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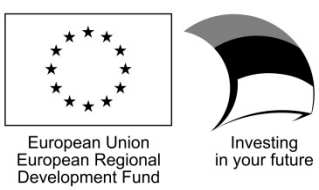

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LIST OF ORIGINAL PUBLICATIONS

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Paper I: Participation in the study design; processing, analyzing, interpreting data, and equal contribution to the manuscript.

Paper II–III: Participation in the study design; processing, analyzing, interpreting data, preparing figures and writing the manuscript.

ABBREVIATIONS

AD	Alzheimer's disease
C6	Complement component 6
CE	Cornified envelope
CNS	Central nervous system
CSF	Cerebrospinal fluid
cDNA	complementary DNA
DEG	differentially expressed gene
EDC	Epidermal differentiation complex
ELISA	Enzyme linked immunosorbent assay
FCS	Functional class scoring
FDR	False discovery rate
G0S2	G0/G1 Switch Regulatory Protein 2
GWAS	Genome-wide association study
HY	Hoehn and Yahr Staging
IL18R1	Interleukin-18 receptor 1
IPA	Ingenuity Pathway Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lewy bodies
LMO3	LIM domain only protein 3
MDS-UPDRS	Movement Disorder Society Unified Parkinson Disease Rating Scale
miRNAs	micro-RNAs
mRNA	Messenger RNA
MMSE	Mini Mental State Examination
ORA	Overrepresentation analysis
PCR	Polymerase chain reaction
PENK	Proenkephalin
PD	Parkinson's disease
PIN	Protein-protein interaction network
PPARGC1A/PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
RIMS3	Regulating Synaptic Membrane Exocytosis 3
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RRA	Robust rank aggregation
RT-qPCR	Real-Time Quantitative PCR
QSBB	Queen Square Brain Bank
SAA1/SAA2	Serum amyloid A1/A2
SE-ADL	Schwab and England Activities of Daily Living Scale
SN	*Substantia nigra*
SNP	Single nucleotide polymorphism

snoRNAs	Small nucleolar RNAs
snRNPRs	Small nuclear ribonucleo proteins
tRNA	Total RNA
UBE2J1	Ubiquitin-conjugating enzyme E2 J1
UPS	Ubiquitin-proteasome system
UV	Ultraviolet

1. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD) with an appreciable rate of clinical misdiagnosis (Poewe et al. 2017). Most notably described by its name bearer, PD presents itself clinically with resting tremor, rigidity and most importantly bradykinesia (Parkinson, 1817). PD is not a homogenous disease biologically, there are sporadic (also called idiopathic – iPD) and familiar, even monogenetic variants. The pathophysiology of PD converges into pathognomonic loss of dopaminergic neurons (Birkmayer and Hornykiewicz 1961) in *Substantia nigra* (SN) of the central nervous system (CNS) and the accumulation of α-synuclein into Lewy bodies in neurons (Lewy, 1912). PD prevalence in the general population is 0.3%, but being a disease of older age, it increases to approximately 0.5 to 1% percent among persons from 65 to 69 years of age and 1 to 3 % among persons above 80 years (Tysnes and Storstein 2017; de Lau and Breteler 2006). With the aging population PD will become more prevalent and already shows a higher prevalence growth than AD (Feigin et al. 2017). Diagnosis of PD is clinical as the definite pathology can only be examined *post mortem* and there are currently no reliable biomarkers for PD *in vivo*. As familiar and monogenetic PD variants make up only around 10% of all PD cases (Bandres-Ciga et al. 2020; Klein, Hattori, and Marras 2018), most of the risk in sporadic PD was thought to come from a large number of common low-risk gene variants (Lohmueller et al. 2003). However, genome wide associations studies (GWAS) that use common genetic variability such as single nucleotide polymorphisms (SNPs) can explain only 1/5 of PD occurrence (Nalls et al. 2019). Next to genetics large scale omics have been thought to be the key in neurodegenerative diseases in filtering large amounts of data and detecting disease relevant changes from this data (Caudle et al. 2010). This body of work investigates transcriptomics of PD in search of feasible diagnostic and prognostic biomarkers. Transcriptomic studies in PD have been previously limited by the canonical notion that PD is solely a disease of the CNS. The current body of work offers a multisystem approach by profiling two peripheral tissues – blood and skin – in well characterized PD patient cohorts. This enables to overcome some major issues related to *post mortem* brain tissue transcriptomics, such as limited application for large-scale biomarker discovery *in vivo*. Another factor that has hampered finding robust changes in PD transcriptomics is the limited comparison across different methods and tissues. The current work tackles also this problem, by offering a method of ranking the relevant transcriptomic changes and provides the first across-method meta-analysis of PD transcriptomics.

2. BACKGROUND

2.1. Parkinson's disease – a quick historical review

The syndrome consisting of slowness of voluntary movement (later known as bradykinesia), rigidity and resting tremor, together known as the parkinsonian triad, was initially recognized in the early 19th century. One of the most eloquent descriptions of this syndrome was from James Parkinson, who named it the shaking palsy, which would be called paralysis agitans in latinized versions (Parkinson 1817). Later in the 19th century Charcot recognized Parkinson as the first descriptor of this syndrome giving the disease his name – *maladie de Parkinson* – Parkinson's disease (PD) (Charcot and Vulpian 1862). During the 20th century key pathological changes of PD were discovered. Lewy described the accumulation of dysfunctional protein– α-synuclein in the brain (Lewy 1912). Deposits of oligomerized α-synuclein are now known as Lewy bodies and PD belongs to a group of synucleinopathies. However, the hallmark clinical manifestation of PD – the parkinsonian triad – is caused by the loss of dopamine producing cells – nigral cells in SN of the midbrain (Birkmayer and Hornykiewicz 1961). Mapping this change became possible due to the discovery of catecholamines (to which dopamine belongs to) as neurotransmitters (Carlsson, Falck, and Hillarp 1962). This discovery enabled the development of the first and still the most used symptomatic treatment of PD – levodopa, to be introduced in early 60s. A myriad of genetic causes of PD were discovered thereafter, most notably in the α-synuclein gene (Polymeropoulos et al. 1997). However, to date, monogenetic PD or even polygenetic risk factors only account for 20% of PD cases (Nalls et al. 2019). It seems, there exist risk modulators of PD for which the genome does not wholly account for. Currently a lot of scientific interest in PD research is towards gene expression studies – transcriptomics including non-coding RNA studies, epigenetics, metabolomics and, perhaps, even enviromics. Many key pathogenic mechanisms in PD have been described, yet it seems that discovering robust biomarkers usable in clinical practice is still a way ahead.

2.2. Parkinson's disease – clinical presentation

2.2.1. Motor symptoms of Parkinson's disease

Since its famous description by Parkinson over 200 years ago the movement disorder with combined motor symptoms has remained the core element of PD. Currently, PD is recognized as a syndrome of bradykinesia in combination with tremor and/or rigidity (Postuma et al. 2015). Bradykinesia is defined in the diagnostic criteria as slowness of movement and additionally decreased movement amplitude, which is sometimes separately referred to a as hypokinesia. Bradykinesia is the pathognomonic sign of PD that correlates best with the

degree of dopamine deficiency in SN (Vingerhoets et al. 1997). Resting tremor is the most easily recognizable symptom of PD. It is usually lateralized with a frequency between 4 and 6 Hz (Deuschl et al. 2000). Rigidity is continuously increased muscle tone and is usually also lateralized (Delwaide 2001). Forth unique symptom next to the triad is postural instability, which has been for a long time one of the main diagnostic criteria (Gibb and Lees 1988). The motor symptoms of PD appear when substantial amount of dopaminergic neurons have already perished (Dauer and Przedborski 2003), yet as there are currently no definite tests the clinical criteria of PD diagnosis have to appreciate the emergence of those cardinal signs. All neurodegenerative diseases have a pre-clinical and prodromal period and PD has a notably long one (>10 years) meaning that the hallmark pathology is already spreading in dopaminergic neurons, but patients display no symptoms or symptoms that are unspecific enough to meet diagnostic threshold.

2.2.2. Non-motor symptoms of Parkinson's disease

Next to the core syndrome of motor deficit in PD there are also several non-motor symptoms of PD that are now recognized as supportive criteria of the clinical diagnosis (Postuma et al. 2015). Some of them are caused by loss of dopamine in other than striatonigral pathway of the brain (Braak et al. 1995), the cause for others is still unknown (Chaudhuri et al. 2006). Some of symptoms antecede the motor involvement in PD such as hyposmia, anxiety, constipation, erectile dysfunction, fatigue and REM-sleep behavior disorder (Postuma and Berg 2019). Sleep disorders, especially rapid-eye-movement sleep behavior disorder is considered a key prodromal sign of PD (Gagnon et al. 2006). An early and wide-known symptom is hyposmia – a decreased sense of smell (Korten and Meulstee 1980) caused by lack of dopaminergic neurons in the mesolimbic pathway. Mesocortical dopaminergic loss causes cognitive and behavioral abnormalities like depression and dementia usually in advanced PD. Drawing the attention to non-motor symptoms of PD in the last 10-20 years (Poewe 2008; Schapira, Chaudhuri, and Jenner 2017) indicated that PD pathology affects a larger scale of neuronal functions than isolated nigral pathways of the basal ganglia. This notion paved the way to investigating other tissues in PD.

When looking into non-motor symptoms of PD, most of them are, indeed, caused by disturbed neuronal functions (autonomic symptoms e.g., constipation, bladder disorders and orthostatic disorder, psychiatric symptoms such as depression or cognitive decline, other brain functions such as sleep disorders or hyposmia). The pathognomonic Lewy bodies have been ever since identified in other parts of the nervous system like enteric nervous system (Wakabayashi et al. 1988), autonomous nervous system (Rajput and Rozdilsky 1976), in the cutaneous nervous system (Ikemura et al. 2008) and their presence in these parts of the nervous system has been correlated to dysfunctions of the latter. Lately,

non-motor symptoms have gained attention such as gastrointestinal dysbiosis and peripheral neuropathy (Zis et al. 2017; Mulak 2015). It has even been proposed that aberrant proteins that cause α-synuclein to misfold might migrate from gut to the brain via the nervous system in a prion-like manner (Hawkes, Del Tredici, and Braak 2007). Over the years it has, indeed, become clear that PD pathology does not begin in the nigral cells of the midbrain, but rather shows an ascending rostrocaudal pattern based on the deposits of α-synuclein showing affection of the lower brain stem regions in earlier stages, which has been clinically correlated to prodromal PD (Braak et al. 2003). However, the exact locus of origin for α-synuclein pathology has remained unclear and the question remains whether non-neuronal peripheral tissues could play a role in it.

2.3. Linking skin pathology and Parkinson's disease

Interestingly, there are a few symptoms of PD in non-neuronal tissues. A non-neuronal peripheral tissue is of particular interest is the skin. PD patients present with several dermatologic problems including seborrhoea, seborrheic dermatitis, hyperhidrosis, cutaneous neuropathy, and impaired wound healing (Beitz 2013; Gregory and Miller 2015). Small unmyelinated fibers are affected in PD causing sensory polyneuropathy and display α-synuclein deposits (Donadio et al. 2014; Wang et al. 2013). Recently, it has even been shown that the rate of α-synuclein aggregation in the skin can differentiate synucleinopathies from other neurodegenerative diseases and is being proposed as a clinical biomarker (Wang et al. 2020). Developmentally skin is an ectodermal derivate like the nervous tissue. A particular subset – the melanocytes – even migrate much later into the skin and display many similar characteristics to nigral cells of the central nervous system (CNS), such as production of pigments from catecholamines – a process which is quite energy consuming and redox-reactive (Fedorow et al. 2005). Neurodegenerative diseases, being related to senescence, are epidemiologically less associated with malignant tumors (Bajaj, Driver, and Schernhammer 2010). However, PD has a notable exception. In a prospective study higher melanoma prevalence compared to the matched general population was found in patients with PD (Bertoni 2010). Even though effects of dopaminergic medication were discussed to be a risk factor for melanoma occurrence, this epidemiologic connection goes beyond simple co-occurrence. Other large-scale studies have shown that in patients who do not have PD, a diagnosis of melanoma or even a family history of melanoma doubles the risk of subsequently developing PD (Olsen, Friis, and Frederiksen 2006; Gao et al. 2009). Additionally, even other types of skin cancer are more common in PD than in the matched general population (Liu et al. 2011). A recent meta-analysis based on pooled genome-wide association study (GWAS) data showed that PD, unlike AD and fronto-temporal dementia show significant disease specific genetic correlation with malignant melanoma (Dube et al. 2020).

Taken together, there is evidence that PD is a multisystem disease with non-neuronal peripheral tissues being disease-specifically affected. Skin is a particularly promising tissue for investigating PD pathology, as it shows epidemiological and clinical disease-specific signs in PD and is relatively easily obtainable allowing for *ante mortem* sampling and even early biomarker discovery.

2.4. The opportunities and challenges of transcriptomics in Parkinson's disease

PD was initially thought to be a typical non-genetic environmentally driven disease (Ward et al. 1983). Upon the discovery of aberrant α-synuclein gene in relation to PD, many genetic studies have discovered more monogenetic causes for PD and genetic risk factors for polygenetic PD. Just recently, a large GWAS discovered 90 independent risk loci associated with PD (Nalls et al. 2019) which accounted for 16–36% of PD heritability. It is a major shift from initial explanation that PD is a non-genetic disease towards genetic causative or modulatory etiologies. However, if PD is compared with another major neurodegenerative disease – Alzheimer's (AD), the differences in how much of disease prevalence can be explained by genetics are still notable. AD twin studies implicated heritability in the range of 60-80% even before the GWAS era (Gatz et al. 1997; Gatz et al. 2006). A GWAS in AD identified 44 risk loci accounting for approximately 75% of heritability (Australian Imaging Biomarkers and Lifestyle (AIBL) Study et al. 2020), implying that AD, unlike PD, has an oligogenic pathology.

It seems, that genetic heritability alone does not account for the majority of PD incidence. After all, gene expression is affected by enzymatic alterations of the DNA or its binding proteins, alternative splicing and non-coding RNAs among many other things. Polyetiological factors can also affect these cellular processes. Some of these variables can be investigated by studying transcription in PD. Detection of gene expression patterns may help with the identification of the molecular mechanisms underlying the disease. Transcription is a process where a part of a DNA molecule that codes a gene is transcribed to an RNA molecule and thus the gene is expressed. Transcriptomics uses different methods to capture RNA molecules and measure them both qualitatively (differential gene expression) and quantitatively (up- or downregulation, usually noted as fold change). Transcriptomic studies provide an option for PD with potential to map pathology-driven patterns and provide dynamic biomarkers in PD.

2.4.1. Transcriptomics from brain tissue in Parkinson's disease

Since gene expression is tissue-dependent and PD is canonically seen as a CNS disease, transcriptomics of PD begun with sampling solid brain tissue. However, acquiring a sample from the brain is very invasive *in vivo*. The first transcriptomic study of PD in humans was, thus, a *post mortem* microarray study of nigral cell populations (Grünblatt et al. 2004). It was performed on 7 end-stage PD patients and 7 controls. The subsequent analysis was limited down to 137 differentially expressed genes (DEGs) with the highest fold change. Then, these DEGs were manually assorted into functional pathways, showing altered gene expression in cell signaling, mitochondrial function and protein degradation which, indeed, correlates with previously postulated disease pathology. Other works from SN mirrored these pathway-level changes (Moran et al. 2006; Cantuti-Castelvetri et al. 2007). Many studies from other CNS tissues followed (Stamper et al. 2008; Dumitriu et al. 2015; Henderson-Smith et al. 2016; Botta-Orfila et al. 2014; Bossers et al. 2009; Zhang et al. 2005). There are significant downsides to investigating brain tissue. First of all, RNA is an unstable molecule, and the tissue is acquired *post mortem*. Long delay between death and sample processing affects the RNA integrity and the disintegration might be cell type specific meaning that cells with a higher energy expenditure, such as the nigral cells, might be especially vulnerable (Zhu et al. 2017). Several *ante mortem* factors also may interfere gene expression such as prolonged agonal state and hypoxia (Ferrer et al. 2008). The samples in brain banks are usually from advanced PD patients with significant disease-specific loss of dopaminergic neuron population. The gene expression profiled from these tissues might not reflect the disease in its prime. Although there is no straightforward connection reported for dopaminergic medication and nigral cell energy metabolism, drugs like levodopa might also affect the transcriptomic profile (Fahn 1999; Schapira 2008). Furthermore, SN consists of mixed cell populations and separating the dopaminergic cells of interest from solid brain tissue becomes a non-trivial task, often yielding samples with varying degrees of dopaminergic cells, leading to differences in transcription. Despite its limitations, transcriptome studies profiled the changes in the brain tissue replicating the hallmark disease pathology but can currently not be used feasibly for finding diagnostic or prognostic biomarkers *in vivo* because of their invasiveness. Taken together, studying the brain tissue is possible only after the death of the individual. These individuals have usually suffered from PD for many years and have extensively taken dopaminergic medications, thus their tissue samples represent late PD, and the samples cannot be used as biomarkers in early PD *in vivo*. As of now, there is a single study in PD from the living brain biopsy from frontal cortex sampled during deep brain stimulation electrodes implantation (Benoit et al. 2020). In this RNA-Seq study 370 DEGs were identified, and they included key members of trophic signaling, apoptosis, inflammation, and cell metabolism pathways.

2.4.2. Transcriptomics from peripheral tissues in Parkinson's disease

Thus, first transcription studies from more accessible tissues emerged, blood being the most prevalent. Scherzer and colleagues (Scherzer et al. 2007) searched for transcriptional biomarkers in PD blood by sampling 50 PD patients predominantly at early disease stages and healthy controls but also controls with neurodegenerative diseases. They found 22 potential marker genes and provided a risk calculation for PD based on eight genes (*VDR, HIP2, CLTB, FPRL2, CA12, CEACAM4, ACRV1*, and *UTX*) that distinguished PD from healthy and disease controls with an odds ratio of 5.1 (95% C.I. 1–27). Also, dopaminergic treatment effects were tested, and none reported. Soon, many different approaches of PD blood transcriptomics emerged (Aguiar and Severino 2010; Shehadeh et al. 2010; Potashkin et al. 2012; Alieva et al. 2014; Infante et al. 2015; Pinho et al. 2016). Kauczynska and colleagues performed a very similar microarray experiment to Scherzer et al. in a slightly bigger cohort (Kauczynska et al. 2013; Scherzer et al. 2007). They also created a prediction model of PD based on a set of 1367 gene probes and added clinical parameters showing that PD can be distinguished from healthy controls with an 88% agreement to clinical diagnosis. However, only 3 of 1367 genes overlapped with the 22 marker genes suggested by Scherzer (Scherzer et al. 2007). Calligaris and colleagues performed thus far the biggest blood RNA microarray analysis on drug naïve PD patients and found 282 DEGs and validated the results with RT-qPCR (Calligaris et al. 2015). A descriptive summary of many of these works underlines the lack of reproducibility of specific gene expression changes (Borrageiro et al. 2017). It has to be mentioned that the datasets of original work are usually not big ranging from 3 to 79 (Vogt et al. 2006; Kauczynska et al. 2013, respectively). Many factors cause the reproducibility to be extremely low: different methods (microarray vs. sequencing), endogenous factors (inherent genetic and transcriptional heterogeneity of PD), other diseases, medication status, and metabolic changes. A particular obstacle in transcriptomics in sampling whole blood is that it consists of a heterogenous mix of different cell linages and calls for additional steps like filtering out hemoglobin-related RNA (Liu et al. 2006). Some attempts have been made to limit the heterogeneity in sampling different lineages of nucleated white blood cells (Kedmi et al. 2011; Soreq et al. 2014) or serum (Botta-Orfila et al. 2014). This, however, also adds analytical steps prior to transcriptional analysis that might cause variations in results and thus may not be suitable for simple biomarker discovery. Other biofluids have been considered, such as the usually low- to non-cellular cerebrospinal fluid (CSF). This yields mostly non-cellular RNA like micro-RNA or fragments of RNA. Another limitation is that lumbar puncture to access this fluid is an invasive and time-consuming procedure. Hossein-nezhad and colleagues found 3521 fragmented transcripts in CSF from coding and non-coding regions from which 201 were differentially expressed, however they did not map these DEGs into pathways (Hossein-nezhad et al. 2016).

Overall, transcriptomics studies from cellular and non-cellular peripheral samples have found changes that are mirrored in CNS suggesting that functional pathway level pathophysiology in PD might be systemic (Cooper-Knock et al. 2012). There have even been single attempts to create a set of DEGs from blood and other moderators to calculate the risk of PD, thus first attempts in finding a diagnostic and prognostic set of DEGs (Scherzer et al. 2007; Kauczynska et al. 2013). However, a general problem with gene expression studies in PD either from CNS or peripheral tissues is the lack of reproducibility of the findings on the DEG level. This is exemplified by an overlap of only 3 DEGs between 2 works from PD blood by Scherzer et al and a subsequent study by Kauczynska et al. Possible cause for the lack of overlap can lie in too low number of transcriptomic studies for generalizing the results onto heterogenic PD cohorts. Other reasons can be the use of incomparable or partly comparable methods, the heterogeneity of signals in the tissue of interest or the heterogeneity of PD pathology, such that genes involved differ, but the pathways they modulate converge not allowing for DEG level overlap.

2.5. Evolution of high throughput methods

Due to the sheer volume of the human transcriptome mapping the changes between disease and controls calls for high throughput methods. Starting with the conventional Sanger method of sequencing (Sanger and Coulson 1975) determining of a particular DNA sequence has come a long way. With the advent of converting the unstable RNA molecule to complementary DNA (cDNA), RNA sequences could also be decoded (Temin and Mizutani 1970). Currently, there are two main methods to determine gene expression both qualitatively and quantitatively – RNA microarray and RNA-Sequencing (RNA-Seq). RNA microarray is a high throughput approach for mapping gene expression (Schena et al. 1995). It is a hybridization-based approach that visualizes fluorescently labelled cDNA if anneals onto a complementary DNA strand. These DNA strands are commercially produced and organized onto a chip representing up to ~30 000 known and relevant gene expressions. Most of the work done on PD transcriptomics relies on microarrays from different manufacturers. However, this method has several limitations. It relies upon existing knowledge about DNA sequences, that rules out the discovery of *de novo* or alternatively spliced transcripts (Wang, Gerstein, and Snyder 2009). Compared to whole transcriptome sequencing a specific chip is needed for either mRNA, micro RNA (miRNA) or other RNA species, so the whole transcriptome cannot be determined in one experiment and the results show a clear batch-specific bias making the comparison between different experiments limited (Kitchen et al. 2010). Further limitations include low dynamic range causing genes with low expression to be difficult to account for and signal from genes with very high expression to be underestimated (Tu, Stolovitzky, and Klein 2002, Mutch et al. 2002). Unlike hybridization, high throughput next-

generation RNA sequencing is based on arranging the cDNA molecule order single nucleotide (or a pair, depending on the technique) at a time. In RNA-Seq fragments of cDNA and converted into library, amplified, and sequenced either from one end (single-end sequencing) or both ends (pair-end sequencing). This creates short reads that are 30…400 base pairs long depending on the manufacturer and RNA species. These reads can be interpreted either by aligning them to reference genome or by *de novo* assembly (Metzker 2010). Thus, unlike hybridization-based approach RNA-Seq is not limited to detection of transcripts that align to existing sequences. It does not create this much background noise allowing a large dynamic range and higher power especially in detecting signals with lower number of transcripts (van Iterson et al. 2009). Also, the number of samples can be starkly reduced and still have the same statistical power as microarray experiments (Guo et al. 2014). RNA-Seq is robust and has high intra- and inter-platform correlation (Stark, Grzelak, and Hadfield 2019). Even though there are some clear-cut benefits to RNA-Seq there are also some limitations. A major source of variation lies in the processing of raw sequencing data. Different computational analysis workflows could introduce imperfections and biases to the subsequent analysis (Sahraeian et al. 2017).

2.5.1. RNA-Seq in Parkinson's disease transcriptomics

Being one of the first and few, Riley et al. compared different brain tissues in PD using both microarray and RNA-Seq (Riley et al. 2014). They found many more significantly changed DEGs with RNA-Seq compared to microarray, however, DEGs did not completely overlap, which they interpreted as differences in sample cell population. The first RNA-Seq array from PD blood acknowledged the limitations of microarray in splice variant discovery and bypassed it by using total RNA-Seq from leukocytes (Soreq et al. 2014). They found novel splice variants and replicated some of the findings in brain tissue. However, the current number of RNA-Seq studies in PD tissues is very low, for example out of all transcriptomic studies from different tissues in PD listed in this review only 8/63 used RNA-Seq (Borrageiro et al. 2017). There are equally few RNA-Seq studies that focus on non-coding RNA like miRNA in PD (Borrageiro et al. 2017). There are even fewer studies of whole transcriptome RNA-Seq which captures all the transcribed RNA species (Soreq et al. 2014).

2.5.2. Interpretation of high throughput methods

Even though high throughput methods allow detection of many gene expressions, an output list of DEGs does not provide pathophysiological insight into the underlying biology of the condition. A pathway analysis has more explanatory power because it links the DEGs with known functions into a network of reactions giving them meaning in the context of cellular processes. Therefore,

most of DEG discovery studies have also included a pathway analysis. There are many databases offering this ranging from open source (Fabregat et al. 2017) to proprietary (Krämer et al. 2014), and many computational algorithms defining a significantly changed pathway in disease (Robinson, McCarthy, and Smyth 2010; Subramanian et al. 2005; Tarca et al. 2009). Most used pathway analysis methods are overrepresentation analysis (ORA), functional class scoring (FCS) and protein-protein interaction network (PIN). These range from mapping which DEGs in a predefined metabolic pathway are present more than expected (ORA) to using complex interactions analysis to determine much weight their differential expression has on biological functioning of the pathway (PIN) (Khatri, Sirota, and Butte 2012). On the one hand, using pathway analysis allows drawing meaningful conclusions from large volumes of transcriptional data, but on the other hand, it is another source of variability in interpreting the results.

Another level of looking at transcriptional data is conducting a meta-analysis by reanalyzing the raw output data. However, the output files are too large (in gigabytes) and usually available upon request. Alternatively, uploaded data from previous experiments can be downloaded from online databases, for example Gene Expression Omnibus (GEO) or ArrayExpress public functional genomics data repositories (Barrett et al. 2009; Athar et al. 2019). Mariani and colleagues pooled 123 PD SN samples versus pooled 104 control SN samples from 9 different RNA microarray experiments and reported top 20 up- and downregulated DEGs with mitochondrial function and signal transduction in Gene Ontology (GO) enrichment analysis (Mariani et al. 2016). Similar meta-analyses have been conducted from cortical tissue microarray data in PD (Feng and Wang 2017; Kelly et al. 2019). Currently a single meta-analysis from RNA-Seq pooled data is conducted from miRNAs of brain and blood in PD (Chatterjee and Roy 2017). A downside of using datasets from repositories is currently the low number of uploaded datasets, that limits the meta-analysis and, perhaps, causes a bias by the reanalysis of the same samples over and over. There are no whole transcriptome RNA-Seq data meta-analyses nor analyses comparing RNA-Seq and microarray data. As aforementioned, there is a review summarizing the compendium of PD transcriptomics, but it does not provide a reanalysis of the data (Borrageiro et al. 2017). Major issues that have been hampering this are the use of different methods, annotations and the publication of incomplete lists (e.g. when only significantly changed DEGs are reported). Secondly, the number of transcriptomic studies could be simply too low to allow the mapping of biological innate, tissue- and time-specific variations in PD transcriptomics. A meta-analysis that could overcome the issue of comparing microarrays and RNA-Seq data and the publication of incomplete DEGs lists would provide more robust results on DEGs across different methods and populations in PD.

2.6. Summary of reviewed literature and rationale for this study

PD is a slowly progressing neurodegenerative disease with a long prodromal period. The emergence of the cardinal movement disorder that allows for the fulfillment of the diagnostic criteria for PD happens after 80% of the dopaminergic neurons have perished. There are currently no robust biomarkers that allow for discovering the pathology earlier. It may be a lost opportunity for future neuroprotective therapies in PD because there may not be enough neurons to preserve in order to make a clinical difference. Therefore, it is of utmost importance that research advances into finding biomarkers that identify PD as early as possible. Many previous studies have investigated the CNS tissue to find pathomechanistic clues for PD, because the loss of dopaminergic cells in the midbrain correlates with movement disorder progression. However, the CNS is a tissue that does not allow for large scale *ante mortem* sampling. Another question is whether PD pathology emerges only in the neuronal cell population or could disease specific changes be found in other tissues as well. The blood is a peripheral tissue that has been previously sampled in PD but has not yet provided reproducible biomarkers. Not many other peripheral tissues have been sampled. The current study therefore approaches PD transcriptomics by sampling two peripheral tissues – skin and blood. The aim of this study is to address the following knowledge gaps: 1) do these peripheral tissues display PD specific pathology; 2) are peripheral tissues therefore usable for large scale *ante mortem* biomarker discovery. The larger rationale is to advance the understanding of PD pathology.

3. AIMS OF THE STUDY

The primary aim of the current thesis was to analyze the skin and blood transcriptomic profiles in PD using RNA-Seq in the hopes of finding disease-specific patterns of gene expression changes in these peripheral tissues in a well-established PD cohort.

The specific aims were the following:

1) Profile the transcriptomic changes in PD patients the skin for the first time and categorize them into functional pathways. Investigate the transcriptomic implications of the known epidemiological link between PD and melanoma. Distinguish disease-specific patterns.
2) Profile the transcriptomic changes of the whole blood in PD and categorize these changes into functional pathways. Compare the blood results with findings from skin. Distinguish disease-specific patterns.
3) Investigate the most significant finding from PD skin RNA-Seq – serum amyloid alpha (SAA), and define whether the downregulation in gene expression level is replicated in another sample set in PD blood and skin, thus translated to protein level changes in blood and skin.
4) Compare the transcriptomic changes in our data set with previously published studies from CNS tissues and blood, by using a novel method of robust rank aggregation to allow for comparison between microarrays and RNA-Seq, in order to find robust and overlapping changes in gene expression of PD.

4. MATERIAL AND METHODS

4.1. Study design

The study examines two peripheral tissues of patients with clinically diagnosed PD and healthy matched controls. The main experimental part consists of RNA-Seq from whole blood and skin biopsy. The top DEG results of the RNA-Seq are validated in a larger cohort of PD patients and matched controls. Further ELISA-analysis from whole blood and skin immunohistochemistry is conducted for a specific gene expression of interest – serum amyloid alpha (SAA).

The study was conducted in accordance with the Declaration of Helsinki and approval was granted by the Tartu University Ethics Committee. Informed consent was obtained from all patients and controls (Certificates No 196/T-10 and 216/M-29).

4.2. Clinical characteristics

The PD patients recruited for this study stem from a larger epidemiological PD cohort that was conducted in the county of Tartu during the period 2010–2016 (Kadastik-Eerme et al. 2018; Kadastik-Eerme et al. 2019). Main studies in this body of work consist of study I – skin RNA-Seq; study II – blood RNA-Seq; III – SAA gene expression study. The inclusion of all PD patients was based on: (1) a diagnosis of idiopathic PD according to the QSBB criteria (Lees, Hardy, and Revesz 2009); (2) on standard medical treatment for PD; and (3) no other severe diagnoses based on medical interview; (4) without major cognitive decline based on MMSE. Control participants were age and sex matched volunteers without a history of CNS diseases. Disease severity, disability and cognitive state in PD patients were assessed using validated instruments including the Movement Disorders Unified Parkinson's Disease Rating Scale (MDS-UPDRS) (Goetz et al. 2008), the Hoehn and Yahr Scale (HY) (Hoehn and Yahr 1967), the Schwab and England Activities of Daily Living Scale (SE-ADL) (Schwab et. al 1969) and the Mini Mental State Examination (MMSE) (Folstein, Folstein, and McHugh 1975). The presence of familial PD and cancers were excluded for all patients at the time of inclusion. In addition, all concomitant medications were documented and none of the patients were taking any medications other than the commonly used dopaminergic medications.

The I RNA-Seq study from skin included other set 12 patients with clinically diagnosed idiopathic PD with mean age of 71.9 (±7.5) years, mean disease duration of 6.1 (±4.3) years and mean HY stage of 3.1 (±0.9). The II RNA-Seq study from whole blood included 12 patients with clinically diagnosed idiopathic PD with mean age of 72.2 (±10.0) years, mean disease duration of 6.9 (±6.5) years and mean HY stage of 2.7 (±1). The results of both RNA-Seqs were validated with qRT-PCR in a larger matched cohort (including the 12+12 from RNA-Seq). In the III study a major finding from study I from skin – SAA gene expression was explored in another cohort with ELISA from blood which

included 36 clinically diagnosed idiopathic PD with mean age of 72.1 (± 8.4) years, mean disease duration of 5.7 (±6.3) years and mean HY stage of 2.7 (± 1.0). Subsequently skin immunohistochemistry was explored in 13 PD patients with mean age of 72.0 (± 7.2) years, mean disease duration of 6.5 (±4.3) years and mean HY stage of 3.1 (± 0.8). A thorough breakdown of clinical characteristics of participants is in Table 1.

Table 1. Clinical characteristic of all study participants.

Study	Study group	Size	Gender; n= male (% of males)	Age (± SD)	Disease onset age (± SD)	Duration of disease (± SD)	1st degree relatives with PD (%)	HY (± SD)	SE-ADL (± SD)	MMSE (± SD)	MDS-UPDRS (± SD)
Skin RNA-Seq	Seq PD patients	12	5 (42%)	71.9 (±7.5)	65.8 (±9.6)	6.1 (±4.3)	0	3.1 (±0.9)	80.4 (±13.2)	28.7 (±1.8)	66.7 (±28.1)
	Seq HC	12	6 (50%)	71.5 (na/)	-	-	n/a	-	-	-	-
	Validation PD patients	37	18 (49%)	61.7 (±8,7)	61.7 (±8.7)	8.01 (±5.2)	n/a	2.5 (n/a)	90 (n/a)	28.8 (±1,7)	62.4 (±25.8)
	Validation HC	33	15 (47%)	68.3 (n/a)	-	-	n/a	-	-	-	-
Blood RNA-Seq	Seq PD patients	12	6 (50%)	72.2 (±10.0)	65.5 (±8.6)	6.9 (±6.5)	1 (8%)	2.7 (±1)	76.3 (±17.2)	27.6 (±2.5)	65 (±38.7)
	Seq HC	12	6 (50%)	68.9±6.9	-	-	n/a	-	-	-	-
	Validation PD patients	59	18 (49%)	69.5 (±7.3)	61.4 (±8.3)	8.2 (±5.1)	3 (8.1%)	2.5 (±0.8)	82.0 (±11.3)	28.8 (±1.7)	62.7 (±24.8)
	Validation HC	33	12 (36%)	72.1 (±7.9)	-	-	n/a	-	-	-	-
SAA	Blood ELISA PD patients	36	12 (33%)	72.1 (± 8.4)	66.5 (± 10.8)	5.7 (±6.3)	4 (11.4%)	2.7 (± 1.0)	77.3 (±14.9)	27.1 (±3.2)	71.8 (± 30.7)
	Blood ELISA HC	27	10 (37%)	72.7 (± 9.6)	-	-	n/a	-	-	-	-
	Immunohistochemistry PD	13	6 (46%)	72.0 (± 7.2)	65.6 (± 9.2)	6.5 (±4.3)	0	3.1 (± 0.8)	79.6 (±13.0)	28.4 (±2.0)	65.8 (± 27.1)
	Immunohistochemistry HC	12	3 (25%)	71.8 (± 8.6)	-	-	n/a	-	-	-	-

4.3. Sampling

For skin RNA-Seq study a 4 mm punch-biopsy was taken from non-sun-exposed skin of each participant in study I at the time of the medical interview. All biopsy specimens were instantly frozen in liquid nitrogen and stored at -80C° until RNA extraction. Biopsies for RNA-Seq and validation were homogenized with Precellys24 homogenizer with the Cryolys system (Bertin Technologies). RNeasy Fibrous Tissue Mini Kit (Qiagen) was used for total RNA extraction, according to the manufacturer's protocol. During the purification on-column DNase I treatment was performed (Qiagen). The RNA quality was assessed using Agilent 2100 Bioanalyzer, the RNA 6000 Nano kit (Agilent Technologies) and the Qubit fluorometer (Life Technologies). The lowest RIN of study samples was 6.7.

For blood RNA-Seq study venous blood of each participant in study II was collected into Tempus Blood RNA Tubes (Thermo Fisher Scientific Inc, CA, USA). The tubes were instantly frozen in liquid nitrogen and stored at -80 ºC. Total RNA was extracted applying Tempus Spin RNA Isolation Kit (Thermo Fisher Scientific Inc, CA, USA) combined with DNase treatment (RNase-Free DNase Set, Qiagen, Hilde, Germany), according to the manufacturers' protocols. The globin mRNA was removed from the extracted total RNA using GLOBINclear Kit, human (Thermo Fisher Scientific Inc, CA, USA). The lowest acceptable concentration of globin clear RNA was 36.5 ng/µL for PD patients and 39.5 ng/µL for the control group. For SAA study skin biopsies were fixed in neutral buffered formalin, processed with ethanol and xylene and then embedded in paraffin. For ELISA serum was extracted from blood samples and instantly frozen in liquid nitrogen and stored at -80 ºC.

4.4. Whole transcriptome sequencing (RNA-Seq)

50 ng of each total RNA sample was amplified with Ovation RNA-Seq System V2 Kit (NuGen Technologies Inc, CA, USA), specific RNA species were not enriched. The output double stranded DNA was used to prepare SOLiD 5500 W System DNA fragment libraries according to the manufacturers' protocols (Thermo Fisher Scientific Inc, CA, USA). Barcoding adapters were applied, and the 12 libraries were pooled prior to sequencing. For blood RNA-Seq fragment (single-end) sequencing chemistry was applied with SOLiD 5500 W XL platform resulting in reads with length of 75 bp. For skin RNA-Seq sequencing on the SOLiD 5500 XL platform paired-end sequencing chemistry was applied (75 bp in forward and 35 bp in reverse directions). SOLiD uses sequencing by ligation with two-base-encoded probes (Metzker 2010) which lessens sequencing errors. No prior filters for sequenced fragments were used. In skin RNA-Seq approximately 40 million reads were mapped per one sample, in blood. 21.4 ± 3.3 million reads (mean ± SD) per sample, out of which 73.6 ± 0.8 percent aligned to the reference genome at least once.

4.5. RNA sequencing data analysis

For initial alignment analysis of RNA-Seq reads to the hg19 reference genome Lifescope software (Thermo Fisher Scientific Inc, CA, USA) was used with recommended default parameters. Gene-level read counts were obtained from LifeScope alignment summary statistics. For differential gene expression statistical analysis DeSeq2 package for R (Love, Huber, and Anders 2014) was used. DeSeq package performs samples comparison and adjusts P-value using the Benjamini-Hochberg procedure, which controls for false discovery rate (FDR) to overcome multiple testing problem. Detected differential expression of genes was considered statistically significant at an FDR≤0.05, no cut-off fold change was applied. Followingly, the obtained DEGs were subjected to a functional pathway analysis. For the initial study from skin the analysis was performed using the proprietary QIAGEN's Ingenuity® Pathway Analysis (QIAGEN Redwood Citytool). A further manual classification of DEGs into broad functional categories followed. This was based on the major functional networks provided by Ingenuity Pathway Analysis and supplemented by PubMed searches initially for the role of the specific DEG and then searching for the association with keywords such as "Parkinson's disease", "neurodegeneration", "neuro", "Alzheimer's disease", "brain". Each DEG was categorized only once, according to the more prominent functional role in association to PD. For the blood RNA-Seq data functional pathway analysis was performed using the hypergeometric test implemented by ClusterProfiler in R(Yu et al. 2012) and based on KEGG pathway annotations. Due to the heterogeneity of blood tissue low number of DEGs at FDR≤0.05 was found. To overcome that issue in pathway analysis, input DEGs for blood were p-adj. ≤0.1, cut-off for pathways was at FDR ≤0.05. A reanalysis of the skin data with the same method was applied with input DEGs with an FDR of ≤0.05, and pathway results were considered significant a p-adjusted≤0.05.

4.6. Validation with RT-qPCR

For validation of RNA sequencing data total RNA from larger cohort was sampled. In the case of skin 37 patients and 32 controls (including the 12 + 12 samples from RNAseq) were included. In the case of blood 59 patients and 33 controls (including the RNA-Seq samples) were included. Total RNA was converted to cDNA using random primers and High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). Duplex quantitative real-time PCR (qRT-PCR) analysis was performed using TaqMan Gene Expression Assays with VIC (housekeeping gene ActinB) and FAM (gene of interest) probes and TaqMan® Gene Expression Master Mix (Applied Biosystems). For skin, the following gene expressions were validated: *SAA-1, SAA-2, HBA-2, CALML-6, DGCR-6 L, CST E/M, OR2HR, ROMO-1, ADAMDEC, HCRT, KLRC-3, APOC-1*. For blood *OGT, UBE2J1, KIR2DL3, ENOSF1,*

FAM219B, IL8R1 and *MIAT*, were validated. RT-PCR was performed using ABI PRISM 7900HT Fast Real-Time PCR System equipment (Applied Biosystems) and the ABI PRISM 7900 SDS 2.2.2 Software. Each reaction was made in four replicates in skin and three replicates in blood to minimize technical errors. The samples were normalized to the corresponding housekeeping gene Actin-B and the comparative ΔΔCT method was used to calculate the fold change for all the samples.

4.7. Enzyme-linked immunosorbent assay of SAA

Serum from 36 patients and 27 controls was defrosted and centrifuged at 1500 g at room temperature for 10 min. ELISA kit for human SAA1/2 (Invitrogen Corporation) was used. 200-fold diluted human serum SAA1/2 standard was calibrated to a highly purified Escherichia coli-expressed recombinant protein. The sample measurements were performed with Tecan GeniOS Pro luminometer in duplicate and repeated 3 times on separate plates. Optical density (450 nm) readings were used to quantitatively express serum SAA1/2 results. The Human SAA1/2 concentrations for samples and controls were plotted based on the standard curve. Values obtained for serum were multiplied by 200 to correct for the overall dilution. The data for mean concentration followed normal distribution, was plotted on a barplot and parametrically tested by unpaired t-test.

4.8. Skin immunohistochemistry

Skin biopsies were deparaffinized with 2× 4 min. xylene, 4 min. isopropanol, 2× 4 min. 96% alcohol. The samples were next blocked with 3% hydrogen peroxide for 7 min and processed with proteinase K for 5 min. The slides were incubated with primary mouse monoclonal serum amyloid A antibody (Novus Biologicals) in 1:100 dilution for 30 min. and processed with detection antibodies (DAKO REAL EnVision+ Dual Link, Single Reagents, HRP Rabbit/Mouse) for 30 min. The sections were immersed in 3.3% diaminobenzidine (Dako Company) chromogen dye and hydrogen peroxide buffer solution for 4 min. This created a brownish staining in the location of detection antibody. The background was dyed with hematoxylin, dehydrated with 2x 96% alcohol and 2x xylene, covered with aqueous resin. The immunohistochemistry visual validation procedure was carried out by an independent pathologist.

4.9. Meta-analysis of previously published gene expression datasets

The rationale for this procedure is apparent lack of overlap in DEGs when comparing previous studies in PD transcriptomics across tissues and transcriptomic methods (predominantly RNA microarray and RNA-Seq). To compare the current work with previous studies and provide a reanalysis of the data a search was conducted on PubMed. Minimal criteria for including previous gene expression results in our reanalysis were: 1) the list of DEGs is openly accessible 2) the list is original data, 3) list does not contain selectively presented gene expressions, lists with statistical significance cut-offs were allowed, 4) sample set includes idiopathic PD vs control comparison, 5) there are enough lists per tissue type for further analysis (at least 3). The search yielded 3 lists from cortex (Henderson-Smith et al. 2016; Dumitriu et al. 2015; Stamper et al. 2008), 5 from SN (Bossers et al. 2009; Simunovic et al. 2009; Cantuti-Castelvetri et al. 2007; Dijkstra et al. 2015; Durrenberger et al. 2012) and 5 from blood (Santiago and Potashkin 2015; Kedmi et al. 2011; Infante et al. 2015; Calligaris et al. 2015; Soreq et al. 2008). Details of the input lists can be found in Table 2. The meta-analysis of datasets was conducted using the robust rank aggregation (RRA) method by Kolde et al.(Kolde et al. 2012). This method bypasses many obstacles that have not allowed for any reanalysis previously due to lack of complete DEG lists or different methods of significance calculation. RRA aggregates lists of DEGs by assigning a significance score to DEGs that appear more frequently in the top of ranked lists. RRA can be applied even with incomplete input data for example if only DEGs with significant p-values were published or a fold change threshold was applied. Another issue hampering aggregating DEG lists has been incongruent gene labeling, therefore all gene identifiers were converted to Entrez IDs prior to analysis with RRA. RRA analysis was conducted separately in each type of tissue (three in total: cortex, SN and blood). In order to allow for comparison between RNA microarray chips that vary in the number of DEGs identified with RNA-Seq data that identifies many more gene expressions an N parameter was calculated as $N(i) + N(avg.\ u)$. Here $N(i)$ denotes the number of genes present on all the different microarray chips used by the studies included in our meta-analysis and $N(avg.\ u)$ denotes the average number of genes per microarray that were not included in the intersected $N(i)$. The N was calculated separately for each set of ranked gene lists in different tissues. DEGs in the tissue specific RRA list were considered statistically significant at FDR ≤ 0.05. A functional pathway level reanalysis was conducted from these lists with ClusterProfiler KEGG package for R. Here, DEGs with a score ≤ 0.05 were included. Score is a parameter of RRA that is not corrected against bias coming from multiple hypothesis testing and allows for more DEGs with lower significance to be added to pathway level analysis. The output pathways were considered statistically significant at stringent cutoff of FDR ≤ 0.05.

Table 2. List of input RNA-Seq and microarray studies for RRA meta-analysis from different central and peripheral tissues in PD. Reasons for exclusion are listed in the last column mostly being comparison between PD patients, selective presentation of DEGs or missing of p-values in the published version.

Group	Paper	Platform	Methods	Identifier	Tissue	Limitations	Conclusion
Cortex	Henderson-Smith 2016	RNA-seq	DESeq; FDR	Gene symbol	posterior cingulate **cortex**	Gene list pruned by fold change (fc) $\geq \lvert 0.2 \rvert$ and $p \leq 0.001$; but only top20 presented and sorted by FC	**Included to analysis**; restricted list
	Dumitriu 2016	RNA-seq	DESeq2; FDR	Ensembl ID	prefrontal **cortex** Brodmann Area 9	None, full gene list presented	**Included to analysis**
	Stamper 2008	microarray	two-tailed t-tests; no correction	Probe ID; gene symbol	posterior cingulate **cortex**	$P < 0.01$ filtered, but P seems to be not adjusted for multiple testing	**Included to analysis**; raw $p < 0.01$ restricted list
	Dimitriu 2012	microarray	linear models; FDR	Probe ID; gene symbol	Prefrontal **cortex**	FDR < 0.05 filtered	**Cannot use – same samples in 2016 data**
	Zhang 2005	microarray	ANOVA; FDR	Probe ID; gene symbol	Combined	P-values only for combined tissues	**Cannot use – absent p-values for individual tissues**
Puta-men	Botta-Orfila 2012	microarray	Limma; FDR	Gene symbol	**Putamen**	FC cutoff, but FDR < 0.05 list usable	**Not included to analysis due to <3 lists found**; FDR < 0.05 restricted list
	Zhang 2005	microarray	ANOVA; FDR	Probe ID; gene symbol	**Putamen**	P-values only for combined tissues	**Cannot use – absent p-values for individual tissues**
	Bossers 2009	microarray	Limma; Bonferroni	Gene symbol; transcript ID	**Putamen**	Small number of significant DE genes probably because Bonferroni correction; no raw p-values	**Not included to analysis due to <3 lists found**; very limited (4 genes)

Group	Paper	Platform	Methods	Identifier	Tissue	Limitations	Conclusion
SN	Bossers 2009	microarray	Limma; Bonferroni	Gene symbol; transcript ID	**SN**	DE gene list limited by Bonferroni correction; no raw p-values	**Included to analysis**; bonf. $P < 0.05$ restricted list
	Hauser 2005	microarray	T-test; no correction	Probe ID; gene symbol	**SN**	List limited to only PD-related genes	**Cannot use – selective presentation of genes**
	Simunovc 2009	microarray	3-way ANOVA; FDR	Probe ID; gene symbol	**SN**	List reportedly filtered by 0.1 FDR; actual list ends at ~0.005, problematic use of 3-way ANOVA to correct for batch effects, possibly not compatible with other studies	**Included to analysis**; FDR < 0.1 restricted list
	Cantuti-Castelvetri 2007	microarray	T-test; no correction	Probe ID; gene symbol; GenBank	**SN**	$P < 0.05$; possibly excluded genes that were DE between genders	**Included to analysis**; $p < 0.05$ restricted list
	Djikstra 2015	microarray	ANCOVA; FDR	Probe ID; gene symbol	**SN**	Gene list pruned by fold change (fc) $\geq \|1.5\|$	**Included to analysis**; restricted list
	Durrenberger 2012	microarray	T-test; no correction	Probe ID; gene symbol	**SN**	Gene list pruned by fold change (fc) $\geq \|1.5\|$	**Included to analysis**; restricted list
	Elstner 2011	microarray	ANOVA; FDR	Probe ID; gene symbol	**SN**	Gene list pruned by custom criteria - enrichment factor (EF) < 0.5	**Cannot use – selective presentation of genes**
Blood	Soreq 2008	microarray	ANOVA; no correction	Probe ID; gene symbol	**Blood**	The presented gene list is a result of ANOVA between 3 groups: PD, HC and NC	**Cannot use – 3-group comparison**
	Alieva 2014	microarray	possibly FDR	Probe ID; gene symbol	**Blood**	No correct gene list published, no summarization at gene level found	**Cannot use – gene list not correct**

Group	Paper	Platform	Methods	Identifier	Tissue	Limitations	Conclusion
	Santiago & Potashkin 2015	microarray meta	T-test; FDR	Entrez ID; gene symbol	**Blood**	This is a meta-analysis. Gene list pruned by FDR < 0.05	**Included to analysis;** FDR < 0.05 restricted list
	Infante 2015	RNA-seq	DESeq; FDR	Gene symbol	**Blood**	PD patients and controls with G2019S mutation – possibly not comparable with other studies. Gene list pruned by FDR < 0.05	**Included to analysis**
	Infante 2016	RNA-seq	DESeq; FDR	Gene symbol	**Blood**	FDR < 0.05 pruned	**Included to analysis;** FDR < 0.05 restricted list
	Pinho 2016	microarray	FDR	Probe ID; gene symbol	**Blood**	Comparison of rapid vs slow progression of PD	**Cannot use – PD vs PD comparison**
	Calligaris 2015	microarray	PUMA; FDR	Probe ID; gene symbol	**Blood**	Gene list pruned by what seems to be FDR < 0.01	**Included to analysis;** FDR < 0.01 restricted list
	Kedmi 2011	microarray	3-way ANOVA; no correction	Gene symbol; RefSeq	**Blood**	Gene list pruned by p < 0.01. Problematic use of 3-way ANOVA to correct for batch effects, possibly not compatible with other studies	**Included to analysis;** **p** < 0.01 restricted list

5. RESULTS

5.1. Differential expression of genes and pathways in Parkinson's disease skin

RNA-Seq from 12 PD patients versus 12 controls from skin resulted in 1074 differentially expressed genes at FDR≤0.05. A reanalysis excluded duplicate genes that yielded, in total 1068 unique DEGs. A pattern emerged with 82% (874 genes) of the DEGs being downregulated in PD skin. A heatmap of 50 most significant DEGs is shown in Figure 1. Following pathway analysis conducted initially with Ingenuity Pathway Analysis (IPA) resulted in 10 major functional networks being with significantly altered gene expression in PD. These functional pathway networks include:

1) gene expression, protein synthesis, dermatological disease and conditions (46/28 – first number is number of genes in the network, second is the number of significantly altered ones in our dataset);
2) dermatological diseases and conditions, immunological disease and inflammatory disease (35/23);
3) cellular assembly and organization, behavior, cell signaling (34/23);
4) cancer, immunological disease, cellular development (33/22);
5) connective tissue disorder, dermatological diseases and conditions, developmental disorder (30/21);
6) lipid metabolism, molecular transport, small molecule biochemistry (28/21);
7) molecular transport, neurological disease, psychological disorders (28/20);
8) cellular movement, hematological system development and function, immune cell trafficking (21/18);
9) cellular growth and proliferation, hematological system development and function, tissue development (21/16);
10) lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism (19/16).

The IPA categorized only a small proportion of DEGs discovered, therefore a further manual classification followed. This yielded 6 broad functional categories including:

1) cellular metabolism/mitochondrial dysfunction (23% of genes);
2) protein metabolism/transport (16%);
3) regulation of nuclear processes (12%);
4) skin homeostasis (11%);
5) cellular signaling and tumorigenesis (7%);
6) immunological processes (7%).

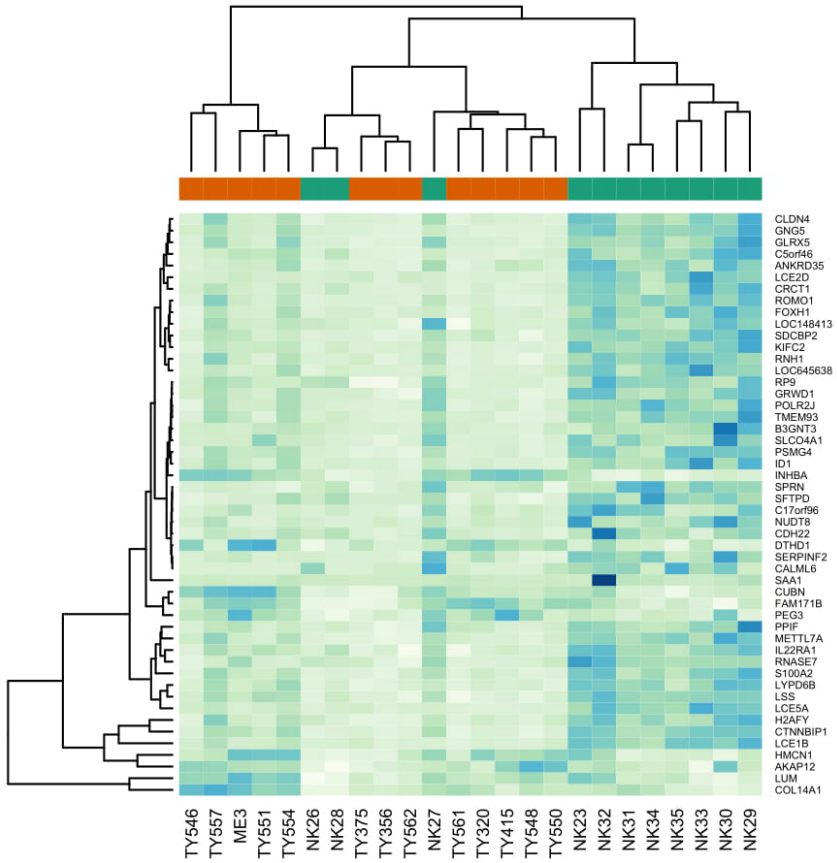

Figure 1. 50 top DEGs (on the right; in rows) visualized in a cluster heat map. PD samples (in columns) are labeled s TY_ or ME_ (below) and coded orange (on the top). Control samples (in columns) are labeled NK_ (below) and coded green (on the top). Gene expression levels are illustrated dark blue for high expression and pale green for low expression.

The largest group of dysregulated genes were involved in mitochondrial functioning with 252 DEGs classified in this group, most of them downregulated (see Table 3). Genes (36/96) involved in mitochondrial respiration were suppressed across all respiratory complexes. An elevated expression of peroxisome proliferator-activated receptor gamma coactivator-1α (*PPARGC1A* or *PGC-1α*) was found. *PGC-1α* is considered to be the master regulator of mitochondrial biogenesis and oxidative metabolism in mammalian cells (Lin, Handschin, and Spiegelman 2005). Auxiliary genes that regulate oxidative metabolism and antioxidant responses were also predominantly downregulated. Other basal cell metabolism alterations included DEGs associated with fatty acid metabolism, oxidation of aldehydes, central mitochondrial transport genes, differential expression of mitochondrial ribosomal proteins (12 members), purine/pyrimidine

metabolism, steroidogenesis, glucose/carbohydrate, amino acid and iron/metal metabolism and detoxification. The second largest group of manually categorized DEGs were involved in protein metabolism with 170 DEGs predominantly downregulated (Table 4). These include DEGs that code for ribosomal proteins, implicating translation. Another major change was found in protein degradation by ubiquitin-proteasome system (UPS) with downregulation of 11 members of the proteasome complex. Next to downregulation of proteosomal genes lysosomal gene expressions were also predominantly downregulated in the skin. Another group consisted of downregulated extracellular proteases like ADAM metallopeptidase family members. Third large group (115 DEGs) of genes linked to epidermal and dermal homeostasis were deregulated in PD (Table 5). Genes involved in epidermal differentiation pathways such as keratins and keratinocyte differentiation factors were downregulated and some of their receptors upregulated. 20 DEGs belong to epidermal differentiation complex (EDC) – a specialized unit involved in the process of epidermal cornification and desquamation with a key DEG coding loricrin being downregulated. These genes code for proteins that generate the cornified envelope (CE) – a structure inside a fully mature keratinocyte that provides the mechanical resistance, elasticity, and stability of the intact epidermis. Furthermore, DEGs coding for intracellular junction proteins showed altered expression. From DEGs expressed in the dermal layers of skin multiple members of the collagen family showed dysregulation. DEGs regulated cytoskeletal morphology were altered. Downregulation of several defencins, mucins and rnases was documented. Also, a few melanocytic DEGs were profiled including d-dopachrome tautomerase and macrophage migration inhibitory factor, both downregulated. Fourth group of DEGs (128 DEGs) in PD skin affect nuclear processes and epigenetic regulation. 14 DEGs associated with cell cycle functioning were downregulated, many more DEGs associated with basal transcription and degradation of DNA were mostly downregulated. DEGs related to chromatin remodeling and DNA binding, transcriptional/post-transcriptional modification and RNA splicing were differentially regulated, as well as a large group of miRNAs, snRNPRs and snoRNAs (Table 6).

Next to reduced cell functioning reflected by alterations in protein and energy metabolism, proliferation and differentiation of skin cells pathways associated with tumorigenesis were altered. Examples include suppression of Ras signaling pathway, Wnt and NOTCH signaling pathways. Alterations in multiple growth factors were found such as fibroblast growth factor, insulin-like growth factor, transforming growth factor-β, nuclear factor-κβ, and other central signaling proteins, such as carcinoembryonic antigen proteins, epidermal growth factor and vascular endothelial growth factor family proteins (Table 7). Next to tumorigenesis immune pathways were affected with the most statistically significantly downregulated DEG in our study – serum amyloid A1 (*SAA1*) and its isoform *SAA2* (also downregulated), which are known to be major acute phase proteins. Their functions span from inflammatory responses to cholesterol metabolism and amyloid aggregation. DEGs from various

immune cascades like chemokines, cytokines, tumor-necrosis-factor (TNF) family signaling, complement genes, immunoglobulins, interleukins, interferon signaling and T-cell signaling showed alterations in PD skin. Specific DEG from humoral immunity the *HLA-DQA2* was upregulated in PD skin being previously associated with an increased risk for sporadic PD (Wissemann et al. 2013).

Followingly a functional pathway reanalysis based on KEGG pathways was conducted with ClusterProfiler R package yielding 9 significantly altered pathways at FDR ≤ 0.05 (Table 8). Three KEGG pathways were associated with neurodegeneration. Huntington disease (HD) pathway contains genes that are involved in vesicular transport, Ca2+ signaling and mitochondrial dysfunction. AD pathway contains genes that increase in the production of amyloidogenic A-beta peptides or affect posttranslational processing of proteins. PD pathway involves the aforementioned processes like UPS and mitochondrial functioning and axonal transport. A significant DEG level overlap between neurodegenerative disease in the CNS has previously been established (Labadorf, Choi, and Myers 2018). Further KEGG pathways associated with different metabolic functions are significantly altered. Oxidative phosphorylation pathway entails downregulated DEGs in mitochondrial respiratory complex. Non-alcoholic fatty liver disease (NAFLD) pathway leads to lipid accumulation through insulin resistance and elevated oxidative process. Another pathway related to lipid metabolism affected in PD skin is thermogenesis pathway in which chemical energy is converted into heat in brown adipose tissue that is controlled by sympathetic nervous system. Retrograde endocannabinoid signaling pathway affects synaptic plasticity and neurotransmission in the CNS. In the skin, changes in endocannabinoid system are linked to melanoma (Río et al. 2018), which is interesting considering the epidemiological bidirectional link between PD and melanoma (Liu et al. 2011).

Table 3. The cellular and mitochondrial metabolism pathways affected in Parkinson's disease versus normal skin. The gene names in bold text reflect the upregulated genes, while all others were downregulated.

MITOCHONDRIAL BIOGENESIS	*ATF3, CEBPA, CEBPB,* ***CREB5****,* ***PPARGC1A/PGCα1****, POLRMT*
MITOCHONDRIAL RIBOSOMAL PROTEINS	*MRPL14, MRPL15, MRPL23, MRPL27, MRPL33, MRPL41, MRPL52, MRPL54, MRPL55, MRPS12, MRPS17, MRPS24*
MITOCHONDRIAL ELECTRON TRANSPORT CHAIN	COMPLEX I**:** *NDUFA1, NDUFA2, NDUFA4, NDUFA7, NDUFA8, NDUFA11, NDUFA13, NDUFAB1, NDUFB1, NDUFB2, NDUFB10, NDUFB11, NDUFC1, NDUFC2, NDUFS5, NDUFS6, NDUFS7, NDUFV1* COMPLEX III**:** *CYC1, UQCR10, UQCR11, UQCRC1, UQCRQ* COMPLEX IV**:** *COX5B, COX6A1, COX6C, COX7A1, COX7A2, COX7A2L, COX7B* COMPLEX V**:** *ATP5E, ATP5G2, ATP5G3, ATP5I, ATP5J2, ATP5L*
CELLULAR RESPIRATION/ENERGY METABOLISM	***ADCY8, ADCYAP1****, ADCK2, ADCK3, ATPIF1, HINT2,* ***OXGR1,*** *SURF1*
OXIDATIVE STRESS/ PEROXISOME/ PEROXIDATION/ ANTIOXIDANTS	*ATOX1, BLVRB, CCDC56, COQ4, CST6, CSTB, FDXR, GLRX2, GLRX5, GPX1,* ***GSTM1****, GSTO1, GSTO2, GSTP1, HAO2, HSPB2, MT1G, MT1L, MT1M, MT1X, MT2A, MT4, PAOX, PEX16, PRDX1, PRDX2, PRDX5,* ***PXDNL****, ROMO1, SEPW1, SNN, SPR, TST, TXNL4A, TXNRD2, VNN2*
MITOCHONDRIAL DYNAMICS AND TRANSPORT MEMBRANE/ PORE/ FISSION	*ABCC8, FIS1, MTCH1, NKAIN2, PAM16, PPIF, SLC14A1, SLC25A20, SLC25A29, SLC25A5, SLC26A7, SLC34A2, SLC52A1, SLC9A4, SMCR7, TIMM10, TIMM13, TIMM8B, TOMM7*
FATTY ACID BIOSYNTHESIS	*ACAA1, ACADVL, ACOX3, ACSBG1, ACSS1,* ***ADIPOQ****, APOC1,* ***ATP8A2****, AWAT2, CBR3, CES4A, COASY, CYB561D2, CYB5A, CYB5R1, DEGS2, DGAT2, ECHDC3, ELOVL1, ELOVL3, FA2H, FABP4, FABP7,* ***GLYATL2****, LBP, LEP, MLXIPL, MOGAT2, PLA2G2A, PLA2G2F, PLA2G3, PLIN2, PLIN3, PLIN4, PLIN5, PXMP2, SPHK1,* ***SPHKAP****, SPNS2, SREBF1,* ***TECRL***
GLYCOPROTEIN METABOLISM	***B3GALT5****, B3GNT3,* ***B3GNT7****, CLEC4E, FSTL3,* ***GALNT5****, GLTPD1, GP1BB, GPC1, GPC2,* ***GPC3****,* ***GPC6,*** *HS3ST4, HS3ST6, LFNG, LGALS1, LGALS12, LGALS7, LGALS7B, MGAT1, NAGLU, OSGEP, PSG9,* ***RELN****, RFNG, RHBG, SDCBP2, SGSH*
PURINE/PYRIMIDINE METABOLISM	*APRT, CKB, GUCY1B2, GUCY2D, GUK1, NME1, NME1-NME2, NME2, NME3, NT5C, NT5DC2, NUBP2, UPB1*
AMINO ACID METABOLISM	*BCAT2,* ***CDO1****, HMGCL, HGD, FAH, GAMT, GGT5, GPT2, MPST, OAZ1, PRODH,* ***PRRG3****, THNSL2*
OXIDATION OF ALDEHYDES	*ALDH16A1, ALDH1L1, ALDH3B1*
STEROIDOGENESIS/ STEROID METABOLISM	*AKR1C3, CYP4F2, CYP4F8, HSD17B2, HSD17B8, LSS, MVD, MVK, PMVK*
GLUCOSE/ CARBOHYDRATE METABOLISM	*CSN1S1, FBP1, PMM1, TALDO1, TSTA3*
CALCIUM HOMEOSTASIS	*CACNA1H, CALML3, CALML5, CALML6,* ***CAPS2****, CIB1, MS4A4A, ORAI1, S100A12, S100A16, S100A2, S100A4, S100A6,* ***S100A7A,*** *S100A9, SOLH/CAPN15*
IRON/METAL METABOLISM	*SLC11A1, TF*
OTHER	***ST6GALNAC5, ST8SIA6****, SULT1A1,* ***SULT1C4****, SULT4A1*

Table 4. The protein metabolism pathways affected in Parkinson's disease versus normal skin. The gene names in bold text reflect the upregulated genes, while all others were downregulated.

EUCARYOTIC TRANSLATION	*EEF1A1P9, EEF1D, EIF1AY, EIF1B, EIF4A3, EIF4EBP1, ELL, FAU*
RIBSOSOMAL PROTEINS	*RPL11, RPL13, RPL21, RPL21P28, RPL22, RPL22L1, RPL23A, RPL27, RPL27A, RPL29, RPL35, RPL36A, RPL37A, RPL39, RPL39L, RPL41, RPL8, RPLP0, RPLP1, RPS12, RPS19BP1, RPS20, RPS29, RPS4Y1, RPS9, RRP9*
POST-TRANSLATIONAL MODIFICATION	*ADPRHL2, GALNS, NUDT14, NUDT16L1, NUDT8, NAA10, NAT6*
PROTEIN FOLDING/ ER PROTEINS/UPR	*CNIH4, DPM3, EBP, EBPL, ERP27, ERP29, OSGEP, PFDN2, PFDN5, SDF2, SDF2L1, SERF2, SEC61B, SEC61G, TRAPPC2L, TRAPPC4, TRAPPC6A, TMEM147, MANF*
VESICULAR TRANSPORT/ PROTEIN TRAFFICKING/ GOLGI PROTEINS/ ENDOSOMES	*ALS2CL, AP2S1, AP4M1, ARF5, CCDC64B, CLTB, CUTA, CYTH4, DNAH5, DNAH6, DNAJB2, DNAJC5, DNAL4, DYNLL1, EXOSC7, GALNTL2, GOLGA7B, KIFC2, LFNG, MGAT1, MYL6, RAB20, RAB40C, RIMS1, SCAMP3,* ***SCG2****, SLC35A2, SLC35D2, SNF8, SNX15, SPRN, SSR4, SYNGR2,* ***SYT13****, SYT8, TMED3, UNC119, VAMP5, VAMP8,* ***VAT1L****, VPS28, YIF1B*
UBIQUITILATION/ NEDDYLATION	*NEDD8, NOSIP, OTUB1, RBX1, REEP4, RNF126, RNF181, UBA52,* ***UBASH3A****, UBB, UBL4A, UBL5, UBXN1, USP6*
PROTEIN DEGRADATION/ PROTEOSOME	*POMP, PSMA6, PSMB1, PSMB3, PSMB6, PSMB9, PSMC5, PSMD9, PSMG3, PSMG4, SHFM1/DSS1*
AUTOPHAGY	*ATG16L2, ATG4D,* ***CA8****, MAP1LC3A, RAB24, ROBLD3, TSPO, ULK3*
PHOSPHATASES	*ACYP1, DUSP2, DUSP22, DUSP23, ITPA, MDP1, PPP1R12C, PPP1R14B, PPP1R16A*
PROTEASES/ PEPTIDASES	***ADAMDEC1, ADAMTS3****, ADAMTS15, ASPRV1,* ***CAPN6****, CAPN10, CAPN13, CLPP, ELANE, HTRA3, KLK11, KLK8, MMP3,* ***MMP16****,* ***PI16****, PLAU, PRSS3, SERPINA1, SERPINF2, SGSH, SPINK1, SPINT1, SPINT2,* ***TMPRSS11A****,* ***TMPRSS4*** *TPSG1*

Table 5. The skin homeostasis pathways affected in Parkinson's disease versus normal skin. The gene names in bold text reflect the upregulated genes, while all others were downregulated.

EPIDERMAL HOMEOSTASIS	*EFNA1, EPHA6, KRT15, KRT17, KRT27, KRT31, KRT4, KRT71, KRT74, KRT79, KRT85,* ***KRT9, KRTAP1-5, KRTAP4-1****, KRTAP5-10, KRTCAP2, KRTCAP3,* ***KC****,* ***LUM, UPK1A***
EPIDERMAL DIFFERENTIATION COMPLEX	*LOR,* ***SPRR2A****, SPRR2B, SPRR2E, SPRR2G,* ***S100A7A****, S100A12, S100A16, S100A2, S100A4, S100A6, S100A9, LCE1A, LCE1B, LCE1C, LCE1D, LCE1E, LCE1F, LCE2C, LCE2D, LCE3D, LCE5A*
CORNIFICATION AND DESQUAMATION PATHWAY	*CST6, CSTB, CTSD, CTSF, CTSL1, TGM5*
STRATIFIED EPITHELIUM SECRETED PEPTIDES COMPLEX	*DMKN, SBSN, KRTDAP*
DESMOSOMAL AND TIGHT JUNCTION PROTEINS	*CLDN4, CLDN5, CDH22, CTNNBIP1,* ***MPP4****, PKP3*
DERMAL HOMEOSTASIS	*COL18A1, COL5A3, COL9A3****, COL14A1, COL1A1, COL1A2, COL24A1, COL3A1, COL6A5, COL6A6****, PSORS1C2*
CYTOSKELETAL DYNAMICS AND MORPHOLOGY	***ACTC1****, ARPC1B, ARPC5L, CAPG, COMP, CORO6,* ***FILIP1, KLHL4, LMOD3****, MGP,* ***MXRA5, MYOT, PCDH9****, TCAP, TMSB15B, TMSB4X, TNNT1,* ***TPPP****, TSPAN4, TTLL12, TUBA1C, TUBB2A, TWF2, UNKL*
ANTIMICROBIAL DEFENCE	*DEFA6, DEFB1, DCD, MUC1,* ***MUC16****, PNLDC1, RNASE2, RNASE7, RNH1, SFTPD*
MELANOCYTE SPECIFIC GENES	*DDT, MIF*

Table 6. The nuclear pathways affected in Parkinson's disease versus normal skin. The gene names in bold text reflect the upregulated genes, while all others were downregulated.

REGULATION OF CELL CYCLE	*CCND3, CCNF, CCNO, CDC34, CDCA3, CDK10, CDK5RAP3, CDKN1C, CHTF18, CKS2, G0S2,* ***HORMAD1****, MZT2B, ORC6, SERTAD1*
REGULATION OF BASAL RNA TRANSCRIPTION	*ABT1, CPSF3L, DAZAP1, POLD4, POLR2G, POLR2H, POLR2J, POLR2L, POP5, POP7, SSU72, TAF1C, TCEB2*
TRANSCRIPTION FACTORS/ IMMEDIATE EARLY RESPONSE	*ARGLU1, c-FOS, CITED4, CTBP1, DNASE1L2, E4F1,* ***ETV1****, ETV7,* ***FOXF1****, FOXF2, FOXH1, HOXB2, ID1, IER2, IER3, IER5, KLF2, OLIG3,* ***SIX4****,* ***TCF24****, TEF*
ZINC FINGER PROTEINS	***ZC3H12D****,* ***ZNF114****, ZNF219,* ***ZNF319****,* ***ZNF334****, ZNF446, ZNF524, ZNF598,* ***ZNF713****, ZNHIT1, ZNHIT3*
NUCLEAR RECEPTORS	*NR1H3,* ***NUP210L****,* ***RORB***
CHROMATIN REMODELLING/DNA BINDING	*AES, CTBP1,* ***HFM1****,* ***HIST1H1D****,* ***HIST1H3C****, HMGN3, INO80C, PNLDC1, SSBP4*
DNA REPAIR MECHANISMS/mtDNA REPAIR/ENDONUCLEASES	*AEN, DDIT4/REDD1, DNASE1L2, ENDOG, ENDOU, FANCE, GADD45B, GADD45GIP1, NEIL2, PNKP, TREX1*
RNA DNA PROCESSING AND MODIFICATION/EPIGENETIC REGULATION	*ASCL2, COMTD1, CRNDE, DMAP1, DUS3L, LSM4, MED10, METTL7A,* ***PEG10****,* ***PEG3****,* ***PEG3-AS1****, PUF60, PUSL1, RNPS1, RP9, RUVBL2, SAP25, SF3B5,* ***SOX2OT****, TARBP2, TAX1BP3/TIP1, THOC6, TRPT1,* ***TTTY10****, WBSCR22*
miRNAS/snRNPRs/snoRNAs	*MIR1244, MIR1282, MIR147B, MIR205, NHP2, NHP2L1, NOP10, SNRNP25, SNRNP70, SNRPB, SNRPD2, SNRPD3, SNRPE, SNRPF, SNHG5, SNHG9, SNHG8*
OTHER	*CECR5, MUSTN1, QTRT1*

Table 7. The signaling/tumorigenicity pathways affected in Parkinson's disease versus normal skin. The gene names in bold text reflect the upregulated genes, while all others were downregulated.

TUMOR SUPRESSORS/ ONCOGENES	*AIP, ASPSCR1, BLCAP, FAU/FBR-MuSV, FGR, GLTSCR2, LZTS2, MYEOV2, OVCA2, PIM3, SPDEF, TP53TG1, TUSC1, YPEL3*
REGULATION OF SIGNAL TRANSDUCTION	***AKAP12***, *DOK1, DOK2,* ***GRAP2***, *LIN7B, PDZK1IP1, SIRPB1,* ***SPHKAP***, ***STAP1***, *STAP2, TNK2*
RAS PATHWAY	*HRAS, PITX1, RAB20, RAB40C, RAB24, RASSF1, RASSF7, RIN1*
SMALL GTPASE SIGNALLING	*AGAP3, ARAP1, ARHGAP40, ARHGEF10L,* ***ARHGEF38,*** *ARHGEF5, RAPGEF3, RHOB, RHOC, RHOT2, RHOV*
G-PROTEIN SIGNALLING	*GNG11, GNG5,* ***GPRC5A***, *GPSM3, RAMP2, RGS10, SGSM3*
WNT PATHWAY	***APCDD1L***, *CTNNBIP1, DKK1, DVL1, HESX1, WISP2, WNT11*
NOTCH PATHWAY	*DLK1, HES5, RFNG*
FGF SIGNALLING	*CNPY1,* ***FGF7***, ***FGF9***, *FGFBP2, FGFR3*
IGF SIGNALLING	***IGF2***, *IGFBP6, IGFL2,* ***INS-IGF2***, *TMEM219*
TGFβ SIGNALLING	***ACVR1C, INHBA***
NFκβ SIGNALLING	***NKAPL***, *TMEM101, CCDC22*
OTHERS	*BOP1, BRMS1, CEACAM3,* ***CEACAM6***, *CSNK2B, EGFL6,* ***GNRHR***, ***HCCAT5***, ***NHEG1***, ***OGN***, ***PTTG3P***, *SCGB2A2, VEGFB*

Table 8. ClusterProfiler KEGG overrepresentation analysis in PD skin. GeneRatio stands for the number of genes in the input list that are major contributors to a specific KEGG pathway/ total number of input genes. BgRatio refers to the number of all input genes annotated to a pathway / all genes included in the database which corresponds to 7528 elements. Pathways are sorted based on the adjusted p-value (FDR).

ID	Description	GeneRatio	BgRatio	pvalue	p.adjust	Count
hsa05016	Huntington disease	44/434	193/7528	9.35e-16	2.68206743957876e-13	44
hsa04932	Non-alcoholic fatty liver disease	36/434	149/7528	6.63e-14	9.5174421652579e-12	36
hsa05012	Parkinson disease	33/434	142/7528	2.47e-12	2.36246616998621e-10	33
hsa00190	Oxidative phosphorylation	31/434	133/7528	1.08e-11	7.72925848756688e-10	31
hsa03010	Ribosome	31/434	153/7528	4.82e-10	2.38471885093517e-08	31
hsa05010	Alzheimer disease	33/434	171/7528	4.99e-10	2.38471885093517e-08	33
hsa04714	Thermogenesis	36/434	231/7528	3.28e-08	1.34452900642647e-06	36
hsa04723	Retrograde endocannabinoid signaling	24/434	148/7528	3.36e-06	0.000120446294335069	24
hsa04260	Cardiac muscle contraction	13/434	78/7528	0.0005	0.0146337547964213	13

5.2. Differential expression of genes and pathways in Parkinson's disease blood

RNA-Seq from 12 PD patients versus 12 controls from blood resulted in 25 differentially expressed genes at FDR≤0.05 (Table 9). Most of DEGs in blood are upregulated, but notably *UBE2J1* (also known as *HIP2*) is downregulated, with a foldchange of 0.63, which has previously even been proposed as a blood-based biomarker in iPD (Su et al. 2018). *OGT* gene product plays a role in protein glycosylation and has been associated with neuroprotection and its dysfunction with neurodegenerative diseases such as AD (Wani et al. 2017) and intellectual disability (Pravata et al. 2020). *LUC7L3* with functions also associated with protein metabolism has been associated with AD (Tang and Liu 2019). *STAT4* has been proposed a biomarker in PD from post mortem microarray reanalysis work (Diao et al. 2012). *PTPN4* is associated with protein tyrosine phosphatases functioning and has shown deregulation in CSF in PD (Hossein-nezhad et al. 2016). *A2M* gene variants are associated with increased risk in PD in a meta-analysis (Guo et al. 2016). *CD36* has been implicated in fatty acid metabolism in PD (Abumrad and Moore 2011). *IL18R1* is a key receptor in neuroinflammation and has been associated with multiple sclerosis (Gillett et al. 2010) and its agonist IL18 has been associated with PD (Alboni et al. 2010). This finding is of particular interest, as it stays relevant in our meta-analysis (see below). *PYHIN1* is associated, again with neuroinflammation and is implicated in PD (Sarkar et al. 2020). *PPT1* is the key protein implicated in neuronal ceroid lipofuscinosis type 1, a young onset neurodegenerative disease, and contributes to parkinsonian features (Dearborn et al. 2015). *PCGF3* has been proposed as a candidate gene in PD in an eQTL analysis (Kia et al. 2019). *PKM2* has been proposed as a biomarker in PD (Wei et al. 2020). *RAB32* is implicated in PD (Waschbüsch et al. 2019). *PRPF4B* is deregulated in PD (Pinho et al. 2016). *STARD9* is implicated in AD (Saad, Brkanac, and Wijsman 2015). Most DEGs are associated either with protein metabolism or neuroinflammation. Functional pathway analysis was conducted based on KEGG pathways with a lowered significance cutoff at p-adj. ≤0.1 yielding one significantly altered pathway in PD blood – cholesterol metabolism pathway.

Table 9. All differentially expressed genes in PD blood. The gene names in bold text reflect the upregulated genes, while all others were downregulated.

DEG Name	Adjusted p-value
UBE2J1	0,0025
OGT	0,0045
C15orf17	0,0056
LUC7L3	0,0056
C12orf33	0,0086
STAT4	0,0086
PTPN4	0,0086
A2M	0,0097
ENOSF1	0,0112
MIAT	0,0121
CD36	0,0169
IL18R1	0,0189
PYHIN1	0,0189
PPT1	0,0189
LOC253039	0,0189
SYTL2	0,0230
EPM2AIP1	0,0232
PCGF3	0,0254
PKM2	0,0254
ZNF767	0,0325
RAB32	0,0371
PRPF4B	0,0375
STARD9	0,0385
AGPAT4	0,0487
GMPPA	0,0487

5.3. Comparison of the peripheral tissue RNA-Seq results

Using the same strict criteria for statistical significance for RNA-Seq results from whole venous blood and skin showed how different the results in these tissues are with skin yielding >1000 DEGs and blood only 25 DEGS at FDR≤0.05. There was no overlap of unique DEGs in PD blood and skin. There was no overlap in the comparable pathway analysis with KEGG pathways (Figure 2)

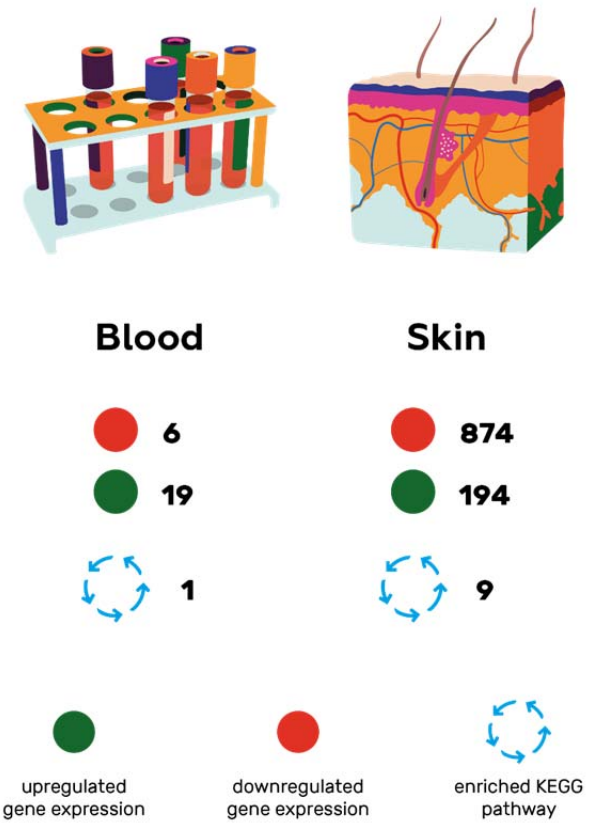

Figure 2. Illustrative comparison of RNA-Seq data from PD blood and skin showing no overlap.

5.4. Results of the meta-analysis of transcriptomic studies from blood, cortex and *Substantia nigra*

PubMed search of previous transcriptomic datasets yielded enough original studies for three different tissue types – blood, SN, and cortical brain tissue. Five previously published lists from PD blood (Santiago and Potashkin 2015; Kedmi et al. 2011; Infante et al. 2015; Calligaris et al. 2015; Soreq et al. 2008) using either microarray or RNA-Seq methods and the current blood RNA-Seq results were reanalyzed using the RRA method. This created an aggregated ranked DEG list, where only one gene from PD blood remained significant: *IL18R1*. It is an interleukin receptor linked with proinflammatory responses that belongs to the immunoglobulin superfamily. Functional pathway analysis of the top 163 DEGs from blood RRA list (score ≤0.05) found two altered KEGG pathways in PD: hematopoietic cell lineage (FDR = 0.001) and prion diseases (FDR = 0.037). Three previously published lists from PD *post mortem* cortex (Henderson-Smith et al. 2016; Dumitriu et al. 2015; Stamper et al. 2008) using either microarray or RNA-Seq methods were reanalyzed despite differences in the exact cortical location and cell composition of samples. The aggregated ranked list yielded 43 DEGs. Notable DEG is *PENK*, which has been implicated in PD mouse models (Bissonnette et al. 2014). Heat shock proteins *HSPA1B, HSPA6* and *SERPINH1* were significant and have been associated with neurodegeneration (Paul and Mahanta 2014). Many growth factors like *VGF* and *CSF3* were elevated, especially *BDNF* which has been proposed as a potential therapeutic agent in PD (Palasz et al. 2020). Functional pathway analysis based on 475 DEGs revealed one altered KEGG pathway: legionellosis, which implicates toll like receptor signaling, phagocytosis, heat shock proteins and apoptosis. Five previously published lists from PD SN (Bossers et al. 2009; Simunovic et al. 2009; Cantuti-Castelvetri et al. 2007; Dijkstra et al. 2015; Durrenberger et al. 2012) using either microarray or RNA-Seq methods were reanalyzed despite differences in extraction of cells of interest (laser microdissection vs. whole tissue). Here, two significant DEGs were found: *LMO3* and *RIMS3. LMO3* affects neurogenesis and inhibits tumor suppressor p53 and has been implicated in SN of PD (Briggs et al. 2015). *RIMS3* regulates synaptic vesicle transport and is relevant in neurotransmitter release and has also shown to be affected in PD, previously (Chandrasekaran and Bonchev 2013). Both DEGs are associated with aberrant microRNA regulatory network functioning. Functional pathway analysis from 390 input DEGs yielded four KEGG pathways in PD: calcium signaling pathway, synaptic vesicle cycle, proteoglycans in cancer and dopaminergic synapse. All these pathways have been previously associated with PD (Zaichick, McGrath, and Caraveo 2017; Esposito, Ana Clara, and Verstreken 2012; Heindryckx and Li 2018). An illustration from the aggregation of previous CNS tissues can be seen in Figure 3. A direct comparison of the three RRA lists from blood, cortex and SN showed no overlap in either DEGs or pathways of these aggregated lists. A comparison of the RRA lists with the RNA-Seq results from skin showed two DEGs overlapping between cortex and

skin: *G0S2* and *C6*, but no pathway level overlap (Figure 4). *G0S2* regulates cell cycle and its differential expression has been found in PD monocytes (Grozdanov et al. 2014). Complement component 6 (*C6*) is implicated in immunomodulatory pathways and has been associated with PD (McGeer and McGeer 2004).

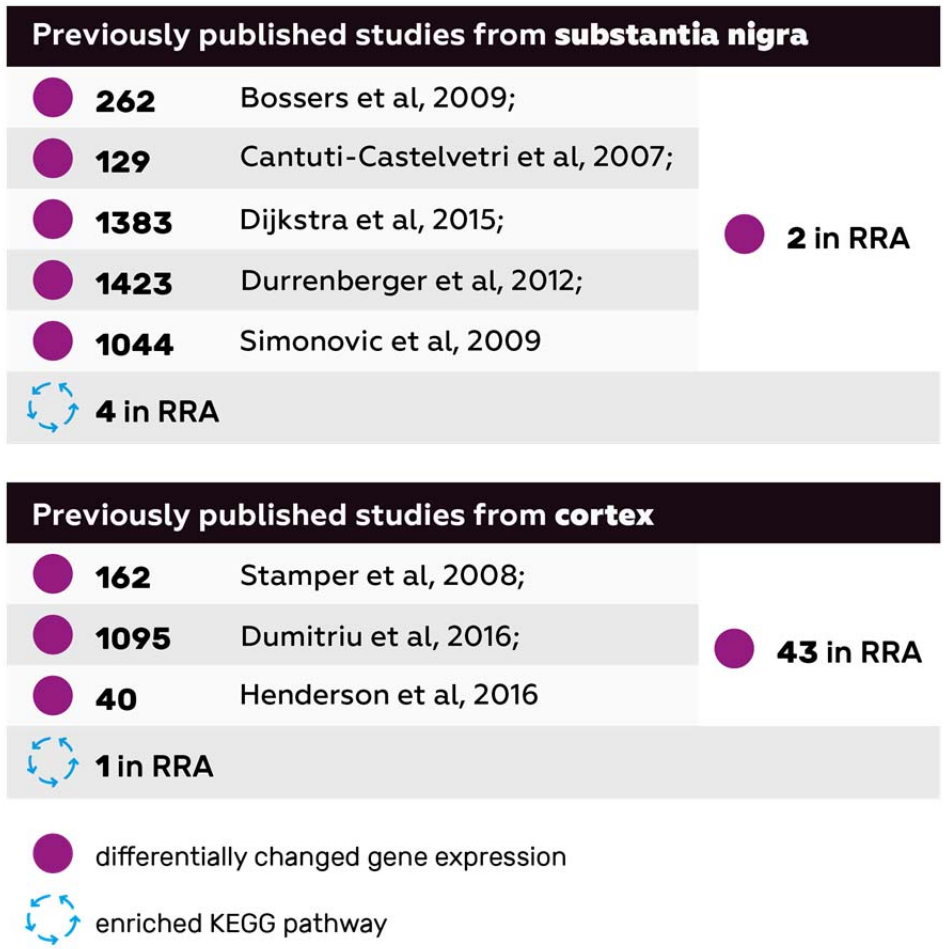

Figure 3. A schematic overview of previously published RNA-Seq and microarray studies from central nervous tissues in PD – cortex and SN showing the number DEGs in each original study, the number of DEGs in RRA and the number of enriched functional pathways in RRA.

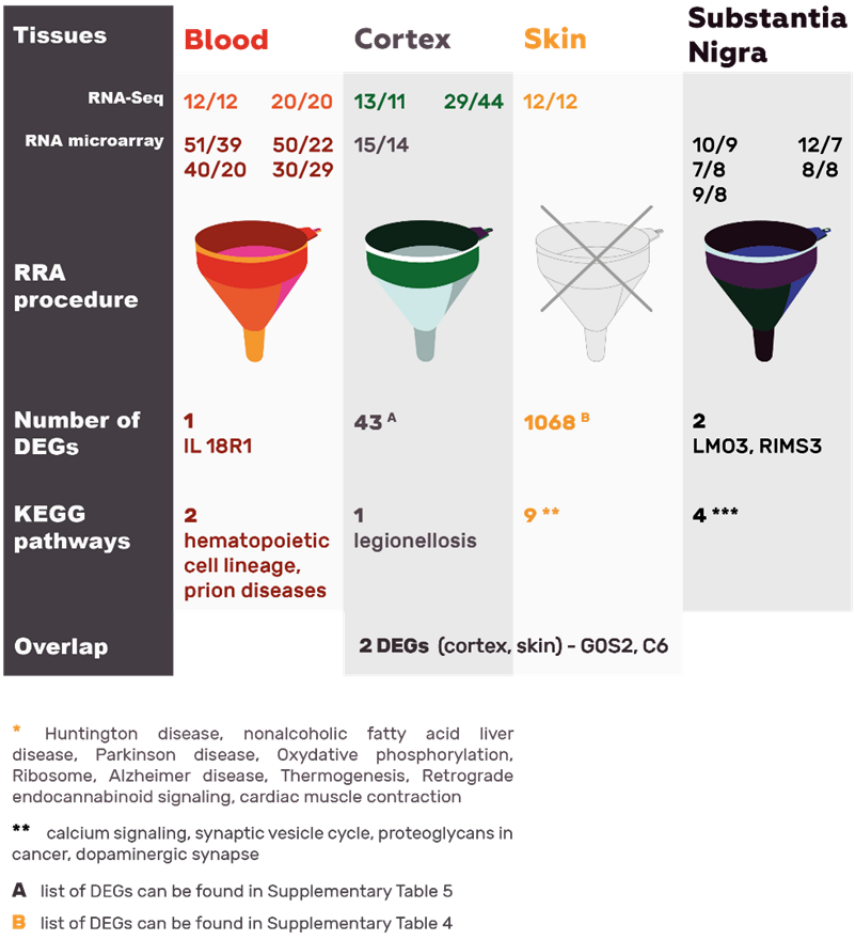

Figure 4. A schematic overview of the studies involved in RRA meta-analysis (51/39 is the number of Parkinson's disease patients and healthy controls in the original study, respectively). List of DEGs for cortex and SN are not included.

5.5. Results of serum amyloid alpha profiling in Parkinson's disease using serum ELISA and skin immunohistochemistry

The skin RNA-Seq showed a top significant downregulation of two gene expressions coding isoformic proteins that prompted a further investigation of their translation and accumulation in a larger PD cohort. These DEGs of particular interest were serum amyloid alpha 1 (*SAA1*) and *SAA2* with lowered expression levels in PD patients (logFC -2.75 and -1.65, respectively). *SAA1* and *-2* gene expression levels in skin were validated using qRT-PCR, followed by ELISA from blood serum measuring protein levels of SAA1/2 combined. Lastly SAA1/2 protein was visualized in skin using immunohistochemistry. The validation qRT-PCR analysis from skin demonstrated a 1.68-fold downregulation of *SAA2* gene (p = 0.0372) and 1.34-fold downregulation of *SAA1* gene in PD, however the result was not statistically significant between groups using this method (Figure 5). ELISA analysis of serum SAA 1/2 protein showed statistically significantly decreased protein concentration in PD patients by 50.9% compared to controls (Figure 6). Immunohistochemistry from skin biopsy samples did not detect visualizable SAA 1/2 protein in either of the two groups (Figure 7).

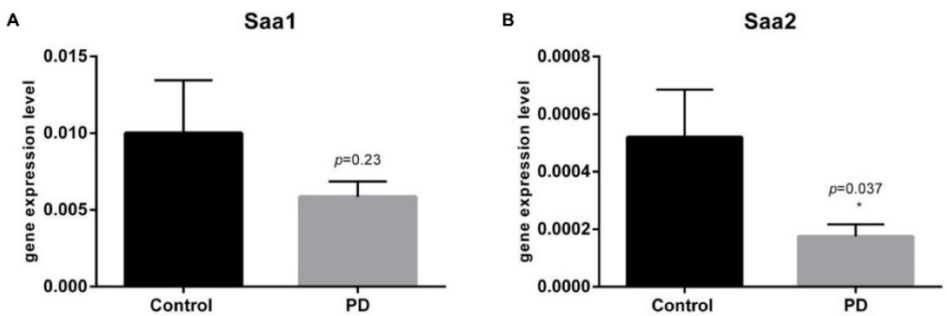

Figure 5. qRT-PCR results from skin showing relative gene expression levels of *SAA1* (A) and *SAA2* (B) genes. Barplots show mean fold changes. (A) Demonstrating not significantly (p = 0.25) lowered *SAA1* levels in PD patients by 1.34-fold. (B) Demonstrating significantly (p = 0.037) lowered *SAA2* levels in PD patients by 1.68-fold.

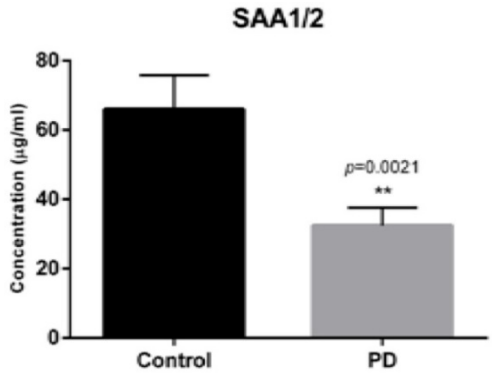

Figure 6. Serum ELISA SAA1/2 concentration showing decreased concentration of SAA1/2 by 50.9%. Bars show mean concentration with upper percentiles, 66.8 µg/ml for healthy controls and 32.8 µg/ml for PD patients (p = 0.0054).

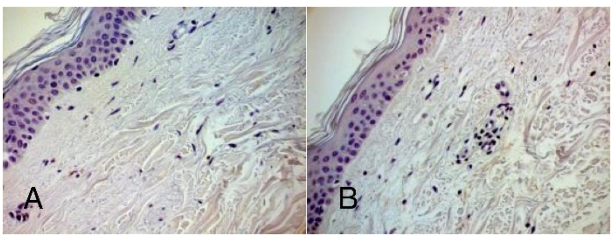

Figure 7. Representative immunohistochemistry results for SAA 1/2. Control (A) and PD patient (B). Magnification: 40×. Dye: Hematoxylin and immunohistochemical labeling for serum amyloid alpha. Both in control and PD patient no detectable visual staining can be demonstrated.

6. DISCUSSION

6.1. Transcriptomics from skin in Parkinson's disease

The current and first RNA-Seq from PD skin (Study I) reveals a state of global suppression with downregulation of over 80% of differentially expressed genes (Planken et al. 2017). These changes categorize into interdependent and disease relevant functional pathways that reveal impaired homeostasis of the skin tissue. This study in the skin supports the assumptions that molecular level changes in PD are systemic and mirrored in non-neuronal peripheral tissues. The skin study had two main focuses – establishing that changes in CNS are mirrored in skin and finding molecular level mechanisms that explain the elevated prevalence of melanoma and non-melanoma skin cancers in PD. A third objective was to demonstrate the applicability of skin biopsies as an accessible source *in vivo* for biomarker discovery studies.

6.1.1. Overlapping changes in Parkinson's disease skin and brain

Molecular PD pathogenesis in dopaminergic cells of the CNS is driven by impairment in two major biological functions: mitochondrial dysfunction (Michel, Hirsch, and Hunot 2016) and protein metabolism (and the accumulation of pathological α-synuclein) (Olanow and McNaught 2011). In our study we found gene expression changes supporting these pathologies in the skin tissue. For example, a large group of DEGs categorized into pathways associated with mitochondrial functions (23% of DEGs) is affected. This is exemplified by the suppression of 1/3 of mitochondrial electron transfer chain components in PD skin. When these genes are downregulated, less ATP is produced while generation of reactive oxygen species (ROS) remains or is even elevated (Drechsel and Patel 2008). These changes have been consistently shown in SN and blood platelets of PD, and less consistently shown in other non-neuronal tissues (Winklhofer and Haass 2010). Another set of observed downregulated genes is responsible for mitochondrial proliferation and transport, for example a key enzyme, the mitochondrial polymerase *POLRMT* which is known to cause neurological diseases (Oláhová et al. 2021). Aberrant mitochondrial proliferation has mostly been linked to key genes (like *parkin, PINK1, DJ1*) associated with familial Parkinson (Büeler 2010) but we describe converging changes in iPD. Probable cause here is the oxidative damage from ROS to mitochondrial DNA (mtDNA) leading to less functional mitochondrial proteins that creates a *circulus vitiosus* of energy failure (Winklhofer and Haass 2010). Other found changes that fuel this circle are alterations in beta-oxidation of fatty acids creating an influx of ROS. Suboptimally functioning mitochondria create a backlog of incompletely oxidized lipid metabolites which, in turn, has been associated with speeding up the accumulation of α-synuclein (Ruipérez, Darios, and Davletov 2010). An important concordance in the skin to previous CNS

tissue studies in PD is altered transcription of *PGC-1α*, which is the central inducer of mitochondrial biogenesis (Lin, Handschin, and Spiegelman 2005). *PGC-1α* has been shown be downregulated in SN and peripheral blood in PD (Su et al. 2015; Yang et al. 2018). It could be hypothesized that the observed upregulation of *PGC-1α* in the skin is reactive to counter failing mitochondria. Induced expression of *PGC-1α* has been considered a potential therapeutic approach and could ameliorate phenotype, but on the other hand could create elevated toxicity in model animals (Clark et al. 2012; Martin et al. 2012).

The second most robust finding in is the involvement of DEGs associated with protein misfolding, aggregation and deposition – known disease mechanisms in PD (Ebrahimi-Fakhari, Wahlster, and McLean 2012). The downregulation begins already at the level of ribosomal proteins, as well as translation initiation and elongation factors. These changes could be result from oxidative stress and can be considered as compensatory to limit energy expenditure in the already compromised cell (Jenner 2003). Major processes linked to PD are degradation of soluble intracellular proteins by the UPS (Betarbet, Sherer, and Greenamyre 2005), showcased in the skin by downregulation of its components. Other key changes were found in the autophagy–lysosomal pathway indicating aberrant processing of larger subcellular components. Downregulation of genes in these two major cellular processes in the skin leads to dysfunctional protein quality control and turnover which contributes to the misfolded protein buildup and cytotoxicity. The differential KEGG pathways from skin point towards neurodegeneration-specific changes underlining the similarities between neurodegenerative processes. The fact that pan-neurodegenerative pathways and key DEGs are mapped in skin in PD show that disease-specific changes are taking place in a non-neuronal peripheral tissue.

6.1.2. Perturbation of basal skin homeostasis and tumor vulnerability

The next connection we sought out to investigate was if epidemiologically found predisposition to melanoma and skin cancer in PD patients is backed by changes in basal homeostasis of the skin. Many DEGs profiled regulate molecular composition of epidermis and dermis that could lead to skin tissue fragility and lower tolerance to environmental stressors. For example, major changes in epidermis shows a downregulation of parallel pathways of the cornification and desquamation processes, highlighted by the suppression of the epidermal differentiation complex (EDC), which contains 57 genes crucial for the differentiation process located within a tight cluster on chromosome 1q21 (20 genes of EDC suppressed) and ephrin A1, which is a central regulator of epidermal growth, located near the EDC on chromosome 1q arm. In addition, we observed the decreased expression of all genes of the stratified epithelium-secreted peptide complex. These are genes that regulate the cross-linking of the cornified envelope (CE) proteins that form a protective structure in a mature keratinocyte. Furthermore, the cystatin/cathepsin/transglutaminase pathway, which showed downregulation, influences the desquamation of the stratum corneum and its

dysregulation causes disturbance of skin barrier function (Zeeuwen, Cheng, and Schalkwijk 2009). Next, we observed suppression of several different junction and desmosome proteins and deregulation of the antimicrobial defense in PD, indicating that the desmosomal adhesions and anchoring junctions are defective in PD, thereby also contributing to impairment of structural integrity and barrier function of the skin. In addition, the dermal genes were affected, characterized by altered levels of several members of the collagen family, as well as deregulation of cytoskeletal remodeling and dynamics. These changes contribute to impairment of tissue elasticity predisposing to premature aging of skin by impacting the structural and compositional remodeling.

From another focal point, many DEGs were affected that regulate antitumor mechanisms of the skin. For example, several cyclins and cyclin-dependent kinases and their activator were downregulated. DEGs related to DNA/mtDNA repair and degradation were downregulated as well. This leads to buildup of damaged DNA and is another tumor predisposing factor. Last group of genes were associated with epigenetic regulation of gene expression. For example, transcriptional and posttranscriptional modifications and non-coding RNA processing. Next to general downregulation of normal nuclear processes a large set of predominantly downregulated tumor suppressor and oncogenes were seen. Major oncogenes, like Ras and other G-protein signaling, WNT, NOTCH pathways were affected. The WNT signaling pathway is especially relevant because it plays a major role in skin differentiation and proliferation, especially of the melanocytes, and its alterations have been associated with the development and progression of both melanoma and non-melanoma cancers (Lim and Nusse 2013; Gajos-Michniewicz and Czyz 2020). WNT signaling has, notably, been associated with development and maintenance of midbrain dopaminergic neurons and its perturbations with neurodegeneration (Berwick and Harvey 2012; Arenas 2014) underlining the similarities between melanocytes and dopaminergic neuromelanin producing neurons of the midbrain. Another signaling pathway affected is the retrograde endocannabinoid signaling pathway which has been linked to melanoma (Río et al. 2018) and is affected in PD brain (Giuffrida and Martinez 2017; Castillo et al. 2012). The findings from PD skin indicate a state of chronic inflammation.

Taken together, these findings indicate towards elevated oncogenic stress in the skin. We also highlighted some pathways that have been especially linked to melanoma. The changes registered in epidermis and dermis show an impairment in homeostasis, differentiation, maturation and structural integrity of the skin, leading to an increase in vulnerability in PD skin to mutagenic hazards, and thus provide an explanation for the risk of melanoma/skin cancer in this patient population. A combination of these internal and external tumorigenic factor might lead to observed cell cycle withdrawal as a sign of stress. Oncogenes trigger senescence or, potentially, terminal differentiation in rapidly proliferating tissues such as the skin (Gandarillas 2012; Gorgoulis and Halazonetis 2010) which in itself could contribute to clinically observed skin ailments such as impaired wound healing or tumorigenesis in PD (Figure 8).

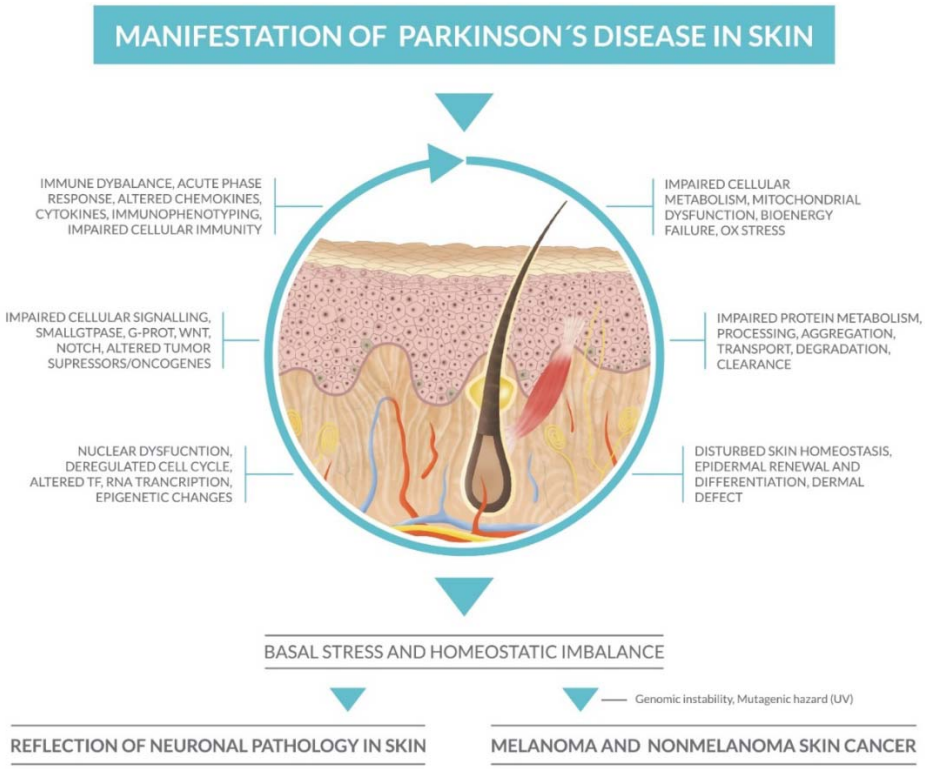

Figure 8. Illustration of central processes in PD skin converging into basal cellular stress and homeostatic imbalance that contributes to skin conditions in PD and elevated risk for tumorigenesis.

6.1.3. Applicability of skin biopsies in transcriptomics

Most previous studies have emphasized the connection of melanocytes and neuromelanin producing dopaminergic neurons in PD. However, similarly to SN, where dopaminergic cells comprise a minority of the total cell population, melanocytes comprise only around 10% in the skin (Yamaguchi, Brenner, and Hearing 2007). The current study was not set up to evaluate the gene expression changes occurring specifically in the small population of melanocytes. We made our focal point to profile the skin as a whole tissue with mixed cell populations, making sampling and sample processing steps more robust. However, applied setup comes with some limitations. In every mixed cell population gene expression levels vary between different cell types and observed changes could be due to differences in cell type proportions between samples (Kuhn et al. 2012). One limitation of our study is the relatively low log2FC levels observed for gene expression, which might pose difficulties in distinguishing the true signal from noise, thus it cannot be excluded that some of the genes with milder expression levels in our pathway analysis might be attributable to noise. A

factor that affects the skin particularly is the gene expression change caused by UV radiation (Goldinger et al. 2015; Weinkauf et al. 2012). In the current work the biopsy was collected from non-exposed side of the underarm in hopes of diminishing that factor. Furthermore, creams or solutions that have a biological effect can affect gene expression in skin (Namkoong, Kern, and Knaggs 2018). This is a factor we did not account for in our discovery study but if follow ups are conducted it might be reasonable to limit these factors. All in all, taking a punch biopsy from non-lesional skin tissue is an easy procedure that does not require ample preparation or experience (Nischal, Nischal, and Khopkar 2008). Its availability and low risk for the patient together with the promising findings of the current paper should allow skin biopsies to be used more often in PD transcriptomics.

6.2. Transcriptomics from blood in Parkinson's disease

RNA-seq results from blood and skin yielded very different number of significantly changed DEGs (25 vs 1068), but no overlap (Kurvits et al. 2021). With only a few DEGs from blood no clear up- or downregulation pattern was found (Study II). Perhaps most notable DEG in the blood was *UBE2J1* which has previously shown to have altered expression in PD venous blood – (Scherzer et al. 2007; Kauczynska et al. 2013; Molochnikov et al. 2012) and also shows links to HD (Kalchman et al. 1996). *UBE2J1* is involved in UPS, which targets proteins for degradation (Smith et al. 2012). Another DEG to especially highlight is *PKM2* which codes pyruvate kinase M2, a rate-limiting glycolytic enzyme which mediates cellular antioxidant responses by diverting glucose flux into the pentose phosphate pathway and generating reducing potential for detoxification of ROS (Anastasiou et al. 2011). In model animals modifying *PKM2* functioning has shown beneficial effects on neuron functions by reducing ROS induced stress. *PKM2* has been proposed as a potential target for PD treatment (Wei et al. 2020). Other DEGs are discussed in the results section. It is remarkable that even though we found only very few genes with significantly changes expression from PD blood, many have previous specific PD related implications. Some of them point towards common mechanisms between neurodegenerative disease, especially AD. Even though the observed differential gene expressions are few, the fact that most of them are previously described in PD support the hypothesis of PD as a multisystem disorder. As aforementioned there is very low concordance of DEG level changes from PD blood, even though previous, mostly microarray studies have reported significantly more DEGs (Borrageiro et al. 2017). This limits the potential of finding reproducible biomarkers. Blood has tissue specific factors which limit discerning signal from noise such as heterogenous cell populations, systemic factors like metabolic changes and medications. If pathway level associations are considered, only one KEGG pathway from blood was significantly enriched – the cholesterol metabolism. Even though brain and peripheral tissue cholesterol

metabolism are seperated by the blood-brain barrier, epidemiological studies of serum cholesterol levels have shown an association between higher levels of cholesterol and lower risk of PD (Guo et al. 2015; Wei et al. 2013), although these findings are not constant and even opposite associations have been reported (Gudala, Bansal, and Muthyala 2013). Currently there are no human studies that directly investigate brain cholesterol metabolism in PD, but a brain-derived cholesterol metabolite 24-HC is decreased in the plasma of patients with PD (Huang et al. 2019). In neuronal cultures cholesterol metabolism is impacted by α-synuclein and leads to impaired formation of synapses and less neurotransmitter release (Alecu and Bennett 2019).

6.3. Meta-analysis with RRA

Although blood is an easily obtainable tissue, the scarcity of DEGs necessitated a comparison of our results with previous studies. Since in this study RNA-Seq was performed only from peripheral tissues in comparing these results with transcriptomic studies from the CNS was in order. Therefore, a meta-analysis aggregating the results of previous studies was conducted in hopes of finding robust changes, especially from blood. In order to be able to analyze both microarray and RNA-Seq data the RRA method was chosen (Kolde et al. 2012). In the analysis an aggregated list of 6 studies (including the current one) from blood yielded only one significant DEG in blood – *IL18R1*. This protein mediates neuroinflammatory processes, but currently has very limited links to PD (Kosloski et al. 2013). We propose *IL18R1* as a potential robust marker, but functional studies revealing its central and peripheral pathomechanisms are still needed. Also, a pathway level reanalysis of the aggregated DEG list was conducted. Two significant pathways were found: hematopoietic cell lineage and the prion diseases pathway. Hematopoietic cell lineage pathway is implicated in immune system functions and has been linked to PD blood (Schlachetzki et al. 2018; Soreq et al. 2008). Since this work concentrated only on profiling the gene expression changes, future functional studies for this pathway and its relation to PD pathology are needed. The other significant pathway, the prion diseases pathway has a more straightforward connection to PD pathology as α-synuclein has prion-like propagating qualities (Brundin and Melki 2017). In order to better link the proposed multisystem disorder, current peripheral tissue results as well as the aggregated list from blood were compared with combined findings from CNS. Two specific localizations yielded enough studies for the meta-analysis- the cortex and SN. Surprisingly, no overlap in DEGs or pathways between these tissues was seen. An aggregated list of three cortical tissues showed many significant DEGs, notable ones being *PENK*, heat shock proteins *HSPA1B, HSPA6* and *SERPINH1* and growth factors *VGF, CSF3* and *BDNF*. A single KEGG pathway was significant – legionellosis. This pathway underlines the importance of cellular stress and proinflammatory responses in PD. It must be considered that a relatively higher number of DEG level findings that do not

converge on the pathway level might be due to lower number of input studies (3 vs. 5 and 6 in other tissues). The meta-analysis from the canonically PD-pathology affected SN tissue found only two DEGs – *LMO3* and *RIMS3* that warrant further functional studies. Considering that dopaminergic cells comprise a minority of SN cell populations, differences in extraction of cells of interest (laser microdissection vs. whole tissue) could be the limiting factor to finding more DEGs. On the other hand, functional pathway analysis yielded four KEGG pathways (calcium signaling pathway, synaptic vesicle cycle, proteoglycans in cancer and dopaminergic synapse) that align with previously described PD pathogenic mechanisms. Comparison across central and peripheral tissues did not show the expected overlap between specific DEGs nor pathways. The only overlapping findings were changes in *G0S2* and *C6* gene expressions between the aggregated list of cortical tissues and the current skin study. This underlines the tissue-specificity of gene expression but does not invalidate the notion of PD as a multisystem disorder. PD pathology seems to be quite complex based on DEG profiles between studies and tissues. The possibility of different converging pathways that lead to core dysfunction in PD might be the reason why transcriptomic studies so far have failed to yield robust reproducible results. Another factor in the light of this heterogeneity is the lack of original studies allowing for reproducible results. We report RRA to a be a useful tool in aggregating the future original studies in order to find robust changes instead or increasing the sample size in a single study.

6.4. Serum amyloid alpha in Parkinson's disease peripheral tissues

The top downregulated gene expressed in PD skin was *SAA1*, with its isoform *SAA2* also being downregulated. This prompted a follow-up study (Study III) to elaborate how robust and translatable these DEG level changes are (Kurvits et al. 2019). Indeed, the downregulation was validated by qRT-PCR from skin. *SAA1/2* in blood were below the detection levels using RNA-sequencing, but on protein levels showed lower concentrations in PD serum, implying SAA1/2 is secreted into blood. SAA1/2 proteins were not detectable from PD skin using immunohistochemistry and Western blot methods as the quantity is physiologically low in normal skin and positive staining is seen only in patients suffering from AA-amyloidosis in chronic inflammation, certain cancers, or genetic defects (James et al., 2011). Notwithstanding its low quantities in skin SAA is an important autocrine modulatory protein and is induced by inflammatory signals (de Seny et al. 2013). Elevated SAA levels have been found in patients of all stages of melanoma (Findeisen et al. 2009). This is an interesting link considering the higher prevalence of melanoma in PD patients. Another quality of SAA might render it being especially vulnerable to ROS mediated cellular stress. SAA is intrinsically disordered protein lacking fixed three-dimensional structures under physiological conditions, allowing the same polypeptide to

undertake different interactions with different consequences (Zhang et al. 2013). Another intrinsically disordered protein is α-synuclein, which causes PD pathology (Kirik and Björklund 2003). It could be that SAA has similar vulnerabilities as α-synuclein causing it to aggregate. Being an acute phase protein SAA is elevated in the CNS in AD (Liang et al. 1997; Kindy et al. 1999) The observed downregulation of peripheral *SAA* expression could be reactive to mitigate the neuroinflammatory responses or the aggregation potential of SAA. How SAA acts in the CNS in case of PD and whether its expression in the periphery affects its central functioning, is largely unknown.

7. CONCLUSIONS

1) We profiled PD skin using RNA-Seq. The results show a pattern of global downregulation of differentially expressed genes that implicate:
   a) cellular processes affected in PD – mitochondrial function and protein metabolism;
   b) epidermal and dermal homeostasis, differentiation, immune responses, and tumorigenesis.
   - This observed vulnerability in PD skin to mutagenic hazards provides an explanation for the elevated risk of melanoma/skin cancer and other skin ailments, such as impaired wound healing in PD.
   - The mirroring of disease-specific transcriptional patterns in the skin compared to PD brain points towards a multisystem impairment in PD.

2) We profiled PD whole blood using RNA-Seq. The results show only very few differentially expressed genes, but, on the other hand most of these are previously implicated in PD pathology.
   - Single significantly affected pathway is the cholesterol metabolism pathway.
   - Total number of differentially expressed genes could be low due to heterogenous and dynamic nature of blood as a tissue, but the concordance with previous findings in PD implies the multisystem impairment in PD.
   - Blood and skin did not yield a single overlapping differentially expressed gene or pathway upon direct comparison.

3) We investigated two particularly interesting DEGs from skin: SAA1 and SAA2 and found that the downregulation on transcriptional level was mirrored in lowered quantities of SAA-protein in blood. However, due to low quantities we were not able to visualize SAA protein in the skin.

4) We compared previous transcriptomic studies using a novel approach of RRA that enabled comparing microarrays and RNA-Seqs with each other regardless of applied statistical tests.
   - Three different tissues of mixed cell populations – cortex, SN, and blood – had enough original datasets to be included in the analysis.
   - The results showed surprisingly few robust DEGs and pathways underlining:

   a) the importance of tissue-specific differences in gene expression;
   b) calling for more original studies and meta-analyses to produce reproducible robust findings.

4.1) Notable DEGs of interest from our sample set and from the RRA analysis are:
PGC-1α and SAA1/2 from skin,
UBE2J1 and IL18R1 from blood,
LMO3 and RIMS3 from SN,
PENK from cortex and
G0S2 and C6 from cortex and skin.
These DEGs showed most robust changes and warrant further functional studies to evaluate their biomarker potential.

4.2) Notable KEGG pathways are:
hematopoietic cell lineage and prion diseases pathway from blood;
calcium signaling, synaptic vesicle cycle, proteoglycans in cancer and dopaminergic synapse pathway from SN;
and legionellosis pathway from cortex.

- Taken together, there is promise in sampling peripheral tissues for transcriptome studies in PD in hopes of finding biomarkers or transcripts that drive key pathological mechanisms because of

a) accessibility of these peripheral tissues *in vivo* and
b) their comparability to PD-specific changes in CNS.

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9. SUMMARY IN ESTONIAN

Parkinsoni tõbi kui multisüsteemne haigus: Parkinsoni tõve patsientide naha ja vere kogu transkriptoomi uuring

Parkinsoni tõbi (PT) on Alzheimeri tõve järel sageduselt teine neurodegeneratiivne haigus, mida iseloomustab liigutushäire, mille kliinilisteks sümptomiteks on bradükineesia, treemor ja rigiidsus (Lees, Hardy, and Revesz 2009). Liigutushäire on PT puhul tingitud ajukoe mustaines (*Substantia nigra*) paiknevate dopaminergiliste neuronite hukkumisest. Lisaks on haigustunnuseks patoloogilise valgu – α-sünukleiini kuhjumine ajukoes. Täna pole täielikult selge, mis vallandab ja määrab patoloogiliste mehhanismide kulgu. Samuti pole olemas kindlaid biomarkereid, mis aitaksid haigust avastada varases faasis. Seetõttu saab haigust diagnoosida alles siis, kui PT avaldub kliiniliselt. Avaldumise hetkeks on aga umbkaudu 80% dopaminergilistest neuronitest juba hukkunud. Liigutushäirele lisaks on PT-l teisigi spetsiifilisi sümptome, mida nimetatakse mittemotoorseteks sümptomiteks. Antud töö fookuses on nahaprobleemide sage esinemine, näiteks seborroiline dermatiit, haavaparanemise probleemid ja hüperhidroos (Gregory and Miller 2015). Samuti on PT-ga patsientidel oluliselt kõrgem risk melanoomi tekkeks võrreldes samaealise üldpopulatsiooniga (R. Liu et al. 2011). Ja vastupidi, nendel patsientidel, kellel endil või kelle lähisugulastel on esinenud melanoom, on kõrgenenud risk PT tekkeks (Olsen, Friis, and Frederiksen 2006). See epidemioloogiline seos viitab selgelt, et nahk ning eriti melanotsüüdid on PT-spetsiifiliselt mõjutatud. Kuigi PT saab vaadelda kui keskaju dopaminergiliste neuronite haigust, siis käeoleva töö hüpotees väidab, et ka perifeersed mitteneuronaalsed koed on haigusspetsiifiliselt mõjutatud.

Ainult ca 10%-l patsientidest on perekondlik PT (Klein and Westenberger 2012). Enamik ehk 90% kannatab sporaadilise PT all, mille riski suurendavad sagedased madala patogeensusega geenide variandid (Lohmueller et al. 2003). Teaduskirjanduse põhjal ilmneb, et patogeensed geenivariandid kirjeldavad ainult 20% kogu PT riskist (Nalls et al. 2019). Siit järeldub, et PT patoloogias on suur roll paljudel muudel faktoritel, näiteks geeniekspressiooni muutustel või nende produktide, valkude funktsioneerimisel. Tuginedes eelöeldule on PT muutuste profileerimiseks antud töös valitud transkriptoomika ehk geeniekspressiooni muutuste meetod. Transkriptoomika puhul on oluline, millisest koest proov võtta, kuna geeniekspressioon on koespetsiifiline. Arvestades, et transkriptoomikat saab keskajust teha ainult peale surma, on teiste ligipääsetavate kudede leidmine eluspuhuseks diagnostikaks oluline. Potentsiaalsete huvipakkuvate kudede hulka võib lugeda naha, sest PT patsientidel esineb üldpopulatsioonist rohkem nii melanoomi kui ka teisi spetsiifilisi nahaprobleeme (R. Liu et al. 2011; Gregory and Miller 2015). Naha kõrval on teine huvipakkuv kude veri, sest see on kõige lihtsamini kogutav kude ja ringeldes kogu organismis, sisaldab ta endas palju süsteemset informatsiooni. Selgitamaks, kas PT

on multisüsteemne haigus, on meie eesmärgiks kaardistada PT geeniekspressiooni RNA sekveneerimise abil mitteneuronaalsest kudedest – nahast ja verest. Uurimuse eesmärgiks on selgitada, kas nimetatud koed on haigusspetsiifiliselt muutnud ja kas nendest kudedest leitud biomarkereid saaks kasutada PT diagnostikas ja prognoosi hindamisel.

Töö eesmärk

Peamine eesmärk oli PT patsientidel analüüsida naha ja vere transkriptoomilisi profiile RNA sekveneerimise meetodil selleks, et leida haigus-spetsiifilise geeniekspressiooni muutusi perifeersetes mitteneuronaalsetes kudedes.

Alaeesmärgid olid:

1) Esmakordselt kaardistada geeniekspressiooni muutused PT patsientide nahas ning liigitada need funktsionaalsetesse ainevahetusradadesse. Uurida, kas nahas kui mitteneuronaalses koes toimuvad haiguse-spetsiifilised muutused. Uurida, kas geeniekspressiooni uuringu abil saab lahti mõtestada epidemioloogilist seost PT ja melanoomi vahel.
2) Kaardistada geeniekspressiooni muutused PT puhul teises mitteneuronaalses perifeerses koes – veres ning liigitada need funktsionaalsetesse ainevahetusradadesse. Võrrelda vere uuringu tulemusi naha omadega. Uurida, kas veres leidub haigus-spetsiifilisi muutusi.
3) Süvendatult uurida naha RNA sekveneerimisel statistiliselt kõige enam muutunud geeniekspressiooni – seerumi amüloid alfat (*SAA*). Uurida, kas see tulemus on korratav ning avaldub ka veres. Uurida, kas see muutus transleerub valgu tasemele veres ja nahas ning on vaadeldav.
4) Võrrelda antud geeniekspressiooni uuringut eelnevalt publitseeritud uuringutega ajukudedest ja verest kasutades robustse järgu liitmise (robust rank aggregation – RRA) meetodit (Kolde et al. 2012), mis võimaldab võrrelda omavahel mikrokiipe ja RNA sekveneerimise tulemusi. Eesmärk oli leida kattuvaid muutusi PT erinevate kudede vahel geeniekspressioonide ja funktsionaalsete ainevahetusradade tasemel.

Materjalid ja meetodid

Patsiendid

Käesolev uuring koosnes kolmest põhiosast: I – naha RNA sekveneerimine (artikkel I); II – vere RNA sekveneerimine (artikkel II); III –*SAA* geeniekspressiooni uuring (artikkel III). Uuringu läbiviimine kooskõlastati Tartu Ülikooli inimuuringute eetikakomiteega. Uuringus osalesid PT patsiendid, kes kaasati Tartus ja Tartu maakonnas toimunud epidemioloogilise uurimistöö raames aastatel 2010–2016 (L. Kadastik-Eerme et al. 2018). PT patsiendid kaasati uuringusse järgmiste tunnuste alusel: (1) PT diagnoos vastas QSBB (Queen Square Brain Bank) diagnoosikriteeriumitele (Lees, Hardy, and Revesz 2009);

(2) patsiendid võtsid tüüpilisi dopaminergilisi PT ravimeid; (3) teisi raskeid diagnoose ei tuvastatud meditsiinilise intervjuu käigus; (4) patsientidel ei esinenud dementsust. Kontrollgrupi patsiendid olid kas PT patsientide partnerid või patsiendid, kes viibisid Tartu Ülikooli neuroloogia osakonnas ravil muudel põhjustel kui haigestumine neurodegeneratiivsesse haigusesse. PT raskusastet, kognitiivset toimetulekut ja igapäevaste tegevustega toimetulekut hinnati kasutades kontrollitud instrumente: Liigutushäirete Seltsi Ühtlustatud Parkinsoni Tõve Hindamise Skaala (MDS-UPDRS) (Goetz et al. 2008), Hoehn&Yahr'i skaala (HY) (Hoehn and Yahr 1967), Schwabi ja Englandi igapäevaste tegevustega toimetuleku skaala (SE-ADL) (Schwab et. al 1969) ja Vaimse Võimekuse Miniuuringu test (MMSE) (Folstein, Folstein, and McHugh 1975). Perekondliku PT ja vähkkasvajate anamneesiga patsiendid välistati uuringust. Naha RNA sekveneerimise uuringusse kaasati 12 PT patsienti keskmise vanusega 71,9 (±7.5) aastat ja 12 soo ja vanuse poolest sobitatud kontrolli. Vere RNA sekveneerimise uuringusse kaasati 12 PT patsienti vanuses 72.2 (±10,0) aastat ja 12 soo ja vanuse poolest sobitatud kontrolli. Mõlema uuringu tulemused valideeriti suuremas PT patsientide ja kontrollpatsientide kohordis. ELISA meetodiga läbi viidud SAA uuringusse kaasati 36 PT patsiendi ja 27 sobitatud kontrollpatsiendi vereproovid. Lisaks uuriti SAA taset immunohistokeemia meetoditel 13 PT patsiendi nahas.

Meetodid

Nahast võeti päikesele mitteeksponeeritud kohast (õlavarre seespoolelt) 4mm suurune löökbiopsia. Vere analüüsideks koguti veeniverd. Proovid säilitati -80C° juures. Nahast RNA eraldamiseks proovid homogeniseeriti kasutades Cryolys meetodit (Bertin Technologies). RNA eraldati homogenisaadist ning puhastati kasutades Qiageni vastavaid testi komplekte. Vere puhul kasutati Thermo Fischeri ja Quiageni vastavaid testi komplekte ning mRNA eraldamiseks lisasammu globiini (Thermo Fischer). RNA kvaliteeti hinnati spektrofotomeetriliselt (Nanodrop) ja elektroforeetiliselt (Agilent 2100 Bioanalyzer). RNA sekveneerimiseks amplifiseeriti 50ng tRNAd ning saadud kaheahelalisest cDNA-st loodi fragmentide raamatukogu SOLiD 5500 W (Thermo Fisher Scientific Inc, CA, USA) sekvenaatori jaoks. Erinevatele proovidele lisati identifitseerivad märgised, proovid ühendati ja sekveneeriti. Sekveneerimine oli edukas, andes nahast umbkaudu 40 miljonit ja verest 21.4 miljonit lugemit (reads). Toorandmete analüüs tehti Lifescope'i tarkvaraga, kus saadi >70% lugemite joondumine referentsgenoomile (Thermo Fisher Scientific Inc, CA, USA). Differentsiaalselt ekspresseerunud geenide analüüsiks kasutati tarkvara R paketti DeSeq2 (Love, Huber, and Anders 2014). Olulisuse määr oli FDR≤0.05 (false discovery rate, mitmese testimise korrektsiooni meetod), geeniekspressiooni muutuse ulatust (fold change) analüüsis ei arvestatud. Enim muutnud tulemused valideeriti kvantitatiivse RNA ahelreaktsiooni tehnoloogia abil kasutades majapidamisgeeni aktiin-B ja fluorestseeruvaid märgiseid VIC ja FAM (Applied Biosystems). Igat reaktsiooni korrati 4 korda, et vigu minimeerida. Geeniekspressiooni muutuse määra arvutati ΔΔCT meetodi abil.

Järgnevalt tehti muutunud geeniekspressioonidele ainevahetusradade analüüs kasutades naha puhul QIAGEN's Ingenuity® ainevahetusradade analüsaatorit (QIAGEN Redwood Citytool). Lisaks klassifitseeriti nahas geeniekspressioone ainevaheturadadesse otsides PubMed andmebaasist vastavaid artikleid, otsisõnadega "Parkinson's Disease" "neurodegeneration" "neuro" "Alzheimer's Disease" ja "brain". Vere ainevahetusradade analüüs tehti R tarkvara ClusterProfiler paketiga (Yu et al. 2012). Sama paketti kasutades analüüsiti uuesti ka naha andmed, et neid saaks verega paremini võrrelda. Ainevahetusradade analüüsi kaasati nahas esinenud geeniekspressioonid piirväärtuste juures FDR≤0.05 ja veres p-adj. ≤0.1. Ainevahetusrajad loeti oluliseks, kui testi tulemus oli FDR ≤0,05.

Selleks, et mõõta nahas kõige enam muutunud geeniekspressioni, *SAA*, valgu tasandil, kasutati vereseerumit ja ELISA testi komplekti inimese SAA1/2 valguga (Invitrogen Corporation). Valgu hulka mõõdeti Tecan GeniOS Pro luminomeetriga kolmes korduses optilise tiheduse 450 nm juures. Algse lahjeduse korrigeerimiseks korrutati tulemus 200-ga. Muutuste olulisust mõõdeti paaritu t-testi abil. Naha SAA immunohistokeemia uuringuks deparafiniseeriti nahabiopsiad alkoholi lahustes ning töödeldi 3% vesinikperoksiidi ja proteinaas K-ga. Seejärel inkubeeriti lõike primaarsete hiire monoklonaalsete SAA antikehadega (Novus Biologicals) 1:100 lahjenduses 30 minutit. Seejärel töödeldi lõike tuvastusantikehadega 30 minutit (DAKO REAL EnVision+ Dual Link, Single Reagents, HRP Rabbit/Mouse) ning värviti kromogeenvärviga (Dako Company) ja hematoksüliiniga. Tulemusi hinnati visuaalselt.

Lisaks teostati metaanalüüs eelnevatest töödest, et hinnata kattuvusi eri kudede ja RNA sekvenatsiooni ning mikrokiibi tulemuste vahel. Selleks otsiti PubMedi andmebaasist varem avaldatud töid. Minimaalsed kriteeriumid töö kaasamiseks olid: 1) geeniekspressiooni nimekiri on avalikult kättesaadav, 2) tegemist on originaalandmetega, 3) nimekiri ei sisalda selektiivselt esitatud geeniekspressioone; lubatud olid nimekirjad, kus olid ainult statistiliselt oluliselt muutunud geeniekspressioonid, 4) proovid on võetud idiopaatilistelt PT patsientidelt ja 5) erinevaid töid samast koest on metaanalüüsi jaoks piisavalt (vähemalt 3). Andmebaasist leiti 3 geeniekspressiooni nimekirja ajukoorest (Henderson-Smith et al. 2016; Dumitriu et al. 2015; Stamper et al. 2008), 5 mustainest (Bossers et al. 2009; Simunovic et al. 2009; Cantuti-Castelvetri et al. 2007; Dijkstra et al. 2015; Durrenberger et al. 2012) ja 5 verest (Santiago and Potashkin 2015; Kedmi et al. 2011; Infante et al. 2015b; Calligaris et al. 2015; Soreq et al. 2008). Metaanalüüs viidi läbi kasutadest RRA meetodit (Kolde et al. 2012). See võimaldab võrrelda ebatäielikke geeniekspressiooni nimekirju ja erinevate meetoditega saadud tulemusi (mikrokiip vs. RNA sekveneerimine). Selleks, et liita erinevate annotatsioonidega geeniekspressioone, viidi eelnevalt kõik tulemused üle Entrez ID-dele. Selleks, et võrrelda RNA sekveneerimise tulemusi mikrokiipidega, kus on erinev arv uuritavaid geeniekspressioone peal, kasutati iga koe kohta eraldi N parameetrit, kus *N(i) + N(avg. u)*. *N(i)* märgistab kattuvat hulka geene kõikidelt mikrokiipidelt antud analüüsis ja *N(avg. u)* märgistab keskmist geenide arvu ühe mikrokiibi kohta, mis ei sisaldunud arvus

N(i). Geenieksрressiooni loeti oluliseks, kui RRA tulemus oli FDR ≤ 0.05. Funktsionaalsete ainevahetusradade analüüs tehti kasutades tarkvara R ClusterProfiler paketti. Sel juhul oli geenieksрressioonide analüüsi kaasamise piir *score* ≤0.05. *Score* on RRA meetodi p-väärtus, mis ei ole korrigeeritud mitmesele testimisele. Ainevahetusrajad loeti oluliseks FDR ≤ 0.05 juures.

Tulemused ja diskussioon

Naha geenieksрressiooni muutused kattuvad tsentraalse PT patoloogiaga

Nahast leiti 1068 oluliselt muutunud ekspressiooniga geeni (artikkel I). Tekkis muster, kus 82% geenidest (874) olid allareguleeritud. Järgnevast analüüsist selgus, et suur osa neid geene jagunevad ainevahetusradadesse, mis tegelevad valgu ainevahetuse ja mitokondriaalse hingamise protsessidega. Need on funktsioonid, mida on eelnevates uuringutes seostatud PT-ga. Lisaks kuulus suur hulk geene naha proliferatsiooni ja diferentseerumise radadesse, Samuti tulid esile mõned kasvaja tekke signaalrajad, immunoloogilised ja raku homöostaasi rajad. Suurim hulk muutunud geenieksрressioone, mis pärines nahast, liigitati mitokondri funktsioneerimise radadesse, kusjuures 36/96 geenidest, mis osalevad respiratoorse hingamise ahelas, olid allareguleeritud. Eriti tähelepanuväärne on ülesreguleeritud peroksisoomi proliferator-aktiveeritud retseptori gamma koaktivaator-1α (*PPARGC1A* või *PGC-1α*), mis on väga oluline mitokondrite biogeneesi ja oksüdatiivse ainevahetuse reguleerija ning mida on eelnevalt seostatud PT-ga (Lin, Handschin, and Spiegelman 2005). Kaasuvalt oli muutunud ka paljude teiste biomolekulide ainevahetus, näiteks rasvhapete beeta oksüdatsioon, lämmastikaluste, steroidide ja süsivesikute ainevahetus. See kattub aju mustainest ja vereliistakutest saadud tulemustega PT puhul (Winklhofer and Haass 2010). Nende muutuste põhjal järeldub, et PT puhul on mitokondrite jõudlus nahas allareguleeritud. See võib olla tingitud kahjustavate reaktiivsete hapnikuühendite kuhjumisest puudulike hingamisprotsesside käigus, mis omakorda viib mitokondite kahjustumisele. Teine suur grupp naha geenieksрressioone on seotud valgu ainevahetusega. Näiteks 11 ubikvitiiniproteasoomi süsteemi valku olid allareguleeritud. See on süsteem, mille häired on PT-ga eelnevalt seostatud (Betarbet, Sherer, and Greenamyre 2005). Lisaks olid mitmed ribosoomi valgud allareguleeritud. Vigane valgu ainevahetus ja mitokondri efektiivsuse langus ning reaktiivsete hapnikuühendite kuhjumine on omavahel seotud (Jenner 2003) ning viib valesti voltunud valkude kuhjumiseni, mis on tsütotoksiline.

Naha geenieksрressiooni muutused viitavad homöostaasi häiretele ja toetavad PT ja melanoomi seost

Lisaks nendele kahele olulisele raku ainevahetusprotessi muutusele leidsime nahast suure hulga geenieksрressioone, mis on seotud marrasnaha ja pärisnaha homöostaasi ning uuenemisprotessidega. Marrasnahas olid mitmeid geenid allareguleeritud, mis on seotud funktsionaalses nahas toimuvate sarvestumis- ja

rakuliiduste protessidega. Lisaks olid mitmeid kaitsevalke (defensiinid, mutsiinid) tootvad geenid allareguleeritud. Pärisnahas olid mitmed kollageeniga seotud geenid muutunud ekspressiooniga. Kokku tähendavad need nahaspetsiifilised geeniekspressiooni muutused, et nahk on vähem stressi taluv ning vastuvõtlikum endo- ja eksogeensete kantserogeenide suhtes. Samuti oli suur hulk rakutsüklit mõjutavatest geenidest muutunud ekspressiooniga, mis mõjutavad naha puhul eriti olulisi rakkude paljunemis- ja differentseerumisprotesse. Olles eeskätt allareguleeritud, võivad need muutused viidata kiirendatud vananemisprotsessile ja osaliselt põhjendada epidemioloogiliselt leitud kõrgenenud melanoomi ja nahavähi riski. Kõrgenenud pahaloomulise kasvaja riski võivad seletada ka muutnud kasvaja tekke signalisatsiooni rajad nagu Ras, Wnt ja NOTCH Eriti Wnt kasvaja tekke rada on seostatud melanoomiga (Lim and Nusse 2013; Gajos-Michniewicz and Czyz 2020). Leitud muutused proinflammatoorsetes ainevahetusradades võivad samuti soodustada kartsinogeneesi PT nahas. Kokkuvõtvalt leidsime nahas hulgaliselt muutusi, mida võib pidada haiguspetsiifiliseks ning mis osaliselt põhjendavad epidemioloogiliselt leitud sagedasi nahakaebusi ning melanoomi ja teiste nahavähkide sagedasemat esinemist PT patsientide seas.

Metaanalüüs varem avaldatud transkriptoomika töödest

Seda toetab järgnenud metaanalüüs (artikkel II), mille läbiviimine oli tingitud üllatuslikust avastusest, et läbiviidud uuringutes (artiklid I ja II) ei leitud kahest eri koest samas populatsioonis ja sama meetodit kasutades mingisugust kattuvust. Metanalüüsi RRA meetodil kaasasime seega närvikoest tehtud uuringuid, et leida kesknärvisüsteemi ja perifeersete kudede vahelisi seoseid. Lisaks huvitas meid varem avaldatud vere transkriptoomika tööde võrdlus. Ajukoorest leidsime kolme töö võrdluses 43 oluliselt muutunud ekspressiooniga geeni. Ainevahetusradadest oli oluline legionelloosi rada, mille üks komponentidest, TLR/MYD88 signaalrada, on eelnevalt seostatud neurodegeneratiivsusega (Xiang, Chao, and Feng 2015). Mustaines RRA nimekirjas olid viie töö võrdluses olulised kaks geeni: *LMO3* ja *RIMS3*. *LMO3* mõjutab neurogeenesi ning *RIMS3* on oluline neurotransmitterite vabanemisel; mõlemaid on eelnevalt seostatud PT-ga. Mõlemad geenid osalevad mikro-RNA regulatoorses võrgustikus. Mustaine RRAs osutusid oluliseks neli funktsionaalset ainevahetusrada: kaltsiumi signaalrada, sünaptilise vesiikli tsükli, proteoglükaanide ja dopaminergilise sünapsi ainevahetusrada, mille kõikide puhul on eelnevaid assotsiatsioone PT-ga. Vere RRA nimekirjas jäi kuue töö (kaasarvatud käesolev töö) metaanalüüsis oluliseks ainult üks geeniekspressioooni muutus – *IL18R1*, mis on interleukiini retseptor ja vahendab proinflammatoorseid vastuseid ning mille robustne muutus veres näitlikustab immuunprotsesside olulisust neurodegeneratiivse haiguse nagu PT korral. Vere RRA tulemuste ainevahetusradade analüüsil jäid oluliseks kaks ainevahetusrada: hematopoeetiliste rakkude loomega seotud protsessid ning prioonhaigustega seotud ainevahetusrada. Mõlemat protsessi on eelnevalt seostatud PT-ga (Schlachetzki et al. 2018; Soreq et al. 2008; Brundin and Melki 2017). Huvitaval kombel kui võrrelda erinevad PT kudesid,

siis ei kattu ei ainevahetusradade ega geeniekspressioonide tasemel eriti palju. Märkimisväärsed erandid on *G0S2* ja *C6*, mis kattuvad ajukoore ja käesoleva naha transkriptoomi tööga. G0/G1 *switch* geen 2 (*G0S2*) mõjutab lipiidide ainevahetust adipotsüütides (Heckmann et al. 2013). Komplementsüsteemi komponent 6 geeni (*C6*) toode on valk lüütilises makromolekulis – membraani ründekompleksis, mille kõrgenenud esinemist on näidatud neurodegeneratiivetes ajukudedes (McGeer and McGeer 2004) ja taaskord viitab see inflammatsiooni olulisusele neurodegeneratsiooni protsessides. Väga väheste geeniekspressioonide ja ainevahetusradade kattumine eri kudede vahel otses võrdluses ja RRA meetodit kasutades võib viidata mitmele asjaolule. Esiteks, võib olla, et eri kudede transkriptoomiline profiil on niivõrd erinev, et neid ei saa võrreleda. Kõik uuritavad koed on pigem heterogeensed, koosnedes mitmest eri rakupopulatsioonist, mis võib tingida uuringutevahelisi erisusi ja raskenda praeguste uuringute arvu juures oluliste muutuste avastamist. On oluline märkida, et erinevate metoodikate kasutamine preanalüütilises, analüütilises ja statistilise andmetöötluse faasis põhjustab palju erisusi, mida on raske arvesse võtta ja normaliseerida. Kasutades RRAd vältisime algandmete analüüsi vajadust. See on lihtne uus meetod, mis võimaldab RNA sekveneerimise ja mikrokiipide andmete võrdlust. Samas võib-olla nii, et selle meetodi kasutamisel lisandus müra, mis varjutas olulisi muutusi. Samuti võib olla, et PT patognoomilised muutused (mitokondrite düsfunktsioon, valkude ainevahetuse defitsiidid) on mitmete ainevahetusradade ja üksikute geeniekspressioonide konvergeeruvate muutuste tulem.

SAA kui huvipakkuv geen PT patoloogias

Lisaks uurisime süvendatult *SAA*-d kui potentsiaalset biomarkerit (Artikkel III) ja kaardistasime püsiva allareguleerituse RNA ja valgu tasandil nii PT veres kui nahas. SAA on akuutse faasi valk ning tal on α-sünukleiiniga sarnaseid omadusi, olles ebastabiilse tertsiaarse struktuuriga valk. See võib olla põhjus, miks ta on haavatav rakusisese redoks-stressi tõusule ning võib kergesti kaotada oma funktsionaalse struktuuri. Varasemates uuringutes on leitud, et *SAA* on ajus neuroinflammatsiooni puhul kõrgenenud tasemega. See võib viidata, et käesolevas uuringus leitud allareguleeritus veres on reaktiivne. Selleks, et *SAA* olulisust PT patogeneesis uurida, on vaja lisakatseid.

Järeldused

1) Naha RNA sekveneerimise tulemused näitavad paljude geenide ekspressiooni allareguleeritust nahas, mis seonduvad
   a) rakuprotsessidega, mis on PT puhul tsentraalse tähtsusega nagu mitokondri funktsioneerimine ja valgu ainevahetus,
   b) epidermise ja dermise homöostaasi, diferentseerumise, immuunvastuse ja kasvaja tekke protsessidega.

- Naha haavatavus mutageensete riskide suhtes selgitab melanoomi ja teiste nahavähkide ning muude PT-spetsiifiliste nahahädade sagedast esinemist.
- Haigus-spetsiifilised transkriptsioonimustrid nahas on võrreldavad ajust leitud muutustega ja viitavad, et PT on multisüsteemne häire.

2) Vere RNA sekveneerimise tulemused näitavad väga väheste geenide diferentsiaalset ekspressiooni, kuid nimetatud geenid on eelnevalt seondatud PT-ga.
    - Differentsiaalselt on mõjutatud kolesterooli ainevahetusrada.
    - Madal oluliselt muutunud geenide arv veres võib olla põhjustatud vere kui koe heterogeensusest ja dünaamilistest omadustest, kuid kattuvus varem avaldatud PT transkriptoomika töödega toetab multisüsteemse häire hüpoteesi.
    - Nahas ja veres ei olnud kattuvaid geeniekspressiooni ega ainevahetusraja muutusi.

3) Kahte huvipakkuvad geeniekspressiooni nahast – SAA1 ja SAA2 uuriti süvitsi. Geeniekspressiooni allareguleeritus nahas peegeldus verest mõõdetud madala valgukoguses, kui SAA valgu visualiseerimine nahas ebaõnnestus valgu madala koguse tõttu.

4) Eelnevaid uuringuid võrreldi metaanalüüsis, mis kasutas uudset RRA meetodid, mis võimaldab võrrelda mikrokiipe ja RNA sekveneerimist omavahel sõltumata kasutatud statistilistest meetoditest. Kolmest eri koes – ajukoorest, SN ja verest – oli piisavalt tõid, et kaasata nad analüüsi. Tulemused näitasid üllatavalt vähe veenvaid geeniekspressiooni ja ainevahetusradade muutusi, mis
    a) rõhutavad koespetsiifilise geeniekspressiooni erinevusi;
    b) näitavad, et on vaja rohkem geeniekspresiooni uuringuid ja metaanalüüse, et leida korratavaid tulemusi.
        4.1) Huvipakkuvad diferentsiaalselt muutunud ekspressiooniga geenid on
        nahas PGC-1α ja SAA1/2,
        veres UBE2J1 ja IL18R1,
        mustaines LMO3 ja RIMS3,
        ajukoores PENK ning
        ajukoore ja naha võrdlusel G0S2 ja C6.

    Need geeniekspresioonid näitasid käesolevas uuringus kõige selgemaid/ veenvamaid muutusi ning eelnimetatud geene tasuks edasi uurida, et hinnata nende kasutatavust PT biomarkeritena.
        4.2) Märkimisväärsed KEGG ainevahetusrajad on
        hematopoeetilise raku ja priionhaiguste ainevahetusrajad verest;
        kaltsiumi signaliseerimise, sünaptiliste vesiiklite tsükli,
        proteoglükaanide vähi puhul ja dopaminergilise sünapsi
        ainevahetusrajad SN-st;
        ning legionelloosi ainevahetusrada verest.

- Kokkuvõtvalt leiti, et perifeersetest kudedest on mõttekas koguda proove transkriptoomika uuringuteks PT puhul, et leida biomarkereid ning transkripte, mis mõjutavad PT patoloogilisi mehhanisme, sest

a) neid kudesid saab uurida eluspuhuselt ja
b) tulemused kattuvad PT-spetsiifiliste muutustega kesknärvisüsteemis.

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