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The metabolomic profiling of psoriasis, atopic dermatitis and atherosclerosis

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Department of Biochemistry, Institute of Biomedicine and Translational medicine, University of Tartu, Tartu, Estonia

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To my son Albert

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–III)

I. Ottas A, Fishman D, Okas TL, Kingo K, Soomets U. The metabolic analysis of psoriasis identifies the associated metabolites while providing computational models for the monitoring of the disease. *Arch Dermatol Res*. 2017;309(7):519–528.

II. Ottas A, Fishman D, Okas TL, et al. Blood serum metabolome of atopic dermatitis: Altered energy cycle and the markers of systemic inflammation. *PLoS One*. 2017;12(11):e0188580. Published 2017 Nov 27. doi:10.1371/journal.pone.0188580

III. K. Paapstel, J. Kals, J. Eha, K. Tootsi, **A. Ottas**, A. Piir, M. Jakobson, J. Lieberg, M. Zilmer. Inverse relations of serum phosphatidylcholines and lysophosphatidylcholines with vascular damage and heart rate in patients with atherosclerosis, Nutrition, Metabolism and Cardiovascular Diseases, Volume 28, Issue 1,2018, Pages 44–52, ISSN 0939–4753, https://doi.org/10.1016/j.numecd.2017.07.011.

The author of this current dissertation contributed to the publications as follows: Papers I and II: the author designed the studies; planned, coordinated and participated in clinical sample collection (where possible), prepared and measured the samples, performed parts of statistical analysis and figure generation, interpreted the results and wrote the papers. In paper III the author performed the measurements for the targeted analysis of serum samples and critically reviewed the paper before submission.

ABBREVIATIONS

[C16 + C18]/C0	ratio of long-chain acyl-carnitines to free carnitine
a	acyl
aa	diacyl
AD	atopic dermatitis
ADMA	asymmetric dimethylarginine
ae	acyl-alkyl
AIx@75	augmentation index adjusted to a heart rate of 75 beats per minute
AKG	alpha ketoglutaric acid
ANOVA	analysis of variance
ASCA	ANOVA-simultaneous component analysis
AUC	area under the curve
C	carnitine
C10.2	decadienylcarnitine
C12	dodecanoylcarnitine
C2	acetylcarnitine
C2...C0	ratio of acetylcarnitine to free carnitine
C7.DC	pimelylcarnitine
C9	nonaylcarnitine
CAD	coronary artery disease
CAMP	cathelicidin antimicrobial peptide
CE	capillary electrophoresis
CE	cholesterol ester
cf-PWV	carotid- femoral pulse wave velocity
Cit...Orn	ratio of citrulline to ornithine
CRP	C-reactive protein
CV	cardiovascular
CVD	cardiovascular disease
DBP	diastolic blood pressure
ECG	electrocardiogram
eGFR	estimated glomerular filtration rate
FIA	flow injection analysis
GC	gas chromatography
Glu	glutamate
HCA	hierarchical cluster analysis
HDL	high-density lipoprotein
HILIC	Hydrophilic interaction chromatography
HPLC	high-performance liquid chromatography
HR	heart rate
hsCRP	high-sensitivity C-reactive protein
IFN	interferon
IL	interleukin
IS	internal standards

LC	liquid chromatography
LDL	low-density lipoprotein
LPC	lysophosphatidylcholines
m/z	mass-to-charge ratio
MAP	mean arterial pressure
MDA-LDL	Malondialdehyde-modified low-density lipoprotein
Met.SO	sulfoxidized methionine
Met.SO...Met	fraction of sulfoxidized methionine of the unmodified methionine pool
MI	myocardial infarction
MS	mass-spectrometry
MWW	Mann-Whitney-Wilcoxon
ND	not detectable
NFKB	nuclear factor κB
NMR	nuclear magnetic resonance
Orn	ornithine
PAD	peripheral arterial disease
PC	principal component
PCA	principal component analysis
PC aa Cx:y	diacyl-phosphatidylcholine
PC ae Cx:y	acyl-alkyl-phosphatidylcholine
PCs	principal components
PE	phosphatidylethanolamine
Phe	phenylalanine
PLS-DA	partial least squares discriminant analysis
PsA	psoriatic arthritis
PSOR1	psoriasis susceptibility region 1
PS	psoriasis
Putrescine...Orn	ratio of putrescine to ornithine
QC	quality control
RF	random forest
RT	retention time
SBP	systolic blood pressure
SNP	single nucleotide polymorphisms
SVM	support vector machine
TAG	triacylglycerol
Th	T-helper
TMAO	trimethylamine N-oxide
TNF	tumour necrosis factor
Total.AC...C0	ratio of esterifed carnitine to free carnitine
Total.AC.DC...Total.AC	fraction of dicarboxylic acylcarnitines of the total acylcarnitines
UHPLC	ultra-high performance liquid chromatography
WBC	white blood cell count
X.C2.C3...C0	ratio of short chain acylcarnitines to free carnitine

1. INTRODUCTION

Metabolomics concerns with the measurement and analysis of small molecule compounds (< 1 kDa, *e.g.* amino acids, biogenic amines, carbohydrates, fatty acids, nucleic acids, peptides) of both exogenous and endogenous origins. These are the substrates and products of various chemical reactions within metabolic pathways (Cajka and Fiehn 2016). The simultaneous measurement of these compounds provides a direct reflection of the metabolic network – the so-called "metabolic fingerprint", which provides the information about the status of the biological system in question (Kosmides et al. 2013). Due to the comprehensiveness of metabolomics it is widely used in biomedical sciences to study various diseases like cancer (Jové et al. 2017; Uchiyama et al. 2017; Bachmayr-Heyda et al. 2017), cardiovascular diseases (Guasch-Ferré et al. 2017; García-Fontana et al. 2016), diabetes (Murfitt et al. 2018; Niewczas et al. 2017; Brugnara et al. 2012) and many others (Yilmaz et al. 2017; Trezzi et al. 2017; Naz et al. 2017). Overall, the methods used in metabolomics have been invaluable to many fields of science and have provided multiple disease-specific markers to help with the better diagnosis and treatment of diseases.

Psoriasis (PS) is a widespread chronic inflammatory skin disease affecting 2%–3% of the population in the world (Parisi et al. 2013). The disease is considered to be multifactorial with a number of key contributing factors including genetic predisposition and susceptibility, environmental influences along with immune dysfunction and the disruption of the skin barrier (Raychaudhuri, Maverakis, and Raychaudhuri 2014). Psoriasis onset usually starts between the ages of 20–30 years and/or a later onset at 50–60 years (Cameron and Van Voorhees 2014; Perera, Di Meglio, and Nestle 2012) The five subtypes of psoriasis are erythrodermic, guttate, inverse, pustular and vulgaris (plaque) of which the latter is the most common affecting up to 90% of psoriatic patients (Griffiths and Barker 2007). In addition to the effects on the skin, psoriasis bestows noticeable psychological distress and impairment, which often results in an adverse impact on the quality of the patient's life (Chapman and Moynihan 2009; Steven R. Feldman, Malakouti, and Koo 2014). The disease can have a negative social influence in the workplace manifesting as discrimination and challenge in finding employment. Absence from work over the previous year has been reported in up to 60% of patients with psoriasis (S. R. Feldman et al. 1997). Psoriatic arthritis (PsA), which is a chronic, progressive inflammatory arthritis affects 6% to 41% of psoriasis patients (Ogdie and Weiss 2015). Approximately 40% of patients with skin psoriasis have nail disease while in patients with PsA the rate increases up to 80% (Sobolewski, Walecka, and Dopytalska 2017). As an added risk factor, psoriasis contributes to numerous concurrent physical comorbidities like diabetes, cardiovascular disease, inflammatory bowel disease, metabolic syndrome, and obesity; all leading to decreasing longevity (Armstrong, Gelfand, and Garg 2014; J. M. Gelfand and Yeung 2012; Kimball et al. 2008; Love et al. 2011) or even death (Pearce et al. 2006).

Atopic dermatitis (AD) is a widespread and complex condition that affects up to 15% adults and children worldwide. Although children have an increased prevalence of atopic dermatitis, many adults remain affected throughout their life (Abuabara and Margolis 2013). The pathogenesis of AD is multifactorial combining skin barrier dysfunction and immune system dysregulation (Czarnowicki, Krueger, and Guttman-Yassky 2014; Barton and Sidbury 2015) in addition to higher odds of developing AD in patients who were overweight or obese (Zhang and Silverberg 2015). The clinical forms of adulthood AD are divided into the following categories although commonly they appear together: head-and-neck eczema, hand eczema, generalized eczema, nummular eczema, erythroderma, nodular prurigo, lichen simplex, psoriasiform dermatitis and miscellaneous (Salvador, Romero-Pérez, and Encabo-Durán 2017). As is with psoriasis, the skin effects of the disease have a severe impact on the quality of life of the patients, which in turn places a burden on the economy (Drucker, Wang, and Qureshi 2016). Comorbidities of AD range from the long-associated food allergies, asthma and hay fever, while more recent data has expanded the list to attention deficit-hyperactivity disorder (Pelsser, Buitelaar, and Savelkoul 2009; Riis et al. 2016; O'Brien and Jones 2013) and anemia (Drury, Schaeffer, and Silverberg 2016).

Atherosclerosis is classified as an inflammatory disease that involves the arterial wall and is characterized by the continuous accumulation of inflammatory cells and lipids within the intima of large arteries (Badimón, Vilahur, and Padró 2009). Given that the pathological process endures and macrophages do not remove cholesterol that is accumulated in the vessel, they become apoptotic, which in turn causes the release of cholesterol, metalloproteinases and prothrombotic molecules to the wall of the vessel (Ghosh et al. 2010). Plaque disruption and the following exposure to thrombogenic substrates initiates the adhesion, activation and aggregation of platelets on the surface of the vascule, which in turn activates the coagulation cascade. This, through thrombus activation, leads to the clinical manifestation of the atherosclerotic disease, acute myocardial infarction or sudden death (Vilahur and Badimon 2013; Legein et al. 2013). The most common clinical manifestations of atherosclerosis are peripheral arterial disease (PAD) and coronary arterial disease (CAD), both of which have been noted as major public health problems (Criqui and Aboyans 2015; Wong 2014).

Psoriasis and atopic dermatitis have both been associated with cardiovascular diseases (CVD) (Prey et al. 2010; Standl et al. 2017). The exact correlations of PS and AD with CVD is not fully understood yet although it can be hypothesized that the chronic inflammation prevalent in both diseases leads to the loss of elasticity of arteries through atherosclerosis, which in turn increases the risk odds for stroke and myocardial infarction (MI) (Dattilo et al. 2018; Brunner et al. 2017).

In the present thesis the metabolomic profiles of patients with psoriasis and atopic dermatitis were explored to find possible disease-specific metabolites that could be used to characterize and better understand the underlying mechanisms

of the disease pathogenesis. Since both skin conditions have been associated with cardiovascular diseases (Dattilo et al. 2018; Radner et al. 2017; Standl et al. 2017; Brunner et al. 2017), the application of the established methods was expanded to peripheral arterial disease and coronary arterial disease to further search for similarities and differences in the metabolomic profiles of the diseases.

2. REVIEW OF THE LITERATURE

2.1. Metabolomics

Metabolomics (also called metabonomics) is among the latest of the so-called "omics" research fields and it focuses on the intermediates and products of metabolism including amino acids, antioxidants, biogenic amines, carbohydrates, fatty acids, nucleotides, organic acids, vitamins and many others. The term "metabolome" is analogous to the "proteome" and "transcriptome", and describes the complete set of metabolites, both endogenous and exogenous, present in a biological system and it can be depicted on all the levels of biological complexity: organisms, tissues, cells and cell compartments (Nicholson and Lindon 2008). In comparison to the amount of proteins, genes and transcript isoforms the number of metabolites present in humans is relatively small with an estimated size of 8000, but since the metabolome is very dynamic and more susceptible to natural fluctuations and external stimuli, it characterises the final response of an organism to genetic alterations, gut microflora and the environment extremely well (Holmes, Wilson, and Nicholson 2008; Vermeersch and Styczynski 2013). This in turn explains the rise in popularity of metabolomics methods used in research.

2.1.1. Analytical techniques in metabolomics

Currently, three different techniques are used in metabolomics: metabolomic fingerprinting, metabolite profiling and targeted metabolomics. In the first approach the identification of metabolites is not necessary since the goal of fingerprinting is to represent various classes of compounds that might be interesting to the study. This technique is used primarily for the classification of a sample (case/control) since it does not use exhaustive sample pre-preparation or advanced chromatographic separation techniques and the measured metabolites are not known *a priori* (Drexler, Reily, and Shipkova 2011; Blekherman et al. 2011). Metabolite profiling aims to identify and quantify as many metabolites as possible. The compounds are not known beforehand and therefore require high-resolution chromatographic separations with precise mass spectrometry detection. Metabolite profiling enables the detection of novel compounds and the metabolic pathways they are associated with, thus often leading to the conceptualization of new scientific hypotheses (Blekherman et al. 2011; Schuhmacher et al. 2013). In the case of targeted metabolomics, the measured compounds and their retention time windows are known beforehand thus enabling the precise quantification of the metabolites of interest. These are usually selected based on their biological availability, metabolic pathways of interest or their potential as a biological marker for a disease. Sample preparation procedures usually involve derivatization and adding of isotopic internal

standards. Chromatographic separations are also included in most cases (Zhou and Yin 2016).

Two of the most widely used analytical platforms used in metabolomics are mass spectrometry and nuclear magnetic resonance (NMR) of which ^{1}H-NMR is the most common (Spratlin, Serkova, and Eckhardt 2009; Gika et al. 2014) to analyze a multitude of small coexisting molecules with either of these techniques, including metabolite identification and quantitation. Both however have their own strengths and limitations. The use of mass spectrometry usually necessitates the use of a separation technique for biological fluids thereby high resolution techniques are applied including capillary electrophoresis (CE), gas chromatography (GC) or liquid chromatography (LC) (Gika et al. 2014; Putri et al. 2013; Lenz and Wilson 2007; Ban et al. 2011) CE enables the analysis of polar, ionic molecules. The main advantage of this technique is the excellent separation capacity while low repeatability remains as the major limitation of CE. While using GC the compounds are heated to the gas state, which is why non-volatile substances require the use of derivatization prior to use. LC on the other hand provides the possibility to analyze a wide range of metabolites ranging from hydrophobic to hydrophilic. Since the introduction of ultra-high performance liquid chromatography (UHPLC), increased peak capacity, reduced time of analysis and higher sensitivity has been achieved through the use of smaller particles in columns and higher pressures in LC (Gika et al. 2014; Putri et al. 2013; Lenz and Wilson 2007). The use of NMR spectroscopy provides other advantages including minimal preparation of samples and the non-dependence on analyte separation. Since the use of NMR does not ruin the sample, it can be recovered and reused in future studies. This makes NMR ideal for the analysis of biopsies and cell samples. Furthermore, when compared to MS the intensity of the NMR signal is not so greatly affected by the matrix components of a sample. This makes NMR a very appropriate tool for the analysis of very complex biological samples. However, one drawback of NMR is its low sensitivity, thus requiring a larger amount of sample (Bu et al. 2012; Putri et al. 2013).

2.1.2. Statistical techniques in metabolomics

The analysis of data acquired from MS and NMR measurements can be divided in two: the pre-processing and the statistical analysis.

The pre-processing of MS data includes *peak picking* – each measured ion is detected and assigned to a feature (mass-to-charge ratio (*m/z)* / retention time (RT) pair) and smoothed where necessary using vendor software; *alignment* – due to the changes in the column stationary phase and the mobile phase, RT shifts occur and are aligned using alignment algorithms (Mitra et al. 2018); *gap filling* – badly-shaped peaks can cause missing values, which are corrected using raw data and algorithms (Hrydziuszko and Viant 2011); *quality control (*QC*) correction* – when analyzing a large number of samples the use of pooled

samples from all study samples are used, called QCs. These are applied to ensure the correction of intensity drifts and repeatability of measurements (Dunn et al. 2011; Kamleh et al. 2012). The pre-processing of ^{1}H-NMR experiment data are usually automated by the instrument's vendor software and include apodization, baseline correction, Fourier transform, chemical shift calibration and phasing. After the pre-processing of data is complete a data matrix is generated where rows correspond to the study samples while spectral data points are stored in columns.

The statistical analysis of the acquired and pre-processed data usually starts with principal component analysis (PCA), which is an unsupervised linear mixture model. The main advantage of PCA is that it takes the whole dataset as input and calculates new principal components (PCs) in a way that they are pairwise orthogonal (i.e. decorrelated) to each other and reordered by the amount of variance explained. The newly calculated PCs can then be used to visualize the clustering and sample group discrimination of data (Lindon, Holmes, and Nicholson 2006; Tharwat 2016). Similarly, other unsupervised multivariate methods like ANOVA-simultaneous component analysis (ASCA) (Smilde et al. 2005), hierarchical cluster analysis (HCA) (Smilde et al. 2005; Beckonert et al. 2003) and k-means clustering (Hageman et al. 2006) can be used. Supervised linear mixture models, i.e. where the response variable (class of sample in case-control study) is specified include partial least squares discriminant analysis (PLS-DA) (Hageman et al. 2006; Boulesteix and Strimmer 2007), orthogonal-PLS (Trygg and Wold 2002). These methods are useful when the classification of samples is needed, however the results are often prone to overfitting (Broadhurst and Kell 2006), which can be overcome by including certain validation techniques like bootstrapping (Wehrens, Putter, and Buydens 2000) or cross validation (Westerhuis et al. 2008). Machine learning methods for the classification of samples include random forest (RF) (Breiman 2001), support vector machine (SVM) (Burges 1998), and elastic net regression (Zou and Hastie 2005) among many others (Tibshirani 1996; Hoerl and Kennard 2000; Trygg and Wold 2002). For hypothesis testing, which is central in the analysis of metabolomics data, metabolite intensities are compared between cases and controls. If the data is normally distributed a Student's *t*-test can be used or Mann-Whitney Wilcoxon test when the data does not follow a normal distribution. When two or more conditions are compared, an analysis of variance (ANOVA) or Kruskal-Wallis test can be used for normally or not-normally distributed data respectively. Regardless of the test used, most metabolic studies consist of a great number of metabolic features and therefore the calculated *p*-values need to be adjusted for multiple hypothesis testing. Two of the most widely used include the Benjamini and Hochberg (Benjamini and Hochberg 1995) and the stricter Bonferroni correction (Goodier 2007).

2.2. Psoriasis overview

Plaque psoriasis is an immune-mediated inflammatory skin disease that is characterised by red, scaly and distinct skin plaques, which result from the hyperproliferation of keratinocytes in addition to inflammatory cell infiltration and neovascularization (Nestle, Kaplan, and Barker 2009). Experimental and clinical evidence point to the immune system having a central role in the pathogenesis of the disease. The disease can be triggered in genetically susceptible individuals by numerous factors that include infection (e.g. streptococcal), medications (β-blockers, IFNα and lithium) and trauma (Goldsmith et al. 2012). The initiation phase involves the activation of dermal dendritic cells by activated plasmacytoid dendritic cells. This in turn activates T helper (Th) and cytotoxic T cells that are essential in the maintenance of the disease (Nestle, Kaplan, and Barker 2009). Th1, Th17 and Th22 all contribute to the disease by the continued production of cytokines. (Di Meglio et al. 2011). IL-17A is responsible for both of these processes (Suzuki et al. 2014). A chronic inflammatory phase follows the initiation phase and is sustained by amplification signals and feedback loops. These key mediators include cationic proteins, antimicrobial peptides and members of the innate immune system such as pro-inflammatory chemokines and cytokines (for example, TNF, IL-17, IL-22 and CC-chemokine ligand 20 (CCL20), among numerous others), cathelicidin antimicrobial peptide (CAMP) and angiogenic factors (Lande et al. 2007). Psoriasis is subdivided into erythrodermic, guttate, inverse, pustular and vulgaris (plaque) (Griffiths and Barker 2007) of which the latter is the most common. Psoriatic arthritis (PsA) affects 6%–41% of patients with psoriasis (Joel M. Gelfand et al. 2005; Reich et al. 2009; Mease et al. 2013) and the onset of PsA is between the ages of 35 and 45 whereas men and women are affected equally. In most cases the onset of arthritis happens roughly 10 years after the onset of psoriasis, however in 15% of patients arthritis develops before any noticeable changes in the skin happen. Currently there haven't been noted any correlations between the severity of skin and joint manifestations (Gladman et al. 2005). In addition to PsA, psoriasis patients have been noted to have an increased prevalence of systemic and vascular inflammation along with clinical atherosclerosis (Ahlehoff et al. 2013).

2.2.1. Psoriasis biomarkers

A biomarker is defined as a biological characteristic that can be measured and evaluated objectively as an indicator of normal and pathogenic biological processes or pharmacological response to therapeutic treatment ("Biomarkers and Surrogate Endpoints: Preferred Definitions and Conceptual Framework" 2001). Biomarkers can also be used to pinpoint disease risk factors, which consequently can lead to an improved understanding of the pathogenesis of a disease. The best possible biomarker is sensitive, specific, reproducible, accurate and predictive. The detection method on the other hand must be standardised,

robust and easy to perform. In clinical practise, if a test for a biomarker is to be used, the sensitivity and specificity have to be ≥ 0.9 (Søreide 2009).

Plaque psoriasis is a multifactorial disease and the search for reliable biomarkers has spanned over multiple fields of research. In genetics for example guttate and early onset psoriasis are associated with psoriasis susceptibility region 1 (PSOR1) (Hébert et al. 2012), but due to the heterogeneity of the disease, no single mutation can be attributed to psoriasis. Instead, a wide range of single nucleotide polymorphisms (SNP) that are in part responsible have been detected using genome-wide association studies (Tsoi et al. 2012). In addition, psoriasis-associated genes have been noted in areas that are closely related to the function of the skin barrier (e.g. LCE3B/3C), nuclear factor κB pathway (e.g. NFKBIA), type I interferon (IFN) induction (e.g. IFHIH1) and Th17 cell activation (e.g. IL23R) (Tsoi et al. 2012; Capon and Barker 2012). Most of the named genes are involved in inflammatory and immunological processes, which further supports the immune system's central role in psoriasis pathogenesis. Protein analysis has yielded many pro-inflammatory cytokines including TNF, IFN-γ, IL-6, IL-8, IL-12 and IL-18 (Arican et al. 2005). Additionally patients with psoriasis exhibited unusual levels in blood fibrinolysis and coagulation, such as increased levels of fibrinopeptide A, fibrinogen, D-dimer and C4, and decreased levels of alpha 2-antiplasmin, protein C, and plasminogen (Marongiu et al. 1994). Regarding the metabolomics of psoriasis, lower metabolite levels of glucose and myoinositol were noted while higher levels of taurine and choline were found in tissue biopsies from psoriatic skin when compared to non-lesional skin (Sitter et al. 2013). In other studies psoriasis patients were found to exhibit higher levels of alpha ketoglutaric acid (AKG) and lignoceric acid, and lower levels of asparagine and glutamine, while patients with both psoriasis and psoriatic arthritis had a decreased level of AKG (Armstrong et al. 2014). Another study found significant psoriasis-associated changes in three metabolic pathways that included multiple amino acids: 1) aspartate, alanine, and glutamate, 2) glycine, threonine, and serine, 3) proline and arginine. Post treatment with the anti-TNFα drug Etanercept, a large part of psoriasis-associated changes in circulating metabolites were reversed, thus shifting the metabolic profiles of psoriasis patients closer to that of controls (Kamleh et al. 2015).

2.3. Atopic dermatitis overview

Atopic dermatitis (AD) or atopic eczema is a widespread disorder of the skin that has a multifactorial pathophysiology. AD usually develops before the age of 5 in 85% of patients and usually clears by adolescence. In some patients however the disease can persist into adulthood (Wüthrich 1999). In western countries AD is one of the most common skin diseases that affects up to 1% to 3% of adults and up to 20% of children (Williams et al. 1999). AD develops in individuals that have a genetic predisposition and experience exogenous

provocation factors. More precisely, the barrier defects in the epidermis and the dysregulation of the innate and adaptive immune systems are thought to be the cause (Lodén 2003). The abnormalities in the skin barrier are the result of multiple factors including microorganisms, exposure to chemicals (Nowicka and Grywalska 2018), and low humidity and temperature (Heimall and Spergel 2012). Since the prevalence of AD has been on the rise (Broberg et al. 2000) it is thought that environmental factors have a greater effect on the outbreak of the disease rather than the genetic background (Taïeb 1999). In the study of the pathogenesis of atopic dermatitis multiple genes have been discovered to play a role; these include interleukin (IL)-4, the receptor of IL-4 and IL-13 (He et al. 2003; Liu et al. 2000). The strongest association for a candidate gene for atopic eczema has been shown in multiple studies to be for the gene encoding filaggrin (FLG) (Irvine, Irwin McLean, and Leung 2011; Palmer et al. 2006; Rodríguez et al. 2009). This filament aggregating protein is expressed in the epidermal granular layer and binds to keratin fibers in epithelial cells. Its functions include the contribution of acidic and hygroscopic amino acids to the stratum corneum, in addition to antimicrobial effects (Brown and McLean 2012). Th-2 (IL-4/IL-13/IL-25) and Th22 (IL-22) cytokines are noted to be increased in AD and they suppress the expression of filaggrin in keratinocytes (Gutowska-Owsiak et al. 2011; Hvid et al. 2011; Pellerin et al. 2013). This in turn might cause the barrier defects in skin. Thereby it is hypothesized that barrier dysfunction is triggered by immune activation through a positive feedback loop (Leung and Guttman-Yassky 2014). Additionally the skin lesions of atopic eczema have epidermal hyperplasia, accompanied by large T-cell and dendritic infiltrates, which leads to an increased production of inflammatory intermediates (Guttman-Yassky, Nograles, and Krueger 2011). AD has the following clinical subtypes although commonly they appear together: head-and-neck eczema, hand eczema, generalized eczema, nummular eczema, erythroderma, nodular prurigo, lichen simplex, psoriasiform dermatitis and miscellaneous (Salvador, Romero-Pérez, and Encabo-Durán 2017). AD has a range of comorbidities ranging from the immunoglobulin E (IgE) mediated hay fever, asthma and food allergy (Silverberg and Simpson 2014; Fölster-Holst et al. 2006) to sleep disturbance (Bender, Leung, and Leung 2003), depression (Yu and Silverberg 2015), obesity (Silverberg et al. 2015), lipid abnormalities and atherosclerosis (Ewald et al. 2015; Hjuler et al. 2015)

2.3.1. Atopic dermatitis biomarkers

The need for biomarkers for atopic dermatitis is evident because the disease is highly heterogeneous. AD has been classified as extrinsic (allergic, high total serum IgE levels) or intrinsic (non-allergic, normal total IgE). The classification models also associate T-cell activation with AD, particularly Th2/Th22 polarization, with a Th1 component in patients with chronic AD and a possible contribution of Th17 (Gittler et al. 2012). This indicates that higher levels of the

Th2 cytokines IL-4 and IL-13 in AD skin lesions could influence immunoglobulin class-switching, promoting excessive IgE production (Poulsen and Hummelshoj 2007; Oettgen and Geha 1999). It has been demonstrated however that both groups showed similar expression levels in Th2-related genes, which suggest an overlap in the underlying pathomechanisms (Suárez-Fariñas et al. 2013). Therefore, to further search for biomarkers, it would be useful to characterise the diversity in pathogenesis of patients with atopic eczema. Currently no reliable biomarkers for diagnosis or prediction for the onset of the disease exist (Thijs, de Bruin-Weller, and Hijnen 2017), although various markers exist for screening, prognostic, pharmacodynamic and monitoring purposes including the filaggrin gene (Kezic et al. 2011), IgE (Peters et al. 2010), and transcriptomic changes (Tintle et al. 2011), among others (Staudacher et al. 2015; Kim et al. 2016). The metabolomics research on AD patients has revealed changes with inflammatory response, bile acid metabolism, energy metabolism disorder (Huang et al. 2014), unbalance in multiple pathways of systemic metabolism (Assfalg et al. 2012) and altered serum lipid metabolism (Agrawal et al. 2018).

2.4. Atherosclerosis overview

Atherosclerosis is the narrowing of the arteries due to the buildup of plaque in the innermost layer of the artery, the intima. The plaques consist of connective-tissue elements, cells, lipids and debris (Stary et al. 1995). The thickening of the arterial wall can lead to blood flow disrupture – stenosis, but angiographic studies have identified that in most cases infarction occurs due to the formation of occluding thrombus on the surface of the plaque (Davies 1996). Thrombosis can occur either when the plaque ruptures due to the thin fibrous cap (van der Wal et al. 1994) or endothelial erosion (without plaque rupture) exposing the underlying collagen, which in turn activates the thromb forming cascade (Farb et al. 1996). Plaque rupture is detectable in up to 70% of cases with coronary infarction (Falk, Shah, and Fuster 1995). Ruptures occur when the fibrous cap is thin and partly destroyed. Activated immune cells are abundant at these sites, which produce multiple proteolytic enzymes and inflammatory molecules (van der Wal et al. 1994). This transforms the stable plaque into an unstable and vulnerable structure that can rupture and induce a thrombus, which in turn elicits an acute coronary syndrome (Hansson 2005). Plaques that precipitate endothelial erosion are morphologically different, being more fibrous, having an intact plaque cap and exhibiting either absent or deeply-seated necrotic cores (White, Newby, and Johnson 2016). Interestingly, the plaques in the skin of psoriasis and coronary artery both have similarities including the presence of proinflammatory cytokines TNF- α**,** IFN.ɣ, IL-6, IL-17, IL-8, the extravasation of white blood cells through the endothelium and Th1, Th17 and regulatory T-cells (Kivelevitch, Schussler, and Menter 2017).

2.4.1. Atherosclerosis biomarkers

Since atherosclerosis is regarded as a form of chronic vascular inflammation (Ross and Glomset 1973), C-reactive protein (CRP), more precisely high-sensitivity CRP in combination with Framingham risk score (10-year cardiovascular risk assessment) has been shown to have a high predictive accuracy (Koenig et al. 2004). IL-6, a member of the inflammatory cytokine family released from vascular smooth muscle cells, monocytes, macrophages and endothelial cells has been shown to have a high impact in atherosclerosis (Ridker et al. 2000). Malondialdehyde-modified low-density lipoprotein (MDA-LDL) is widely used as a marker because its chemical structure is readily-identifiable and it is the most common aldehyde resulting from lipid peroxidation. Higher levels are associated with fibroatheromas in patients with coronary artery disease (Matsuo et al. 2013). Regarding low-molecular compounds, lipidomics has yielded many species including triacylglycerol (TAG) 54:2, cholesterol ester (CE) 16:1 and phosphatidylethanolamine (PE) 36:5 (Stegemann et al. 2014). Additionally, machine learning models have been applied to discover plasma lipids closely related to coronary artery disease. Various phosphatidylcholines, triglycerides, sphingomyelins and other lipids were incorporated into statistical models that helped to distinguish cases from controls (Meikle et al. 2011).

2.5. Concluding remarks

The application of the various techniques used in metabolomics to discover new biomarkers in psoriasis, atopic dermatitis and atherosclerosis is very important because it might give new insights into the pathophysiologies of the diseases and help to create a premise for new drug targets. Additionally, the application of various machine learning methods to create new sample-discriminating statistical models could one day prove to be extremely useful in helping doctors with the improvement of diagnosis, and consequently lead to better treatment for patients.

3. AIMS OF THE THESIS

The primary aim of the current thesis was to analyze the metabolomic profiles of patients with plaque psoriasis, atopic dermatitis and atherosclerosis to assess the similarities and differences in the discovered metabolites.

The specific aims were the following:

1) Evaluate the serum metabolomic profiles of plaque psoriasis patients using targeted and untargeted metabolomic methods and to provide statistical models that could be used in the diagnosis and monitoring of treatment.
2) Discover the metabolites that are characteristic to atopic dermatitis and use statistical models that use data from targeted and untargeted metabolomic measurements to classify samples.
3) Analyze the levels of serum phosphatidylcholines and lysophosphatidylcholines and their relations to hemodynamics, endothelial dysfunction and arterial stiffness in patients with coronary artery disease and peripheral artery disease.
4) Discover whether there are similarities in metabolite levels in patients with plaque psoriasis, atopic dermatitis, and atherosclerosis.

4. SUBJECTS AND METHODS

4.1. Ethics approval

The permission to perform studies on the metabolomics of skin diseases and cardiovascular diseases were granted by the Research Ethics Committee of the University of Tartu. The protocols of the Declaration of Helsinki were followed and all of the patients and volunteers gave their informed, written consent. Good clinical practices were followed when collecting the samples.

4.2. Recruitment of volunteers

For the studies on skin diseases the adult patients with either plaque psoriasis or atopic dermatitis were recruited during 2013–2015 at the University Hospital of Tartu, the Clinic of Dermatology. The patients were asked to participate when they first came in and before the beginning of treatment. The controls were age- and sex-matched and asked to participate either from the same clinic or from the Clinic of Traumatology and Orthopaedics. It was not counted how many volunteers refused to participate; also, the dropout-rate was not calculated because subjects only needed to give written consent and samples at the beginning of the study. The exclusion criteria for patients and controls included comorbid chronic inflammatory skin diseases, diabetes and pregnancy. In total the number of patients with plaque psoriasis was 55 (18 women, 37 men, ages 20–75), 25 with atopic dermatitis (19 women, 6 men, ages 20–55) and 63 controls (26 women, 37 men, ages 23–75). All of the patients and controls were adults to increase the probability that the changes seen in metabolite levels are specific to diseases and not due to age. The participants were all Caucasians of Eastern European descent (Ottas, Fishman, Okas, Kingo, et al. 2017; Ottas, Fishman, Okas, Püssa, et al. 2017).

For the study on cardiovascular diseases a total of 124 male subjects, including 32 patients with PAD, 52 patients with CAD, and 40 clinically healthy controls, were enrolled into this study. The patients were recruited from the Department of Vascular Surgery and the Department of Cardiology, University of Tartu, Estonia. All patients had angiographically proven PAD or CAD. The exclusion criteria for both the groups were any comorbid acute or chronic inflammatory disease, diabetes mellitus, myocardial infarction, cerebrovascular events or revascularization operation during the preceding 6 months, unstable angina, cardiac arrhythmias, clinically significant heart failure or valvular disease, reduced kidney function (estimated glomerular filtration rate <60 ml/min/1.73 m^2), presence of cancer, or endocrine pathology. In the PAD group, six (16%) patients with CAD as a comorbidity were included in the analysis. Age- and gender-matched clinically healthy controls were enrolled through local family physicians in the same geographic region. The exclusion

criteria included any comorbid acute or chronic inflammatory disease, CAD, cerebral or peripheral atherosclerotic disease, diabetes mellitus, cardiac arrhythmias, clinically significant heart failure or valvular disease, hypertension, reduced kidney function (estimated glomerular filtration rate <60 ml/min/1.73 m^2), presence of cancer, infectious disease or endocrine pathology, or regular use of any medication (Paapstel et al. 2018).

4.3. Serum collection protocol

Blood samples were collected in the morning into 5 ml Vacutainer (REF 367614) tubes that contain micronized silica particles that help to accelerate the clotting process. The blood was left at room temperature for one hour then centrifuged at 1300 × g for 20 minutes. The supernatant serum was pipetted into 300 µl aliquots and stored in the freezer at –80 °C until needed.

4.4. Cardiovascular diseases study protocol

Data on lifestyle factors and medical history were obtained using an interview and a self-completed questionnaire. Peripheral venous blood samples were collected from all subjects between 8am and 11am after an overnight fast and abstinence from tobacco, alcohol, tea, and coffee. Thereafter, height and weight were assessed and body mass index was calculated. The subjects were studied after 10 min of rest in a supine position in a quiet, temperature- controlled room. Brachial blood pressure and carotid-femoral pulse wave velocity (cf-PWV) were assessed and pulse wave analysis was conducted. All hemodynamic measurements were taken in duplicate and averaged. The study protocol was approved by the Ethics Committee of the University of Tartu, and each subject gave their written informed consent.

4.4.1. Arterial stiffness and central hemodynamic measurements

The SphygmoCor device (AtCor Medical, Sydney, Australia) was used for non-invasive assessment of arterial function and hemodynamics. The cf-PWV was calculated from the pulse transit time using ECG-gated carotid and femoral artery waveform recordings over a known distance (Wilkinson et al. 1998). The distance from the suprasternal notch over the umbilicus to the femoral artery minus carotid arterial length was used for the calculation of the cf-PWV. In addition, aortic augmentation index, corrected for a heart rate (HR) of 75 bpm (AIx@75), and central hemodynamic parameters were evaluated. In brief, after 15 sequential high- quality radial waveforms were recorded at the patient's left wrist using a high-fidelity micromanometer (SPT- 301B; Millar Instruments, Houston, Texas), a validated generalized transfer function for calculation of the

central aortic pressure waveform was used (Pauca, O'Rourke, and Kon 2001; Adji, O'Rourke, and Namasivayam 2011). The augmentation index (%) was defined as the ratio of the difference between the second and first systolic peaks of the central arterial waveform to the central pulse pressure (Laurent et al. 2006).

4.4.2. Angiographic score

In patients with PAD, digital subtraction angiography (Axiom Artis; Siemens Medical Solutions, Forchheim, Germany) of the aorta and lower extremity arteries was performed, using a femoral approach at the Department of Radiology, Tartu University Hospital, Estonia. The Bollinger angiogram scoring method takes into account both the severity and location of atherosclerotic lesions in the lower extremity arteries (Bollinger et al. 1981).

4.4.3. Biochemical analysis

Venous blood samples were collected in serum separator tubes (BD SST" II Advance) and plain tubes (Plain BD Vacutainer Tubes) for clinical biochemical and metabolomic analysis, respectively. All samples were centrifuged and the supernatant was transferred into 1.5 mL Eppendorf tubes. The tubes were frozen at –80°C until assayed. The serum concentrations of interleukin-6, resistin, and insulin were determined using The Evidence Investigator (Metabolic Syndrome Array-1, Randox Laboratories, Crumlin, UK). The plasma levels of total cholesterol, low- density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, glucose, white blood cell count, platelet count, high-sensitivity C-reactive protein, and estimated glomerular filtration rate were measured in a local clinical laboratory using automated analyzers following standard laboratory methods (Paapstel et al. 2018).

4.5. Measurements using mass spectrometer

4.5.1. Targeted analysis of metabolites in serum

Targeted analysis was performed using the Absolute*IDQ*™ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). Before the measurements began, a 96-well plate report detailing the position of samples, quality controls, phosphate buffered saline samples, blank samples and calibration standards was generated in the vendor's software Met*IDQ*™ Carbon 6.0.0. The serum samples were thawed on ice, vortexed and centrifuged at 4 °C for 5 minutes at 2750 × g. The lyophilized internal standards (IS) were prepared by adding 1200 µl of HPLC grade water to the tube, followed by vortexing and shaking for

15 minutes at 1200 rpm. The quality control samples and calibration standards were prepared by adding 100 µl of HPLC grade water to the tubes and vortexed, followed by shaking for 15 minutes at 1200 rpm. 10 µl of IS was added to all wells with the exception of the blank well in position A1 to allow for the calculation of the background noise of the mass-spectrometer. 10 µl of serum samples and other samples were pipetted according to the generated plate report. The samples on the plate were dried using air through evaporator needles for 30 minutes. The samples were derivatized in 20 minutes using 50 µl per well of a solution containing 31.6% ethanol, water, pyridine and 5% of phenylisothiocyanate. The plate was dried again for 60 minutes after, which the metabolites were extracted using 300 µl per well of 5 mM ammonium acetate solution. The measurements consisted of two parts – using flow injection analysis (FIA) and liquid chromatography (LC). For the FIA part, 20 µl of each sample was transferred to a new 96-well plate where 380 µl of methanol with Biocrates Solvent I (one ampule of solvent per 290 µl of methanol) was added. The LC part required the transfer of 50 µl of sample to a new 96-well plate and dilution with 250 µl of 40% methanol in HPLC grade water. The measurements were done on a Sciex 4500 QTRAP mass-spectrometer using an Agilent 1290-series liquid chromatograph with an Agilent Zorbax Eclipse XDB C18, 3.0 × 100 mm, 3.5 µm with Pre-Column SecurityGuard, Phenomenex, C18, 4 × 3 mm for the LC part. The column temperature was set to 50 °C. The solvent used for the FIA part was methanol with Biocrates solvent I; The LC part had two solvents – HPLC grade water and acetonitrile with 0.2% of formic acid. The targeted analysis used multiple reaction monitoring with predetermined retention times and specific mass-to-charge ratios of fragments provided by the vendor. The concentrations for the LC part were calculated using Analyst 1.6.2. and Met*IDQ*™ Carbon 6.0.0. for the FIA part. As a result, the concentrations of 20 amino acids, 40 acylcarnitines, 18 biogenic amines, 15 sphingomyelins, 14 lysophosphatidylcholines, 75 phosphatidylcholines and a sum of hexoses were calculated. In addition, the Met*IDQ*™ Carbon provided 44 ratios of various metabolites that have biological significance (Ottas, Fishman, Okas, Kingo, et al. 2017; Ottas, Fishman, Okas, Püssa, et al. 2017).

4.5.2. Untargeted analysis of metabolites in serum

Untargeted analysis was performed in Papers I and II. For samples in Paper I the protocol is as follows. Serum samples were thawed on ice, 100 µl of sample was transferred to a new Eppendorf tube and 400 µl of acetonitrile was added. The tube was vortexed for 2 minutes and left for 15 minutes at room temperature to allow for the extraction of metabolites. Then the samples were centrifuged for 15 minutes at 15,800 × g at 4 °C. 10 µl of supernatant was used for analysis on an ABSciex 3200 QTRAP coupled to a Shimadzu high-performance liquid chromatograph (HPLC) using a SeQuant® ZIC®-pHILIC (5 µm polymer) PEEK 150 × 4.6 mm metal-free HPLC column and ZIC®-pHILIC Guard

column PEEK 20 × 2.1 m column at room temperature. The parameters for the measurement were: solvents used were water and acetonitrile in 0.1% formic acid, total runtime was 62 minutes, gradient flow rate was 0.3 ml/min starting from 80% acetonitrile to 20% in 32 min, then to 5% in 1 minute where it stayed for 8 minutes, followed by rapid gradient to 100% in 5 minutes and back to 80% for re-equilibration for 8 minutes. The curtain gas of the turbo spray was set to 10 au, ion spray voltage to 4500 V, collision gas to "High", temperature to 300 °C, entrance potential to 10 V, declustering potential to 20 V. The collision energy was set to 10 V in regular measurements and to either 20 V, 30 V or 40 V in fragmentation analysis. All of the samples were randomized and measured in negative and positive modes from 50 to 1500 mass-to-charge ratios (*m/z*). The injection volume was 10 µl. Enhanced Product Ion mode was used for fragmentation analysis using respective values and the same column (Ottas, Fishman, Okas, Kingo, et al. 2017).

In Paper II the samples were prepared according to a protocol by Want *et al (Want et al. 2010)* and is as follows. The serum samples were left to thaw on ice, 20 µl of each sample was pipetted to a separate tube to make a pooled sample for quality control analysis. Per 50 µl of sample, 150 µl of ice cold methanol was added to precipitate proteins and solve metabolites. The samples were vortexed for 30 seconds and left for 20 minutes at –20 °C after, which they were centrifuged at 4 °C for 15 minutes at 16089 × g. 150 µl of supernatant was transferred to a new tube and lyophilized using a SpeedVac lyophilizer. 100 µl of HPLC grade water was added to the freeze-dried samples, vortexed for 30 seconds, centrifuged at 4 °C for 15 minutes at 16089 × g. 90 µl of supernatant was transferred to a glass vial for measurements on a 6450 UHD Accurate Mass Q-TOF tandem liquid chromatograph with 100 series quaternary pump (Agilent, USA) using an EclipsePlus C18 RHD 1.8 µm 2.1 × 50 mm column (Agilent, USA) at 40 °C. The samples were measured according to a slightly modified protocol by Want *et al (Want et al. 2010, 2012)*. The solvents used were methanol and water in 0.1% formic acid, total runtime was 26 minutes, gradient flow rate was 0.3 ml/min starting from 99.9% water for 2 minutes, going to 75% in 4 minutes, then to 20% in 4 minutes, to 10% in 2 minutes and gradually to 0.1% in 9 minutes where it stayed for 2 minutes, ramping back up in 1 minute to 99.9% for the last 2 minutes. Capillary voltage was set to 3.2 kV for ESI+ and 2.4 kV for ESI-, source temperature was 120 °C, desolvation temperature 350 °C, cone gas glow 25 litres/hour, desolvation gas glow 900 litres/h. The injection volume was 5 µl. The collision energy was at 10 V for ESI measurements; 20 V and 40 V for fragmentation analysis (Ottas, Fishman, Okas, Püssa, et al. 2017).

4.5.3. Identification of metabolites

The fragmentation spectra and individual mass-to-charge ratios of peaks from untargeted analysis were compared visually and numerically to spectra from multiple public databases e.g. HMDB (Wishart et al. 2017), MassBank (Horai et al. 2010), METLIN (Smith et al. 2005) and LipidMaps (Fahy et al. 2007). A compound from fragmentation analysis was considered identified when the spectra with its m/z peaks and relative heights were identical to spectrums from online databases.

4.6. Processing and statistical analysis of data

4.6.1. The pre-processing and analysis of untargeted data in the study of skin diseases

The data from Papers I and II were processed similarly where the acquired vendor files (.wiff for QTRAP 3200, .d for Agilent 6450 UHD) were first converted to .mzXML using the MSConvert software (Chambers et al. 2012). RStudio version 0.98.501 (R Development Core Team 2010) was used to extract the peaks with XCMS (Smith et al. 2006) and processed further using mzMatch.R (Scheltema et al. 2011) that allowed the combination of biological replicates, retention time correction, QC correction, Reproducibility Standard deviation filtering, blank filtering with gap filling, filtering the minimum number of detections (at least six) and the matching of related peaks. In Paper I the data was analyzed using Mann-Whitney-Wilcoxon (MWW) test. In Paper II the data was processed further – $\log^{10}$ transformed, mean subtracted and divided by standard deviation, then tested for normality using a Shapiro-test after, which the Mann-Whitney-Wilcoxon test was applied. All of the values from the statistical test were FDR 5% corrected (Ottas, Fishman, Okas, Kingo, et al. 2017; Ottas, Fishman, Okas, Püssa, et al. 2017).

4.6.2. The pre-processing and analysis of targeted data

In Paper I the metabolites that had zero variance were removed. Scaling and centering was applied to all the metabolite values in the dataset. In Paper II the data were $\log^{10}$ transformed and normalized through the subtraction of mean and the division of the standard deviation. In Paper III the data were processed even more thoroughly – to avoid unwanted bias the metabolites, which limits of detection and the lower limits of quantitation were lower than given in the vendor's specifications were discarded. Using the 'boxplot' function in R the metabolites with concentration values smaller or larger than 1.5 × interquartile range were considered as outliers and removed. Using the 'mice' package, the now missing values were imputed based on corresponding metabolite values

from other samples. A batch effect was discovered that was independent of measurement time, gender, phenotype or the time of sample collection. To correct for the batch effect, the samples were normalized within either batch that resulted in comparable groups. In Paper I, MWW test was used, in Paper II the non-paired t-test was used and in Paper III the non-parametric Kruskal-Wallis rank-sum test and Wilcoxon rank-sum tests were applied for the discovery of metabolites that differentiate the phenotype (Ottas, Fishman, Okas, Püssa, et al. 2017; Ottas, Fishman, Okas, Kingo, et al. 2017; Paapstel et al. 2018).

4.6.3. Machine learning and statistical modeling for the discovery and validation of metabolites

Using classical statistical tests is vital and important to discovering phenotype differentiating metabolites. Machine learning and statistical modeling provide the added benefit of cross-validating the results while providing predictive models that can be used for the phenotypic classification of new samples. In Paper I, to discriminate between psoriasis patients and controls the random forest algorithm (Tin Kam Ho and Ho, n.d.) was applied, which uses a variety of decision trees that are built using random subsets from the original data. Three feature selection methods were used in parallel to cross-check and select only relevant metabolite features – a recursive feature elimination method, a genetic algorithm (Mitchell 1998) and a filter method (Saeys, Inza, and Larranaga 2007). Fivefold cross-validation strategy was applied due to the lack of training data as suggested by Ambroise *et al. (Ambroise and McLachlan 2002)*. In Paper II three widely used algorithms were applied for machine learning and to verify the statistical importance of the metabolites found using univariate tests: GLMNET (Friedman, Hastie, and Tibshirani 2010), PDA (Hastie, Buja, and Tibshirani 1995) and RandomForest (Liaw and Wiener 2002). These methods were used for targeted and untargeted data. Using the results from univariate tests as features for a supervised model can lead to the inflation of accuracy for the constructed classifiers; this is called selection bias (Cawley and Talbot 2010). The three classifiers used the whole dataset using a 5-fold cross-validation algorithm (Geisser 1993) for training to avoid selection bias. This was repeated 5 times to validate the metabolites and to avoid overfitting, which is when a model is unable to generalize to unavailable data because of the limited sample size of a training set (Ng, n.d.). In Paper III the machine-learning algorithms used were GLMNET (Friedman, Hastie, and Tibshirani 2010), RandomForest (Liaw and Wiener 2002) and support vector machines (“Support Vector Machines” 2009). The training of algorithms was done without specific feature selection although 5-fold cross validation repeated 5 times was still applied (Ottas, Fishman, Okas, Kingo, et al. 2017; Ottas, Fishman, Okas, Püssa, et al. 2017).

4.6.4. Principal component analysis

Metabolomics analysis yields large amounts of data – over 200 data points when measuring the concentrations of known compounds using targeted metabolomics and thousands of data points when measuring all of the charged ions in a run using untargeted metabolomics methods. Using univariate statistical methods usually provides a handful of metabolites that differ statistically significantly between samples and gives a rough idea of variability in samples. Principal component analysis (PCA) however can be used to reduce the dimensionality of large datasets, which can help with the interpretability while minimizing the loss of information. This is achieved by the creation of new uncorrelated variables called principal components (Jolliffe and Cadima 2016), which can either be used for data visualization or the discovery of relevant biomarkers. The visualization of data using PCA was used in Papers I–II but not for feature selection (Ottas, Fishman, Okas, Püssa, et al. 2017; Ottas, Fishman, Okas, Kingo, et al. 2017).

4.6.5. Statistical analysis in cardiovascular study

In Paper III, the SPSS software for Windows, version 22.0 (SPSS, Chicago, IL, USA), was used for the statistical analyses. One-way analysis of variance was employed or a Kruskal Wallis test was conducted to determine differences between the mean or median values of continuous variables among the groups. The normality of the distribution of variables was assessed using the Shapiro Wilk test. The serum concentrations of glycerophospholipids were logarithmically transformed as they were positively skewed. Analysis of covariance was employed to adjust cf-PWV levels for mean arterial pressure and glycerophospholipid levels for BMI, current smoking status, and statin therapy. For comparing dichotomous measures, the Fisher-Freeman-Halton test was employed. Univariate correlations were reported as the Spearman rank correlation coefficient (rho). The Benjamini Hochberg procedure was used to control the false discovery rate at the level of 0.05 [18]. Multivariate analysis was performed using a stepwise (forward, followed by backward) multiple linear regression. The independent variables entered into the models were chosen on the basis of their biological relevance and/ or statistical significance from univariate analyses (lysoPC a C17:0, lysoPC a C18:0, lysoPC a C20:4, mean age, BMI, mean arterial pressure, interleukin-6, total cholesterol, low-density lipoprotein cholesterol, glucose, resistin, platelet count, antihypertensive treatment, and statin use). All statistical tests were checked for violations of assumptions (Paapstel et al. 2018).

5. RESULTS

5.1. The metabolomic profile of plaque psoriasis

In Paper I where plaque psoriasis patients' serum metabolites were compared to controls the statistically significant differences for targeted analysis are given in Table 1. The levels of acylcarnitines, mainly nonaylcarnitine (C9), dodecanoyl-carnitine (C12), decadienylcarnitine (C10.2), and pimelylcarnitine (C7.DC) had all lower concentrations in psoriasis patients' serums. Phosphatidylcholine diacyls (PC aa) C36:5/C36:6 and phosphatidylcholine acyl-alkyls (PC ae) C38:0/C40.6 were all higher levels of concentration in controls' serum. Glutamate (Glu), ornithine (Orn), phenylalanine (Phe), and methioninesulfoxide (Met.SO) concentrations were higher in psoriasis subjects. Ratios of acyl-carnitine to free carnitine (C2...C0), short-chain acylcarnitines to free carnitine (X.C2.C3...C0), citrulline to ornithine (Cit... Orn), esterifed carnitine to free carnitine (Total.AC...C0), putrescine to ornithine (Putrescine...Orn), and long-chain acyl-carnitines to free carnitine ([C16 + C18]/C0) were all statistically significantly higher in controls, whereas the levels of the fraction of sulfoxidized methionine of the unmodified methionine pool (Met.SO...Met) were higher in psoriasis patients.

Table 1. Statistically significantly different metabolites and their ratios from targeted analysis of serum.

Metabolite abbreviation	Metabolite	p-value	Psoriasis mean μM ± SD	Control mean μM ± SD
Met.SO	Methionine- sulfoxide	6.06E-06	0.88±0.37	0.51±0.27
Met.SO...Met	Fraction of sulfoxidized methionine of the unmodified methionine pool	2.65E-05	0.04±0.02	0.02±0.01
C9	Nonaylcarnitine	0.002	0.04±0.01	0.05±0.01
Glu	Glutamate	0.002	92.85±66.43	49.06±22.76
Cit...Orn	Ratio of citrulline to ornithine	0.002	0.37±0.13	0.44±0.12
C2...C0	ratio of acetylcarnitine to free carnitine	0.004	0.17±0.08	0.22±0.08
X.C2.C3....C0	Ratio of short chain acylcarnitines to free carnitine	0.005	0.18±0.08	0.23±0.08
PC.aa.C36.6	Phosphatidylcholine diacyl C36:6	0.006	0.68±0.27	0.89±0.33
Total.AC...C0	Ratio of esterified to free carnitine	0.006	0.25±0.1	0.31±0.1

Metabolite abbreviation	Metabolite	p-value	Psoriasis mean μM ± SD	Control mean μM ± SD
PC.ae.C38.0	Phosphatidylcholine acyl-alkyl C38:0	0.007	1.73±0.5	2.17±0.69
C7.DC	Pimelylcarnitine	0.011	0.019±0.006	0.024±0.008
Orn	Ornithine	0.011	99.79±29.44	82.28±20.85
PC.ae.C40.6	Phosphatidylcholine acyl-alkyl C40:6	0.011	3.39±0.99	4.02±1.01
Putrescine...Orn	Ratio of putrescine to ornithine	0.013	0.001±0.001	0.002±0.001
PC.aa.C36.5	Phosphatidylcholine diacyl C36:5	0.019	24.78±13.25	34.34±19.98
Phe	Phenylalanine	0.026	82.91±18.96	72.46±13.51
[C16+C18]/C0	Ratio of long chain acylcarnitines to free carnitine	0.027	0.004±0.001	0.005±0.001
C12	Dodecanoylcarnitine	0.036	0.1±0.036	0.124±0.051
C10.2	Decadienylcarnitine	0.044	0.069±0.021	0.076±0.02

The results for untargeted analysis in Paper I are given in Table 2. Out of 22 metabolites, 12 could be identified. Urea, taurine, phytol, 1,11-undecanedicarboxylic acid, glycerophosphocholines PC(16:0/18:2), PC(18:1/0:0), PC(16:0/18:1), PC(16:0/0:0), PC(20:4/0:0), PC(18:1/0:0), and phosphatidylethanolamine PE(20:4/0:0) were all higher in psoriasis patients serum.

Table 2. Statistically significantly different m/z-s from untargeted analysis of serum

Negative ionization	Mass-to-charge ratio	p-value	Intensity levels higher in psoriasis or controls?	Metabolite
	189	2.80E-07	Psoriasis	No match in databases
	802.5	8.28E-05	Psoriasis	PC(16:0/18:2) + FA
	556.32	2.91E-04	Psoriasis	PC(18:1/0:0)
	129.17	4.82E-05	Psoriasis	No match in databases
	249	1.46E-04	Psoriasis	No match in databases
	198	8.36E-05	Psoriasis	No match in databases
	325.5	1.03E-04	Psoriasis	No match in databases
	249	1.30E-04	Psoriasis	No match in databases
	243.12	9.73E-08	Psoriasis	1,11-Undecanedicarboxylic acid

Positive ionization	Mass-to-charge ratio	p-value	Intensity levels higher in psoriasis or controls?	Metabolite
	760.56	7.91E-07	Psoriasis	PC(16:0/18:1)
	496.38	8.15E-07	Psoriasis	PC(16:0/0:0)
	159	3.07E-06	Psoriasis	No match in databases
	126	1.59E-03	Psoriasis	Taurine
	544.38	1.31E-06	Psoriasis	PC(20:4/0:0)
	282	1.58E-06	Psoriasis	No match in databases
	297	1.10E-05	Psoriasis	No match in databases
	297.059	4.30E-03	Psoriasis	Phytol
	522.36	2.79E-03	Psoriasis	PC(18:1/0:0)
	120	3.94E-06	Psoriasis	No match in databases
	502.38	0.0108	Psoriasis	PE(20:4/0:0)
	679.5	5.54E-08	Psoriasis	No match in databases
	60.69	1.23E-05	Psoriasis	Urea

The PCA plot from targeted analysis is based on the summed concentrations of similar metabolite classes (Figure 1). The overlapping group clustering of controls and psoriasis samples is best observed along principal component 1 that accounts for 35% of variability. Biogenic amines, glycerophospholipids, and metabolite ratios are responsible for the clustering. The PCA plot based on the data from positive and negative untargeted analysis data displays very clear clustering of the groups on the axis of PC1 that accounts for 55% of variability (Figure 2).

With recursive feature elimination in Paper I, the random forest model achieved 0.86 AUC, 0.77 sensitivity and 0.74 specificity averaged across five repetitions. 15 metabolites were selected into a final model using this method. Feature selection using internal importance measures that applied inside random forest model yielded 46 metabolites and 0.85 AUC resampling performance with sensitivity and specificity 0.77 and 0.74 correspondingly. Finally, the best model that used genetic algorithm for feature selection kept 90 features and achieved 0.85 AUC. 9 metabolites/ratios was selected by all three methods and these are Met.SO, Cit...Orn, Met.SO...Met, X.C2.C3...C0, C2...C0, C9, Orn, C7.DC, and PC.aa.C38.5. Figure 3 shows the difference in concentrations of nine overlapping metabolites between healthy subjects and psoriasis patients. All of these differences are statistically significant with the exception of PC.aa.C38.5, whereas the maximum Wilcoxon test p-value equals 0.011 for Orn and the minimal 6.06E−06 for Met.SO (Ottas, Fishman, Okas, Kingo, et al. 2017).

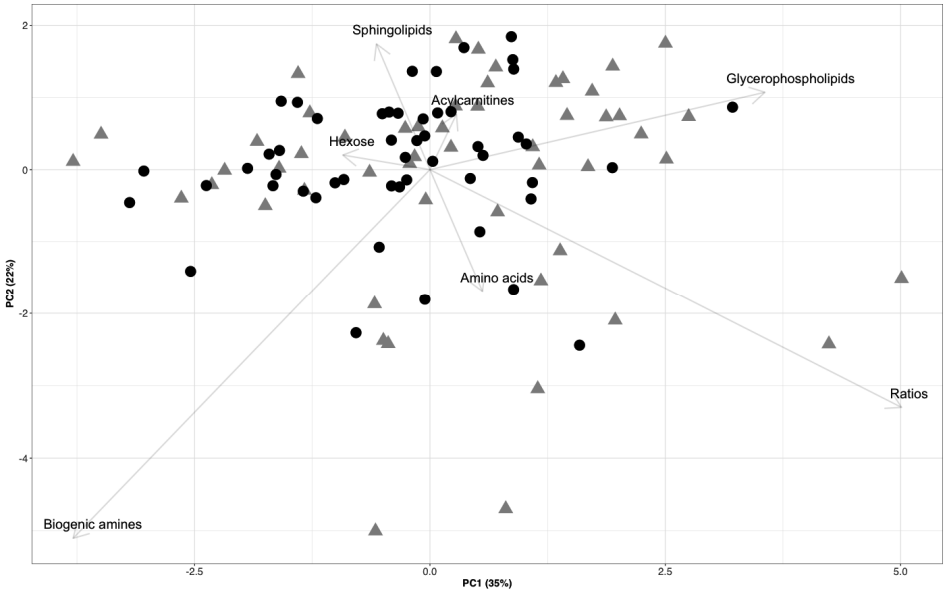

Figure 1. PCA plot of the targeted analysis. Psoriasis samples are marked as gray triangles and control samples as black circles. The metabolite groups responsible for the separation are marked at the end of the arrows. X and Y axes represent the percentage of variability explained by principal components one and two

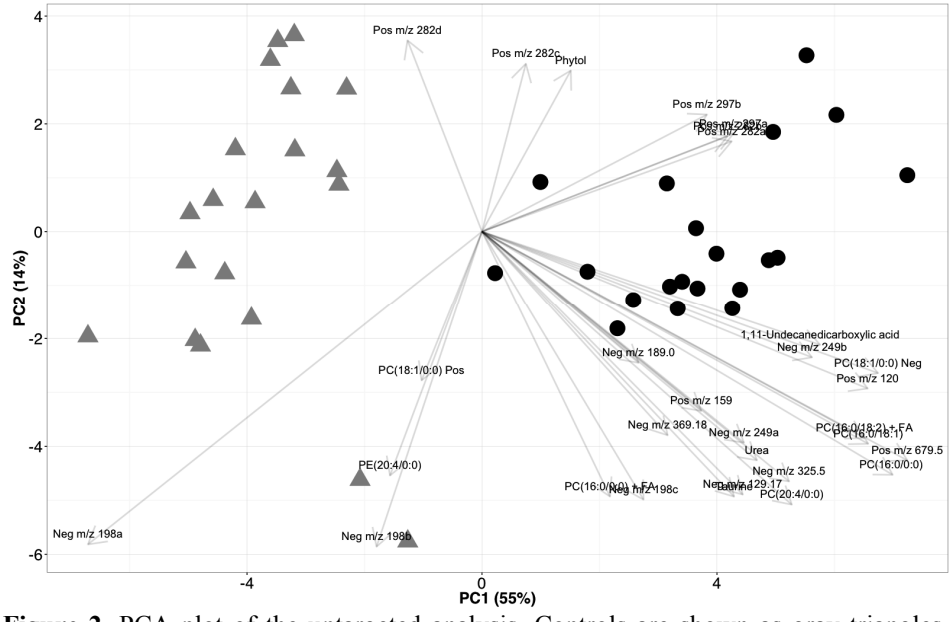

Figure 2. PCA plot of the untargeted analysis. Controls are shown as gray triangles, while psoriasis patients are marked as black circles. The metabolites responsible for the separation are shown at the end of the arrows. X and Y axes represent the percentage of variability explained by principal components one and two

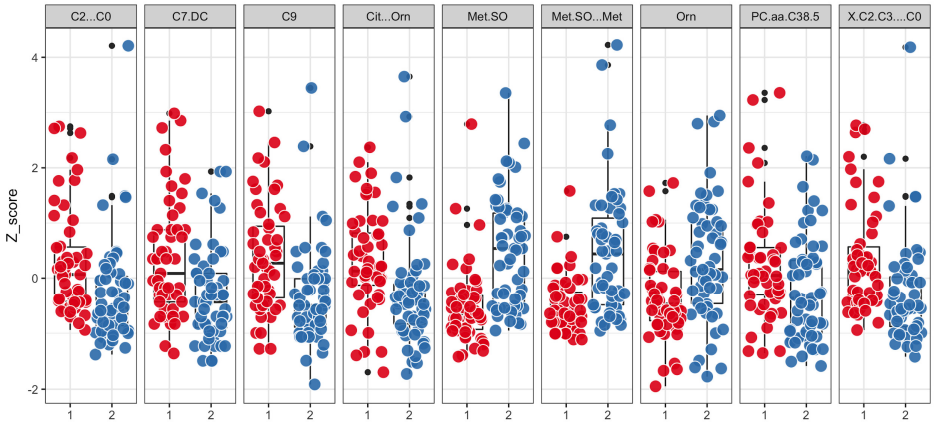

Figure 3. Distribution of standardized signals for nine metabolites overlapping in all three modeling methods. Red dots represent standardized concentrations for psoriasis patients, while blue ones represent controls.

5.2. The metabolomic profile of atopic dermatitis

In Paper II atopic dermatitis patient serum metabolites were compared to controls using both targeted and untargeted methods. The targeted analysis yielded a total of 7 metabolites that differ statistically significantly between groups: acetylcarnitine (C2), phosphatidylcholine diacyl C38:5 (PC.aa.C38.5), phosphatidylcholine diacyl C40:5 (PC. aa.C40.5), ratio of short chain acylcarnitines (acylcarnitine–C2, propionylcarnitine–C3) to free carnitine (C0), ratio of acetylcarnitine to free carnitine (C2. . .C0), fraction of dicarboxylic acylcarnitines of the total acylcarnitines (Total.AC.DC. . .Total.AC) and ratio of esterified acylcarnitines to free carnitine (Total.AC. . .C0). The untargeted analysis resulted in 6 statistically differing metabolites out of which 3 were identified: a peptide DSGEGDFXAEGGGVR and phosphatidylcholine PC(16:0–16:1) /(14:0–18:1) levels in the serum were both significantly higher in AD patients while phosphatidylcholine PC(16:1/20:4) was significantly lower in AD patients' serum. No statistically significant changes could be seen in amino acids, biogenic amines, hexoses or sphingolipids in targeted analysis. The results from targeted analysis are shown in Table 3 while untargeted results are given in Table 4.

Table 3. Targeted analysis results from non-paired t-test where atopic dermatitis patients' serum metabolites were compared to controls

Metabolite	p-value	AD mean	Control mean
C2	0.026	–0.617	0.397
PC.aa.C38.5	0.026	–0.667	0.142
PC.aa.C40.5	0.026	–0.663	0.195
X.C2.C3....C0	0.026	–0.232	0.737
C2...C0	0.026	–0.237	0.736
Total.AC.DC...Total.AC	0.026	0.551	–0.353
Total.AC...C0	0.036	–0.174	0.698

Table 4. Untargeted analysis results from Mann-Whitney Wilcoxon test where atopic dermatitis patients' serum metabolites were compared to controls

Positive mode	m/z	Identification	AD mean	Control mean	p-value
	545.393	unknown	–0.98	0.85	0.0075
	737.735	DSGEGDFXAEGGGVR	0.98	–0.85	0.0075
	780.611	PC(16:1/20:4)	–0.91	0.79	0.0192
	829.891	unknown	–0.91	0.79	0.0192
	641.512	unknown	–0.77	0.67	0.0430
Negative mode	537.507	PC(16:0–16:1) /(14:0–18:1)	0.84	–0.73	0.0351

PCA plots constructed on the whole data from targeted (Figure 4) and untargeted analysis (Figure 5) did not show any hidden batch effects that could bias metabolite identification. The separate clustering of phenotypic groups could also not be seen, suggesting that the use of simple linear models will not differentiate classes. PCA plots generated using only statistically significantly different metabolites produced visually distinct clusters of samples that correspond to studied phenotypic groups (Figures 6 and 7).

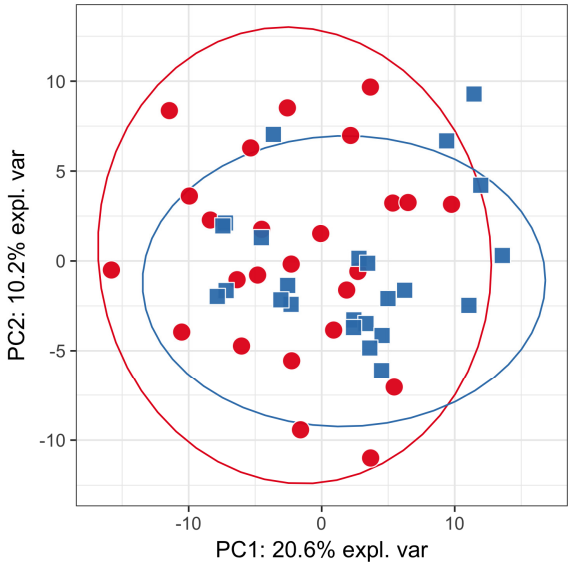

Figure 4. PCA plot for targeted analysis based on the whole data. Red circles—cases; blue squares – controls. Initially, both groups largely overlap, also PCs explain a modest amount of variance.

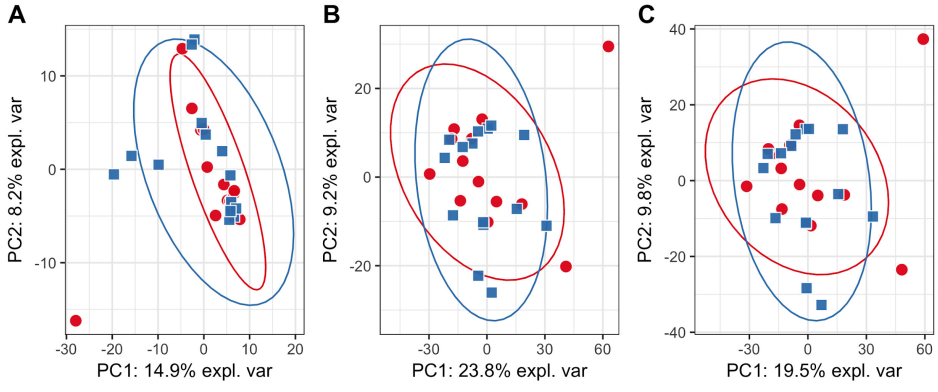

Figure 5. PCA plot for untargeted analysis based on negative (A), positive (B) and a combination of both (C) datasets. Red circles – cases, blue squares – controls. It can be seen that adding the dataset obtained from negative mode does not explain additional variance in the positive mode dataset, on the contrary – the amount of variance explained by the first and second principle components decreases for the combined dataset, which suggests that there is very little important information in the dataset obtained from negative mode.

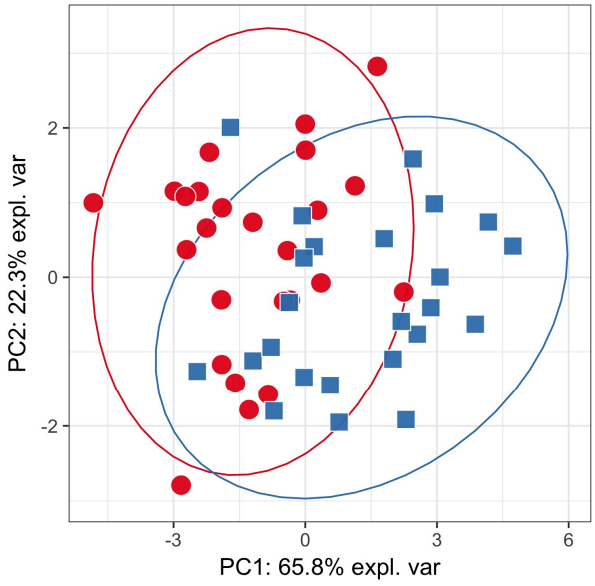

Figure 6. PCA plot for targeted analysis based on the significantly different metabolites. Red circles – cases; blue squares – controls. The groups are visually separable, although there is still a significant overlap between samples. It is important to note that PCs explain much more variance than in Fig 1.

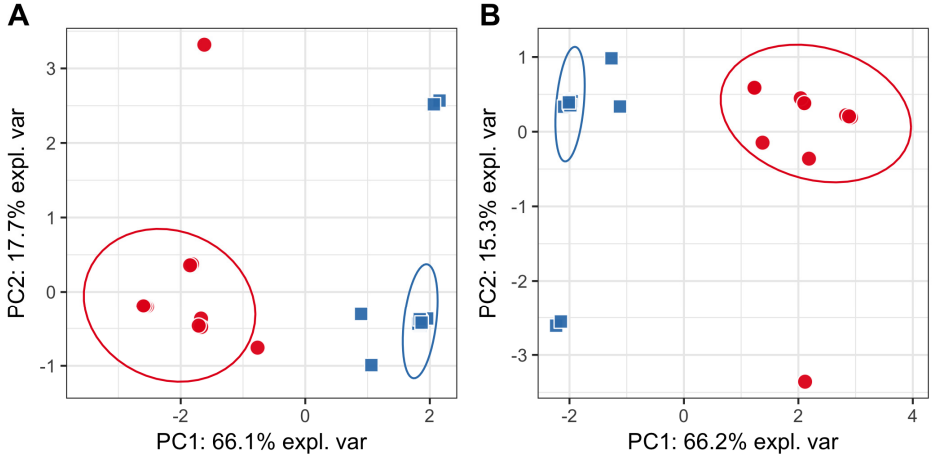

Figure 7. PCA plots based only on metabolites that were found to be significant in untargeted analysis for positive (A) and combined (B) datasets. Red circles – cases; blue squares – controls. Points on both plots are visually separable and form very clear clusters that correlate with phenotypes.

In targeted analysis, machine-learning models achieved an average performance close to 75%, suggesting that it is possible to differentiate between cases and controls using metabolites measured in targeted analysis (Figure 8A). Importantly, metabolites identified as significant in targeted analysis appear in the top of the list of metabolites that were most influential for Random Forest classifier (Figure 8B).

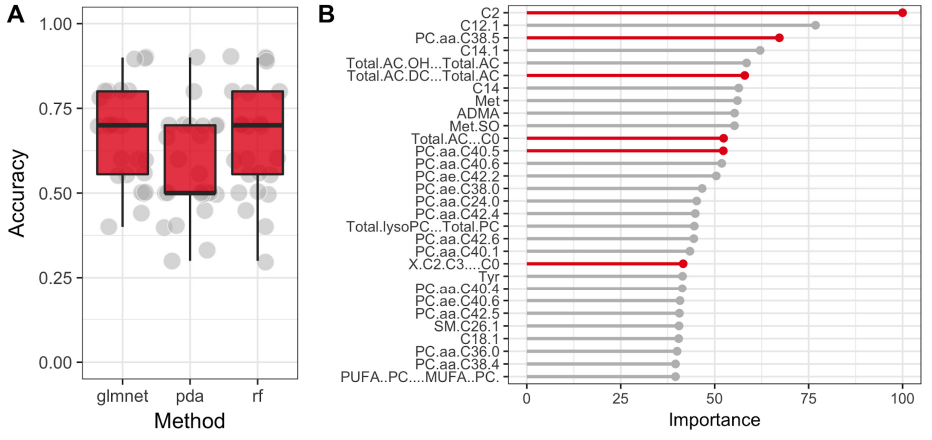

Figure 8. (A) Average classification performance (accuracy) of three distinct classifiers (rf = RandomForest, pda = Penalised Discriminant Analysis and glmnet = Lasso and Elastic-Net Regularized Generalized Linear Model) on data from targeted analysis. The performance was measured with cross-validation algorithm over 5 folds and 5 repetitions. Average performance reaches 70%–75%,, which suggests that metabolites are indeed capable of distinguishing between cases and controls. (B) Top 20 most important metabolites used by RandomForest classifier. Metabolites identified as significantly different in targeted analysis are highlighted. We can see that indeed almost all metabolites (except for C2. . .C0) identified as significantly different are in the top of the list.

For untargeted analysis all three machine-learning models show high accuracy (~90%) on all three modes (negative, positive and combined) (Figure 9). Metabolites that were identified using statistical tests in untargeted analysis were ranked first by the RandomForest algorithm in all three modes (Fig 10), suggesting that these metabolites carry true discriminative power between classes. Also, the constructed PCA plot based on significant metabolites clearly shows the separation between classes of samples (Fig 7) (Ottas, Fishman, Okas, Püssa, et al. 2017).

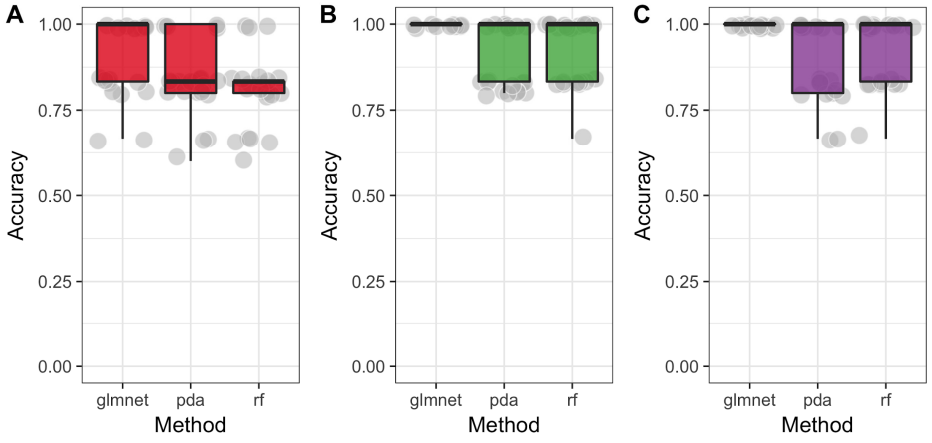

Figure 9. Averaged classification performance (accuracy) of three classifiers (rf = Random Forest, pda = Penalized Discriminant Analysis and glmnet = Lasso and Elastic-Net Regularized Generalized Linear Model) on data from untargeted analysis. Their performance was measured with cross-validation algorithm over 5 folds and 5 repetitions on data obtained with negative mode (A), positive mode (B) and a combination of two modes (C). We can see that on average all three classifiers show high accuracy (for all about 90% on average). Hence, it is possible to conclude that metabolites in untargeted analysis indeed have a discriminative power.

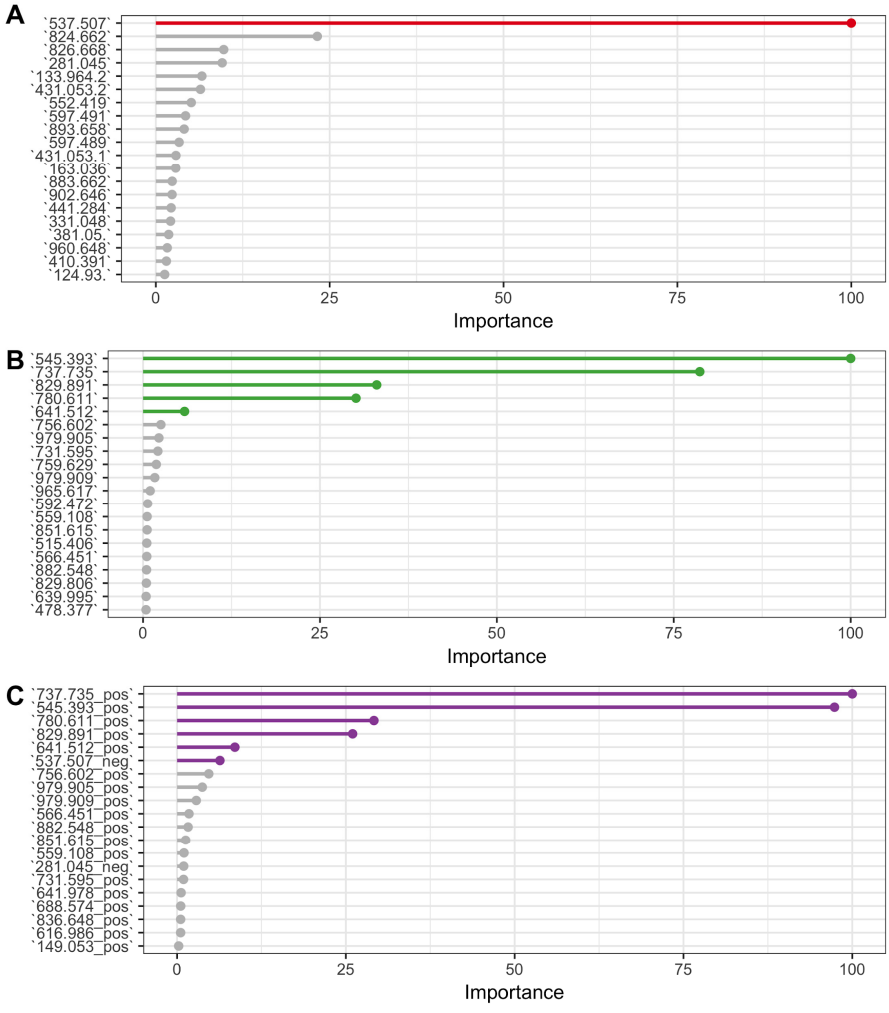

Figure 10. Top 20 of the most influential metabolites used by the RandomForest algorithm trained on data obtained from negative mode (A), positive mode (B) and a combination of two modes (C). Metabolites identified as significantly different from untargeted analysis (highlighted) are at the top of the list that was used by Random Forest classifier to obtain highly accurate classification performance.

5.3. The inverse relations of serum phosphatidylcholines with cardiovascular diseases

5.3.1. Baseline characteristics of the study population

The hemodynamic and biochemical characteristics of patients with PAD, CAD and controls are summarized in Table 5.

The values are presented as means ± standard deviation, medians (interquartile range), or prevalence (%). PAD, peripheral arterial disease; CAD, coronary artery disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; AIx@75, augmentation index adjusted to a heart rate of 75 beats per minute; cf-PWV, carotid–femoral pulse wave velocity; ADMA, asymmetric dimethylarginine; WBC, white blood cell count; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; ND, not detectable.

There were no significant differences in mean age, peripheral diastolic or central diastolic blood pressure, estimated glomerular filtration rate, ADMA, ADMA/arginine, total cholesterol, low-density lipoprotein cholesterol, glucose, insulin levels, or platelet count. The patients with PAD differed statistically significantly from both patients with CAD and the control subjects in relation to central systolic blood pressure, AIx@75, cf-PWV, arginine, triglycerides, white blood cell count, high-sensitivity C-reactive protein, interleukin-6, prevalence of current smoking, antihypertensive therapy, and statin use. Differences in the levels of peripheral systolic blood pressure, mean arterial pressure, HR, high-density lipoprotein cholesterol, and the proportions of antiplatelet therapy only emerged from comparisons with the controls. Higher BMI values were observed in the CAD group than in the PAD group. The CAD group also had significantly increased cf-PWV, HR, and white blood cell count and higher prevalence of antihypertensive, antiplatelet, and statin therapy compared to the controls. After adjusting for multiple comparisons, BMI, current smoking status and statin therapy, the PAD group had significantly lower serum levels of PC aa C28:1, PC aa C30:0, PC aa C32:2, PC ae C30:0, and PC ae C34:2 compared to the healthy controls (Fig. 1). In addition, patients with CAD had lower serum levels of PC aa C32:2 and lysoPC a C18:2 compared to the controls (Fig. 1). The ratio between total lysoPC and total PC (expressed as a percentage) was 18.1 3.5, 19.3 3.5, and 21.4 3.3 for patients with PAD, patients with CAD, and healthy controls, respectively ($p < 0.001$).

Table 5. Baseline characteristics of the study participants.

Variable	PAD patients	CAD patients	Healthy controls	*p*-value
	(*n*=32)	(*n*=52)	(*n*=40)	
Age (years)	61.7 ± 9.0	63.2 ± 9.2	60.3 ± 7.1	0.27
BMI (kg/m^2)	25.8 ± 3[b]	27.9 ± 3.5	26 ± 3.4	0.02
Peripheral SBP (mmHg)	142.5 ± 17.7[a]	135 ± 14.5	129.5 ± 15.0	0.003
Peripheral DBP (mmHg)	79.7 ± 7.5	78.5 ± 8.0	77.8 ± 7.3	0.59
Central SBP (mmHg)	132.7 ± 16.5[a,b]	122.7 ± 11.1	119.3 ± 13.9	<0.001
Central DBP (mmHg)	80.7 ± 7.8	79.7 ± 7.6	78.6 ± 7.3	0.51
MAP (mmHg)	102.5 (95–107.5)[a]	96.0 (91.6–101.4)	92.5 (89.0–100.0)	0.01
Heart rate (bpm)	65.5 (57.5–74.5)[a]	62 (58–68)[a]	57.3 (53–62.5)	0.001
AIx@75 (%)	28 ± 7.4[a,b]	18.9 ± 7.2	15.3 ± 8.0	<0.001
cf-PWV (m/s)[c]	10.5 ± 2.9[a,b]	9.7 ± 2.6[a]	8.2 ± 1.7	0.007
ADMA (μM)	0.46 (0.42–0.57)	0.43 (0.35–0.52)	0.44 (0.37–0.49)	0.07
Arginine (μM)	122.7 ± 22.7[a,b]	102.4 ± 21	99.4 ± 22.3	<0.001
ADMA/arginine	0.004 (0.003–0.005)	0.004 (0.003–0.005)	0.005 (0.003–0.005)	0.36
Total cholesterol (mmol/l)	5.3 ± 1.1	5.1 ± 1.0	5.5 ± 0.9	0.43
HDL-cholesterol (mmol/l)	1.3 ± 0.4[a]	1.5 ± 0.4	1.6 ± 0.4	0.003
LDL-cholesterol (mmol/l)	3.4 (2.7–4.2)	3.2 (2.8–4.1)	3.6 (3.3–4.2)	0.19
Triglycerides (mmol/l)	1.6 (1.4–2.1)[a,b]	1.2 (0.8–1.6)	1.0 (0.7–1.5)	0.002
Glucose (mmol/l)	5.5 (5.2–5.9)	5.8 (5.3–6.3)	5.8 (5.4–6.1)	0.07
Insulin (uIU/mL)	4.9 (3.1–8.2)	5.8 (4.4–8.8)	4.9 (3.3–7.5)	0.13
WBC count (x10^9/L)	8.2 (6.3–9.1)[a,b]	6.4 (5.2–7.9)[a]	5.1 (4.7–5.8)	<0.001
Platelet count (x10^9/L)	240 (192–298)	213 (182–247)	210 (186–239)	0.18
hs-CRP (mg/l)	3.2 (1.1–6.8)[a,b]	1.3 (0.7–2.8)	1.2 (0.7–2.1)	<0.001
Interleukin-6 (pg/mL)	2.81 (1.14–5.57)[a,b]	1.33 (0.74–1.98)	0.95 (0.64–1.29)	<0.001
Resistin (ng/mL)	3.5 (2.6–4.4)	3.4 (2.6–4.5)	2.8 (2.3–3.7)	0.18
eGFR (ml/min/1,73m^2)	94 (78–100)	85 (73–97)	89 (79–93)	0.24
Angiographic score (AU)	27.8 ± 7.1	ND	ND	ND
Current smoking, n (%)	25 (78)[a,b]	13 (25)	5 (13)	<0.001
Medication, n (%)				
Antihypertensive therapy	19 (59)[a,b]	48 (92)[a]	0 (0)	<0.001
Antiplatelet therapy	13 (41)[a]	34 (65)[a]	0 (0)	<0.001
Statin therapy	7 (22)[a,b]	28 (54)[a]	0 (0)	<0.001

a = <0.05 vs control group
b = <0.05 vs CAD group
c = cf-PWV has been adjusted for MAP

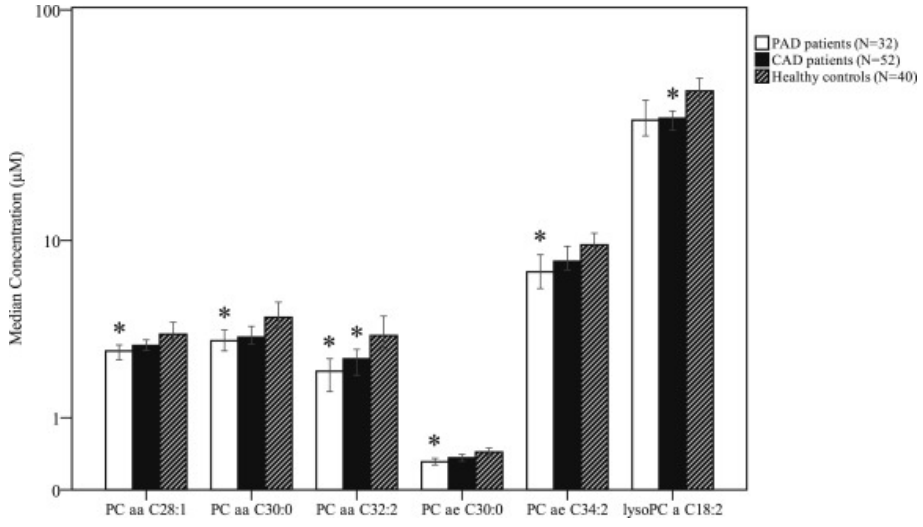

Figure 11. Bar chart showing the median concentrations of selected phosphatidylcholine (PC) and lysophosphatidylcholine (lysoPC) species across the three study groups. *p-values were adjusted for body mass index, current smoking status, and statin use. The Benjamini–Hochberg procedure was used to account for multiple testings. A corrected p-value <0.05 was considered to be statistically significant.

Abbreviations: PAD, peripheral arterial disease; CAD, coronary artery disease.

5.3.2. Relationships of arterial stiffness, endothelial dysfunction biomarkers, and resting heart rate with glycerophospholipids

Table 6 presents the correlation coefficients of lysoPC a C16:0, lysoPC a C17:0, lysoPC a C18:0, lysoPC a C20:4, and PC aa C32:2 for the three study groups.

Table 6. Coefficients of Spearman's rank correlation between clinical characteristics and selected glycerophospholipids for the three study groups.

	lysoPC a C16:0	*lysoPC a C17:0*	*lysoPC a C18:0*	*lysoPC a C20:4*	*PC aa C32:2*
PAD patients (N=32)					
cf-PWV	**–0.57***	–0.16	**–0.45***	–0.28	**–0.6****
Heart rate	–0.34	**–0.56****	**–0.45***	–0.3	**–0.47***
Angiographic score	–0.34	—0.05	–0.2	**–0.41***	0.01
ADMA	**–0.49***	–0.24	**–0.44***	**–0.5***	**–0.42***
ADMA/Arginine	**–0.47***	–0.15	**–0.38***	**–0.44***	–0.21
Total cholesterol	**0.54***	0.09	**0.58***	0.16	**0.66****
LDL-cholesterol	0.45	–0.01	**0.47***	0.07	**0.56****
HDL-cholesterol	0.29	0.13	**0.47***	0.07	0.32

	lysoPC a C16:0	*lysoPC a C17:0*	*lysoPC a C18:0*	*lysoPC a C20:4*	*PC aa C32:2*
Interleukin-6	**–0.54***	**–0.65****	**–0.6****	**–0.49***	**–0.44***
hs-CRP	**–0.42***	**–0.54****	**–0.42***	–0.18	–0.38
Insulin	–0.38	–0.22	**–0.42***	–0.38	–0.31
CAD patients (N=52)					
cf-PWV	–0.08	–0.04	–0.11	**–0.36***	–0.04
Heart rate	–0.07	–0.18	–0.12	–0.26	–0.13
ADMA	**–0.32***	–0.04	–0.26	–0.15	0.01
ADMA/Arginine	–0.28	–0.08	–0.22	–0.1	0.03
Total cholesterol	**0.56****	0.16	**0.55****	0.22	0.37
LDL-cholesterol	**0.47****	0.11	**0.55****	0.24	0.27
HDL-cholesterol	0.11	0.12	–0.04	–0.12	0.27
Interleukin-6	–0.14	**–0.36***	**–0.31***	–0.19	–0.21
hs-CRP	–0.09	–0.21	–0.11	0.05	–0.06
Insulin	–0.07	–0.1	–0.06	–0.24	0.17
Healthy controls (N=40)					
cf-PWV	0.18	0.1	0.1	–0.06	0.05
Heart rate	–0.01	–0.27	–0.13	0.05	0.23
ADMA	–0.18	–0.2	–0.2	–0.09	–0.15
ADMA/Arginine	–0.21	–0.18	–0.2	–0.08	–0.13
Total cholesterol	0.25	0.22	0.31	0.27	0.13
LDL-cholesterol	0.13	0.13	0.26	0.12	0.1
HDL-cholesterol	0.21	0.34	0.29	**0.4***	0.1
Interleukin-6	0.02	–0.26	–0.2	0.01	–0.19
hs-CRP	–0.19	–0.33	–0.17	0.07	–0.01
Insulin	–0.24	**–0.43***	**–0.38***	**–0.37***	0.09

Abbreviations: PAD, peripheral arterial disease; CAD, coronary artery disease; PC, phosphatidylcholine; lysoPC, lysophosphatidylcholine; a, acyl; aa, diacyl; ae, acyl-alkyl; cf-PWV, carotid-femoral pulse wave velocity; ADMA, asymmetric dimethylarginine; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein. Statistically significant correlation coefficients are presented in bold. * Benjamini-Hochberg adjusted p-value <0.05, ** Benjamini-Hochberg adjusted p-value <0.01.

After adjustment for multiple testing, cf-PWV showed negative correlation with serum levels of PC aa C32:2, lysoPC a C16:0, and lysoPC a C18:0 for patients with PAD. The lysoPC a C20:4 exhibited inverse correlation with cf-PWV for the CAD group, while no significant relationships were observed between arterial stiffness and individual lipid species for the controls. In multiple regression analysis, however, the above mentioned lipids lost their significant relationships with cf-PWV for both the patient groups (data not shown). Resting HR correlated negatively with lysoPC a C17:0, lysoPC a C18:0, and PC aa C32:2 for patients with PAD but not for patients with CAD or controls (Table 6). After adjusting for potential confounders, lysoPC a C17:0 retained significance as a determinant of HR for the PAD group (Table 7). The lysoPC a C20:4, lysoPC a C16:0, and lysoPC a C18:0 were inversely related to ADMA/arginine only for patients with PAD. The ADMA correlated negatively with lysoPC a C20:4, lysoPC a C18:0 and PC aa C32:2 for the PAD group, while lysoPC a C16:0 showed inverse relationship with ADMA for both patients with PAD and CAD (Table 6). However, multiple regression analysis revealed that after adjusting for potential confounders, only lysoPC a C20:4 remained a significant determinant of both ADMA/arginine and ADMA for the PAD group, while lysoPC a C18:0 played a similar role for the patients with CAD (Table 8). Finally, lysoPC a C20:4 correlated inversely with angiographic score for patients with PAD (Table 6). However, this relationship lost statistical significance after adjustment for potential confounders (data not shown) (Paapstel et al. 2018).

Table 7. Multiple regression model for PAD group with resting heart rate as the dependent variable.

Variable	Regression coefficient	Standard error	*p*-value
LysoPC a C17:0	–7.962	2.302	0.002
Glucose	9.192	3.110	0.006
Mean arterial pressure	0.309	0.138	0.033

Table 8. Multiple regression analyses for the PAD and CAD patients with ADMA/Arg and ADMA as dependent variables.

Variable	Regression coefficient	Standard error	*p*-value
PAD patients			
ADMA/Arg[a]			
LysoPC a C20:4	–0.0002	6.7^{-5}	0.006
Mean arterial pressure	4.6^{-5}	1.7^{-5}	0.012
LDL-C	0.0004	0.0002	0.025
ADMA[b]			
LysoPC a C20:4	–0.024	0.009	0.010
LDL-C	–0.034	0.023	0.155
Mean age	0.002	0.003	0.426
CAD patients			
ADMA/Arg[c]			
Platelets	-9.7^{-6}	3.0^{-6}	0.005
Interleukin-6	7.4^{-5}	2.6^{-5}	0.008
LysoPC a C18:0	-4.9^{-5}	2.1^{-5}	0.022
ADMA[d]			
Resistin	0.031	0.012	0.010
LysoPC a C18:0	–0.004	0.002	0.013
LDL-C	0.028	0.016	0.086

[a]R2=0.388, p=0.001. [b]R2=0.300, p=0.005. [c]R2=0.273, p<0.001. [d]R2=0.162, p=0.009. PAD, peripheral arterial disease; CAD, coronary artery disease; ADMA, asymmetric dimethylarginine; Arg, arginine; lysoPC, lysophosphatidylcholine; LDL-C, low-density lipoprotein cholesterol. LysoPC a C20:4, lysoPC a C18:0, mean age, body mass index, mean arterial pressure, interleukin-6, total cho-lesterol, LDL-C, glucose, resistin, platelet count, antihypertensive treatment and statin use were entered as independent variables into the regression models.

5.3.3. Glycerophospholipids in relation to biochemical parameters

After adjusting for multiple comparisons, the following relationships were found (Table 6). LysoPC a C16:0 showed a positive correlation with total cholesterol and inverse correlations with interleukin-6 and high-sensitivity C-reactive protein for patients with PAD; it also showed positive correlations with total cholesterol and low-density lipoprotein cholesterol for patients with CAD. LysoPC a C17:0 was inversely correlated with interleukin-6 and high-sensitivity C-reactive protein for the patients with PAD; it also correlated negatively with interleukin-6 and insulin for the CAD and control groups, respectively. LysoPC a C18:0 showed positive correlations with total cholesterol, low-density

lipoprotein cholesterol, and high- density lipoprotein cholesterol and inverse correlations with interleukin-6, high-sensitivity C-reactive protein, and insulin for the PAD group; it showed positive correlations with total cholesterol and low-density lipoprotein cholesterol and an inverse correlation with interleukin-6 for the CAD group; for the healthy controls, it correlated inversely with insulin. LysoPC a C20:4 correlated negatively with interleukin-6 for patients with PAD; it showed a positive correlation with high-density lipoprotein cholesterol and an inverse correlation with insulin for the healthy subjects. PC aa C32:2 correlated positively with total cholesterol and low-density lipoprotein cholesterol and inversely with interleukin-6 only for the PAD group (Paapstel et al. 2018).

5.3.4. The comparative analysis of metabolite levels in patients with plaque psoriasis, atopic dermatitis and atherosclerosis.

Targeted analysis of the same metabolites and metabolite ratios were used in the study on psoriasis (Paper I), atopic dermatitis (Paper II) and atherosclerosis (Paper III) which makes their results comparable to each other. When comparing the metabolomic profiles of PS and AD no metabolites were discovered to be statistically significantly different although three metabolite ratios namely X.C2.C3...C0, C2...C0 and Total.AC...C0 were downregulated in PS and AD when compared to controls. The concentration levels of statistically significant phosphatidylcholines in PS and AD were found to be statistically not significant in atherosclerosis patients and vice versa.

6. DISCUSSION

6.1. The metabolomic profile of plaque psoriasis

The metabolomic profiles of patients with plaque psoriasis differ significantly from controls as evidenced by the multitude of discovered metabolites, the generated principal component analysis figures where the clustering of samples can be noted and the statistical models used to classify samples in Paper I. Many of the discovered metabolites, namely Glu, Orn and Phe were amino acids, which had elevated levels in plaque psoriasis patients thus confirming the results of similar studies where perturbations in the amino acid profile were noted (Kamleh et al. 2015; Kang et al. 2017). Due to the nature of plaque psoriasis, keratinocyte proliferation is upregulated and therefore the increase in the biosynthesis of proteins is necessary, which may account for the increase in amino acid levels. Similarly to Kang *et al.* some amino acids and their ratios (Orn, Putrescine...Orn) were indicative of changes in the urea cycle of psoriatic patients. The urea cycle leads to the polyamine synthesis pathway, the products of which can stimulate cell proliferation (Wei et al. 2001).

Plaque psoriasis is an inflammatory disease and a constant inflammation can lead to oxidative stress in the body. Increased levels of methioninesulfoxide and phytol were discovered in plaque psoriasis patients, which is a direct indication of oxidative stress (Brot and Weissbach 2000; Leipnitz et al. 2010). Arguably the altered levels of phosphatidylcholine levels are also indicative of oxidative damage in the organism. However, due to the novelty of lipidomics, it is not determined yet whether the up- or downregulation of certain PCs is highly specific and indicative of a condition or whether a change in an organism affects many PCs simultaneously and the changes seen vary among individuals.

In plaque psoriasis patients the decrease in acylcarnitine levels like C9, C12, C10.2, C7.DC and ratios C2...C0, X.C2.C3...C0 and [C16 + C18]/C0 hint at changes in overall β-oxidation activity, and it could be argued that the levels decrease due to the increased energy consumption and fatty acid oxidation. The increased activity of carnitine palmitoyltransferase-1, which is responsible for the rate of transport of long-chain fatty acids into mitochondria has been demonstrated in lesional skin (Caspary et al. 2005).

The random forest statistical model that was trained on data from targeted analysis achieved 0.86 AUC, 0.77 sensitivity and 0.74 specificity averaged across five repetitions. The results are promising and if the number of participants increased then a better model could be trained and perhaps one day it could be applied in clinical medicine to help with the diagnosis of plaque psoriasis. However, the amount of data needs to increase beforehand as well as the data on other diseases incorporated into the model for a useful predictive model capable of differential diagnosis.

6.2. The metabolomic profile of atopic dermatitis

The analyzed metabolomic profiles of atopic dermatitis patients were rather similar to controls when looking at PCA figures where the clustering of cases and controls overlapped quite noticeably. Using classical statistical methods, however, a number of metabolites emerged as seen in Paper II. AD patients showed lower levels of acylcarnitine and a number of carnitine ratios, which are all indicative of a suppressed β-oxidation pathway. A study conducted on mice showed that this might lead to lipid accumulation in the liver (Seino et al. 2012) although a similar study in humans to assess the lipid contents of liver in patients with AD is yet to be conducted. To compensate for the suppression of the β-oxidation, other pathways have to be upregulated. In our study on AD we found higher levels of Total.AC.DC. . .Total.AC, which could be indicative of β-oxidation dysregulation, whereas other studies have reported the upregulation of anaerobic glycolysis through the rise of lactate levels in AD patients (Assfalg et al. 2012). It is worth pointing out however that in AD patients only a slight inhibition of β-oxidation is probable and not the complete lack of it.

The datasets from targeted and untargeted analysis in the AD study were subjected to statistical analysis using three different machine learning models, namely glmnet, pda and rf. In targeted analysis, machine-learning models achieved an average performance close to 75% whereas for untargeted analysis all three machine-learning models showed high accuracy (~90%) on all three modes (negative, positive and combined). Repeated cross-validation technique was used but the same conclusions apply here as were with PS study in Paper I that a larger cohort is needed before a predictive model can be applied in a clinical setting to better help diagnose atopic dermatitis.

6.3. The analysis of serum phosphatidylcholines and lysophosphatidylcholines in patients with coronary artery disease and peripheral artery disease

The serum profiles of patients with atherosclerosis were altered in comparison to controls as evidenced by the numerous PCs and LPC that emerged as a result of statistical tests. Interestingly, the coefficients of Spearman's rank correlation between clinical characteristics and selected glycerophospholipids for the three study groups showed similarities in the correlations but were not identical in regards to PCs and a correlating characteristics. This raises the hope that PCs and LPCs are specifically dependent on external and internal stimuli and changes in their serum levels have not happened by chance but more specifically tied to certain conditions and events.

6.4. The comparative metabolomic analysis of plaque psoriasis and atopic dermatitis

In this thesis, the metabolomic profiles of patients with plaque psoriasis and atopic dermatitis were explored using targeted and untargeted methods. Targeted analysis was done using the Absolute*IDQ*™ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria), which uses isotopic internal standards and quality controls for the exact quantification of metabolites while untargeted analysis used two different mass-spectrometers and methods. The targeted method uses isotopic internal standards for the exact quantification of a handful of metabolites and quality controls for the normalization in variation of signal intensities. This allows to compare samples that are measured at different time-points while still remaining comparable despite the variation in the performance of the machine. The data from different untargeted methods used to measure psoriasis and atopic dermatitis samples can still provide insight into the similarities and differences of the two diseases, but the comparison is not exhaustive and fully overlapping.

6.4.1. The similarities in the metabolomic profile of psoriasis and atopic dermatitis

Using the targeted analysis method, 3 metabolite ratios were all discovered to be downregulated in both psoriasis and atopic dermatitis when compared to controls, namely ratios of acylcarnitine to free carnitine (C2...C0), short-chain acylcarnitines to free carnitine (X.C2.C3...C0), and esterifed carnitine to free carnitine (Total.AC...C0). Carnitine can either have an endogenous origin where it is synthesized from lysine and methionine (Kanehisa 2000) or exogenous origin from dietary sources that accounts for up to 75% of carnitine (Rebouche 1992). Acylcarnitines are fatty acid and carnitine esters formed in the cytosol to transport fatty acids into the mitochondrial matrix for β-oxidation and for the structural maintenance of the cell membrane (Famularo et al. 1997). The liver is mostly responsible for the synthesis of various acylcarnitines and they are transported throughout the body for energy production where needed (Xu et al. 2016). The three downregulated ratios are all indicators of overall β-oxidation activity meaning that the metabolites central in β-oxidation (acetyl-CoA and propionyl-CoA) have lower levels in PS and AD serum when compared to controls. This in turn means that less ATP is produced through β-oxidation as has been demonstrated in mice with AD by Seino *et al* (Seino et al. 2012). To compensate the defective β-oxidation and the accumulation of lipids in the cytosol, ω-oxidation has been upregulated in AD patients, indicated by the higher level of fraction of dicarboxylic acylcarnitines of the total acylcarnitines (Total.AC.DC. . .Total.AC) discovered in Paper II. The change was not found to be significant for psoriasis.

6.4.2. The differences in the metabolomic profile of psoriasis and atopic dermatitis

Several differences in metabolite profiles were noted when comparing the results from the serum metabolomic analysis of psoriasis and atopic dermatitis patients. Most notably, the metabolomic profile of psoriasis shows an increase in several circulating amino acids. The levels of glutamate (Glu), ornithine (Orn) and phenylalanine (Phe) are all upregulated in psoriasis samples while the ratios of citrulline to ornithine (Cit...Orn) and putrescine to ornithine (Putrescine...Orn) are downregulated. The increased serum levels of the three amino acids can be associated with a higher demand for amino acids in the hyperproliferative epidermis, where *de novo* synthesis of proteins is upregulated and the rate of mitosis in basal keratinocytes is increased compared to non-lesional skin (Nestle, Kaplan, and Barker 2009). Cit...Orn is lower in psoriasis patients, which indicates the lower activity of ornithine carbamoyltransferase and the accumulation of ornithine. Additionally, Putrescine...Orn is lower in psoriasis patients showing a reduction in the activity of ornithine decarboxylase (EC 4.1.1.17) and to overall changes in the urea cycle, which is concordant with a similar study on urea cycle intermediates in psoriasis (Kang et al. 2017).

Methioninesulfoxide (Met.SO) is the oxidized form of methionine that reacts with free radicals and goes through the oxidation process (Brot and Weissbach 2000). Met.SO and the ratio for the fraction of sulfoxidized methionine of the unmodified methionine pool both had elevated concentrations in psoriasis patient serums, which is indicative of oxidative stress.

While both PS and AD have similar changes in multiple acylcarnitine ratios, both diseases show a reduction in concentrations of different acylcarnitines. PS has decreased levels of nonaylcarnitine (C9), pimelylcarnitine (C7.DC), dodecanoylcarnitine (C12) and decadienylcarnitine (C10.2) while AD has a lower level for acetylcarnitine (C2). The lower levels of various acylcarnitines have been associated with insulin resistance (Bailin et al. 2018), which can be associated with a higher prevalence of metabolic syndrome in PS (Milčić et al. 2017) and AD patients (Lee et al. 2017).

The multiple phospholipids that are up- or downregulated in PS and AD are all different, but due to the high number of endogenous phosphatidylcholines (PCs) it is unclear what the functions of different PCs are beyond functioning as building blocks in cell membranes.

Untargeted analysis revealed more disease specific metabolites in addition to the expected changes in phospholipids. Urea was found to be of higher concentration in psoriasis samples, which correlates well with the changes found in urea cycle related metabolites. The urea cycle leads to the polyamine synthesis pathway, which can stimulate cell proliferation such as keratinocyte expansion, a part of keratinocytosis in psoriasis (Wei et al. 2001). The requirement of polyamines may speed up the mobilization of arginine (an intermediate of the urea cycle) from synthesis sites to the epidermis, which can lead to elevated levels in serum (Abeyakirthi et al. 2010). AD on the other hand had

higher concentrations for a peptide DSGEGDFXAEGGGVR, which is a cleavage peptide of Fibrinogen A-α. The increase in the concentration is not specific to atopic dermatitis but has also been seen in tuberculosis (Weiner et al. 2012), diabetes (Suhre et al. 2010), and Buruli ulcer (Niang et al. 2015). The properties of fibrin clot in AD patients were increased in clot mass and fiber thickness, and rate of clot formation (Nastałek, Wojas-Pelc, and Undas 2010). Another study showed higher cutaneous fibrinolytic activity in the acute phase of AD patients (Lotti et al. 1989).

6.5. Phosphatidylcholines and their role in atherosclerosis

Phosphatidylcholines (PC) belong to a class of phospholipids consisting of choline as a headgroup, glycerophosphoric acid and a variety of fatty acids, which can either be saturated or unsaturated. The synthesis of PCs in eukaryotes includes the condensation reaction of diacylglycerol and cytidine 5'-diphosphocholine, which is mediated by an enzyme called diacylglycerol cholinephosphotransferase. In the liver however the synthesis is done step-by-step through the methylation of phosphatidylethanolamine with S-adenosyl methionine as the methyl group donor (Yeagle 2016).

Lysophosphatidylcholines (LPC) are produced when a PC molecule is partially hydrolyzed mainly by phospholipase A_2 and phospholipase A_1 (Schmitz and Ruebsaamen 2010), hepatic secretion (Sekas et al. 1985) or lecithin-cholesterol acyltransferase (Subbaiah et al. 1980). PCs have long been thought of as being merely cell membranes components, but other functions have recently emerged. For instance, PCs may act as natural surfactants, blood lipoproteins and bile (Furse and de Kroon 2015). Additionally, PCs act as a reservoir for fatty acids (arachidonic acid, docosahexanoic acid) that are used as precursors for lipid-derived signaling molecules. More specifically, arachidonic acid can form inflammatory prostanoids whereas anti-inflammatory and homeostatic prostanoids are derived from ω-3 unsaturated fatty acids. This affects allergy, asthma and systemic inflammation (Wymann and Schneiter 2008). LPCs have many biological roles involved in endothelial dysfunction, monocyte recruitment, vascular smooth muscle cell proliferation (Park et al. 2015). Since the biological roles of PCs and LPCs are so vast it is difficult to pinpoint their cause and effect in atherosclerosis. In mice however a higher uptake of dietary phosphatidylcholine led to higher concentrations of trimethylamine N-oxide (TMAO), choline and betaine, all of which were later associated with a higher risk for CVD in human patients (Wang et al. 2011). Another study found 10 alkenylphosphatidylcholine species and 6 alkyl-phosphatidylcholines that were negatively correlated with myocardial infarction incidence after the adjustment for traditional risk factors and correction for multiple testing (Moxon et al. 2017). The PCs found in Paper III are also correlated with atherosclerosis, which hints at the causative effect of PCs in CVDs. Interestingly, the three PCs found in targeted plaque psoriasis analysis (Paper I), namely PC.ae.C38.0,

PC.ae.C40.6, PC.aa.C36.5, and the PCs that were discovered to be statistically significantly different in the targeted analysis of atopic dermatitis (Paper II), namely PC.aa.C38.5 and PC.aa.C40.5 were all lower in concentrations for PS and AD than controls, similarly to the PCs found in Paper III that were also lower in cases than controls – lysoPC.a.C18:2, PC.aa.C28:1, PC.aa.C30:0, PC.aa.C32:2, PC.ae.C30:0 and PC.ae.C34:2. However no overlap in the exact PCs was noted and the PCs found in PS and AD analysis were not statistically significant in patients with PAD and CAD. This can be explained by the difference in cohorts, their comorbidities and disease-specific risk factors. These findings might also imply that multiple PCs affect the cardiovascular health of patients and finding just a handful of PCs that are solely responsible or indicative of CVDs is not likely. The other PCs found in the untargeted analysis of PS and AD patients were either not measured or statistically not significantly changed in patients with PAD or CAD.

6.6. Psoriasis, atopic dermatitis and their association with a higher risk for cardiovascular diseases

Psoriasis is a chronic immune-mediated inflammatory disease that has been associated with coronary artery disease (CAD) (Prey et al. 2010). Psoriasis in combination with other cardiovascular (CV) risk factors can lead to an increased incidence of CAD (Shaharyar et al. 2014; Joel M. Gelfand et al. 2006). A more recent study shed light on whether psoriasis might increase the chance for CAD as an independent variable where the patients and controls were carefully selected to not be influenced by other common CAD risk factors such as arterial hypertension, chronic kidney disease, diabetes mellitus, dyslipidemia or smoking. It was noted that patients with psoriasis had higher values for arterial stiffness and lower values for global longitudinal strain, which can both be associated with a higher risk for CAD (Dattilo et al. 2018). Another study showed that patients with psoriasis have a higher prevalence and incidence for common CV risk factors including hypertension, obesity, diabetes and hyperlipidemia (Radner et al. 2017). The combination of psoriasis as an independent cofactor and traditional CV risk factors may therefore act synergistically to increase the prevalence of CAD. Several metabolites that could contribute to CV risk were found in Paper I, including methionine-sulfoxide, phosphatidylcholines and phytol. It can be argued that the altered levels of the discovered metabolites are the effect of psoriasis and not the cause explained by their exogenous origin. In Paper I we found several amino acids the levels of which differed from controls, which were implicated in the urea cycle. These findings have been confirmed by other studies (Kang et al. 2017; Kamleh et al. 2015) but the association can be more likely explained by the hyperproliferation of the skin rather than these altered metabolite levels constituting CV risk factors.

The higher risk for cardiovascular diseases in atopic dermatitis patients however is not so clear and remains controversial. AD can be linked to a higher body mass index (BMI), which is a risk factor for CVD, but it seems that the association can also be linked to population differences. For example people living in North America or Asia had higher association of AD and BMI whereas the European population did not (Silverberg 2016; Zhang and Silverberg 2015). To understand the connection of AD with CVD risk a comprehensive meta-analysis was done, which revealed only a marginally increased risk for PAD, hypertension and angina pectoris but an increased risk for myocardial infarction or stroke was not noted in patients with AD (Standl et al. 2017). On the other hand the increase of proteins that are associated with atherosclerosis (fractalkine/CX3CL1, CCL8, M-CSF, HGF), inflammatory mediators (MMP12, IL-12/IL-23p40, CXCL9, CCL22, PI3/Elafin), and proteins associated with T-cell activation and angiogenesis were upregulated in AD patients (Brunner et al. 2017). In Paper II several metabolites were found that could be associated with a higher risk for CVD, namely the peptide DSGEGDFXAEGGGVR, which can contribute to a faster clot formation, disturbance in the levels of phosphatidylcholines that can be associated with systemic inflammation and lactate, which has been associated with hypertension (Juraschek et al. 2015). Overall it can be said that atopic dermatitis might slightly increase the risk for CVD but the association is not as clear or strong as with psoriasis.

6.7. Limitations

The present thesis that is based on Papers I–III has several limitations which must be acknowledged for a more balanced interpretation of the results. Firstly, the number of participants in all of the studies was relatively low, which could skew the data and give a distorted view of the results. Secondly, although the statistical computational models used in Papers I and II were cross-validated, they should be applied to a larger dataset before being implemented in clinical practise. Thirdly, care should be taken when extrapolating the results from Paper III to women because only men were included in the study. Additionally, the recruited men remained on medications and were smokers, which blurs the lines of cause and effect.

7. CONCLUSIONS

1) The metabolomic profiles of psoriasis patient serums were measured using a targeted method on a ABSciex QTRAP 4500 mass-spectrometer with an Absolute*IDQ*™ p180 kit resulting in 19 statistically differing metabolites, and an untargeted method on a ABSciex QTRAP 3200, which yielded 22 metabolites that were statistically significantly different. Data from the targeted analysis was used to build computational models that had high sensitivity and specificity in classifying samples as cases or controls.
2) The analysis of data from targeted and untargeted measurements of atopic dermatitis patient serums resulted in 13 metabolites that characterize AD. The computational models built on the data achieved high precision when classifying samples achieving a 75% and 90% rate of accuracy for targeted and untargeted data respectively.
3) The serum levels of phosphatidylcholines and lysophosphatidylcholines in patients with coronary artery disease and peripheral artery disease were measured and their associations with hemodynamics, endothelial dysfunction and arterial stiffness were analyzed. Multiple correlations of serum metabolite levels to disease severity and progression were discovered
4) The comparative analysis of serum metabolite levels for psoriasis, atopic dermatitis and atherosclerosis resulted in multiple metabolites that differed which is indicative of similarities in the underlying inflammatory processes of the diseases. The differences in the discovered metabolites point to the contrastive pathophysiological mechanisms.

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SUMMARY IN ESTONIAN

Psoriaasi, atoopilise dermatiidi ja ateroskleroosi metaboloomne profileerimine

Metaboloomika on teadusharu, mis tegeleb madalmolekulaarsete ühendite mõõtmise ja analüüsimisega. Nendeks on aminohapped, biogeensed amiinid, süsivesikud, rasvhapped, nukleiinhapped või peptiidid, mis võivad olla nii eksogeensed kui ka endogeensed päritolu. Nende ainete samaaegne mõõtmine võimaldab näha ainevahetusradade otsest peegeldust, nö. metaboloomset sõrmejälge. Selline metaboloomne profileerimine võimaldab klassifitseerida proove juhtudeks või kontrollideks ning jälgida seeläbi haiguse intensiivsust ja kulgu pärast ravi alustamist. Metaboloomika meetodeid on kasutatud paljudes erinevates biomeditsiini-alastes uuringutes sealhulgas ka et uurida vähkkasvajaid, südame-veresoonkonnahaigusi, diabeeti ja paljusid teisi. Üldiselt saab väita, et metaboloomikas kasutatavad meetodid on osutunud hindamatuks paljudes teadusharudes ning on viinud mitmete haigus-spetsiifiliste bioloogiliste markerite avastamiseni, mis omakorda on parandanud haiguste diagnoosi ja ravi.

Psoriaas on laialt levinud krooniline põletikuline nahahaigus, mis esineb kuni 1%-l lastest ja 2%–3% üldpopulatsioonist. Haiguse teke on seotud mitme põhjusega, sealhulgas geneetiline eelsoodumus ja vastuvõtlikkus, keskkonna mõjutegurid koos immuunsüsteemi düsfunktsiooni ja nahabarjääri häirega. Lisaks märgatavatele muutustele nahas, avaldab haigus mõju ka inimese psühholoogilisele ja sotsiaalsele heaolule. Psoriaasi on seostatud ka mitmete kaasuvate haigustega, milleks võivad olla diabeet, südame-veresoonkonnahaigused, põletikulised soolehaigused, metaboolne sündroom, ülekaal ning psoriaatriline artriit.

Atoopiline dermatiit on laialt levinud ja kompleksne nahahaigus, mis mõjutab kuni 15% lapsi ja täiskasvanuid üldpopulatsioonis. Kuigi enamik lapsi kasvab haigusest välja, hõlmab see teatud juhtudel ka täiskasvanuid, mõjutades patsientide heaolu ja põhjustades rida kaasuvaid haigusi, sealhulgas allergiad, astma, tähelepanuhäired ning aneemiat.

Ateroskleroos on põletikuline haigus, hõlmates arterite seinu, kuhu kogunevad põletikulised rakud ja lipiidid. See viib arterite ahenemiseni, mis võib päädida trombi tekkega, põhjustades infarkti. Ateroskleroosi kõige levinumad vormid on perifeerne arterite haigus ja koronaar-arteri haigus, millest mõlemast on saanud suured rahvatervise probleemid.

UURIMUSE EESMÄRGID

Käesoleva doktoritöö peamiseks eesmärgiks oli analüüsida psoriaasi, atoopilise dermatiidi ja ateroskleroosi patsientide metaboloomseid profiile ning hinnata sarnasusi ja erinevusi leitud metaboliitides.

Uurimuse täpsed eesmärgid olid järgnevad:

1) Mõõta psoriaasipatsientide metaboloomsed profiilid kasutades suunatud ja suunamata metaboloomseid meetodeid ning luua statistilised mudelid, mida saaks kasutada haiguse diagnoosimisel ja ravi jälgimisel.
2) Avastada metaboliidid, mis on iseloomulikud atoopilisele dermatiidile ning kasutada statistilisi mudeleid, mis kasutavad andmeid suunatud ja suunamata metaboloomilistest mõõtmistest proovide klassifitseerimiseks.
3) Analüüsida seerumi fosfatidüülkoliinide ja lüsofosfatidüülkoliinide tasemeid ning nende seoseid hemodünaamikaga, endoteeli düsfunktsiooniga ning arterite jäikusega patsientidel, kellel on diagnoositud koronaar-arterite haigus ja perifeerne arterite haigus.
4) Uurida, kas on sarnasusi metaboliitide tasemetes patsientides, kel on diagnoositud psoriaas, atoopiline dermatiit ja ateroskleroos

UURINGUTE MEETODID

Psoriaasiuuringus värvati Tartu Ülikooli Kliinikumis kokku 106 vabatahtlikku, atoopilise dermatiidi uuringus 49 uuritavat ning ateroskleroosiuuringus 124 isikut. Uuritavate vereseerumeid uuriti kasutades mitmeid erinevaid suunatud ja suunamata analüüsi meetodeid. Saadud andmetel rakendati klassikalisi statistilisi meetodeid haigust iseloomustavate metaboliitide avastamiseks ning loodi mitmeid erinevaid statistilisi mudeleid juhtude ja kontrollide klassifitseerimiseks.

TULEMUSED JA JÄRELDUSED

1) Psoriaasipatsientide metaboloomsed profiilid mõõdeti kasutades suunatud meetodit ABSciex QTRAP 4500 mass-spektromeetril rakendades Absolute*IDQ*™ p180 analüüsikomplekti ja suunamata meetodit kasutades ABSciex QTRAP 3200 mass-spektromeetrit. Tulemusena leiti suunatud analüüsist 19 ja suunamata analüüsist 22 statistiliselt oluliselt erinevat metaboliiti. Andmeid, mis saadi suunatud analüüsist, kasutati arvutuslike statistiliste mudelite ehitamiseks, millel oli kõrge sensitiivsus ja spetsiifilisus proovide klassifitseerimisel juhuks või kontrolliks.
2) Atoopilise dermatiidiga patsientide vereseerumite uurimine päädis 13 erineva metaboliidi avastamisega suunatud ja suunamata analüüsist, mis iseloomustavad atoopilist dermatiiti. Arvutuslikud statistilised mudelid saavutasid head tulemused proovide klassifitseerimisel andes 75% ja 90% täpsused vastavalt suunatud ja suunamata analüüsi andmetest.
3) Vereseerumi fosfatidüülkoliinide ja lüsofosfatidüülkoliinide tasemeid mõõdeti patsientides, kellel oli diagnoositud koronaar-arterite haigus ja perifeerne

arterite haigus ning tulemusi analüüsiti seoses hemodünaamikaga, endoteeli düsfunktsiooniga ning arterite jäikusega. Avastati mitmeid seoseid seerumi metaboliitide tasemete ja haiguse tõsiduse ja edenemise vahel.

4) Psoriaasi, atoopilise dermatiidi ja ateroskleroosi võrdlev analüüs viis mitmete metaboliitide avastamiseni, viitamaks sarnasustele erinevate haiguste põletikulises olemuses samas kui erinevused metaboliitides viitavad haiguste erinevatele patofüsioloogiatele.

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PUBLICATIONS

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2010–2012	University of Tartu, Faculty of Science and Technology, Master's studies in Molecular and Cell Biology
2007–2010	University of Tartu, Faculty of Science and Technology, Bachelor's studies in Biology
2004–2007	University of Tartu, Faculty of Medicine, medicine
2001–2007	Tartu Hugo Treffneri Gümnaasium
1992–2001	Tartu Kommertsgümnaasium

Professional employment

01.03.2018– present	Tartu University Hospital, Biobanking Quality Assurance Manager
01.01.2017– present	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Specialist
01.02.2018–31.08.2018	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Junior Research Fellow of Biomedicine and Translational Medicine
01.01.2018–31.08.2018	University of Tartu, Faculty of Medicine, Junior Researcher
01.06.2012–31.12.2016	University of Tartu, Faculty of Medicine, Department of Biochemistry, Chair of Medical Biochemistry, Specialist
01.06.2012–20.05.2013	University of Tartu, Faculty of Medicine, Department of Biochemistry, Specialist
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Publications

Paapstel, Kaido; Kals, Jaak; Eha, Jaan; Tootsi, Kaspar; Ottas, Aigar; Piir, Anneli; Jakobson, Meelis; Lieberg, Jüri; Zilmer, Mihkel (2018). Inverse relations of serum phosphatidylcholines and lysophosphatidylcholines with vascular damage and heart rate in patients with atherosclerosis. Nutrition, Metabolism and Cardiovascular Diseases, 28 (1), 44−52.10.1016/j.numecd.2017.07.011.

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Narvik, Jane; Vanaveski, Taavi; Innos, Jürgen; Philips, Mari-Anne; Ottas, Aigar; Haring, Liina; Zilmer, Mihkel; Vasar, Eero (2018). Metabolic profile associated with distinct behavioral coping strategies of 129Sv and Bl6 mice in repeated motility test. Scientific Reports, 8 (1, 3405), 1−11.10.1038/s41598-018-21752-9.

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2010–2012	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, magistriõpe, eriala: molekulaar- ja rakubioloogia
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2004–2007	Tartu Ülikool, Arstiteaduskond, arstiõpe (lõpetamata)
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1992–2001	Tartu Kommertsgümnaasium

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Publikatsioonid

Paapstel, Kaido; Kals, Jaak; Eha, Jaan; Tootsi, Kaspar; Ottas, Aigar; Piir, Anneli; Jakobson, Meelis; Lieberg, Jüri; Zilmer, Mihkel (2018). Inverse relations of serum phosphatidylcholines and lysophosphatidylcholines with vascular damage and heart rate in patients with atherosclerosis. Nutrition, Metabolism and Cardiovascular Diseases, 28 (1), 44−52.10.1016/j.numecd.2017.07.011.

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