 horses underwent euthanasia soon after the placebo-controlled study, one of these due to another disease and one due to summer eczema and another disease
**including 5 horses without clinical signs
***including 4 horses euthanized due to summer eczema in 1998-2003

In the placebo-controlled and double-blinded study (II), horses were treated for 4 weeks, after which their clinical status was evaluated by the author. Signs were recorded as relieved, unchanged or aggravated. Of autoserum-treated horses, significantly fewer horses showed aggravated clinical signs (Table 4).

Data of new horses on autoserum therapy were collected continuously in 1997-2008 and the total response rate by owners to the questionnaires was 88%, corresponding to information on 301 of the 343 horses in total. One owner had not started the therapy at all, thus only opinions of 300 owners are listed in Table 4. Based on the information from owners, most of the horses had benefited from this treatment (Table 4). Some of the owners could not express clear opinion, e.g. if therapy has commenced in late autumn, a horse had been recently bought or the circumstances of the horse had simultaneously changed. According to owners’ answers, clinical signs usually became milder during the first 4 weeks of therapy; only 13% reported positive responses later (III).
Accelerated healing of skin lesions without the development of new damage was an initial sign of alleviation reported by the owners (III). Oral administration and a single daily dosage for one-week courses, usually twice in every summer month, were regarded as convenient to perform. No harmful side effects were reported during the 12-year period when data were collected.

Of the 28 horses that were originally grouped in the placebo-controlled study (II), 26 were followed for 5 years (Table 4). After 5 years, significantly fewer horses suffered from severe or moderate signs than before autoserum therapy (Table 4). These horses had received autoserum preparation mainly for two summer seasons (III), mean being 2.1±1.1 seasons (range 1-5). Some of the horses with mild signs (Table 4) had also been treated with locally applied ointments during these years. Only one horse had been medicated with glucocorticoids due to simultaneous respiratory signs. However, a total of 5 of the original 28 horses had been euthanized due to summer eczema (Table 4).

Of the 300 horses treated by autoserum therapy in 1997-2008 (III), 16% had received no benefit from this therapy according to owners’ answers (Table 4). Features of these 48 horses were analysed in detail (III). No significant association with breed, gender or age could be found. Neither were clinical results significantly linked to age at disease onset or duration of disease. Additionally, the use of special blankets showed no significant relation to better outcome of therapy. Severity of clinical signs was the only feature that had a significant association with the efficacy of this therapy; significantly fewer horses with severe signs had benefited from the treatment than horses with milder signs (P=0.0245). A similar trait was also observed among the 28 horses that had participated in the first placebo-controlled study (II); all 5 horses that were euthanized had suffered from moderate or severe clinical signs before autoserum therapy (Table 4).

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Phospholipids

Phospholipids in autoserum preparation
Of the PL classes (PC, PE, PS, PI, PA and SM) analysed, PC, SM, lysoPC and PA were detected from the preparations. Of these, PC was found most abundantly, and of the PC species, 34:0, 34:1, 34:2, 36:0, 36:1 and 36:2 displayed the highest concentrations. In addition, traces of many other PC species were detected and all of the PC species are presented in Table 9. The detected SM species were 16:0, 18:0 and 21:0, SM 16:0 showing the most abundant concentrations. Traces of lysolipids (lysoPC 16:0, 18:0, 18:1 and 18:2) were also found in all samples. PA was the only negative-charged lipid that was detected across all samples and comprised species of 32:0 and 34:1. Relative contents of the major PLs detected in autoserum preparations of healthy and affected horses are presented in Table 5.

Horses with summer eczema showed significantly different concentrations of PC, SM and PA in their autoserum preparations compared with preparations from healthy controls (Figures 4, 5 and 6). Both PC and SM were found more abundantly in the preparations of affected than healthy horses (P=0.042 and P=0.0017, respectively), while concentrations of PA were significantly higher in healthy horses (P=0.0075). Concentrations of lysoPC did not differ between affected horses and controls.

Comparisons of clinical signs and concentrations of PC, SM and PA displayed significant associations. Both PC and SM increased according to the severity of clinical signs (both P values <0.001), while concentrations of PA had a negative relationship with the severity of summer eczema (P<0.001).
Table 5. Relative contents of the major phospholipids detected in autoserum preparations of horses with summer eczema (n=10) and healthy control horses (n=6). Phospholipid classes: phosphatidylcholine (PC), sphingomyelin (SM), phosphatidic acid (PA) (IV).

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Relative content mean±sd (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eczema horses</td>
</tr>
<tr>
<td>PC</td>
<td>77.7±25.9</td>
</tr>
<tr>
<td>SM</td>
<td>2.1±0.7</td>
</tr>
<tr>
<td>PA</td>
<td>20.2±26.3</td>
</tr>
</tbody>
</table>

Figure 4. Phosphatidylcholine (PC) concentrations in autoserum preparations. Serum sample of 65 µl derived from horses with summer eczema (n=10) and healthy control horses (n=6) was first diluted 1:100 in distilled water, mixed and from top of this dilution, another 65 µl was further treated at 1:100 dilution with distilled water. From this, 65 µl superficial aliquot was dissolved 1:100 in 48% ethanol. Ethanol was evaporated and finally the residue was dissolved in 0.5 ml of chloroform/methanol for ESI-MS analysis. The concentrations of lipids are expressed as pmol/0.5 ml and not per original serum volume. The figure displays minimum, lower quartile, median, upper quartile and maximum PC levels. PC concentrations differed significantly between eczema and healthy horses (P=0.042) (IV).
Figure 5. Sphingomyelin (SM) concentrations in autoserum preparations. Serum sample of 65 µl derived from horses with summer eczema (n=10) and healthy control horses (n=6) was first diluted 1:100 in distilled water, mixed and from top of this dilution, another 65 µl was further treated at 1:100 dilution with distilled water. From this, 65 µl superficial aliquot was dissolved 1:100 in 48% ethanol. Ethanol was evaporated and finally the residue was dissolved in 0.5 ml of chloroform/methanol for ESI-MS analysis. The concentrations of lipids are expressed as pmol/0.5 ml and not per original serum volume. The figure displays minimum, lower quartile, median, upper quartile and maximum SM levels. SM concentrations differed significantly between eczema and healthy horses (P=0.0017) (IV).

Figure 6. Phosphatidic acid (PA) concentrations in autoserum preparations. Serum sample of 65 µl derived from horses with summer eczema (n=10) and healthy control horses (n=6) was first diluted 1:100 in distilled water, mixed and from top of this dilution, another 65 µl was further treated at 1:100 dilution with distilled water. From this, 65 µl superficial aliquot was dissolved 1:100 in 48% ethanol. Ethanol was evaporated and finally the residue was dissolved in 0.5 ml of chloroform/methanol for ESI-MS analysis. The concentrations of lipids are expressed as pmol/0.5 ml and not per original serum volume. The figure displays minimum, lower quartile, median, upper quartile and maximum PA levels. PA concentrations differed significantly between eczema and healthy horses (P=0.0075) (IV).
Phospholipids in serum

Of the PL classes analysed, all except PS were detected in the sera of healthy and affected horses, although the major classes were present in differing relative contents (Table 6). In addition to those classes presented in Table 6, PA 36:2 and lysoPC species of 16:0, 18:0 and 18:2 were detected. PC was the most abundant of the PL classes found, and its total concentrations in horses with summer eczema and in matched healthy controls are provided in Figure 7. The major molecular species of PC are presented in Figure 8, and all of the detected species are listed in Table 9. The most abundant species was PC 36:2, in both healthy and affected horses. The total serum concentrations of SM in affected horses and matched healthy controls are illustrated in Figure 9, and the detected SM species are presented in Figure 10. The most abundant species in all samples was SM 20:0 (Figure 10). Total concentrations of PC and SM were significantly more abundant in healthy than in affected horses (Table 7), despite the higher relative content of SM in the sera of horses with summer eczema (Table 6). Differences in specific molecular species between healthy and affected horses are provided in Figures 8 and 10. Concentrations of PC and SM increased in the samples that were collected later in the summer, both in affected horses and in their matched controls (horses numbered 7-10 in Figures 8 and 10). PI and PE were found in lower relative contents than PC or SM (Table 6). Concentrations of these minor PLs showed no significant difference between healthy horses and horses with summer eczema (Table 7).

Table 6. Relative contents of the major phospholipids detected in sera of horses with summer eczema (n=10) and their matched healthy controls (n=10). Phospholipid classes: phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylethanolamine (PE) (V).

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Relative content mean±sd (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eczema horses</td>
</tr>
<tr>
<td>PC</td>
<td>76.0±4.8</td>
</tr>
<tr>
<td>SM</td>
<td>21.9±3.8</td>
</tr>
<tr>
<td>PI</td>
<td>1.9±1.4</td>
</tr>
<tr>
<td>PE</td>
<td>0.2±0.2</td>
</tr>
</tbody>
</table>

Figure 7. Total serum phosphatidylcholine (PC) concentrations in horses with summer eczema and in their matched healthy controls (a) before therapy and (b) after a 4-week autoserum therapy of the eczema horses. A horse and its matched control are presented consecutively. Serum sample of 65 μl was first diluted in 4.2 ml of 48% ethanol, which was evaporated and finally the residue was dissolved in 0.5 ml of chloroform/methanol for LC-MS lipid analysis. Lipid concentrations are expressed as pmol/0.5 ml and not per original serum volume. Statistical comparisons between the groups at both time points are presented in Table 7 (V).
Figure 8. Concentrations of major species of phosphatidylcholine (PC) detected in sera of horses with summer eczema (n=10) and their matched healthy controls (n=10, matched pairs numbered similarly from 1 to 10) (a) before and (b) after a 4-week autoserum therapy of the affected horses. Samples were treated and concentrations of species are expressed as presented in Figure 7. All species showed significantly lower concentrations in affected horses before therapy (P<0.0001). After therapy, concentrations of PC 34:1 and PC 36:1 were significantly lower in affected horses, while PC 40:8 was significantly lower in healthy controls (P<0.05) (V).

Figure 9. Total serum sphingomyelin (SM) concentrations in horses with summer eczema and in their matched healthy controls (a) before therapy and (b) after a 4-week autoserum therapy of the eczema horses. A horse and its matched control are presented consecutively. Serum sample of 65 μl was first diluted in 4.2 ml of 48% ethanol, which was evaporated and finally the residue was dissolved in 0.5 ml of chloroform/methanol for LC-MS lipid analysis. Lipid concentrations are expressed as pmol/0.5 ml and not per original serum volume. Statistical comparisons between the groups at both time points are presented in Table 7 (V).
Figure 10. Concentrations of sphingomyelin (SM) species detected in sera of horses with summer eczema (n=10) and their matched healthy controls (n=10, matched pairs numbered similarly from 1 to 10) (a) before and (b) after a 4-week autoserum therapy of the affected horses. Samples were treated as presented in Figure 9. Concentrations of species are expressed as nmol/0.5 ml and not per original serum volume. SM 15:0 and SM 24:1 showed significantly lower concentrations in affected horses before and SM 18:0 and SM 21:0 after therapy than in their healthy controls (P<0.05) (V).
Table 7. Statistical pairwise analyses, according to the Friedman test, of differences in serum concentrations of the major phospholipid classes detected between horses with summer eczema (n=10) and their matched healthy controls (n=10, controls indicated with c). The samples collected before therapy of horses with summer eczema are indicated with 0 in both horses with summer eczema and their matched healthy controls, and the samples collected when the horses with summer eczema have been on autoserum therapy for 4 weeks are indicated with 4 in both horses with summer eczema and their matched healthy controls. Phospholipid classes: phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylethanolamine (PE) (V).

<table>
<thead>
<tr>
<th></th>
<th>PC 0</th>
<th>PC 4</th>
<th>PC 0 c</th>
<th>PC 4 c</th>
</tr>
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<tbody>
<tr>
<td>PC 0</td>
<td>p=0.0007</td>
<td>ns</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
</tr>
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<td>PC 4</td>
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<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PC 0 c</td>
<td>p&lt;0.0001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PC 4 c</td>
<td>p&lt;0.0001</td>
<td>ns</td>
<td>ns</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>SM 0</th>
<th>SM 4</th>
<th>SM 0 c</th>
<th>SM 4 c</th>
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<tbody>
<tr>
<td>SM 0</td>
<td>ns</td>
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</tr>
<tr>
<td>SM 4</td>
<td>ns</td>
<td>p=0.0005</td>
<td>ns</td>
<td>p=0.0186</td>
</tr>
<tr>
<td>SM 0 c</td>
<td>p=0.0115</td>
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<td>p=0.0186</td>
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<td>PI 4</td>
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<th>PE 0 c</th>
<th>PE 4 c</th>
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<tbody>
<tr>
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<td>p=0.0058</td>
<td>ns</td>
<td>p&lt;0.0001</td>
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<td>p=0.0058</td>
<td>ns</td>
<td>ns</td>
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<tr>
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<td>ns</td>
<td>p=0.0018</td>
<td>ns</td>
</tr>
<tr>
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<td>p&lt;0.0001</td>
<td>ns</td>
<td>p=0.0018</td>
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Changes in phospholipids after therapy

After a 4-week autoserum therapy, sera of affected horses and their matched healthy controls were analysed again and the relative contents of the main PL classes detected are provided in Table 8. In healthy horses, relative contents of the PL classes remained virtually unchanged, while in affected horses, concentrations of PC and SM had changed, and the resulting relative contents hardly differed from the values of healthy horses (Table 8). No significant differences could be found in the total concentrations of PC or SM between healthy and affected horses after therapy (Table 7, Figures 7 and 9). In the horses with summer eczema, the total PC concentration had increased significantly (Table 7), whereas healthy horses showed a slightly decreased total concentration (Figure 7). Concentrations of all the major PC molecular species in the affected horses shown in Figure 8 increased significantly (P<0.05) after therapy, while in the healthy horses the only significant change found was a decrease of PC 40:8 (P=0.0007). The total concentrations of SM decreased in both groups (Figure 9), and this change was significant in healthy horses (Table 7). Of the specific molecular species, concentrations of SM 16:0, 18:0, 24:0 and 24:1 decreased significantly (P<0.05) in both healthy and affected horses during the 4 weeks (Figure 10). Concentrations of PI or PE showed no significant differences between healthy and affected horses after therapy (Table 7).
Table 8. Relative contents of the major phospholipids detected in sera of horses with summer eczema (n=10) and matched healthy control horses (n=10) after the affected horses have been on autoserum therapy for 4 weeks. Phospholipid classes: phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylethanolamine (PE) (V).

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Relative content mean±sd (%)</th>
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<tr>
<td></td>
<td>Eczema horses</td>
</tr>
<tr>
<td>PC</td>
<td>87.4±1.7</td>
</tr>
<tr>
<td>SM</td>
<td>11.0±1.6</td>
</tr>
<tr>
<td>PI</td>
<td>1.4±0.7</td>
</tr>
<tr>
<td>PE</td>
<td>0.2±0.2</td>
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Changes in phospholipid concentrations and associations with clinical signs
Alterations in PL concentrations were evaluated in 10 eczema horses according to amelioration or aggravation of clinical signs after autoserum therapy (V). Five of the horses had mild and 5 moderate signs before autoserum therapy. After a 4-week therapy, clinical signs became milder in 6 horses and remained unchanged in 3, whereas one horse suffered from an aggravated disease. Horses with positive clinical outcome displayed a milder decrease in the total SM concentrations than horses with poorer response or controls (Figure 11). The change in these concentrations correlated significantly with alterations in clinical status (P=0.0047). Of the specific molecular species, SM 15:0 showed a significant association with the change of clinical signs (P=0.0268), and actually, SM 15:0 exhibited also a significantly lower concentration in affected horses before therapy than in healthy horses, but not afterwards (Figure 10).
Differences in phospholipid profiles between serum and autoserum preparations

The main PLs that differed in both the serum (V) and autoserum preparations (IV) between healthy horses and horses with summer eczema were PC and SM. Healthy horses showed significantly more abundant concentrations of PC and SM in their sera (V), while in autoserum preparations concentrations of these PLs were significantly lower in healthy horses than in affected horses (IV). Specific molecular species of PC and SM detected in the sera and concentrated in autoserum preparations are provided in Table 9. Autoserum preparations contained more species of PC than present in serum. Most of these species in the autoserum preparations seemed to have two double bonds or fewer; this was especially evident in the species with shorter acyl chains (Table 9). Accordingly, in autoserum preparations those species with more double bonds were found only in species with long acyl chains such as 38:5, 40:5 or 40:6 (IV). In addition, these highly unsaturated species were detected in minor quantities, while in serum (V) they were also found in abundant concentrations, e.g. 36:4 or 40:8 (Figure 8). In contrast to PC, more molecular species of SM were detected in serum than in autoserum preparations (Table 9). In serum, SM 20:0 was the most abundant in all horses (V), whereas SM 16:0 prevailed in autoserum preparations (IV).
Table 9. Phosphatidylcholine (PC) and sphingomyelin (SM) species detected in sera (n=20), autoserum preparations (n=16) or both in healthy horses and horses with summer eczema (IV, V).

<table>
<thead>
<tr>
<th>Sera</th>
<th>PC</th>
<th>Autoserum preparations</th>
<th>Same species in both</th>
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</table>

<table>
<thead>
<tr>
<th>Sera</th>
<th>SM</th>
<th>Autoserum preparations</th>
<th>Same species in both</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:0, 16:0, 18:0, 20:0, 21:0, 24:0, 24:1</td>
<td>16:0, 18:0, 21:0</td>
<td>16:0, 18:0, 21:0</td>
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6. DISCUSSION

General aspects of equine summer eczema

According to this thesis, Finnhorses and Icelandic horses were the breeds most afflicted by equine summer eczema in Finland, the vast majority of horses comprising Finnhorses (I, III). During the 11-year period of Study I the average number of Finnhorses in Finland was 19 500 and the number of Icelandic horses 1300, while the total number of other breeds was continuously about 10 000 greater than the number of Finnhorses over those years (Hippos 2016). Noteworthy is that only 23% of the eczema horses in Study I comprised breeds other than Finnhorses or Icelandic horses, while in many countries this disorder is typical in warmblood horses (Kurotaki et al. 1994, Steinman et al. 2003a).

Icelandic horses imported from Iceland are known to be prone to insect hypersensitivity since they have not been predisposed to bites of *Culicoides* (Broström et al. 1987, Björnsdottir et al. 2006), indicating that horses exposed since birth are less commonly affected than horses without early contacts with insects (Broström et al. 1987, Björnsdottir et al. 2006). In Finland, virtually all horses, even in northern Finland, are exposed to biting insects since birth, and thus, unknown factors are responsible for the susceptibility of Finnhorses to develop summer eczema. Andersson et al. (2012) suggested that in horses not born versus born in Iceland genetic susceptibility may play a more marked role in the outbreak than purely environmental factors. However, genetic studies on Finnhorses have not yet been performed. Due to strict official breeding orders and accurate records maintained for Finnhorses for over a century, this breed could be an interesting target for such studies, as has been shown in the recent study on crib-biting in Finnhorses (Hemmann 2014).

Study I revealed that summer eczema usually manifests by the age of 5 years and seldom after 12 years in Finnhorses. It is therefore a disease of young animals, although it tends to follow a horse for years (Barbet 1992). Findings of the typical age of onset in other breeds are controversial since in some studies summer eczema has been concentrated in grazing seasons and in others the prevalence of the disease has been compared between age groups (Broström et al. 1987, Halldorsdottir & Larsen 1991, Steinman et al. 2003a). However, Icelandic horses not born in Iceland were found to develop summer eczema by the age of 5 years (Halldorsdottir & Larsen 1991, Schurink et al. 2013), which is consistent with the findings in Finnhorses. Interestingly, Wagner et al. (2003) demonstrated that Icelandic horse foals younger than six months did not show any serum IgE when they were exposed to biting insects, in contrast to their simultaneously IBH-affected or healthy dams. Furthermore, in atopic dermatitis of humans, 85% of the outbreaks are found before the age of 5 years, but rarely after adolescence (Kawakami et al. 2009, Dubrac et al. 2010, Rutkowski et al. 2014). This implies that there may be some common immune-mediated mechanisms that predispose to allergies or atopic disorders in youth. Notably, age at onset seemed not to predict severity of clinical signs neither in all horses nor in Finnhorses (I).

Most of the horses participating in this study had moderate clinical signs, and this was also the case among Finnhorses (I, III, IV). The subgroups of horses that significantly more often showed severe signs than Finnhorses were New Forest ponies and Icelandic horses imported from Iceland (I). The latter finding agrees with the observation that imported Icelandic horses show more severe disease than horses not born in Iceland (Broström et al. 1987). Despite the seasonally typical recurrences, clinical signs seemed not to be aggravated over the years, with no significant difference found between duration of the disease in horses with mild, moderate and severe signs (I). This result differs from an earlier cross-sectional survey on Icelandic horses (Broström et al. 1987) in which clinical signs tended to worsen with time, but is in accordance with the later findings of Schurink et al. (2012, 2013), who showed no increase in severity over the years.

The rather small number of horses with mild signs may be partly explained by the strict limits in classification of signs and perhaps owners’ unwillingness to contact veterinarians when their horses suffered only from pruritus without skin lesions, i.e. mild disease (I, II, III). This is supported by the data of van Grevenhof et al. (2007) showing that Shetland ponies suffer more often
from mild than severe signs when mild signs also include signs of dermatitis. In the present study, there were no Shetland ponies with only pruritus without skin lesions (I). Thus, accurate classification of clinical signs when results between various clinical studies are evaluated is critical.

**Autoserum therapy**

The idea to use autoserum therapy in the treatment of equine summer eczema stemmed from our earlier studies on equine sarcoid and its treatment with an autogenous polymerized tumour vaccine prepared from excised sarcoid material and supplemented with serum proteins (Tallberg et al. 1979, Kinnunen et al. 1999, Hallamaa 2007). However, in summer eczema our original hypothesis focused on lipids that were supposed to be excessively produced and thereby released into the circulation, leading to abnormal aggregate complexes of lipid particles (II). According to this hypothesis, these clusters would be incapable of binding their corresponding receptors on the plasma membrane, thus preventing the target signalling pathways. Additionally, lipids as a part of floating lipoproteins (Watson et al. 1991, 1993, Hammad et al. 2012) constitute the main fraction that could manage the serial dilutions with water and finally with highly concentrated ethanol during the processing of serum since water-soluble molecules are dissolved during washings and more insoluble molecules, such as proteins, will be denatured by alcohol (Nelson & Cox 2008).

The results of the first placebo-controlled and double-blinded clinical study on this therapy were encouraging (II). Horses in the placebo group showed significant aggravation during the 4-week follow-up compared with horses on autoserum therapy (II). In general, it is important to explicitly state the criteria for the evaluation of clinical results, especially between the different studies and therapies. In the present study, horses were assessed according to the same 3-graded scale over the entire study period from 1997 to 2014. Based on the long-term information collected about the disease and autoserum therapy (I, II, III and V), this scale proved to be a practical and sufficient method for classifying clinical signs and evaluating therapeutic responses, especially in Finnhorses (I).

The majority of the horses treated by autoserum therapy (III) had suffered from moderate or severe clinical signs before enrolling. Interestingly, horses entering this study after 1998 showed significantly less severe signs than horses included earlier (III). The use of special blankets had simultaneously become more common in Finland (III), which could explain this difference since there were no blanket users when the first placebo-controlled study was carried out. However, no significant difference in the clinical response to autoserum therapy was found between horses with and without a blanket (III). These special blankets may shield horses from developing large skin lesions, although this relationship was not statistically assessed here. In any case, the beneficial effect of the use of a blanket combined with overnight stabling has been demonstrated by Olsén et al. (2011).

After a 5-year follow-up, significantly fewer horses showed moderate or severe signs than before treatment and some of the horses were actually symptom-free (III). According to earlier research, equine summer eczema does not typically improve with age (Scott & Miller 2003, Schaffartzik et al. 2012), although Schurink et al. (2009) did report unexpectedly low repeatability among a Shetland mare population. However, they speculated that these results were probably due to ambiguity in data collection (Schurink et al. 2009), and in their later studies this trait of low repeatability could not be demonstrated (Schurink et al. 2012, 2013). Therefore, this follow-up result of amelioration in 12 horses was a favourable outcome, especially as most of the horses had been affected with summer eczema for several years before enrolment. However, 5 horses of the original 28 horses had been euthanized due to severe clinical signs of summer eczema, which had not improved with autoserum therapy (III). When horses without benefit from this therapy were assessed in detail, severity of initial clinical signs had a significant association with failure of autoserum treatment (III). This is consistent with earlier studies on allergen-specific immunotherapy in both horses and humans; poorer responses were found in the treatment of disorders with more severe signs (Anderson et al. 1996, Ginsberg & Eichenfield 2016, Slavyanakaya et al. 2016). It has been demonstrated recently that various cell surface proteins, especially those linked to eosinophils, were expressed differently depending on the type and severity of allergic disorders...
(Metcalfe et al. 2016). Some of those proteins were up-regulated when patients showed mild or moderate signs, whereas others only in severe clinical manifestations (Metcalfe et al. 2016). Therefore, unknown immunological/biochemical factors may be related to the extent of equine summer eczema, rendering the most affected horses refractory to therapies, including autoserum treatment.

Owners’ opinions of the therapy were mainly positive. Approximately 70% of horses had benefited from this autoserum treatment according to their owners (III). However, the lack of placebo-controlling makes the assessment of this result somewhat unreliable. Owners may evaluate findings without reasonable objectivity or may be willing to give positive answers to please the researcher. Nevertheless, most owners in this study had been involved with this disorder for years, thus being experienced and fairly sceptical of various treatments. In addition, responses of owners who had recently purchased their horses and were unfamiliar with the manifestation of the disease were recorded as ‘no opinion’ by the author. The rather high proportion of horses receiving considerable benefit from this treatment, about 28%, relative to horses receiving some or no benefit, 42% and 16%, respectively (III), suggests that most of the positive answers by owners were based on realistic evaluations. Interestingly, in most of the horses receiving benefit from therapy, clinical signs started to regress within 2-4 weeks of commencing autoserum therapy (III), a time when allergen-induced late cutaneous responses have been observed to be suppressed in humans after allergen-specific immunotherapy (Matsuoka et al. 2013).

The long-term study made it possible to attenuate the impact of annual weather conditions on clinical results since various climatic changes influence the amount of Culicoides (Björnsdottir et al. 2006, van Grevenhof et al. 2007). Furthermore, over the years of this study, horses were enrolled from many areas of Finland, thus minimizing the potential impact of geographical variation (Broström et al. 1987), such as seaside (Riek 1953), altitude (Steinman et al. 2003a) or vegetation (van Grevenhof et al. 2007), on the number of insects.

In general, oral administration was regarded as easy to perform, and no adverse effects related to this therapy were recorded during the series of studies carried out in 1997-2014. The route of administration plays an important role in compliance, especially when we are working with animals. Oral administration is considered more convenient than, for instance, serial injections (Anderson et al. 1996, Valenta et al. 2012, Jonsdottir et al. 2015, Slavyanakaya et al. 2016). One disadvantage of autoserum therapy, besides the poor clinical outcome in some horses, was the 2-week wait period before the blood sample for autoserum preparation could be collected. This delay was especially stressful when a horse showed severe signs soon after onset. However, this time was needed since the prerequisite for blood sampling (II, III, V) was that the allergic reaction be fully initiated, which occurs within 2 weeks of exposure to insect bites (Barbet 1992). Nonetheless, autoserum therapy was continued the following summer, when necessary, with the former preparation stored at room temperature, without renewed collection of blood (III).

### Phospholipids

**Findings in serum**

Of the major PLs in the mammalian serum (Fuchs et al. 2009, Quehenberger & Dennis 2011), PC and SM were detected in healthy and affected horses. However, horses with summer eczema showed significantly lower total concentrations of PC and SM in their sera than healthy controls (V). Due to matching of these horses, environmental circumstances, such as the fodder and feeding regimen, were similar between the affected horses and their counterparts. Moreover, the samples from the affected horse and its matched healthy control were collected at the same time. Therefore, it is unlikely that nutritional factors had markedly impacted on these results, although the composition of serum lipids (Subbaiah & Liu 1996, Vesper et al. 1999, O’Connor et al. 2007, Fuchs et al. 2009, Paassilta et al. 2014) and even of milk lipids (Laitinen et al. 2006, Thijs et al. 2011, Kuhnt et al. 2015) is strongly dependent on the type of diet, and certain diets may per se decrease the risk for allergies (Magnusson et al. 2015). It has been suggested that PC is consumed as a source of arachidonic acid that is further converted to leukotrienes in cells assembling in allergic responses (Metcalfe et al. 2016). Thus, the lower PC levels in the serum of eczema horses could
be explained by the accelerated turnover to eicosanoids. Also concentrations of SM may fluctuate according to the amounts of PC since SM can be converted from PC (Olivera & Rivera 2011, Ried et al. 2013), and these PLs are carried by the same lipoproteins in the circulation (Watson et al. 1991). Furthermore, SM may be consumed via sphingomyelinase action in the synthesis of ceramide, which is the main component of the epidermal lipid barrier (Proksch et al. 2003, Kolter 2011). This barrier needs constant repairing in severe skin afflictions (Proksch et al. 2003). Affected horses in Study V did not show large skin lesions, and therefore, the lower SM levels in these horses were probably not linked to skin injuries. In addition, epidermal lipid synthesis is highly independent of circulating lipids (Proksch et al. 2003). It seems more likely that the decreased SM concentrations reflect immune-mediated changes in lipid metabolism and mediators since SM is a precursor for signalling molecules such as S1P (Olivera & Rivera 2011, Maceyka & Spiegel 2014, Kulinski et al. 2015). However, concentrations of S1P were not analysed in this thesis, and the role of these molecules remains to be elucidated.

Leslie et al. (2008) demonstrated that serum lipids of healthy humans, especially polar lipids such as PLs, regulate expression of CD1 molecules on the APCs, i.e. lipids regulate presentation of lipid antigens, and thereby, the subsequent T cell activation. These serum lipids act in a dose-dependent manner and this action is reversible depending on the state of inflammatory responses (Leslie et al. 2008). Horses may also have lipid antigens involved in the pathogenesis of summer eczema. However, lipid antigens and the adjacent CD1 antigen-presenting proteins related to summer eczema have not been demonstrated thus far, although the role of lipids as a primary or at least a part of protein antigens has been widely recognized in allergies affecting humans (De Libero & Mori 2010, Jyonouchi et al. 2011, Bublin et al. 2014, Layre et al. 2014). The findings of Study V suggest that the lower levels of PC and/or SM in eczema horses compared with healthy horses could predispose these horses to continued antigen presentation, and further, when these concentrations have become restored to levels indistinguishable from healthy horses, also clinical signs have become milder (V). The observations of Leslie et al. (2008) seem to support this speculation.

PA was detected in serum (V), which was a surprising finding. This PL has not been demonstrated earlier in horse serum (Fuchs et al. 2009), and as a minor inner leaflet lipid it is not a usual component of serum (Nelson & Cox 2008, Fuchs et al. 2009). In the present study, sera were harvested without centrifugation; it is thus probable that the samples contained also a few blood cells, leading to these detectable amounts of PA (IV, V). This is also supported by the fact that healthy horses had PA more abundantly in their autoserum preparations than affected horses (IV). The erythrocyte sedimentation rate is generally slower in healthy horses than in horses with inflammatory disorders since fibrinogen increases aggregation of red blood cells (Baskurt et al. 1997), leading to more rapid sedimentation in horses with inflammation. Despite the significant differences in PA detected in autoserum preparations of healthy and affected horses, PA may not have a marked role among the PLs related to this disease. In addition to PA, lysoPC was detected in serum (V). This PL mainly originates from PC after phospholipase degradation during storage at room temperature (Breier et al. 2014) and was thus an expected finding. Therefore, the impact of lysoPC was not evaluated further, especially when its concentrations did not differ significantly between healthy and affected horses (IV, V). Interestingly, in humans with cholestasis induced pruritus, serum concentrations of lysoPA and autotaxin activity were significantly higher than in healthy controls (Oude Elferink et al. 2011). Autotaxin is an enzyme that converts lysoPC into lysoPA, which in turn has receptors on neurons and is thus suggested to be involved in itch signalling (Oude Elferink et al. 2011).

Of the PL classes analysed, only PS was not detected in serum samples (V). This PL has been demonstrated in EVs (Laulagnier et al. 2004, Chaput & Théry 2011), particularly at the surface of exosomes, which are EVs of endosomal origin (Raposo & Stoorvogel 2013). Extracellular vesicles are small, 50-100 nm, membrane-coated particles (Harding et al. 2013, Raposo & Stoorvogel 2013) that are secreted by numerous cell types, especially cells involved in immune responses (Bobrie et al. 2011, Chaput & Théry 2011). Although PS was not detectable in the samples, this does not necessarily rule out the possibility that some part of the detected PLs had stemmed from exosomes present in the circulation. The amount of the analysed serum from these horses was small (V) and consequently also the possible number of vesicles included. Additionally, PC, SM and PE have been demonstrated to show higher relative contents of the PLs usually detected in those vesicles compared with the levels of PS (Laulagnier et al. 2004).
Findings in autoserum preparations in comparison with serum
The presence of lipids in autoserum preparations was the original hypothesis for the treatment of horses with summer eczema in Studies II and IV. Additionally, the finding that affected horses showed more abundant concentrations of PC and SM in these preparations (IV) was in accordance with our preliminary suggestion that horses with summer eczema have abnormally large amounts of lipids in the circulation (II). Therefore, there was a discrepancy between the results from serum and autoserum preparations. In contrast to our preliminary hypothesis (II), affected horses showed significantly lower concentrations of these major serum PLs in their sera than healthy horses (V). This contradictory finding may be related to the changes in hydrophobic/hydrophilic interactions between lipid molecules and their lipoprotein carriers (Watson et al. 1993) among horses with summer eczema. Lipid composition of autoserum preparations showed that hydrophobic lipid classes and their specific species had been concentrated in those preparations (IV). Although minor amounts of PI and PE were detected in serum (V), they were not found in the preparations (IV). Evidently, this resulted not only from their usually low relative content in mammalian sera (Fuchs et al. 2009), but also from their more hydrophilic nature compared with PC or SM (Nelson & Cox 2008). In autoserum preparations, the less water-soluble PC species were prevalent and the most hydrophilic species were found only in low concentrations (IV), whereas in the serum these more water-soluble species were abundantly present (V). This supports the interpretation that the most hydrophilic species were dissolved in water during the processing of serum. However, species of SM seemed to act in a slightly different way. SM 16:0 was the most abundant in autoserum preparations, while SM 20:0 predominated in serum, although the former should be more soluble in water than the latter (Nelson & Cox 2008). This suggests that there are at present some obscure factors influencing hydrophobic/hydrophilic properties of certain PLs and their specific species in horses with summer eczema. Mineral salt interactions have been found to affect the solubility of lipids and make lipids even more insoluble in water (Folch et al. 1957). Moreover, lipid-mineral salt interactions dissociate, but only after several washings (Folch et al. 1957). This could be a potential explanation for eczema horses having lower concentrations of PC and SM in serum but higher concentrations in autoserum preparations than healthy horses, and for certain more water-soluble species being concentrated in autoserum preparations.

Phospholipids and clinical status
In horses with summer eczema, serum concentrations of PC and SM were significantly lower than in healthy control horses, although they per se showed no significant associations with the severity of clinical signs before therapy (V). On the other hand, concentrations of PC and SM in autoserum preparations displayed a significant relation to severity of signs (IV). Based on these findings, PL profiles in serum seemed to reflect “a rough picture” of the clinical status, i.e. healthy or affected, while in autoserum preparations they provided a more detailed fingerprint. This implies that lipid particles linked to the severity of this allergic disorder could have been concentrated in autoserum preparations. However, this interpretation requires further studies with a greater number of horses than here.

Changes in the total SM concentrations correlated significantly with the alterations in clinical status in response to therapy (V). Of the specific SM species, SM 15:0 showed a significant association with the alterations in clinical signs after therapy and also was exhibited at significantly lower levels in eczema horse sera than healthy horse sera before therapy, but not afterwards. Therefore, SM seems to play a dynamic role in the course of this disease.

Mode of action in autoserum therapy
The underlying mechanism of this therapy remains unknown. A rational explanation for the action is probably related to cell signalling pathways. The small amounts of various PLs, detected mainly in pico- or nanomolar concentrations, in the autoserum preparations could be sufficient only as a part of signalling cascades, particularly participating in downstream pathways. On signal transduction, minute amounts of molecules are able to interact not only as inducers but also as amplifiers of the corresponding cascades (Nelson & Cox 2008, van Meer & de Kroon 2011). Nanomolar concentrations are typical for lipid mediators, with these substances being formed during inflammation and its resolution (Serhan & Petasis 2011, Stables & Gilroy 2011); S1P receptors,
for instance, are sensitive to nanomolar affinities (Chun et al. 2010). Sometimes, higher amounts of lipid mediators even decrease the expression of target substances, while lower concentrations accelerate their production (Demetz et al. 2014). Intriguingly, sphingolipids have been detected in other biological substances, e.g. dairy products such as milk and butter, usually in micromolar concentrations (Vesper et al. 1999).

The site of action and the target cells involved could not be established based on the information here. However, some cautious suggestions can be made. In autoserum preparations, lipid particles were absorbed into sugar granules for oral administration. Recently, it has been demonstrated that taste receptors are able to regulate innate immune defences and these taste receptors are also expressed in various tissues beyond the oral cavity, thus supporting their additional function in immune responses (Laffitte et al. 2014, Lee & Cohen 2015, Workman et al. 2015). Of these receptors, bitter and sweet taste receptors are the most important (Lee & Cohen 2015, Workman et al. 2015). Therefore, it seems possible that sweet taste receptors could perceive lipid molecules embedded in sugar granules and provide the primary step for the ongoing action. In the next phase, immune cells, probable basophils, might interact with taste receptors, potentially leading to a modification of FcεRI receptors. Mast cells and basophils are the main cell types assembling in allergic reactions by releasing histamine and a plethora of lipid mediators and cytokines when activated via FcεRI receptors (Kawakami & Galli 2002, Wagner et al. 2006, Kawakami et al. 2009, Cromheecke et al. 2014, Galli et al. 2016, Metcalfe et al. 2016). However, of these cells only basophils are detected in the circulation (Galli et al. 2016, Kratzer & Pickl 2016, Metcalfe et al. 2016). Basophils have been implicated as one of the major cell populations expressing IgE in the peripheral blood of IBH-affected horses (Wagner et al. 2003) and cell response to specific allergen challenge in horses with summer eczema (Langner et al. 2008). Therefore, basophils could be one of the messengers participating in the response during autoserum therapy. This suggestion is based also on the following investigations dealing with basophils and allergic inflammation. Local antigen challenge has been shown to activate circulating basophils, i.e. inducing also systemic allergic influence (Saini et al. 2004). In addition, basophils are able to promote CD4+ T helper cell polarization (Sokol et al. 2008, Kim et al. 2009, Otsuka & Kabashima 2015) and also to act as antigen-presenting cells to CD8+ T cells (Kim et al. 2009). Moreover, Mukai et al. (2005) demonstrated that basophils are essential for the development of IgE-mediated chronic allergic reactions without the intervention of T cells or mast cells. Recently, basophils have been linked to the development of severe cutaneous hypersensitivity to mosquito bites in humans (Sakakibara et al. 2015). Because basophils interact in various phases of immune responses and they possess capacities not only in innate immune responses but also in adaptive immune responses (Schneider et al. 2010), they might be involved in the course of summer eczema and its resolution after autoserum therapy. Further basic research, including cell culture approaches, is needed.

In mast cells, ensuing activation of FcεRI receptors promotes synthesis of S1P (Olivera & Rivera 2005, 2011, Price et al. 2008, Kulinski et al. 2015), and this lipid mediator has certain two-dimensional functions by enhancing or suppressing allergic responses, possibly depending on type of allergen and site of action (Kulinski et al. 2015). An interesting finding was made in the study of Hamza et al. (2008); a supernatant collected from cultured PBMCs of healthy horses and added to cultures from horses with summer eczema abrogated IL-4 production in these cell cultures. They observed that IL-10 and TGF-β, the major cytokines produced by Treg cells (Akdis et al. 2005), possessed this down-regulating effect on IL-4 secretion when these cytokines were simultaneously expressed. But, as stated by Hamza et al. (2008), there may be other factors present in the supernatant that also promote or affect IL-4 down-regulation. Notably, they added autologous serum to these cell cultures, and accordingly, the supernatants probably contained signalling molecules, such as S1P, that are normally present in serum (Christoffersen et al. 2011, Hammad et al. 2012). Afterwards, Hamza et al. (2012) showed that horses with severe eczema were incapable in response allergen challenge of promoting expression of Treg cells, in contrast to healthy horses. Intriguingly, S1P plays a pivotal role in the maturation and function of Treg cells (Cyster & Schwab 2012). Galli et al. (2016) demonstrated that serum collected from animals exposed to honey bee venom prevented detrimental allergic reactions in naïve animals, although injected in minute amounts. When binding to the FcεRI receptor was blocked, the beneficial effect of the donor serum disappeared (Galli et al. 2016). This cautiously supports the speculation
that the autoserum preparation could interpose via FcεRI receptors and the changes found in the SM concentrations of the horses with summer eczema may reflect or influence alterations in S1P production. The findings of significantly higher SM concentrations in the preparations of affected horses than in healthy horses and the significant correlation between the changed SM concentrations and clinical status after therapy are in agreement with this assumption. However, S1P was not analysed in this thesis. Even though alterations in SM concentrations correlated with clinical signs, no conclusions can be drawn from the causality.

Although many research projects associated with S1P signalling are ongoing (Chun et al. 2010), various basic mechanisms between lipid mediators and allergic responses are still unresolved, not only in relation to the present thesis, but also at a more general level (Christoffersen et al. 2011, Cyster & Schwab 2012, Maceyka & Spiegel 2014, Kulinski et al. 2015, Galli et al. 2016). Advanced methods to analyse detailed information on cell biochemistry (Shaner et al. 2009, Hammad et al. 2010, Harkewic & Dennis 2011, Ried et al. 2013, Maceyka & Spiegel 2014, Metcalfe et al. 2016) will help to illuminate the answers.

Limitations of the thesis

In addition to the limitations related to autoserum therapy and discussed earlier in that section, there are limitations in the analysis of the blood samples. By low speed centrifugation for autoserum preparation, red blood cells could have been avoided in serum samples, without compromising lipid recovery. In addition, lipoprotein particles were not analysed in detail. Therefore, water-insoluble components other than PLs carried by lipoproteins remained unidentified in autoserum preparations, likewise their possible therapeutic effect.

Clinical implications

This was the first series of studies focused on serum PLs and their use in treating horses with summer eczema. Autoserum treatment showed beneficial effects on this allergic skin disease and was convenient to apply in clinical practice since serum PLs could be easily extracted and prepared for oral administration. Based on the findings of this thesis and recent literature in human medicine, serum lipoproteins with specific PL species could be an interesting target for further studies on immune-mediated disorders in horses, especially respiratory tract allergies or autoimmune diseases. Accordingly, the utilization of autoserum preparation in the therapy of other allergic manifestations should be explored.
7. CONCLUSIONS

1. This study demonstrated that in Finnhorses summer eczema usually manifests at a young age, and most horses in contact with veterinarians suffer from moderate clinical signs. Severity of clinical signs was not related to either age at onset or duration of disease.

2. Autologous serum preparation containing the major serum phospholipids was shown to be a favourable method to treat equine summer eczema, with no harmful side effects.

3. Horses with summer eczema had an altered phospholipid profile in their sera compared with healthy horses, and these profiles seemed to change according to the clinical status of the horse. In affected horses, phosphatidylcholine and sphingomyelin were more concentrated in autoserum preparations than they were in healthy horses. Of the major serum phospholipids, sphingomyelin might be a potential link between equine summer eczema and its therapy; however, the causal relation has to be verified in future studies.
8. ACKNOWLEDGEMENTS

“Sometimes crazy ideas prove to be useful”. These were the encouraging words of Docent Thomas Tallberg when I first introduced my idea of autoserum therapy to him in 1996. That was the start of my studies on equine summer eczema, and over the next twenty years, I have met some wonderful people, visited numerous stables and seen, of course, many horses. I sincerely thank everyone who has helped me on this journey and contributed to my academic dissertation, not forgetting the owners of the horses and the practising vets. Special thanks go to the following persons and institutions:

My supervisors, Professor Outi Vainio and Docent Marja Raekallio, at the Faculty of Veterinary Medicine have been the main promoters of my dissertation and without their encouragement and positive attitude this work would never have been finished. I am deeply grateful for their scientific help and guidance and also appreciate the good atmosphere that they created for students in their department.

I am indebted to Docent Thomas Tallberg and his kind staff, especially Eeva Lönnqvist, at the Institute of Bio-Immunotherapy, where we collaborated on several long-term research projects. They gave me important support in organizing these studies, and Docent Tallberg had a crucial role in our studies focusing on lipids. Unfortunately, he did not live to see the fruits of our labour.

I warmly thank Krishna Batchu for his valuable work with lipid analyses and for his co-authorship. He always had time for me even though he was writing his own PhD thesis. I am also grateful to Tarja Grundström for extensive laboratory work with the horse samples. I gratefully acknowledge Docent Pentti Somerharju for the opportunity to conduct the lipid analyses at the Department of Developmental Biology and Biochemistry, Institute of Biomedicine, University of Helsinki.

I am sincerely grateful to Docent Petteri Nieminen for kindly accepting the invitation to be my opponent.

My deep gratitude is owed to the official reviewers of this thesis, Docent Peter Mattjus and Docent Matti Jauhiainen, for the expert comments and valuable criticism that greatly improved the manuscript.

I am also thankful to University Lecturer Sami Junnikkala for his contribution to this thesis, especially for his excellent comments to the section dealing with immunology.

My author-editor Carol Ann Pelli revised the language of the thesis, and I deeply appreciate her help.

I am grateful to Docent Reijo Käkelä and all members of the Functional Lipidomics Group for the time spent at Lipidomics seminars. These seminars were worthwhile not only for the abundant breakfasts but also for the intriguing lectures from the diverse field of lipids and the fresh ideas that emerged from our discussions. I also thank Reijo for his advice and useful comments on my thesis.

Professor Seppo Sarna has provided assistance with statistical analyses and Docent Jan Dabek with language issues, for which I am most grateful. I also thank Docent Mirja Ruohoniemi for her advice and attentive comments when I was writing my publications and Karin Hemmann, PhD, for her help with many practical matters.

Docent Lena Huldén and Larry Huldén, PhD, are thanked for cooperation and many interesting discussions about insects, especially Culicoides, in recent years.
This study was supported by a grant from the Albert Lindsay von Julin Foundation, for which I am sincerely grateful.

I deeply thank Ritva Nyman, my friend and one of the co-authors of the first study on autoserum therapy, for her help and advice in preparing the serum. Our inspiring discussions and sometimes even intense arguments during our wanderings in Lapland gave me impetus for this work.

I am grateful to my assistant Leena for her skilful work with autoserum preparations over the years; she has really been my “right hand” with her laboratory and secretarial assistance. My warm thanks also go to Leila and Minna at our veterinary clinic for helping me with day-to-day problems.

Finally, I thank my kind husband Raimo for his support, understanding and love, always.
9. REFERENCES


Batchu, K.C. (2016) Factors regulating the substrate specificity of A-type phospholipases – a mass-spectrometric study. *Academic Diss.*, Faculty of Biological and Environmental Sciences, University of Helsinki, 17-35.


Hemmann, K. (2014). Crib-biting in Horses: A Physiological and Genetic Study of Candidate Causative Factors. Academic Diss., Faculty of Veterinary Medicine, University of Helsinki.


Kainu, V. (2012) Metabolism and Translocation of Aminophospholipids in Mammalian Cells. *Academic Diss.*, Faculty of Medicine, University of Helsinki.


APPENDIX I

Date ________

Owner
Address
Telephone
Name of the horse
Breed
Gender
Date of birth, country
Colour
Use of the horse

Anamnesis:
Onset of clinical signs

Severity of signs before autoserum treatment:

- Itching in the mane ______
- Itching in the tail ______
- Mild skin lesions in the mane ______
- Mild skin lesions in the tail ______
- Mild skin lesions in the body ______
- Large skin lesions in the mane ______
- Large skin lesions in the tail ______
- Large skin lesions in the body ______

Aggravating factors

Hereditary background

Treatments given and their influence
APPENDIX II

FOLLOW-UP

Date__________

Owner

Name of the horse

Clinical signs after start of autoserum therapy:

Pruritus: same ______
milder ______
worse ______

Skin lesions:
same ______
milder ______
worse ______

If pruritus has become milder, when has this occurred?
during the first 2 weeks of therapy ______
during the 1-week pause ______
during the third week of therapy ______
later ______

Has the horse been tired during therapy?
no ______
yes ______

If yes: during the first 2 weeks of therapy ______
during the 1-week pause ______
during the third week of therapy ______
later ______
all the time ______

Has appetite changed?
no ______
yes ______

Has the horse been exposed to?
sun ______
insects ______
grass fodder ______

Use of autoserum preparation:

Autoserum preparation has been administered:
about 1 week a month ______
about 1-2 weeks a month ______
about 2-3 weeks a month ______
about daily ______
Indicate the months of medication:

May       
June      
July      
August    
September 
October   

Do you think that your horse has benefited from this therapy relative to the severity of clinical signs in earlier summers?

no benefit
some benefit
much benefit

How convenient was this therapy to perform?

easy
rather easy
difficult

Has your horse had some adverse effects from this therapy?

yes
no

If yes, what kind of effects? ________________________________________________________________