Polycystic Ovary Syndrome

Androgen Excess and Insulin Resistance in Women:
Identification of Molecular Targets to Improve Glucose
Homeostasis

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Gothenburg 2018

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"We all make mistakes, and it is better to make them before we begin."

Nikola Tesla

ABSTRACT

Polycystic ovary syndrome (PCOS) is the most common endocrine and metabolic disorder in women. Women with PCOS demonstrate metabolic morbidities such as obesity, insulin resistance with compensatory hyperinsulinemia and type 2 diabetes. Physical inactivity in women with PCOS aggravates the metabolic conditions. Pharmacological treatment is efficient but associated with side effects. The pathogenesis of PCOS is largely unknown and current research suggests that genetic and epigenetic factors are implicated. Epigenetics e.g. methylation is an important mechanism that regulates gene transcription and transcriptional alterations could explain metabolic aberrations associated with PCOS.

The aim of this thesis was to profile genome-wide gene expression and methylation patterns in adipose tissue and skeletal muscle in women with PCOS and controls, and to investigate if electroacupuncture could be used to restore altered CpG sites and transcriptional alterations. Furthermore, we aimed to investigate the effect and mechanisms of a single bout of low-frequency electroacupuncture (EA) on whole-body glucose uptake.

The results of the thesis demonstrate that women with PCOS have multiple differentially methylated sites that are associated to gene expression changes in adipose tissue. In subcutaneous adipose tissue, we found 30 differentially methylated genes that are associated with mRNA expression. We have shown that transcriptional alteration in adipose tissue are associated to circulating testosterone, glucose infusion rate and adipocyte size. Furthermore, in adipose tissue electroacupuncture reversed expression of 80 genes to a healthier phenotype. In skeletal muscle, we found 85 genome-wide transcriptional differences and 21 differentially methylated genes. We also showed that mRNA expression levels of *KLF10* and *COL1A1* are under hormonal regulation of insulin and testosterone respectively and both of these genes are involved in controlling glycogen accumulation in human skeletal muscle cells.

Women with PCOS have increased sympathetic nerve activity, which is related to aberrant androgen levels and this can lead to increased insulin resistance and obesity. We have shown that EA lowers protein expression of markers of sympathetic nerve activity: proNGF, serotonin and homovanillic acid in adipose tissue. We measured whole-body glucose uptake by euglycemic-

hyperinslinemic clamp and we demonstrated that EA increases glucose infusion rate in both rats and in women with PCOS and controls. In the rat experiment, we show that administration of α and β blockers during clamp blocks glucose uptake suggesting that adrenergic receptors partly mediates the effect of EA by activating the autonomic nervous system. Overall, EA-induced glucose uptake was controlled by activation of both sympathetic and parasympathetic nervous system in rats. In women with PCOS we identified increased expression of several genes involved in regulation of glucose uptake, including *NR4A2* and *JUNB,* in adipose tissue after EA. We investigated transcriptional changes of those genes in rats receiving α and β adrenergic blockers, and the involvement of the sympathetic nervous system is supported by lowered expression of *Nr4a2* and *Junb* genes, suggesting that EA mediates it effect by affecting mRNA levels of those genes.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Polycystiskt ovariesyndrom (PCOS) är en vanlig hormonell och metabol störning som förekommer hos ungefär 17 % av alla kvinnor i fertil ålder. Syndromet leder till ökning av det manliga könshormonet testosteron, ökad kroppsbehåring, akne och oregelbunden menstruation, vilket leder till nedsatt fertilitet. Många kvinnor med PCOS är överviktiga och har nedsatt insulinkänslighet vilket ofta leder till typ 2-diabetes. Det är okänt vad som orsakar PCOS men miljö, epigenetiska och genetiska faktorer har en inverkan. Epigenetik syftar på förändringarna som sker på DNAt utan att underliggande mekanismer påverkas. Epigenetiska mekanismer kontrollerar hur DNA läses av och hur generna uttrycks. Det finns olika epigenetiska mekanismer som DNA-metylering, histonmodifiering och icke-kodande RNA som reglerar genuttryck. Epigenetik är ett relativt nytt forskningsområde som kan vara involverat i utvecklingen av PCOS.

Målet med denna avhandling var att undersöka om det finns metyleringsförändringar som påverkar genuttryck i fettvävnad och skelettmuskulatur och som därmed kan förklara metabola störningar hos kvinnor med PCOS. Vi undersökte också om akupunktur i kombination med elektrisk stimulering av nålarna, s.k. elektroakupunktur (EA), kan öka glukosupptag och insulinokänslighet hos överviktiga och obesa kvinnor, samt om epigenetiska förändringar kan återställas av akupunktur.

Vi fann att kvinnor med PCOS har epigenetiska förändringar som kan påverka ett stort antal gener som är relevanta för utvecklingen av PCOS i både i fettvävnad och skelettmuskulatur. I fettväv hittades 30 och i skelettmuskel 21 gener som var skilda i både metylering och genuttryck och som kunde associeras till kliniska PCOS-karakteristika. En gen som regleras av insulin (*KLF10)* ökade i expressionen i både muskel och fett i kvinnor med PCOS, och det kan leda till både lägre glukosupptaget och insulinkänsliget.

Lågfrekvent elektroakupunktur under 45 minuter ökade glukosupptaget hos både friska kontroller och kvinnor med PCOS. Samtidigt återställdes DNA-metylering och genuttryck så att det till stor del liknade det som ses hos friska kontroller. Effekterna av elektroakupunktur förmedlades (åtminstone delvis) av det autonoma nervsystemet eftersom effekterna blockerades när ett läkemedel som blockerar sympatikus och parasympatikus administrerades till

råttor. Hos kvinnor med PCOS har vi dessutom identifierat ökat genuttryck i *NR4A2* och *JUNB* och ökat glukosupptag efter efter 45 minuters behandling med elektroakupunktur. I råttor har vi undersökt hur genuttrycket förändras när råttorna fick sympatikus- och parasympatikusblockare. Vi fann att genuttrycket av *Nr4a2 och Junb* sänktes i gruppen som fick sympatikus- och parasympatikusblockare, vilket tyder på att elektroakupunktur medierar effekten genom att påverka mRNA-nivåer av dessa två gener. *NR4A2* och *JUNB* är transkritionsfaktorer som är kopplade till inflammatoriska respektive metabola störningar. Ytterligare stöd för att effekten av elektroakupunktur förmedlas via modulering av autonom aktivitet stöds av fynden att proteinuttrycket av nervtillväxtfaktorn (NGF), en markör för sympatikusaktivitet, efter 45 minuters behandling med elektroakupunktur hade minskat hos kvinnor både med och utan PCOS.

PAPERS INCLUDED IN THIS THESIS

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

I. **Epigenetic and transcriptional alterations in human adipose tissue of polycystic ovary syndrome**

   Milana Kokosar, Anna Benrick, Alexander Perfilyev, Romina Fornes, Emma Nilsson, Manuel Maliqueo, Carl Johan Behre, Antonina Sazonova, Claes Ohlsson, Charlotte Ling, Elisabet Stener-Victorin. *Sci Rep. 2016*; 6: 22883 │ DOI: PMCID: PMC4791632

II. **Transcriptional and epigenetic changes influencing skeletal muscle metabolism in women with polycystic ovary syndrome**

   Emma Nilsson, Anna Benrick, **Milana Kokosar,** Anna Krook, Eva Lindgren, Thomas Källman, Michaela Martis, Kurt Højlund, Charlotte Ling, Elisabet Stener- Victorin. **Manuscript 2017**

III. **Autonomic nervous system activation mediates increase in whole-body glucose uptake by electroacupuncture**

   Anna Benrick, **Milana Kokosar,** Min Hu, M.D, Martin Larsson, Manuel Maliqueo, Rodrigo Marcondes, Marzia Soligo, Virginia Protto, Elisabet Jerlhag, Antonina Sazonova, Carl Johan Behre, Kurt Højlund, Peter Thorén, Elisabet Stener-Victorin. *FASEB J*. 2017 Apr 12. pii: fj.201601381R. doi: 10.1096/fj.201601381R.

IV. **Single bout of electroacupuncture remodels epigenetic and transcriptional changes in adipose tissue in polycystic ovary syndrome**

   Milana Kokosar, Anna Benrick, Alexander Perfilyev, Emma A Nilsson, Thomas Källman, Claes Ohlsson, Charlotte Ling, Elisabet Stener-Victorin. **Accepted,** *Sci Rep.* **2017**

PAPERS NOT INCLUDED IN THIS THESIS

I. **Changes in HbA1c and circulating and adipose tissue androgen levels in overweight-obese women with polycystic ovary syndrome in response to electroacupuncture**

    Stener-Victorin E, Maliqueo M, Soligo M, Protto V, Manni L, Jerlhag E, **Kokosar M**, Sazonova A, Behre CJ, Lind M, Ohlsson C, Højlund K, Benrick A. *Obes Sci Pract*. 2016 Dec;2(4):426-435. doi: 10.1002/osp4.78

II. **Maternal testosterone and placental function: Effect of electroacupuncture on placental expression of angiogenic markers and fetal growth**

    Fornes R , Hu M, Maliqueo M, **Kokosar M**, Benrick A, Carr D, Billig H, Jansson T, Manni L, Stener-Victorin E. Mol Cell Endocrinol. 2016 Sep 15;433:1-11. doi: 10.1016/j.mce

III. **Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring**

    Hu M, Richard JE, Maliqueo M, **Kokosar M,** Fornes R, Benrick A, Jansson T, Ohlsson C, Wu X, Skibicka KP, Stener-Victorin E. *Proc Natl Acad Sci U S A*. 2015 Nov 17; 112(46):14348-53.

IV. **Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood**

    Rönn T, Volkov P, Gillberg L, **Kokosar M**, Perfilyev A, Jacobsen AL, Jørgensen SW, Brøns C, Jansson PA, Eriksson KF, Pedersen O, Hansen T, Groop L, Stener-Victorin E, Vaag A, Nilsson E, Ling C. *Hum Mol Genet*. 2015 Jul 1;24(13):3792-813.

CONTENTS

BMI	Body Mass Index
CRH	Corticotrophin releasing hormone
DNMT	DNA methyltransferases
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
EA	Electroacupuncture
E1	Estrone
E2	Estradiol
FDR	False Discovery Rate
FSH	Follicle stimulating hormone
FG	Ferriman-Gallwey Score
GLUT4	Glucose transporter 4
GSEA	Gene Set Enrichment Analysis
HOMA	Homeostasis model assessment
HbA1C	Glycated haemoglobin
IRS-1	Insulin receptor substrate 1
IPA	Ingenuity pathway Analysis
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA

ncRNA	Non-coding RNA
NIH	National Institute of Health
OGTT	Oral Glucose Tolerance Test
PCOS	Polycystic Ovary Syndrome
PCOM	Polycystic ovarian morphology
PI3K	Phosphatidylinositide 3-kinases
piRNA	Piwi-interacting RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
SHBG	Sex hormone binding globulin
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNAs
WHO	World Health Organization

1 INTRODUCTION

1.1 POLYCYSTIC OVARY SYNDROME

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder that affects between 5−17% of women worldwide (*1, 2*). The aetiology of PCOS is poorly understood and high prevalence might be due to the genetic predisposition, environmental and epigenetic factors (*3*). Up to 70% can be attributed to heredity and genetic factors. Recent genome wide association studies (GWAS) have identified around 15 to 19 susceptible genes, which may be involved in the development of PCOS (*4, 5*).

1.2 DIAGNOSIS AND PREVALENCE

Diagnostic criteria of PCOS are under debate. According to the National Institute of Health (NIH) requirement for diagnosis of PCOS are hyperandrogenism and ovulatory dysfunctions (*6*). European Society for Human Reproduction and Embryology and American Society for Reproductive Medicine require the presence of at least two out of three following: hyperandrogenism, ovulatory dysfunction and/or PCO morphology (*7*), and Androgen Excess and PCOS Society require presence of hyperandrogenism and ovarian dysfunction or PCO morphology (*8*). In the Rotterdam Consensus report from 2003 (*7*) PCO morphology was included in the diagnostic criteria which have resulted in more phenotypes (*9*) (Table 1).

Table 1. Diagnostic criteria in PCOS

NIH 1990	Rotterdam 2003	AE-PCOS 2006
HA	HA	HA
OD	OD	OD and/or PCOM
	PCOM	
Both criteria needed	Two out of three criteria needed	Both criteria needed

Identification of specific phenotypes includes: 1: HA + OD + PCOM; 2: HA + OD; 3: HA + PCOM; 4: OD + PCOM. Hyperandrogenism; HA, ovulatory dysfunctions; OD, PCO morphology; PCOM.

1.3   PATHOPHYSIOLOGY

The pathogenesis of PCOS is complex and not elucidated. The hallmarks of PCOS are hyperandrogenemia and hyperinsulinemia. The hyperandrogenemia, is a direct result of excessive androgen production from the theca cells in the ovaries and to a small extent production from the adrenal gland (*10*). Women with PCOS have impaired steroidogenesis, P450 aromatase activity in thecal cells is decreased contributing to high levels of androgens and increased CYP17 activity (*11*). It is not understood if hypersecretion of androgens from the ovaries starts the early age or at the puberty (*12*). However, there are convincing evidence that foetal hyperandrogenisation *in utero* might lead to epigenetic reprogramming in foetal reproductive tissue which in adulthood leads to development of PCOS (*13*). Furthermore, excessive androgen exposure during foetal life may contribute to development of insulin resistance and visceral adiposity (*14*). Insulin resistance with compensatory hyperinsulinemia controls availability of androgens where hyperinsulinemia leads to decreased secretion of sex hormone binding globulin (SHBG) from the liver which subsequently leads to increased levels of circulating free androgens (*15*). Moreover, increased levels of free androgens in the circulation affect insulin action in both skeletal muscle and adipose tissue (*16*).

Women with PCOS exhibit neuroendocrine defects where increased ovarian androgen production is, in part, a result of increased ratio between luteinizing hormone (LH) and follicle stimulating hormone (FSH) (*17*) resulting with elevated LH/FSH ratio. Gonadotropin releasing hormone (GnRH) plays an essential role in pathogenesis of the PCOS where, either increased pituitary sensitivity to GnRH or increased pulse frequency of GnRH secretions leads to aberrant secretion of gonadotropins (*18*). In addition, it is common that women with PCOS are overweight or obese which leads to aggravation of other PCOS symptoms (*19*) (Figure 1).
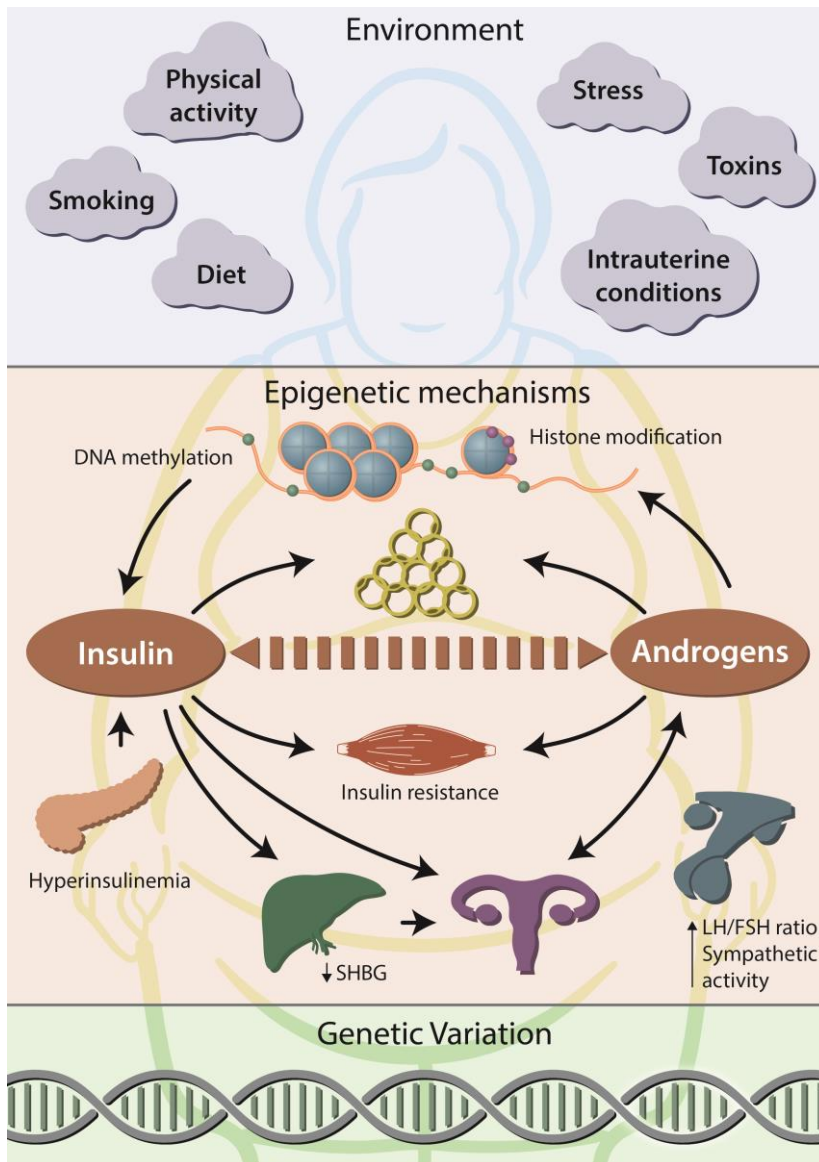
Figure 1. **Hypothetical model of the pathophysiology of PCOS includes:**
Genetic and epigenetic defects, excessive ovarian and adrenal steroidogenesis,
neuroendocrine defects, increased sympathetic activity, metabolic disturbances,
insulin resistance with compensatory hyperinsulinemia and overweight/obesity.
Illustration by Igor Cervenka

1.4 METABOLIC DYSFUNCTION IN PCOS

1.4.1 Adipose tissue

Adipose tissue is an endocrine organ and the main function is to store triglycerides as an endogenous fuel that are used upon metabolic demand and for body insulation. Together with liver and skeletal muscle, adipose tissue is responsible for the maintenance of insulin stimulated glucose uptake. There are two different types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (20). The knowledge in mechanisms that control browning of the white adipose tissue is advancing and it might have potential therapeutic applications (21). Largest WAT depots in humans are the subcutaneous and the visceral depots. WAT is composed of different cells types: adipocytes and connective stroma-vascular fraction which contains pre-adipocytes, fibroblasts, leukocytes, adipose tissue macrophages, lymphocytes, endothelia and mesenchymal stem cells where all have different functions (22). Furthermore, WAT is involved in production and secretion of adipokines (cytokines) such as leptin, resistin and adiponectin that are involved in regulation of metabolic homeostasis and vascular health (23). Normal function of adipose tissue is disturbed by increase in body weight (24). According to the World Health Organization (WHO) obesity is causing death of 2.8 million people each year in high income countries, which is as a direct result of abnormal fat accumulation (25). In October 2017 WHO released new numbers concerning overweight, obesity, and the new number calls for rapid measures:

- Worldwide obesity has nearly tripled since 1975
- In 2016, more than 1.9 billion adults, 18 years and older, were overweight. Of these over 650 million were obese
- 39% of adults aged 18 years and over were overweight in 2016, and 13% were obese
- Most of the world's population live in countries where overweight and obesity kills more people than underweight
- 41 million children under the age of 5 were overweight or obese in 2016.
- Over 340 million children and adolescents aged 5-19 were overweight or obese in 2016

Women with PCOS are often overweight or obese and adiposity leads to metabolic complications such as insulin resistance with compensatory hyperinsulinemia and glucose intolerance, which increases risk of developing type 2 diabetes (*26*). There is evidence that several regulatory processes in WAT such as lipolysis, adipose tissue morphology and vascularization are altered, which associates white adipose tissue changes with PCOS pathophysiology (*27*).The strongest factors explaining insulin resistance in women with PCOS are the increased adipocyte size together with decreased circulating levels of adiponectin (*28*). Furthermore, women with PCOS have alterations in adipokine levels. Adiponectin is produced only in adipose tissue and has insulin-sensitizing, anti-inflammatory and anti-atherosclerotic properties (*29*). Levels of adiponectin are inversely related to body weight, insulin resistance and type 2 diabetes (*30*). Several studies have shown that women with PCOS have lower adiponectin levels when compared to controls (*31, 32*). Moreover, it was demonstrated that low adiponectin levels were associated with insulin resistance and not with testosterone suggesting that hyperinsulinemia is a major factor contributing to aberrant levels of androgens from the ovaries (*32*). Leptin is another adipokine that regulates energy homeostasis and controls reproductive functions. High levels of leptin are associated to insulin resistance, obesity and metabolic syndrome (*33*). In women with PCOS the results are conflicting. Some studies suggest there is no difference in circulating leptin levels (*34*), while other studies showed increased levels of leptin in women with PCOS in comparison to the controls (*35*).

Overweight and obesity together with lack of exercise and physical inactivity have become a problem in developed countries that often lead to development of metabolic disorders such as: dyslipidemia, hypertension, inflammation, development of cardiovascular diseases and increased production of testosterone and estradiol (*36, 37*).

1.4.2   Skeletal muscle

Skeletal muscle is one of the most dynamic tissues in human body that controls multiple body functions such as the movement and different metabolic process. Human body consists of around 600 muscles, which are controlled by the nervous system (*38*). Skeletal muscle is involved in regulating energy metabolism, storage of amino acids and carbohydrates. Furthermore, it

accounts for 75% of insulin stimulated glucose uptake from the blood and is a key factor in regulating whole-body glucose homeostasis (*39*). Together with brown adipose tissue and liver, skeletal muscle regulates the heat production and controls the peripheral glucose uptake (*40*). Therefore, it is important to maintaining optimal skeletal muscle health through life and prevent development of metabolic disturbances such as insulin resistance, metabolic syndrome, obesity and chronic diseases.

Insulin resistance is characterized as decreased ability of cells to respond to insulin. PCOS is likely to be complicated into other comorbidities such as type 2 diabetes and cardiovascular diseases because of disturbances in glucose uptake in both adipose tissue and skeletal muscle. (*41, 42*). The mechanisms of insulin resistance are partly known and the factors that contribute to the development of prediabetes and insulin resistance are attributed to genetics, age and life style (*43*). The prevalence of insulin resistance in women with PCOS is between 50-70% (*44*). Interestingly, insulin resistance in women with PCOS is independent of obesity. This indicates that there are other drivers of insulin resistance than overweight or obesity. Furthermore, elevated insulin levels lead to increase in both the production and secretion of androgens from the theca cells in the ovaries. In addition, hyperinsulinemia lead to reduction of hepatic biosynthesis of sex hormone-binding globulin which subsequently increase levels of circulating free testosterone (*15*).

Previous studies in skeletal muscle in women with PCOS showed that insulin action is reduced and activity of insulin-signaling pathways along with glucose uptake was decreased (*10*). It is believed that the two central mediators in development of insulin resistance in PCOS are hyperandrogenemia together with hyperinsulinemia (*45*). The molecular mechanism of insulin resistance and the theory that insulin derives hyperandrogenemia in women with PCOS are not well investigated and a variety of different factors, genes and pathways have been suggested to play an important role (*46*). Transcriptional alterations in insulin signaling pathways phosphatidylinositide 3-kinases (PI3K) that regulates insulin stimulated glucose uptake have been demonstrated (*47*). It was shown that women with PCOS have altered activity of two upstream mediators in PI3K signaling pathway: IRS-1 and AKT, which could explain low insulin sensitivity (*47*). Data suggested that increased phosphorylation of IRS-1 might be connected to hyperandrogenemia and hyperinsulinemia and contribute to development of insulin resistance (*48*). Another pathway that has

altered activity in women with PCOS is mitogen-activated protein kinase pathway (MAPK) and those disturbances might contribute to insulin resistance development (*49*). In addition, insulin resistance in skeletal muscle in women with PCOS has been associated with reduction in mRNA expression in genes involved in mitochondrial oxidative metabolism, disturbances in fatty acid metabolism and calcium homeostasis (*50*). It was suggested that insulin resistance in women with PCOS is due to the decreased mitochondrial respiration (*51*), however this finding is not supported by other studies, which showed that mitochondrial ATP synthesis was not altered in women with PCOS (*52*). Furthermore, it was proposed that myostatin, a common regulator of metabolism, is involved in regulation of metabolism of androgens in skeletal muscle cells but this finding have not been confirmed (*53*). It was shown that testosterone affects expression of aromatase and androgen receptor without affecting insulin sensitivity in women with PCOS (*54*). The effect of androgens on insulin sensitivity in skeletal muscle in humans is unknown and this require further research.

1.5   EPIGENETICS

In 1956, British developmental biologist Conrad Waddington published a paper in the Journal of Evolution where he laid down the principles of today´s filed of Epigenetic. He defined epigenetics as ''*the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being*''. Waddington created the model of "Epigenetic landscape" (Figure 2) which is a process of cellular differentiation where a marble rolls from the top of the hill (which represents the pluripotent state of the cell), and during the movement the cell has a potential of differentiating into any cell type in the body.
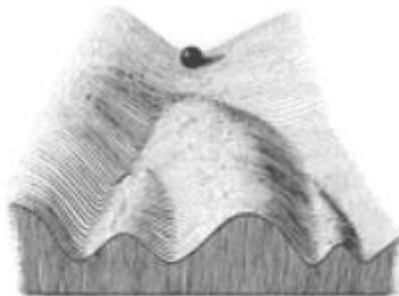

Figure 2. Waddington´s Epigenetic Landscape. Image under public domain.

Since then, a numerous of scientific meeting and more than 22 000 published papers have studied the *epigenome*. The modern definition of Epigenetics where the concept of inheritance play a big part have been introduced and is well accepted by others.

 "*The study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence*" (*55*).

Epigenetic marks can be influenced by the information coming from the environment supporting the theory that epigenetic changes can be rapidly altered and therefore are involved in controlling the gene expression (*56*). There are different epigenetic mechanisms that are involved in controlling the gene expression:

- DNA methylation
- Post- translational histone modification and
- Non-coding RNA (ncRNA)

1.5.1   DNA Methylation

DNA methylation is the most studied epigenetic mechanism that regulates gene transcription and expression. It involves transfer of methyl group from S-adenosylmethionine to the top of the fifth carbon on the cytosine base (*57*). Addition of methyl group on the top of genomic information controls gene transcription and expression without changing underlying DNA sequence. Methylation marks are laid down by *de novo* DNA methyltransferases DNMT3A, DNMT3B and DNMT3L (*58*). After cell division hemi-methylated DNA is maintained by DNMT1 methyltransferase enzyme (*59*). DNA methylation can occur in regulatory region of the gene (promoter) but it is more traditional to find methyl marks on gene body, intergenic regions and repetitive elements. The function of methylation in intergenic regions and repetitive elements is to stabilize chromatin, maintain genomic integrity and provide genomic stability. Cytosine bases that are followed by guanidine (CpG) are usually located in the promoter region and in general, it is believed that these CpG regions are protected from methylation. Most cells have a stabile DNA methylation pattern where between 70–80% of CpG sites are methylated (*60*). The field of epigenetics is progressing; however, it is still largely unknown how many CpGs are involved in genomic regulation. It has been reported that

around 22% of autosomal CpGs within a normal developmental context are used and many of those were distal to transcription start sites (*61*).

1.5.2   Histone Modification

Histone modification is another type of epigenetic mechanism that is involved in regulation o gene expression. In eukaryotic cells, DNA is wrapped around histone octamers (H2A, H2B, H3 and H4) and it forms DNA-histone protein complex called nucleosome. In this way, two meter of DNA is tightly packed into a chromatin structure. A posttranslational modification of histone N-terminals is a subject to several modifications including methylation, acetylation, phosphorylation, ubiquitination and sumoylation (*62*). Most studies on histone modification are performed on histone acetylation and methylation. Histone methylation is associated with both gene activity and gene silencing and it comes in different states (mono, di and tri methylation). Histone acetylation is a chromatin modification that is associated with gene activity only (*63*).

1.5.3   Non- coding RNAs

Non-coding RNAs (ncRNA) have important function in epigenetic regulation of the gene transcription at the post-transcriptional level. There are several different types of ncRNA that are involved in the epigenetic control: small non-coding RNAs such as micro RNA, siRNAs, snoRNAs, piRNAs and long non-coding RNAs such as XIST and HOTAIR (*64*). The role of non-coding RNAs in PCOS is limiting. Some studies aimed to identify differentially expressed micro RNAs and associate them to impaired glucose metabolism, reduced insulin sensitivity and disturbances in steroidogenesis (*65, 66*). However, these finding are not well established and, further research has to be carried through to demonstrate the involvement of non-coding RNAs in pathophysiology of PCOS.

1.5.4   Epigenetics in PCOS

PCOS is a heritable disorder, however, no genes responsible for the development have been found. Therefore, it has been proposed that the origin and the development of PCOS could be a result between an interaction in genetic and epigenetic alterations (*67*). There are limited numbers of human studies that have profiled epigenome in women with PCOS although, with the new emerging techniques being developed, new data is being generated

constantly. The first implications of epigenetic involvement in development of PCOS came from studies performed on PCOS rhesus monkey model (*68*) which hypothesized that foetal hyperandrogenemia might be responsible for altering the epigenome and lead to development of PCOS in adolescence (*69, 70*).

This theory suggest that PCOS might originate in fetal life, where elevated maternal androgens might have been implicated to play a role in altering the faith of foliculogenesis and this would lead to development of PCOS however, the mechanisms are largely unknown (*71, 72*). It is known that abnormal exposure to androgens and glucocorticoids during critical intrauterine periods during pregnancy leads to development of different PCOS phenotypes (*73*). Another theory suggested that PCOS might be a disorder related to environmental conditions such as diet (*74*). Caloric restriction during pregnancy leads to hypoxia consequently leading to restricted intrauterine growth and therefore smaller birth weight (*75*). Therefore, PCOS would manifest itself later in adolescent life as a way to compensate for insufficient intrauterine growth (*75*). The genome-wide screen epigenetic studies suggested that methylation disturbances in skeletal muscle, adipose tissue (*76*) and ovaries (*77*) are due to the diet, obesity, sedentary life and environmental factors (*78*). The enormous efforts have been put into generating extensive amount of methylation microarray data. However, the results from different epigenetic studies in tissues such as adipose tissue (*76, 79*) and blood (*80-82*), are conflicting and further research is needed to elucidate if methylation changes are leading to transcriptional changes that could explain pathogenesis of PCOS..

1.6   TREATMENT FOR PCOS

There is no cure for PCOS but there are different strategies to treat the symptoms. The first line treatment for women with PCOS, independent of obesity, is lifestyle modifications including weight reduction and physical exercise to improve reproductive and metabolic function (*83*). Sedentary lifestyle, nutrient overload and poor physical activity lead to excessive fat accumulation in some PCOS phenotypes. Overweight or obesity can be prevented with regular physical activity/exercise (60 minutes a day for children and 150 minutes spread through the week for adults, WHO, 2017). Women with PCOS have often decreased insulin sensitivity and lifestyle changes are

the first line of treatment (*84*). Furthermore, physical activity and exercise improve metabolic health and beneficial effects of exercise have been shown on the mental health of women with PCOS (*85*). Pharmacological administration is a conventional management to control the symptoms. However, it has many side effects and the goal is not to use pharma as a lifelong treatment. Oral contraceptives are effective but they cannot be used in women that are trying to conceive. Anti-androgen treatment improves clinical symptoms of hirsutism, acne and restores menstrual cyclicity, however, it aggravates metabolic profile (*86*). In women with PCOS metformin is used to inhibit ovarian androgen production and improve ovulation (*87*). Furthermore metformin improves whole-body glucose uptake by decreasing hepatic glucose production and lowering free fatty acid in liver, skeletal muscle and adipose tissue in women with PCOS (*88*). The exact mechanism of metformin is unknown but it enhance glucose uptake in the skeletal muscle, liver and adipose tissue (*89*). In addition, metformin is used when patients do not respond to lifestyle modifications and when they do not follow exercise regimen (*90*). Since pharmacological treatment has adverse effect, we are searching for other alternative methods to improve metabolic profile.

1.7 ELECTROACUPUNCTURE

Acupuncture is as ancient Chinese practice that is defined as insertion of needles into the body at specific points (acupoints). In the western world patients usually seek for the treatment of acupuncture when pharmacological treatment is unsuccessful. Since pharmacological managements are often accompanied by negative side effects, acupuncture has a status of being safe and efficient and therefore widely used (*91*). Acupuncture needles can be stimulated either manually by rotation or with low-frequency electrical current (1-15Hz), so called electroacupuncture. Low-frequency electroacupuncture results in muscle contractions and theoretically, it resembles the effect of exercise (*92*). The mechanisms of acupuncture are not completely known but hypothetical models have been proposed. Both manual and low-frequency electroacupuncture stimulation activates special type of mechanoreceptors in skeletal muscle, so called ergoreceptors, which activates sensory nerve fibers; myelinated Aα, β, δ, and unmyelinated C-fibers (*93*). When needles are stimulated, it evokes an activation of sensory nerve afferents. The stimulation cause a direct release of a number of neurotransmittors including calcitonin gene related peptide, vasointestine peptide, and nerve growth factor to mention a few, from the peripheral nerve endings, resulting in a direct, local effect in the tissue including increased microcirculation as well as glucose transporter 4 (GLUT4) translocation to the cell membrane and increased glucose uptake (*94*). In the spinal cord the stimulation modulates spinal reflexes, including sympathetic output to organs located in the same somatic innervation area. All spinal reflexes are under control of the central nervous system (*95*). Activation of sensory nerve afferent are transmitted to thalamus, hypothalamus and higher control centers including limbic structures (*96-98*). In the hypothalamus is β-endorphin secreted which has a high affinity for μ-receptor and has been demonstrated to regulate general sympathetic activity by decreasing blood pressure (*99-101*). That β-endorphin is involved in the regulation of blood pressure is evidenced by the fact that administration of μ-receptor blocker diminish the effect of low-frequency electrical stimulation on blood pressure (*102*). Further, β-endorphin is also thought to regulate the gonadotrophin releasing hormone secretion pattern which consequently affect he secretion of luteinizing hormone and follicle stimulation hormone, and might therefore, hypothetically has a regulatory effect on the testosterone production in the theca cells (*103*) (Figure 3).
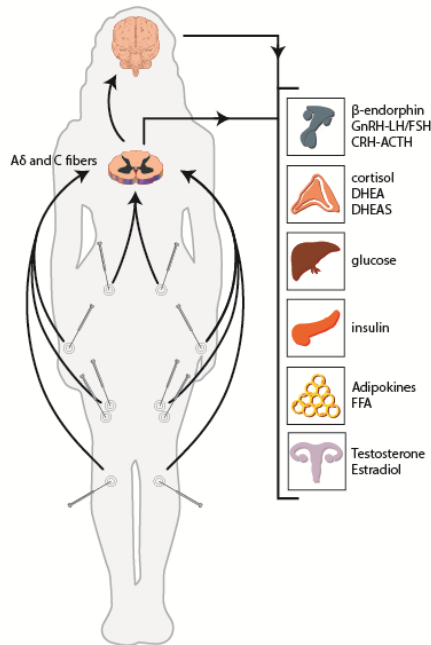
Figure 3. A hypothetical model of the low-frequency electroacupuncture (EA). Needles are inserted in abdominal and quadriceps muscle and in muscle below the knee. Muscle contractions induces by electrical stimulation activates ergoreceptors that in turn activates afferent Aδ and C nerve fibers. Thereafter, the signal of EA is transmitted to the spinal cord where sympathetic reflexes can modulates organs that are in the same innervation area. From the spinal cord the signal of electroacupuncture can modulated the central nervous system and affect the endocrine system (*GnRH: Gonadotropin releasing hormone; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; CRH: Corticotrophin releasing hormone; DHEA: Dehydroepiandrosterone; DHEAS: Dehydroepiandrosterone Sulfate*).
Illustration by Igor Cervenka

In a dihydrotestosterone-induced rat PCOS model, exhibiting irregular cycles, obesity and insulin resistance, both a single bout of low-frequency electroacupuncture and repeated electroacupuncture treatment have been shown to increase whole-body glucose uptake and restore dysfunctional adipose tissue gene expression (*104-106*). Whether acupuncture improves whole- body glucose regulation and cellular function in skeletal muscle and adipose tissue of women with PCOS is unknown. Repeated combined manual and low-frequency electroacupuncture has been shown to ameliorate excessive sex steroid secretion and to improve ovulatory function (*107, 108*), an effect that has been attributed to a decrease in a high sympathetic nerve activity (*109, 110*). However, in a recent large randomized controlled trial investigating whether low-frequency electroacupuncture is as good as pharmacological treatment with clomiphene citrate, or if it potentiates the effect of

pharmacological treatment for live birth rate in infertile women with PCOS, it was shown that clomiphene citrate was superior to acupuncture on live birth rate (*111*).

Human obesity is characterized by increased sympathetic nervous resulting that leads to hypertension, increased lipolysis, and elevated free fatty acids in circulation, which is related with insulin resistance, overweight and obesity (*112, 113*). It is largely unknown if increased sympathetic activity is a cause or a consequence of obesity and insulin resistance results (*114*). There are clinical evidence of acupuncture regulating sympathetic nervous system (*109*). However, the clinical effectiveness together with mechanistic evidence of acupuncture leading to improvement of insulin sensitivity and other metabolic abnormalities in women with PCOS are sparse. Therefore, in **paper III** and **IV** in this thesis, we have studied the effect and possible mechanisms of action of a single bout of low-frequency electroacupuncture on whole-body glucose uptake.

2   AIM

The overall aim of this thesis is to study if women with PCOS have altered methylation and expression patterns in adipose tissue and skeletal muscle. Subsequently we aim to examine if a single bout of low-frequency electroacupuncture has the potential to improve insulin sensitivity in overweight and obese women with PCOS. Moreover, whether epigenetic alterations in subcutaneous adipose tissue in women with PCOS can be remodeled by the stimulation. In addition, we have studied the mechanisms by which electroacupuncture mediates its effect.

SPECIFIC AIMS

Study I   To analyze genome-wide DNA methylation and gene expression levels in adipose tissue from women with PCOS and controls. In addition, to replicate these findings we used unrelated subjects and a new case-control cohort.

Study II   To analyze genome-wide DNA methylation and gene expression levels in skeletal muscle tissue from women with PCOS and controls. In validation *in vitro* experiments in human female myotubes we aimed to study the effect of insulin and testosterone on expression of selected genes as well as glycogen synthesis.

Study III   To study the effect of a single bout of low-frequency electroacupuncture on whole-body glucose uptake in overweight and obese women with and without PCOS. Furthermore, we aimed to test if increased glucose uptake by electroacupuncture is mediated via activation of the autonomic nervous system and the opioid system in rodents.

Study IV   To test if a single bout of low-frequency electroacupuncture remodels genome-wide DNA methylation and gene expression pattern in subcutaneous adipose tissue in women with PCOS. In addition, we aimed to study if changes in gene expression are mediated via activation of the sympathetic nervous system.

3 PATIENTS AND METHODS

3.1 ETHICS

The Regional Ethical Review Board of the University of Gothenburg approved all human studies (**paper I-IV**) in this thesis. All studies were conducted at the Sahlgrenska Academy, University of Gothenburg and at the Sahlgrenska University Hospital, Gothenburg, Sweden, in accordance with the Declaration of Helsinki. All participants gave oral and written consent before inclusion.

The animal experiment (**paper IV**) was approved by the Animal Ethics Committee of the University of Gothenburg, Sweden, and followed the principles of the Guide for the Care and Use of Laboratory Animals.

3.2 HUMAN STUDY

3.2.1 Subjects

We used two different cohorts of women. Subjects in cohort 1 are presented in **paper I** and subjects from cohort 2 are presented in **paper II-IV**. All women were recruited by advertisements in local newspapers and in frequently visited places in the community. A thorough anamnesis was taken before the participants were included in the study. The eligibility criteria for women with PCOS were at least two of the following three signs:

1. Polycystic ovarian morphology (PCOM)
2. Clinical and/or biochemical signs of hyperandrogenism
3. Oligo/amenorrhea.

The PCOS inclusion criteria for cohort 1 were:
   a) Ultrasound-verified polycystic ovaries with 12 or more 2–9 mm follicles and/or ovarian volume ≥10 ml in one or both ovaries.
   b) A self-reported Ferriman-Gallwey (FG) score ≥ 8 was defined as hirsutism with or without acne as defined by a positive response to the question *Do you have acne Yes/No*?
   and/or
   c) Oligomenorrhea was defined as an intermenstrual interval >45 days and < 8 menstrual bleedings in the past year. Amenorrhea was defined as < 3 menstruations per year.

The inclusion criteria for subjects in cohort 2 were the same but PCO morphology was not mandatory i.e. a) and/or b) and/or c).

The exclusion criteria for controls were:

a) Evidence of PCO morphology
b) Clinical signs of excessive acne or hirsutism
c) Menstrual irregularities (cycles >35 days)

The exclusion criteria for all women were:

a) Endocrine disorders such as hyperprolactinemia, non-classic congenital adrenal hyperplasia, androgen-secreting tumors, Cushing´s syndrome
b) Autoimmune disorder
c) Cancer
d) Type I or type II diabetes
e) Cardiovascular disease
f) Pharmacological treatment within 12 weeks before inclusion
g) Breastfeeding for 6 months before inclusion

In **paper I** we included 30 controls and 64 women with PCOS over a wide range of BMI. Out of 64 women with PCOS, 49 women met all three criteria for the PCOS diagnosis: PCO morphology; clinical signs of hyperandrogenism and ooligo/ amenorrhea and 15 women had hyperandrogemia and PCO morphology.

In **paper II−IV** was 21 women with PCOS and 21 controls included. Seventeen out of 21 women with PCOS met all three PCOS criteria. Cases and controls were matched for, age, weight and BMI.

3.2.2   Assessment of Insulin Resistance

There are different methods to test for disturbances in insulin sensitivity. Insulin resistance is simply diagnosed by oral glucose tolerance test (OGTT), which is a non-invasive method. Insulin sensitivity is also assessed by calculations of different sensitivity indexes such as: homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index. These methods are regularly used in a routine clinical practice however; they are not as consistent and reliable as direct measurement of insulin resistance by euglycemic- hyperinsulinemic clamp technique, which is considered the gold standard method (*115*).

The euglycemic- hyperinsulinemic clamp technique has been described in detail by others (*116*). In brief, after an overnight fast, insulin is infused at constant rate until a new hyperinsulinemic steady-state is reached. In theory this leads to a complete suppression of hepatic glucose production. Insulin is infused in a constant rate while exogenous glucose is infused at variable levels in order to avoid hypoglycemia and maintain euglycemia. The clamped levels of glucose provide information on how much of glucose is being metabolized per kilo of body weight when insulin levels are at the constant rate.

In **paper I during** euglycemic- hyperinsulinemic clamp insulin was continuously infused at 120 mU/m^2/min in **paper II** insulin was infused at 40mU/m^2/min for 120 min to reach steady state.

Therefore, insulin resistance is proportional to the amount of glucose cleared from the blood and low glucose infusion rate indicates insulin resistant patients. In all papers in this thesis, we estimated insulin sensitivity by calculating HOMA-IR and HOMA-B indexes and by euglycemic–hyperinsulinemic clamp technique.

3.2.3   Human Samples

Tissue biopsies were collected from *musculus vastus lateralis* and abdominal subcutaneous adipose. All biopsies were collected under local anesthesia (Xylocain, 20mg/ml, AstraZeneca AB, Södertälje, Sweden). Human skeletal muscle biopsies were collected by using a Bergström needle, and the subcutaneous adipose tissue were collected with needle biopsy. Adipose tissue biopsies were immediately rinsed with saline and prepared for further analyses.

After overnight fast, blood was collected from all participants and circulating hormones, lipid profile and adipokines were analyzed in an accredited laboratory. Clinical characteristics of all subjects and study design are presented in **papers I-IV** respectively.

3.2.4   Laboratory analyses

In **study I** Serum SHBG was measured with a chemiluminescent microparticle immunoassay (CMIA) (Architect SHBG reagent kit; Abbott Laboratories, Diagnostic Division, Chicago, IL). Gas chromatography/mass spectroscopy (GC-MS) was used to analyze serum testosterone and serum 17β-estradiol. Free testosterone and free estradiol were calculated as described by Vermeulen et al. (23) and Van den Beld et al. (24).

In **study II** and **IV** plasma glucose was measured by One Touch Ultra2 (LifeScan). Insulin was analyzed at an accredited laboratory. Serum insulin was measured with immunometric two-step sandwich method (Advia Centraur Insulin Ready Pack; Bayer HealthCare) where two antibodies are used: anti-insulin (Lite Reagent) and a monoclonal mouse anti-insulin antibody (Solid Phase) to initiate a chemiluminescence reaction. Serum C-peptide was measured with a human diabetes C-peptide magnetic bead set (Bio-Rad, USA). Plasma noradrenaline, dopamine, DOPAC, HVA, serotonin, and 5HIAA were measured on a split-fraction HPLC-ED system (*117*). Serum testosterone and estradiol were measured by gas chromatography-tandem mass spectrometry (GC-MS/MS) (*118*). In **study IV** subcutaneous adipose tissue levels of dehydroepiandrosterone (DHEA), androstenedione, testosterone, dihydrotestosterone (DHT), estrone (E1), estradiol (E2), and progesterone were measured by GC-MS/MS as previously described (*119, 120*).

3.2.5   Adipocyte size

To determine the adipocyte size we used a relatively quick method based on collagenase treatment accompanied by computer image analysis. Fresh adipose tissue (approximately 500mg in paper I and 250mg in paper II and IV) was cut and treated with collagenase (Typ A; Roche, Mannheim, Germany) in essential medium (Invitrogen, Carlsbad, CA) for 50 minutes at 37°C in a shaking water bath. Adipocytes were filtered and washed through 250 μm nylon mesh, then suspended in fresh medium. A drop of adipocyte cell suspension was placed between a siliconized glass slide and a cover slip and transferred to the microscope stage (Axioplan 2 imaging, Carl Zeiss, Oberkochen, Germany; ×5 objective). Mean adipocyte diameter was determined by using computerized image analysis as previously described (20). Nine random fields were photographed with a CCD camera (Axiocam, Carl Zeiss). Images were analyzed with Leica software (QWin V3, Leica Microsystems, Wetzlar, Germany). Mean adipocyte volume was calculated with the Goldrick formula (21). Although the method is convenient and provides reliable measurement of mean cell size, it has some disadvantages. During preparation of adipocyte suspension, collagenase treatment can lead to rapture of large adipocytes resulting with lipid droplets, which are then manually removed during computerized images analysis. This process is time consuming and it can be difficult to distinguish between small adipocytes and lipid droplets. Furthermore, this method does not provide information on the size distribution of the adipocytes.

3.2.6   Human Skeletal Muscle Cells Experiments

One objective of the cell experiment was to investigate if the two key features of PCOS, hyperandrogenism and hyperinsulinemia are controlling the gene expression. For these experiments, we selected in total 13 genes to study: *DYRK1A*, *SYNPO2*, *KLF10*, *SCP2*, *NAMPT, FOXO3*, *ING2*, *TTN*, *PPP2R2D*, *THY1*, *MAP2K6*, *YWHAE* and *COL1A1*. Cells from four healthy young donors were exposed to testosterone and/or insulin for three days and gene expression levels were compared to women with PCOS. We used following substances to stimulate cells: Testosterone, CDX (Bicalutamide, androgen antagonist) and insulin.

The second objective of these experiments was to investigate the effect of androgens on glycogen accumulation. Human female myotubes were stimulated with insulin, testosterone, and CDX and the effect of the substances

on the glycogen synthesis was measured. The conditions used in the human cells experiment and the process of myotube stimulation are described under Material and Methods in **paper II**.

3.3   ANIMAL STUDY PROCEDURE

The aim of the animal experiment in **paper III** was to study the mechanisms by which electroacupuncture mediates its effect on whole-body glucose uptake. All experiments were performed in the estrous phase and rats were randomly divided into treatment or no-treatment group. We conducted an experiment where we performed euglycemic-hyperinsulinemic clamp on rats and measured if one single bout of electroacupuncture affects whole-body glucose uptake in controls and DHT-induced PCOS rats. To investigate if the effect of acupuncture is mediated via autonomic nervous system and/or via the opioid system, we administrated different pharmacological agents. The dosage of the blockers used in this study were selected from the previous defined and established experiments (*121, 122*). Firstly, we identified responders to electroacupuncture as those that have increased in glucose infusion rate by 15% at 25 minutes of stimulation. Bolus dose of blocking agents of naltrexone (10mg/kg), Atropin (1mg/kg), Butaclamol (2mg/kg), Phentolamine (5mg/kg), Propranolol (1mg/kg) and saline were administrated at 30 minutes. After the bolus dose, atropine (0.5mg/kg), butaclamol (1mg/kg), phentolamine (7mg/kg/h), propranolol (0.5mg/kg/h) were continuously infused. Study design can be find in **paper III** Figure 1B. At steady-state of euglycemic-hyperinsulinemic clamp, two acupuncture needles were placed in the *rectus abdominis* muscle- which corresponds to the acupuncture points ST27, ST28, and ST29- and two were placed in the *triceps surae* muscle bilaterally– which corresponds to SP6 and SP9. The needles were attached to an electrical stimulator (CEFAR ACU II; Cefar-Compex Scandinavia, Sweden) and stimulated with low-frequency (2 Hz) at an intensity high enough to evoke muscle twitches, which varied from 0.8 mA to 2.2 mA due to receptor adaptation. The euglycemic-hyperinsulinemic clamp was performed under general anesthesia. Insulin was continuously infused at 8 mU/kg/min, and glucose was infused to maintain euglycemia of 6 mM.

3.4 SUMMARY OF THE METHODS (PAPER I–IV)

Table 2. Summary of methods used in the thesis.

Methods	Paper I	Paper II	Paper III	Paper IV
Euglycemic–hyperinsulinemic clamp	✓	✓	✓	✓
Adipocyte Size	✓			✓
LC/MS and GC/MS	✓	✓		
HPLC-ED		✓		
TaqMan Micro Fluid Cards	✓			
RT-qPCR /SYBR		✓	✓	✓
Immunoblot Analysis	✓			
Illumian HumanHT-12 v4 Expression BeadChip	✓	✓		✓
Illumina Infinium Human Methylation450k BeadChips	✓	✓		✓
Ingenuity Pathway Analysis	✓	✓		✓
Gene Set Enrichment Analysis		✓		
ELISA	✓	✓	✓	✓
Human Skeletal Muscle Cells		✓		
Electroacupuncture			✓	✓

3.5 GENOME-WIDE EXPRESSION ARRAY AND REAL TIME QPCR

In this thesis, we studied genome-wide gene expression in adipose tissue (**paper I and IV**) and in skeletal muscle (**paper II**) by using Illumina HumanHT-12 v4 Expression Bead Chip array. This array provides genome-wide transcriptional coverage of 47 000 probes that represent well-characterized genes, gene candidates and unknown splice variants.

In all papers in this thesis RNA was extracted with commercial column based RNA extraction kit (Qiagen) using the Trizol reagent. The RNA concentrations and purity were assessed with a NanoDrop spectrophotometer 1000 (Thermo Fisher Scientific) and RNA integrity was determined with automated electrophoresis method (Experion, Bio-Rad).

In **paper I, II** and **IV**, cRNA synthesis for genome- wide expressions studies were carried out at Genomic Core facility at University of Gothenburg, by using Illumina TotalPrep RNA Amplification Kit (Life Technologies & Invitrogen).

In validation experiment in paper I, Taq-Man assay micro fluid cards (Life technologies) were used to measure mRNA abundance of selected genes. We selected to use Taq-Man probes to measure mRNA abundance of selected genes due to the high specificity and efficiency.

In **paper II** and **IV**, we synthesized a stabile cDNA by using High- capacity cDNA Kit (Thermo Fisher Scientific). Abundance of the mRNA transcripts in human cell experiment (**paper II**) and animal experiment (**paper IV**) were measured by SYBR Green real-time PCR reactions (Applied Biosystem).

In all papers, we used reference housekeeping genes that are stable in the tissues and not affected by the treatment. We used the algorithms of the NormFinder (*123*) to find the best combination of the housekeeping genes for the normalization.

3.6 GLOBAL METHYLATION ARRAY

The analysis of DNA methylation levels in adipose tissue (**paper I** and **IV**) and skeletal muscle **(paper II)** are separated into two major steps: a) bisulfide conversion and, b) genome-wide methylation DNA analysis.

Prior to the DNA methylation screening, as recommended, DNA was converted from unmethylated cytosines to uracil. We used ´of-the-shelf´ bisulfite conversion kit (Zymo Research). The bisulfite conversion involves four major chemical steps: denaturation where DNA strands are separated; Sulfonation and a treatment with sodium bisulfite (NaHSO$_3$) that results with addition of SO$_3^-$ and creation of cytosinsulphonate. In a reaction of hydrolytic deamination, loss of water and ammonia leads to formation of uracilsulphonate. In a last step in a reaction of desulphonation uracil is formed. The methylated cytosines remain unchanged during the bisulfite conversion. During the downstream reaction of PCR, uracil is replaced by thymine, which during the sequencing step is read out as unmethylated cytosine. The bisulfite conversion is a critical step and we have performed a random quality check to confirm the successful bisulfite conversion Figure 4.
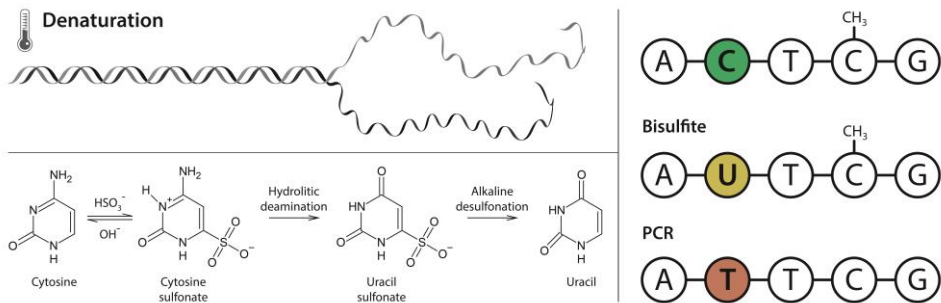
Figure 4. Chemical reaction of bisulfite conversion of cytosine to uracil.
   Illustration: Igor Cervenka

To analyze multiple methylation sites we used a high-throughput methylation array from Illumina Infinium® HumanMethylation450k BeadChip array. The array covers 485577 cytosine methylation sites which cover 99% of the reference sequence (RefSeq) genes and 96% of CpG islands. The array also gives information on location of the non- CpG sites and SNPs. HumanMethylation450k BeadChip is a two color assay that is based on Infinium I assay and Infinium II study design. Infinium I assay depends upon the base preceding the CpG locus being queried. Infinium II design depends on a single base extension at the 3' end of the probe sequence. Both designs will result in either red or green signal, depending on if CpG sites is methylated (red) or unmethylated (green). Methylation levels in BeadChip array are

measured for each CpG locus by calculation of a beta value (β). To estimate the levels of methylation and the beta value, the ration between intensity of methylated and unmethylated allele is calculated using $\beta = \text{Im}/(\text{Iu}+\text{Im}+\alpha)$, where Im represents methylated probe and Iu unmethylated probe, α is a constant. (*124*). Beta values range is between 0-1 and which represents the level of methylation between 0-100% within the specific cell pool.

3.7   WESTERN BLOT ANALYSIS

Protein concentrations were measured by Direct Detect™ spectrometer (Millipore, Billerica, USA). In **paper I** we used monoclonal antibodies that were specific against CYP1B1 (AV51761, Sigma-Aldrich, Stockholm Sweden) and PPARG (ab191407, Abcam, Cambride, UK). The protein analysis is challenging, comprehensive and time-consuming technique and we experienced different problems. In **paper I**, we optimized the signal strength of the PPARγ antibody in adipose tissue by increasing protein concentration and optimizing the concentration of our primary and secondary antibodies.

In **paper III** protein concentration was quantified by the Bradford assay. The protein levels of mNGF and proNGF were measured using MAB5260Z clone 27/21 and EP1318Y antibodies (Merck Millipore, Billerica, MA, USA) in a homemade sandwich ELISA.

3.8   PATHWAY ANALYSIS

3.8.1   Ingenuity Pathway Analysis

Ingenuity pathway Analysis (IPA) is an online research tool based on experimental knowledge libraries that was used for functional characterization of DNA methylation and genome-wide expression microarray data. We used IPAs canonical core analysis to investigate functional enrichment in expression and methylation differences between cases and controls (**paper I** and **II**) linked with metabolic disturbances in women with PCOS. In **paper IV** we used IPA to characterize pathways before *vs* after single bout of electroacupuncture that might be affected by treatment.

3.8.2 Gene Set Enrichment Analysis (GSEA)

GSEA is also known as functional enrichment analysis that we used in **paper II** on genome-wide expression array. Here we aimed to provide evidence that there is an association between a certain biological pathways (differentially expressed genes within the pathway) and the disease that we study (PCOS). Data analysis is based on their gene set ranking rather than on the individual genes that were ranked according to differences of expression between PCOS and controls.

3.9 STATISTICAL ANALYSIS

Statistical details for each clinical and experimental study are presented in respective manuscript. Gene expression data and DNA methylation levels in all experiments are expressed as Mean ± SD. In **study I, II** and **IV** we applied false discovery rate corrections (FDR) to account for different probe types on the array type I and type II when testing for multiple comparisons. We used t-test to identify differences in DNA methylation data between women with PCOS and controls (**Paper I**, **II** and **IV**). Furthermore, we applied the chi–square test to calculate whether the changed methylated sites were more than the expected number by chance. Spearman correlation coefficient were calculated to determine association between gene expression and DNA methylation.

4   RESULTS AND DISCUSSION

DNA methylation and gene expression differences in subcutaneous adipose tissue and skeletal muscle

There is a growing interest in understanding the involvement of epigenetic mechanisms in the development of PCOS (*125*). Data produced with new available technologies are still limited and it is difficult to connect methylation differences to the pathophysiology of PCOS. Further, no mechanistic studies have been done to verify the involvement of e.g. methylation mechanism in PCOS. From epigenetic studies in e.g. cancer (*126*), it became obvious that genetic and epigenetic mechanism cooperate together to control the genomic transcriptions and development of a disease (*127*). Therefore, it should be held in mind that all methylation difference identified in our studies are connected to underlying genetic disturbances in women with PCOS.

Firstly, we demonstrate that women with PCOS are more insulin resistant and have low glucose disposal rate independent of BMI. Insulin sensitivity was measured by the gold standard method; euglycemic- hyperinsulinemic clamp and by calculating HOMA-B and HOMA-IR indexes. We have confirmed previous findings that women with PCOS have; enlarged adipocytes and higher circulating HbA1c, triglycerides, LH/FSH ratio, C-peptide index, and testosterone in comparison to age and BMI the controls (*18, 128-130*). Increased levels of testosterone could explain increased BMI (*131*) because overweight and obesity are associated to increased levels of androgens and this in addition explains insulin resistance in some PCOS phenotypes (*132*). However, in our studies we have not seen association between increased testosterone and insulin resistance (*19*).

In this work we established that absolute differences in gene expression in skeletal muscle are larger (expression range 21–186%) than gene expression in subcutaneous tissue (expression range 28–58%) suggesting that skeletal muscle is more sensitive to transcriptional changes than adipose tissue. Whether DNA methylation changes in skeletal muscle are involved in inducing the large transcriptomic changes we observed is not clear. Over 17 000 CpG sites were differently methylated but only two genes differed after FDR corrections; (annotated to C3orf58 and cg10074626 (intergenic)). As ~30% of the significantly methylated CpG sites were in or near the gene correlated with

27

differently expressed genes in women with PCOS, this supports a functional role of DNA methylation in the skeletal muscle phenotype in these individuals. Although smaller expression differences, we identified more alterations in both genome-wide methylation and mRNA expression in adipose tissue than in skeletal muscle. Our requirements after corrections for the multiple testing in adipose tissue were set to FDR of 15% (or $q < 0.15$). When those requirements were fulfilled, we identified 440 differentially methylated sites with the absolute difference between 0.24–6.28% in adipose tissue. Out of those, we found 33 differentially methylated sites to have corresponding transcriptional changes in 30 genes. In skeletal muscle we found 24 differentially methylated genes ($p < 0.05$ and $q = 0.84$) to correspond to 21 differentially expressed genes with the absolute changes in methylation were between 0.48–4.35%. This shows that there might be methylation differences that are involved in regulation of phenotypic presentation of the PCOS syndrome in subcutaneous adipose tissue. Previous methylation studies have demonstrated that small alterations in DNA methylation within the regulatory regions of the genes might result with large phenotypical alterations (*133*). Even though we identified small changes in DNA methylation in adipose tissue, they still might have biological significance and contribute to the development of PCOS.

We identified 1720 differentially expressed genes with FDR below 5% in adipose tissue and differences were up to 58% between women with PCOS and controls. We focused on finding the genes with alteration in mRNA that might be the main contributors to development of metabolic alterations in PCOS. For the first time we identified transcriptional alterations in adipose tissue in women with PCOS in: *GPT*, *RTN4*, *BCKDHA* and *SVEP1* which, were associated with increased adipocytes size, testosterone and glucose disposal rate in women with PCOS. Genome–Wide Association Studies (GWAS) identified *BCKDHA* as a candidate gene for obesity (*134*). It belongs to a family of branched-chain amino acids genes (BCAAs) and it might be involved in development of insulin resistance (*135*). Furthermore, we also found a positive correlation between methylation and gene expression in *GPT* and negative correlation in *BCKDHA,* suggesting that epigenetic alteration might contribute to development of insulin resistance by altering the mRNA levels of these genes in adipose tissue.

Furthermore, we found that methylation levels of Ras-related protein (*RAB5B)* were increased in average by 1.2% that were associated to decreased mRNA

expression by 5% in adipose tissue. This goes in line with findings that increase in methylation lead to silencing of gene expression (*136*). GWAS identified *RAB5B* as a PCOS candidate gene (*137*). Hyperandrogenism is a major driver of PCOS (*138*). It has been proposed that *RAB5B* is involved in the signaling network regulating androgen synthesis (*139*). Here we could speculate that mRNA expression alterations may play an important role in contributing to development of hyperandrogenemia. However, additional studies are needed to firstly determine the exact function of *RAB5B* and, if so, how methylation differences in *RAB5B* play a role on transcriptional level that subsequently can be connected to alterations in androgen levels and development of insulin resistance in PCOS.

Also in skeletal muscle we wanted to associate differences in mRNA expression to the genes that might be involved in development of metabolic phenotype in women with PCOS. Skeletal muscle is a major organ involved in regulating whole-body glucose uptake and we intended to interrogate glucose metabolism in skeletal muscle. Exact molecular mechanisms explaining insulin resistance in skeletal muscle in women with PCOS has not been identified. Firstly, we performed correlations between genes with largest expression differences and clinical characteristics of PCOS. In skeletal muscle, we identified several significant correlations between gene expression in *KLF10, ING2*, *GGTL3*, *MAPKAP1*, *COL1A1*, *PPP2R2D* and *NAMPT,* and circulating testosterone, insulin levels, C-peptide, HOMA-IR and HOMA-B and adipocyte size. *ING2* is a chromatin remodeling protein involved in muscle differentiation (*140*). The family of *GGT* enzymes is important for metabolism of glutathione, however the function of *GGTL3* has to be elucidated (*141*). The role of *MAPKAP1* is not defined in skeletal muscle. Previous studies have showed that mRNA levels of *COL1A1* are affected by over-nutrition, which induces changes on muscle extracellular matrix and leads to obesity and metabolic dysfunctions (*142*). *PPP2R2D* is involved in regulation of a number of metabolic processes such as lipid metabolism and lipid biosynthesis (*143*). *NAMPT* positively regulates glucose homeostasis and this gene plays a role in several biological processes (*144*). These are the genes with the largest mRNA expression differences in skeletal muscle between cases and controls and these transcriptional alterations might be associated with low glucose uptake and insulin resistance. Therefore, we propose that these genes should be elucidated in future studies.

Further, we overlapped differentially expressed genes in adipose tissue and skeletal muscle from the gene expression array in order to find targets to study. We overlapped the 85 differentially expressed genes in skeletal muscle with 1720 unique genes in adipose tissue and found sixteen common genes, whereof nine genes changed in the same direction e.g. *KLF10* that is involved in glucose metabolism.

We generated large amount of data from both DNA methylation and gene expression arrays in paper I and paper II and it is not the scope of this thesis to elucidate them all. Whether it is hyperandrogenemia and/or hyperinsulinemia that drives the PCOS phenotype is not completely understood (*145*). There are clear indications that androgens contribute to development of metabolic alterations in PCOS (*146*). Likewise, studies propose that hyperinsulinemia is the main player contributing to the PCOS phenotype by stimulating the ovarian theca cells to produce testosterone and to be involved in development of metabolic and reproductive alterations in women with PCOS (*147*). Support for the latter hypothesis comes from studies demonstrating that low insulin lowers aberrant levels of androgens (*148*). However, it is still unknown if it is the hyperandrogenemia and/or hyperinsulinemia that drives the development of metabolic alterations in PCOS. Therefore, we aimed to explore this 'phenomenon' by investigating effects of insulin and testosterone on glycogen synthesis and the expression of selected genes in myotubes of human female donors.

Firstly, as expected, in study IV we confirm that insulin is a strong regulator in the conversion of glucose to glycogen in skeletal muscle whereas we found no effect of testosterone on glycogen accumulation, as this has been shown before (*149*). The role of *KLF10* in different diseases is unclear. Overexpression of *KLF10* in liver leads to increased gluconeogenesis and to hyperinsulinemia (*150*). Furthermore, animal studies have shown that KLF10 knockdown in hepatocytes lead to decreased glucose levels and improvement of glucose tolerance (*151*) . Next we investigated if the expression of *KLF10,* a transcriptional repressor regulating the circadian expression of genes involved in lipogenesis, gluconeogenesis, and glycolysis in liver (*151*), is regulated by insulin and/or testosterone in myotubes. Interestingly, insulin stimulation increased the mRNA expression of *KLF10,* a gene linked to conversion of glucose to glycogen in myotubes (*152*). Upregulation of *KLF10* in response to insulin in skeletal muscle of women with PCOS goes in line with

previous findings (*153*). Because we do not see an effect of testosterone on *KLF10* expression, it suggests that hyperinsulinemia is a key factor in the regulation of this gene in PCOS. Further, as mentioned, *KLF10* was upregulated in both adipose tissue and skeletal muscle in women with PCOS (Figure 5).
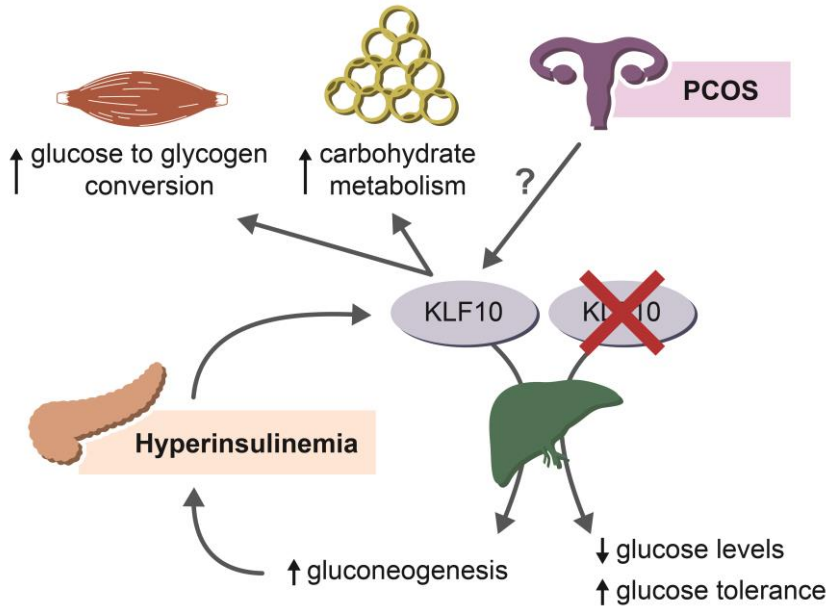
Figur 5. Schematic illustration of KLF10 activity in different tissues.
Illustration: Igor Cervenka

It can be hypothesized that increased levels of androgen can have an indirect effect on aggravating hyperinsulinemia (*154*). This theory is supported by our findings from human muscle cell experiment where we showed that testosterone was involved in regulation of the gene expression of *COL1A1* and *MAP2K6*. Furthermore, we found that changes in gene expression of these genes are under epigenetic control.

We identified large number of genes that were altered in methylation and expression (paper I and II) and there are other interesting candidates that require further *in-vitro and in-vivo* studies in order to demonstrate that differential expression are related to low glucose infusion rate, increased testosterone levels and large adipocyte size in women with PCOS.

In general, we observed that gene expression changes are more pronounced than DNA methylation changes in both adipose tissue and skeletal muscle, which presumably is a result of interplay between other epigenetic and genetic mechanisms.

The first challenge in running genome-wide methylation and gene expression arrays in all studies was to pre-process bioinformatics data an analyze Illuminas 450k BeadChip platform. We addressed problems with batch effect and removed the technical influence without removing the biological signal. The potential influence of technical effect on the biological results were avoided by running all genome-wide methylation and gene expression chips at the same time, and, by placing samples randomly on the cards. Furthermore, we have filtered out problematic probes such as the ones that have targeted the Y-chromosome. Methylation and gene expression data were scanned with probes. Therefore, we applied a method of quantile normalization in order to remove for unwanted technical variation. As expected, in paper I and II there was an effect of age and BMI on methylation data. Therefore, in all array analyses the corrections for age and BMI were made.

Although the clinical studies were carefully designed, we have not taken into the account that different cell types can be confounding factor. Adipose tissue and skeletal muscle biopsies are composed of many different cell types. In our global methylation and gene expression studies, we used whole tissue biopsies to study methylome and transcriptome. Women with PCOS are often overweight and obese which leads to inflammation and it could explain why we identified genes involved in mediating inflammatory responses, such as *IL6* and *CD74*. In parallel to the publication of paper I, researchers from USA demonstrated that women with PCOS have methylation alterations in subcutaneous adipose tissue (*79*). However, results from these two studies are differing which might be due to the sample size and differential clinical characteristic between the two cohorts (*76, 79*). Surprisingly, we have not identified alterations in either *INSR* or *LHCGR* gene expression; however, both studies identified expression alterations in *RAB5B*. Furthermore, the other study found increased methylation of *INSR* in region that is largely unmethylated which explains decrease in gene expression. Furthermore, they have shown that genetic variations regulate methylation quantitative trait loci (meQTL) and expression quantitative trait loci (eQTL) suggesting that genetic variation regulates gene expression.

Effect of Electroacupuncture on whole-body glucose uptake and protein and gene expression

We here demonstrate for the first time that a single bout of low-frequency electroacupuncture, via muscle contractions, increase whole-body glucose uptake in overweight and obese women with and without PCOS (study III). Single bout of low-frequency acupuncture also lowered adipose tissue expression of markers of sympathetic nerve activity: proNGF, serotonin and homovanillic acid. Recently we established that five weeks of low-frequency electroacupuncture treatment improves HbA1c and lowers circulating and adipose tissue androgens, which might improve ovulation (*155*), indicating that it might be used in the clinic to assist muscle contractions and increase whole-body glucose uptake. Furthermore, we have shown that electroacupuncture restores aberrant gene expressions in adipose tissue that were -identified in study I (*76*). In total, we found 80 genes including *PPARγ*, *CD74* and *ADIPOR2* to be restored to healthier phenotype by one single bout of electroacupuncture.

PPARγ is a master regulator of adipocyte differentiation, and it controls the expression of many genes that are regulating different metabolic processes, it also have therapeutic application (*156*). In paper I we showed that there is a correlation between disturbances in methylation and transcriptomic changes in *PPARγ* in subcutaneous adipose tissue. We demonstrated that DNA methylation of *PPARγ* was increased and expression was downregulated by 13.5 %, supporting the fact that increased methylation leads to downregulation of gene expression and, at least in part, contribute to the development of insulin resistance (*76*). Interestingly, one single bout of electroacupuncture decreased methylation marks in the body regions of *PPARγ* by 5.7% and increased the expression by 49.7%, which indicates that electroacupuncture exerts similar changes on *PPARγ* mRNA levels as exercise and can in part explains the increased glucose uptake measured by the clamp (*157, 158*).

Next, we showed that major histocompatibility complex, class II invariant chain (*CD74*) is involved in the regulation of adipogenesis and inflammation (*159*). The expression of *CD74* was increased in adipose tissue from women with PCOS, which might contribute to the unhealthy PCOS phenotype (*76*), and there was a positive correlation between gene expression and DNA methylation in *CD74*. Furthermore, we found gene expression of *CD74* to

correlate positively with increased testosterone. In study IV, the *CD74* gene was hypomethylated by 2.4%, and mRNA levels were decreased by ~23%, supporting the idea, that electroacupuncture may have the capacity to restore methylation alterations that could partly improve insulin sensitivity.

Adipocyte size together with circulating adiponectin are the strongest factors explaining insulin resistance in women with PCOS (*160*). There is evidence that disruption of adiponectin and adiponectin signaling plays a role in pathogenesis of PCOS (*161*). Previous studies reported that mRNA expression of adiponectin receptor 2 (*ADIPOR2)* in women with PCOS is downregulated by hyperinsulinemia and overweight (*162, 163*). In our paper I was *ADIPOR2* downregulated by 12% in adipose tissue in women with PCOS in line with other studies (*162*). In animal models of PCOS, the role of adiponectin has been shown to play an important role in protecting against increase in weight gain, insulin resistance and adipocyte hypertrophy (*164*). We demonstrate that electroacupuncture increases the *ADIPOR2* mRNA expressions by 21% and therefore might be considered as an alternative treatment to improve glucose and lipid uptake *via* activation of adiponectin signaling.

Because the positive effect of electroacupuncture have been demonstrated on several metabolic parameters e.g. HbA1c in women with PCOS we conducted a study where we aimed to elucidate the mechanism of action of electroacupuncture (*165*). As the effect of acupuncture has shown to be mediated via modulation of autonomic activity (among many factors) we decided to investigate the involvement of autonomy nervous system by administrating unselective adrenergic α and β-blockers, butaclamol and atropine during a euglycemic-hyperinsulinemic clamp and low-frequency electrical stimulation. We found that a single bout of electroacupuncture increases whole-body glucose uptake by activation of the sympathetic and partly the parasympathetic nervous systems, which could have important clinical implications for the treatment of insulin resistance. Next, we investigated if the expression of selected genes that changed by electroacupuncture was blocked when administrating alpha- and beta-adrenergic blockers to rats during a euglycemic hyperinsulinemic clamp.

Some animal studies showed that hyperinsulinemia exerts excitatory effect on sympathetic nervous system and might lead to elevated sympathetic output to other organs (*166*). However, high sympathetic nervous activity is measured in animal PCOS models, which is accompanied by elevated levels of nerve

growth factor (NGF) that are involved with ovarian pathology (*167*). In an animal experiment, we have shown that electroacupuncture lowered the expression of *Nr4a2* and *Junb* indicating that sympathetic nervous system in humans might mediates it signal via altering the gene expression of *NR4A2* and *JUNB*. The biological function of NR4A4 in unknown and the role of *JUNB* (*168*) on adipogenesis remains to be elucidated. However, the nuclear receptor subfamily 4 (NR4A) has been associated with alterations in glucose utilization and oxidative phosphorylation in liver and skeletal muscle (*169*).

5   CONCLUSIONS AND FUTURE PERSPECTIVES

Current studies on methylation in women with PCOS rely on microarrays in order to provide the information on methylation differences. The field of epigenetics is constantly advancing. With new emerging techniques approaching we will be able to make new discovers that will help to advance the knowledge of how epigenetic disturbances contribute to pathophysiology of PCOS.

In the four studies of this thesis, we revealed that the methylation pattern of women with PCOS is altered in both adipose tissue and skeletal muscle and that the methylation mechanisms are involved in controlling the gene expression. Furthermore, we show that one single bout of electroacupuncture induces rapid changes on methylome supporting the idea that epigenetic changes can by rapidly reversed by electroacupuncture in the similar way as exercise. The relationship between the two hallmarks of PCOS, hyperinsulinemia and hyperandrogenemia is still puzzling. Overall, our results point out that hyperinsulinemia is responsible for transcriptional changes on genes that are involved in regulating different metabolic processes. If the transcriptional changes are due to the epigenetic differences remains to be evaluated in the future studies. However, hyperinsulinemia may lead to increase in androgen concentration and furthermore to development of insulin resistance.

The main conclusions of the papers are:

Study I   Women with PCOS have genome-wide DNA methylation alterations that are associated with transcriptional changes in adipose tissue. Methylation changes correlate with gene expression changes in specific genes, which are associated with hyperandrogenemia, glucose homeostasis and increased adipocyte size.

Study II   Women with PCOS have DNA methylation alterations that are associated with transcriptional changes in skeletal muscle. In human myotubes we demonstrated that mRNA expression levels are under hormonal regulation of insulin and testosterone.

Study III   Single bout of low- frequency electroacupuncture increased whole-body glucose uptake in overweight and obese women with and without PCOS. Increased glucose uptake by electroacupuncture is mediated via

activation of the autonomic nervous system. Electroacupuncture lowers protein expression of markers of sympathetic nerve activity in plasma serotonin and homovanillic acid and a proNGF in adipose tissue.

Study IV   **S**ingle bout of low-frequency electroacupuncture remodels genome-wide DNA methylation and gene expression changes in subcutaneous adipose tissue in women with PCOS. Changes in gene expression are mediated via activation of the sympathetic nervous system. Women with PCOS have increased levels of androgens in subcutaneous adipose tissue.

new lab members **Yang Qian** and **Zhiyi Zhano** I enjoyed our short, but great talks. **Sanjiv Risal**, thank you for collaboration. To our research engineer **Eva Lindgren**, you entered the lab in a perfect moment. I could not have asked for better support! Thank you for all your expertise and support during my final year!

To the colleagues at the Physiology Department in Gothenburg: **Peidi**, **Jeanette**, **Pete Smith**, **Mayte**, **Christina**, **Heike**, **Cuicui**, **Fredrik**, **Rozita**, **Markus**, **Vilborg, Veronika Golubinskaya, Emman, Jennifer** and **Kerstin Ebeförs.** It was a pleasure meting you and working with you.

To research leaders in other groups at Karolinska Institutet: **Camilla Svensson, Johanna Lanner, Lennart Lindbom, Daniel Andersson, Bo Rydqvist and Alfredo Gimenez- Casssina.** It was a privilege to interact with you. Thank you **Kent Jardemark**, **Anna Feldreich** and **Irene Lund** for the guidance and for always having time for discussions. Thank you Professor **Jorge L. Ruas**, Your knowledge and genuine interest in science is inspiring. I will always remember my discussion with Jonathan Mudry on confusing and maybe not so exciting data on DNA methylation. You joined us for five minutes; discussed God knows what about you´re tryptophan metabolism. When you left Jonathan said: "This guy makes me wanna stay in research". I feel the same! Thank you for the motivating chats. **Jonathan Mudry** thanks for the friendship and for being the only person willing to go with me to SR☺.

To the wonderful people at KI: **Ellinor Kenne**, **Joel Rasmuson**, **Paolo Janing, Liu Zhengye, Gianluigi Pironti, Maarten Steinz, Duarte Ferreira, Vinko Palada** and **Teresa Fernandes**. Thanks for fun chats and sharing your experience. To my dear **Alex-Bersellini Farinotti** for being nice and caring person! Thank you **Sally Abdelmoaty** for kindness, honesty, and making bad days good. To **Thais**, **Milena**, **Petter**, **Mladen, Laura, Sara, Mona and Giacomo**, It was nice seeing you in the corridors and outside of them. To the new cool girl I got to know in the past few weeks **Theresa Mader**, what fun it is to have you around!

Without you guys I would have given up a long time ago:
To **Igor Cervenka**, and **Jana Valnohova**. I am amazed by the creativity of you two. You guys are upright funny, entertaining and I love chatting with you! Igore, many thanks for creating nice figures for my thesis. **Rocio,** it´s nice to be around a joyful and genuine person like you. Thank you **Arthur Cheng** for not judging! To my kind of girl, good-looking blondie **Maja Schlitter,** lunches at FyFa becomes super fun since you joined them☺. Thanks for all dinners, after-works, nights out, pushing me to work out and everything else to come. I

Хвала мојој породици ♥: Мојом брату- **Чаславу**. Брате, what would I do without you!? Нема тих речи које би описале колико сам захвална за све! **Yrsa** och **Nikola**, livet är mycket roligare nu när ni finns här! **Jelena,** tack så mycket för att sreca mala- **Emilia** finns i våra liv. Хвала мојој мами **Ради** на бескрајној подршци све ове године.

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